Chemical Modification of Acyl-CoA:Cholesterol *O*-Acyltransferase. 2. Identification of a Coenzyme A Regulatory Site by *p*-Mercuribenzoate Modification†

Paula M. Kinnunen, Curtis A. Spilburg, and Louis G. Lange*

Cardiology Division, Jewish Hospital of St. Louis at the Washington University Medical Center, St. Louis, Missouri 63110

Received February 22, 1988; Revised Manuscript Received June 6, 1988

ABSTRACT: Acyl-CoA:cholesterol *O*-acyltransferase (EC 2.3.1.26, ACAT) is the major intracellular cholesterol-esterifying activity in vascular tissue and is potentially a key regulator of intracellular cholesterol homeostasis during atherogenesis. We have previously reported inhibition of microsomal ACAT by histidine and sulfhydryl-selective chemical modification reagents and present here a more detailed analysis of the effect of sulfhydryl modification on ACAT activity. This analysis indicated two effects of sulfhydryl modification on ACAT activity. Modification of aortic microsomes with relatively low concentrations of *p*-mercuribenzoate (PMB) (100–200 μM) identified an inhibitory coenzyme A binding site on ACAT which contains a modifiable sulfhydryl group. This site binds CoA tightly (K_i = 20 μM), and PMB modification prevented subsequent ACAT inhibition by CoA without itself inhibiting enzyme activity. At higher concentrations (1–2 mM), PMB inhibited ACAT activity, indicating the presence of a modifiable sulfhydryl group necessary for cholesterol esterification by ACAT. Modification of both sites by PMB was reversible by thiols, and protection against modification was afforded in both cases by oleoyl-CoA, indicating that these sites may also bind oleoyl-CoA. Thus, at least two sulfhydryl groups influence ACAT activity: one is necessary for cholesterol esterification by ACAT, and one is at or near an inhibitory CoA binding site, which may be occupied at intracellular concentrations of CoA.

Regulation of cholesterol esterification by acyl-CoA:cholesterol *O*-acyltransferase (EC 2.3.1.26, ACAT)[1] is a key element in intracellular cholesterol homeostasis. Increased ACAT activity may lead to intracellular cholesterol ester accumulation in vascular tissue during atherogenesis (Spector et al., 1979). ACAT activity is also markedly induced during cholesterol feeding in liver, intestine, and pancreas, organs intimately involved in absorption and processing of dietary cholesterol (Kinnunen et al., 1988), suggesting as well a possible role for ACAT regulation in determining total body cholesterol balance. Because the enzyme polypeptide has neither been identified nor purified to homogeneity, we designed chemical modification studies to explore the catalytic mechanism of ACAT and to obtain structural chemical information potentially useful for its identification (Kinnunen et al., 1988).

This general survey of the effects of protein modification reagents on ACAT activity showed that liver and aortic ACATs were inhibited by reagents which modify histidyl residues, sulfhydryl groups, and arginyl residues. Two subtypes of ACAT were identified on the basis of their markedly different sensitivities to the histidine-modifying reagent diethyl pyrocarbonate (DEP). One subtype, typified by liver ACAT, had a K_i of 1500 μM for inhibition by DEP, and the other subtype, typified by aortic ACAT, had a K_i of 40 μM. In addition, both liver and aortic ACAT activities were highly sensitive to sulfhydryl-modifying reagents, such as *p*-mercuribenzoate. In this work, a more detailed analysis is presented of the effects of sulfhydryl modification on ACAT activity, and these studies show that in addition to a modifiable sulfhydryl necessary for cholesterol esterification by ACAT, there is an inhibitory coenzyme A binding site which modulates ACAT activity and is also modifiable by *p*-mercuribenzoate. This inhibitory site binds CoA tightly (K_i = 20 μM), indicating that physiological concentrations of CoA may regulate ACAT activity.

Materials and Methods

Materials. All reagents 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.

p-Mercuribenzoate, dithiothreitol, 2-mercaptoethanol, coenzyme A, ATP, oleic acid, ethyl oleate, cholesteryl oleate, and oxalyl chloride were obtained from Sigma. Fatty acid poor bovine serum albumin was purchased from Calbiochem, and DEAE-cellulose was from Whatman. Two percent cholesterol rabbit chow was purchased from Purina, and silica gel 0 plates were purchased from Analabs.

Synthesis of [^{3}H]Ethyl Oleate. [^{3}H]Ethyl oleate was synthesized by acid-catalyzed esterification of [^{3}H]oleic acid in ethanol as previously described (Lange et al., 1981) and then diluted with ethyl oleate to a final specific radioactivity of 40 dpm/pmol before use as an internal standard in ACAT assays.

Synthesis and Purification of [^{14}C]Oleoyl-CoA and Oleoyl-CoA. [1-^{14}C]Oleoyl-CoA (107 dpm/pmol) and oleoyl-CoA

† This work was supported in part by grants funded by the Missouri Affiliate grant-in-aid, American Heart Association; Alcoholic Beverage Medical Research Foundation; NIH/NHLBI Clinical Investigator K08-HL01846; NIH/NIAAA R01-AA06989; NIH/NIAAA R01-AA07656; and American Cancer Society BC-610.

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

[1] Abbreviations: PMB, *p*-mercuribenzoate; ATP, adenosine 5′-triphosphate; DEAE-cellulose, (diethylaminoethyl)cellulose; DEP, diethyl pyrocarbonate; BME, 2-mercaptoethanol; DTT, dithiothreitol; CoA, coenzyme A; ACAT, acyl-CoA:cholesterol *O*-acyltransferase.

0006-2960/88/0427-7351$01.50/0

were synthesized via their acid chlorides (Bishop & Hajra, 1980) and then purified before use by chromatography over DEAE-cellulose (Steinman & Hill, 1973), as described in the preceding paper (Kinnunen et al., 1988).

Microsome Preparation. Male New Zealand white rabbits, normal or fed 2% cholesterol chow for 1–2 months, were sacrificed by cervical dislocation followed by rapid excision of aortic and liver tissue into ice-cold 0.1 M potassium phosphate, pH 7.4. After the tissues were cleaned, they were homogenized (10%, w/v) in 0.1 M potassium phosphate, pH 7.4, using a Polytron (Brinkmann) at half-maximal power for 60 s. Microsomes were prepared by sequential centrifugation at 800*g* for 10 min, 13300*g* for 10 min, and 100000*g* for 60 min, and the microsomal pellets were resuspended in 0.1 M potassium phosphate, pH 7.4, using a Duall tissue homogenizer at a final protein concentration of 2–20 mg of protein/mL and then stored at −70 °C until used. Microsomal protein was determined by the method of Lowry (1951) using BSA as a standard.

ACAT Assay. Microsomal ACAT activity was measured by first preincubating microsomal membranes (30–160 μg of protein) in a total volume of 0.45 mL of 0.1 M potassium phosphate, pH 7.4, containing 0.88 mg/mL bovine serum albumin, 5.5 mM ATP, and 5.5 mM magnesium chloride, with or without coenzyme A and/or a thiol (2-mercaptoethanol or dithiothreitol). After a 30-min preincubation at 37 °C, 50 μL of 100 μM [^{14}C]oleoyl-CoA (107 dpm/pmol) was added to the assay mixture, which was incubated at 37 °C for 30 min. The reaction was quenched by the addition of 4 mL of methanol at 4 °C. Lipids were extracted (Bligh & Dyer, 1959) with addition of cold carrier cholesteryl oleate (1 μmol) and [^{3}H]ethyl oleate (1 μmol, 40 dpm/nmol) as a yield marker. Extracted lipids were separated by thin-layer chromatography on silica gel 0 in an apolar solvent system (petroleum ether/diethyl ether/acetic acid, 75:5:1) and exposed to iodine vapor to identify cholesteryl esters and fatty acid ethyl esters. After removal of iodine by gentle heating, the areas of silica gel containing cholesteryl esters and fatty acid ethyl esters were scraped separately into scintillation vials, 10 mL of Aquasol (New England Nuclear) was added, and incorporated radioactivity was quantitated by liquid scintillation counting. Quenching was monitored by using carbon-14 as reference. Yield was determined by recovery of [^{3}H]ethyl oleate, which migrates just below cholesteryl ester in this solvent system (Lange et al., 1981), and ACAT activity was calculated as picomoles of cholesteryl [^{14}C]oleate formed per milligram of microsomal protein per minute. Blanks were determined in parallel incubations containing buffer instead of microsomes.

Protein Modification with p-Mercuribenzoate. Ten microliters of *p*-mercuribenzoate (PMB) solution was added to 90 μL of microsomes (2–8 mg/mL) in 0.1 M potassium phosphate, pH 7.4. After 0–30 min at 22 °C, 20-μL aliquots were diluted into standard ACAT assay mixtures (total volume 0.5 mL), and ACAT activity was determined as described above.

Results

ACAT Assay Conditions. Numerous assays for ACAT have been reported in the literature, including those employing as substrate [^{14}C]oleate plus coenzyme A and ATP or, alternatively, commercially available [^{14}C]oleoyl-CoA (Sgoutas, 1970; Kritchevsky & Kothari, 1978). Our analysis after ion-exchange chromatography of oleoyl-CoA over DEAE-cellulose (Kinnunen et al., 1988; data not shown) indicated that commercially available oleoyl-CoA was contaminated with as much as 50% CoA. Hence, in the present experiments, oleoyl-CoA was synthesized every several weeks and purified before use by ion-exchange chromatography over DEAE-cellulose to eliminate contaminating substances. The importance of substrate purity was underscored by our subsequent experiments, which examined the effect of added coenzyme A on ACAT activity, data not previously reported.

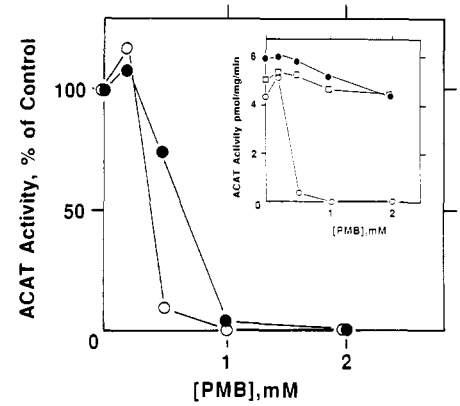

FIGURE 1: PMB inhibition of aortic and liver ACATs: concentration dependence and reversibility. Aortic (○, 2 mg/mL) and liver (●, 8 mg/mL) microsomes in 0.1 M potassium phosphate, pH 7.4, were incubated with 0–2 mM PMB at 22 °C for 1 min; then 20-μL aliquots were added to 0.43 mL of ACAT preincubation mixtures. After incubation at 37 °C for 30 min, 50 μL of 100 μM [^{14}C]oleoyl-CoA (107 dpm/pmol) was added to initiate ACAT assays. Assays were incubated at 37 °C for 30 min, followed by quenching, lipid extraction, and TLC as described under Materials and Methods. Results are expressed as percent of control ACAT activity, measured in the absence of PMB, which was 10.8 pmol of cholesteryl [^{14}C]oleate synthesized mg^{-1} min^{-1} for aorta and 21.6 pmol mg^{-1} min^{-1} for liver. Each point represents the average of duplicate assays. Blank values were assayed in parallel incubations containing no microsomes. Insert: Reversibility with thiols. Aortic microsomes (2 mg/mL) in 0.1 M potassium phosphate, pH 7.4, were incubated with 0–2 mM PMB at 22 °C for 1 min; then 20-μL aliquots were added to 0.43 mL of standard ACAT preincubation mixtures with no thiols (○), DTT (□, 1.3 mM), or BME (●, 1.3 mM) and incubated at 37 °C for 30 min. ACAT assays were then initiated by addition of [^{14}C]oleoyl-CoA (final concentration 10 μM, 107 dpm/pmol) and incubated for 30 min more at 37 °C, followed by quenching, lipid extraction, and TLC as described under Materials and Methods. Each point represents the average of duplicate assays.

ACAT Inhibition by PMB. A general survey of the effects of protein modification on ACAT activity revealed that both liver and aortic ACATs were highly sensitive to the sulfhydryl-modifying reagent *p*-mercuribenzoate (PMB), which fully inhibited both liver and aortic ACAT activities at a concentration of 1 mM (Kinnunen et al., 1988). These data suggested the presence of a sulfhydryl group essential for ACAT activity and modifiable by PMB. More detailed analysis of the concentration dependence of inhibition by PMB indicated that aortic and liver ACAT activities were similarly inhibited by PMB (Figure 1). For both, sharp decreases in ACAT activity were observed at PMB concentrations above 0.2 mM, with apparent K_i's of 400 and 600 μM for inhibition of aortic and liver ACATs, respectively. These data were reproducible using protein concentrations in the range of 2–10 mg/mL. Inhibition was reversible by thiol reagents, as shown for aortic ACAT (Figure 1, insert). Thus, 2-mercaptoethanol and DTT (both 1.3 mM) fully reversed aortic ACAT inhibition after exposure to up to 2 mM PMB, confirming that PMB inhibition of ACAT was occurring through sulfhydryl modification.

Further characterization of aortic ACAT inhibition by PMB indicated that it was time dependent, being 90% complete after 5 min at 22 °C, and that protection against inhibition was afforded by excess oleoyl-CoA present during modification. In the absence of oleoyl-CoA, 200 μM PMB inhibited aortic

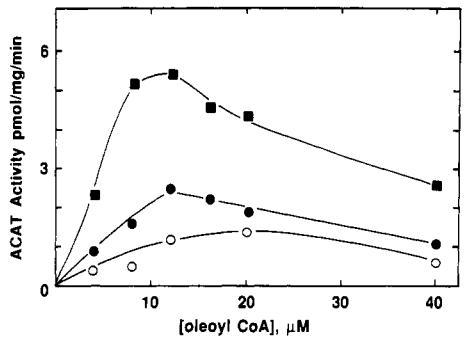

FIGURE 2: PMB activation of aortic ACAT in the presence of CoA. Aortic microsomes (2 mg/mL) in 0.1 M potassium phosphate, pH 7.4, were incubated without (●) or with (○, ■) 200 μM PMB at 22 °C for 1 min; then 20-μL aliquots were added to 0.43 mL of standard ACAT preincubation mixtures without (○) or with (●, ■) 100 μM coenzyme A and preincubated at 37 °C for 30 min. [^{14}C]Oleoyl-CoA (4–40 μM, 100 dpm/pmol) was then added to initiate ACAT assays, which were incubated for 30 min at 37 °C, followed by quenching, lipid extraction, and TLC as described under Materials and Methods. Results are expressed as picomoles of cholesteryl [^{14}C]oleate synthesized per minute per milligram of protein.

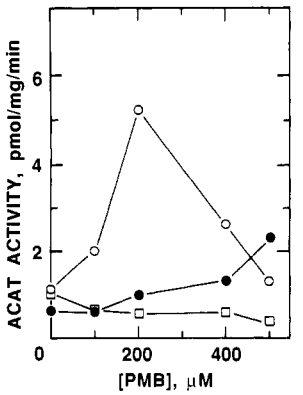

FIGURE 3: Activation of aortic microsomal ACAT activity by PMB in the presence of coenzyme A. Aortic microsomes (5 mg/mL) were incubated at 22 °C for 1 min with (●) or without (○) 100 μM oleoyl-CoA and then modified with 0–500 μM PMB at 22 °C for 1 min. Twenty-microliter aliquots were then added to 0.43 mL of standard ACAT preincubation mixtures containing 100 μM coenzyme A with (□) or without (○, ●) 1.3 mM dithiothreitol, which were incubated at 37 °C for 30 min. [^{14}C]Oleoyl-CoA (final concentration 10 μM, 100 dpm/pmol) was then added to initiate ACAT assays, which were incubated at 37 °C for 30 min. ACAT activity was calculated as picomoles of cholesteryl [^{14}C]oleate formed per minute per milligram of of microsomal protein after lipid extraction and TLC as described under Materials and Methods.

ACAT 15% compared to unmodified ACAT, but in the presence of 100 μM oleoyl-CoA, there was no inhibition of aortic ACAT by 200 μM PMB compared to microsomes incubated with 100 μM oleoyl-CoA alone. Thus, PMB inhibition of ACAT was prevented by substrate, suggesting that the modified sulfhydryl may be at or near the active site of ACAT.

When PMB-modified ACAT was incubated with coenzyme A (initially added to assess its effect as a thiol reagent), we noted marked activation of ACAT activity. Thus, treatment of aortic microsomes with 200 μM PMB, followed by preincubation and assay in the presence of 100 μM coenzyme A, produced marked activation of ACAT activity compared to control microsomes assayed in the presence of CoA alone (Figure 2). At the oleoyl-CoA concentration which yielded maximal ACAT activity ([oleoyl-CoA] = 12 μM), there was a 2.2-fold activation of ACAT activity in aortic microsomes treated with PMB and assayed with CoA, compared to ACAT activity in untreated microsomes assayed with the same concentration of CoA.

PMB activation of ACAT activity when assayed in the presence of CoA was concentration dependent with respect to [PMB] (Figure 3). Maximal activation of ACAT activity (5-fold) was observed after modification of aortic microsomes with 200 μM PMB, and this effect was diminished at higher PMB concentrations (approaching 500 μM). Activation of aortic ACAT by PMB in the presence of CoA was virtually abolished either by inclusion of DTT (1.3 mM) in the ACAT assay or by inclusion of oleoyl-CoA (100 μM) during PMB modification, indicating that a modified sulfhydryl is present and interacts with oleoyl-CoA as well as CoA. These results are consistent with the presence of a modifiable sulfhydryl group on ACAT that interacts with coenzyme A and whose modification by PMB leads to ACAT activation in the presence of CoA.

Coenzyme A Inhibition of ACAT. To further examine the basis for the apparent activation of PMB-modified aortic ACAT in the presence of coenzyme A, the effect of added coenzyme A on ACAT activity was characterized. After incubation of normal aortic microsomes with 100 μM CoA for 60 min, measurement of ACAT activity demonstrated 68% inhibition of ACAT activity compared to control ACAT activity measured in microsomes incubated without CoA. Liver and cholesterol-fed aortic ACATs were similarly inhibited by coenzyme A (61% and 56%, respectively). More detailed analysis of ACAT inhibition by CoA indicated that inhibition was time dependent; that is, incubation of aortic microsomes with 100 μM CoA at 37 °C resulted in maximal inhibition only after 45 min (Table I, rows 1–5). ACAT inhibition produced by 2-h incubation with CoA was fully reversed by removal of CoA (Table I, row 6). These results indicated that CoA inhibition of ACAT is time dependent but reversible.

Table I: Aortic ACAT Inhibition by Coenzyme A: Time Dependence and Reversibility ([Coenzyme A] = 100 μM)[a]

time (min)	ACAT act. (pmol mg^{-1} min^{-1})	time (min)	ACAT act. (pmol mg^{-1} min^{-1})
0	5.5	90	1.9
15	2.5	120	1.9
45	1.8	120[b]	6.2

[a] Twenty-microliter aliquots of normal rabbit aortic microsomes (2.7 mg/mL) were added to 0.43 mL of standard ACAT preincubation mixtures containing in addition 100 μM coenzyme A and then incubated at 37 °C. At the indicated times (0–120 min), 50 μL of [^{14}C]-oleoyl-CoA was added to initiate ACAT assays, which were then incubated for 30 min at 37 °C, followed by quenching, lipid extraction, and TLC as described under Materials and Methods. A parallel sample was incubated at 37 °C for 120 min and then centrifuged at 100000*g* for 60 min to repellet microsomes. The supernatant was removed after centrifugation, and the microsomes were resuspended in 0.1 M potassium phosphate, pH 7.4. ACAT activity in the resuspended microsomes was measured in the absence of added coenzyme A and was corrected for protein concentration in the reconstituted microsomes. [b] Followed by spin and resuspension.

To assess a possible mechanism for CoA inhibition of ACAT, the concentration dependence and kinetics of ACAT inhibition by CoA were next explored. Incubation of aortic microsomes with up to 200 μM CoA for 30 min resulted in concentration-dependent inhibition of ACAT activity up to 50 μM CoA, with a maximum of 60% inhibition of ACAT activity (data not shown). To determine whether CoA inhibition was occurring through competition with substrate, i.e., oleoyl-CoA binding, Lineweaver–Burk analysis of the inhibition of ACAT activity by coenzyme A was performed (Figure 4A). This analysis indicated an apparent noncompetitive mechanism for ACAT inhibition by CoA, with no alteration in the apparent K_m of 10 μM for oleoyl-CoA. The calculated

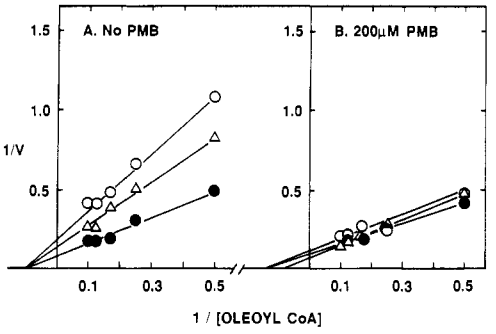

FIGURE 4: Aortic ACAT inhibition by coenzyme A: effect of PMB. Aortic microsomes (3.7 mg/mL) were incubated for 1 min at 22 °C without (A) or with (B) 200 μM PMB; then 20-μL aliquots were added to 0.43 mL of standard ACAT preincubation mixtures containing 0 (●), 10 (△), or 200 (○) μM coenzyme A and then incubated at 37 °C for 30 min. [^{14}C]Oleoyl-CoA (2–10 μM, 107 dpm/pmol) was then added to initiate ACAT assays, which were incubated for 30 min at 37 °C. ACAT activity was determined as picomoles of cholesteryl [^{14}C]oleate synthesized per minute per milligram of microsomal protein, and the reciprocal was plotted versus 1/[oleoyl-CoA]. The apparent K_m for oleoyl-CoA calculated from these data was 10 μM, and the apparent K_i for ACAT inhibition by coenzyme A was 20 μM.

apparent K_i for ACAT inhibition by coenzyme A was 20 μM when these data were used. Thus, ACAT inhibition by CoA occurs via a time-dependent, reversible, apparently noncompetitive mechanism.

A similar analysis of PMB-modified ACAT was also performed (Figure 4B) to characterize other potential alterations of ACAT activity produced by PMB modification. In this experiment, aortic microsomes were modified with 200 μM PMB for 2 min prior to ACAT preincubation and assayed in the presence of 0–200 μM CoA. Under these conditions, PMB modification did not inhibit ACAT activity but did abolish the concentration-dependent inhibition of ACAT by CoA (Figure 4B). Thus, incubation of PMB-modified microsomes in the presence of 10–200 μM coenzyme A for 30 min did not inhibit ACAT activity, whereas these concentrations of CoA resulted in 33% and 60% inhibition, respectively, of the unmodified enzyme. PMB modification and subsequent assay in the absence of CoA did not change either the apparent K_m of the enzyme for oleoyl-CoA (10 μM) or the calculated V_{max} (3.3 pmol min^{-1} mg^{-1}). Interestingly, PMB modification also did not alter the observed substrate inhibition of ACAT by oleoyl-CoA consistently observed at concentrations above 20 μM (data not shown). Thus, low-concentration PMB modification of aortic microsomes prevented concentration-dependent inhibition of ACAT by coenzyme A, without alteration of the apparent K_m for oleoyl-CoA, the calculated V_{max}, or substrate inhibition of ACAT by oleoyl-CoA.

Effect of CoA on PMB-Modified and Unmodified ACAT. Our results thus demonstrated that CoA inhibition of ACAT was likely not occurring by competition with substrate (oleoyl-CoA) for binding at the active site. PMB modification, however, both could prevent inhibitory CoA binding and could at higher concentrations inhibit cholesterol esterification by ACAT. Clarification that these two effects of PMB on ACAT activity were occurring through modification of two distinct sulfhydryl groups was obtained by examination of the effect of coenzyme A on unmodified and on PMB-modified ACAT (Figure 5). Thus, aortic microsomes were modified with 0, 100, or 200 μM PMB, followed by preincubation and measurement of ACAT activity with or without coenzyme A (100 μM).

In unmodified aortic microsomes, coenzyme A (100 μM) inhibited ACAT activity 50% compared to unmodified microsomes incubated without CoA, as previously observed. Modification of microsomes with 100 μM PMB prevented CoA inhibition of ACAT activity without itself inhibiting ACAT activity. Two hundred micromolar PMB inhibited ACAT activity 75% compared to unmodified ACAT, and CoA *reactivated* the PMB-modifiefd enzyme to full activity. These results indicate, first, that the two effects of PMB modification on ACAT have different concentration dependences since prevention of CoA inhibition occurred at a lower concentration than inhibition of cholesterol esterification. Second, two effects of CoA were seen: inhibition of unmodified ACAT and reactivation of PMB-inhibited ACAT, possibly through the action of CoA as a thiol reagent. Thus, CoA can reverse PMB modification at the ACAT sulfhydryl essential for cholesterol esterification, but not at its own inhibitory binding site. These two observations support the conclusion that two separate SH groups modifiable by PMB are responsible for the two observed effects of PMB on ACAT activity.

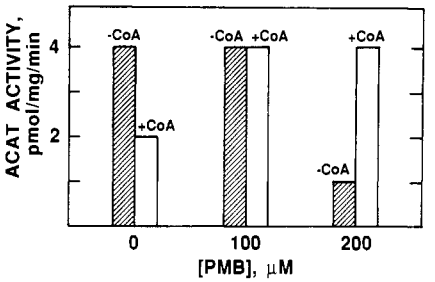

FIGURE 5: PMB inhibition of aortic ACAT: reversibility by coenzyme A. Normal aortic microsomes (2 mg/mL) were incubated with 0, 100, or 200 μM PMB for 1 min at 22 °C; then 20-μL aliquots were added to standard ACAT preincubation mixtures (0.43 mL) with or without 100 μM coenzyme A. After 30 min at 37 °C, ACAT assays were initiated by addition of [^{14}C]oleoyl-CoA (final concentration 10 μM, 107 dpm/pmol) and then incubated at 37 °C for 30 min. ACAT activity was measured as picomoles of cholesteryl [^{14}C]oleate synthesized per minute per milligram of protein after lipid extraction and TLC as described under Materials and Methods.

PMB Modification of Normal and Cholesterol-Fed Rabbit Aortic ACAT. Prior studies have demonstrated up to 50-fold increases in aortic ACAT activity in vivo during induction of experimental atherosclerosis. To quantitate the magnitude of CoA-mediated product inhibition on ACAT activity in aortic microsomes and to assess any potential changes in this inhibition or in the susceptibility of ACAT to PMB modification occurring during atherogenesis, we compared normal and cholesterol-fed aortic ACAT activities with respect to PMB modification and CoA inhibition. Normal and cholesterol-fed aortic ACAT had identical responses to CoA with respect to time and concentration dependence (not shown). In both cases, inhibition was maximal at CoA concentrations greater than 50 μM, resulting in approximately 60% inhibition of enzyme activity. Thus, CoA inhibition was not found to be significantly different between normal and cholesterol-fed aortic ACATs.

Normal and cholesterol-fed aortic ACATs were also identical with respect to the concentration dependence of PMB inhibition. For both, plots of ACAT inhibition vs [PMB] were linear, with 50% inhibition occurring at 400 μM PMB. The inhibition of both by PMB was also similarly time dependent, being virtually complete by 10 min. However, cholesterol-fed aortic ACAT was more sensitive to irreversible inhibition by PMB than normal aortic ACAT. For example, normal aortic ACAT was fully reactivated by 1.3 mM 2-mercaptoethanol after up to 30-min exposure to 500 μM PMB, but only 60% of cholesterol-fed aortic ACAT activity was restored by 2-mercaptoethanol (1.3 mM) after 10-min exposure to PMB.

Similarly, although normal aortic ACAT could be fully reactivated by 2-mercaptoethanol at all PMB concentrations up to 2 mM, cholesterol-fed aortic ACAT was reactivated to only 60% of control activity after modification with 500 μM PMB. Thus, normal and cholesterol-fed aortic ACATs were similarly susceptible to inhibition by PMB with respect to concentration and time dependence. However, the cholesterol-fed enzyme could only be partially reactivated by 2-mercaptoethanol after exposure to PMB for prolonged periods or at higher concentrations, possibly reflecting more rapid denaturation of the cholesterol-fed enzyme following sulfhydryl modification.

Discussion

In this report, we identify two modifiable sulfhydryl groups which separately modulate ACAT activity. First, there is a specific, inhibitory coenzyme A binding site. PMB modification of a sulfhydryl group at or near this site prevents ACAT inhibition by coenzyme A. Thiol reagents such as DTT reverse PMB modification at this site, and oleoyl-CoA affords protection against PMB modification, placing it at or near the active site of ACAT or at a site influenced by oleoyl-CoA-mediated conformational changes. A second, separate effect of PMB modification on ACAT activity is inhibition of cholesterol esterification, which requires higher concentrations of PMB and is also reversed by thiols and prevented by oleoyl-CoA, indicating a second modifiable sulfhydryl group at or near the active site of ACAT. Coenzyme A, presumably acting as a thiol, can reactivate PMB-inhibited ACAT but cannot reverse PMB modification at its own inhibitory binding site.

Although these sulfhydryl groups clearly influence ACAT activity, they have not been conclusively localized to the ACAT polypeptide itself. Thus, alterations in ACAT activity by CoA and PMB may be due to effects on essential ACAT cofactor(s) or other microsomal components which influence ACAT activity. Although the effects of oleoyl-CoA in protecting against PMB modification strongly suggest that PMB modification occurs at or near the active site of ACAT, definitive characterization of ACAT in this regard must await chemical modification studies of the active, homogeneous enzyme.

Coenzyme A inhibition of ACAT has not been previously reported. The apparent noncompetitive mechanism and relatively slow, time-dependent process of ACAT inhibition by CoA are most compatible with a conformational change in the enzyme, which is slow to occur, but fully and quickly reversible. The calculated K_i of 20 μM indicates tight binding of this reaction product. Since cytosolic CoA concentrations range from 14 to 60 μM (Robishaw & Neely, 1985), this reaction product may act as an intracellular regulator of ACAT activity. Inhibition of ACAT by CoA is also distinct from the previously described substrate inhibition of ACAT by fatty acyl-CoA (Chang & Doolittle, 1983), because the latter is not altered by PMB modification.

Although ACAT inhibition by free coenzyme A has not been previously reported, similar time-dependent, reversible inhibition by CoA has been described for HMG-CoA reductase, another key enzyme in intracellular cholesterol homeostasis. For example, Tan-Wilson and Kohlhaw (1978) demonstrated 80% inhibition of yeast HMG-CoA reductase by 100 μM CoA which required 10 min to occur and yet was reversible with dialysis. Additionally, inhibition of HMG-CoA reductase by oleoyl-CoA and by sulfhydryl-modifying reagents has been reported (Lippe et al., 1985; Durr & Rudney, 1960). These similarities between ACAT and HMG-CoA reductase may simply reflect similar mechanisms for substrate binding and/or product inhibition by free CoA or, alternatively, may reflect a major common mechanism for intracellular regulation of cholesterol metabolism. Both of these enzymes are inhibited by free CoA in concentration ranges found intracellularly, indicating that factors determining cellular CoA concentration may be critical for overall regulation of cellular cholesterol synthesis and esterification.

In addition to identifying a possible regulatory role for coenzyme A, the tight binding of CoA to ACAT emphasizes the importance of substrate purity when assaying ACAT activity in vitro. ACAT assays commonly employ commercially available fatty acyl-CoA, which we found to be substantially contaminated by free CoA. Even small amounts of contaminating CoA may introduce artifactual variations in measured ACAT activity. Thus, purification of commercially obtained fatty acyl-CoA by DEAE chromatography to remove inhibitory contaminants is mandatory prior to their use as ACAT substrates.

Normal aortic ACAT, cholesterol-fed aortic ACAT, and liver ACAT are all similarly sensitive to inhibition by PMB, with apparent K_i's of 400–600 μM. Cholesterol-fed aortic ACAT is more susceptible than normal aortic ACAT to irreversible denaturation after exposure for long periods or to high PMB concentrations, perhaps reflecting differences in protein stability or membrane compositional differences. However, all three are alike with respect to inhibition by coenzyme A, indicating that their active-site or regulatory-site configurations may be similar in this respect. Notably, on direct measurement, normal and cholesterol-fed aortic microsomes behaved similarly with respect to CoA and PMB. Thus, changes in CoA binding likely are not sufficient to account for the marked changes in ACAT activity occurring during atherogenesis.

In conclusion, our chemical modification studies have identified histidyl and sulfhydryl residues necessary for ACAT activity and have delineated unique properties of each. A histidine, likely located at the active site, manifests markedly different reactivities in aortic and liver ACATs, thus identifying two subtypes of ACAT activity (Kinnunen et al., 1988). Further, two types of sulfhydryl groups affecting ACAT activity were identified: one involved in inhibitory coenzyme A binding and the other essential for cholesterol esterification by ACAT. Although the direct involvement of these residues in catalysis of cholesteryl ester formation by ACAT has not been definitively proved, their properties suggest similarities to LCAT and HMG-CoA reductase, indicating possible common catalytic and/or regulatory features. These specific properties of ACAT may additionally be exploited in differential labeling experiments to provide necessary information leading to identification of the ACAT polypeptide.

Acknowledgments

We thank Dr. Philip Majerus for advice regarding fatty acyl-CoA synthesis and Helen Nikolaisen for preparation of the manuscript.

References

Bishop, J. E., & Hajra, A. K. (1980) *Anal. Biochem. 106*, 344–350.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Chang, T. Y., & Doolittle, G. M. (1983) *Enzymes (3rd Ed.) 16*, 523–539.

Durr, I. F., & Rudney, H. (1960) *J. Biol. Chem. 235*, 2572–2578.

Kinnunen, P. M., DeMichele, A., & Lange, L. G. (1988)

Biochemistry (preceding paper in this issue).
Kritchevsky, D., & Kothari, H. V. (1978) *Adv. Lipid Res. 16*, 221–266.
Lange, L. G., Bergmann, S. R., & Sobel, B. E. (1981) *J. Biol. Chem. 256*, 12968–12973.
Lippe, G., Deana, R., Cavallini, L., & Galzigna, L. (1985) *Biochem. Pharmacol. 34*, 3293–3297.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Robishaw, J. D., & Neely, J. R. (1985) *Am. J. Physiol. 248*, E1–E9.
Sgoutas, D. S. (1970) *Biochemistry 9*, 1826–1833.
Spector, A. A., Mathur, S. N., & Kaduce, T. L. (1979) *Prog. Lipid Res. 18*, 31–53.
Steinman, H. M., & Hill, R. L. (1973) *J. Biol. Chem. 248*, 892–900.
Tan-Wilson, A., & Kohlhaw, G. B. (1978) *Biochem. Biophys. Res. Commun. 85*, 70–76.

Identification of Aspartate-184 as an Essential Residue in the Catalytic Subunit of cAMP-Dependent Protein Kinase†

Joseph A. Buechler‡ and Susan S. Taylor*,§
Department of Chemistry, University of California, San Diego, La Jolla, California 92093
Received February 22, 1988; Revised Manuscript Received May 18, 1988

ABSTRACT: The hydrophobic carbodiimide dicyclohexylcarbodiimide (DCCD) was previously shown to be an irreversible inhibitor of the catalytic subunit of cAMP-dependent protein kinase, and MgATP protected against inactivation [Toner-Webb, J., & Taylor, S. S. (1987) *Biochemistry 26*, 7371]. This inhibition by DCCD indicated that an essential carboxyl group was present at the active site of the enzyme even though identification of that carboxyl group was not possible. This presumably was because a nucleophile on the protein cross-linked to the electrophilic intermediate formed when the carbodiimide reacted with the carboxyl group. To circumvent this problem, the catalytic subunit first was treated with acetic anhydride to block accessible lysine residues, thus preventing intramolecular cross-linking. The DCCD reaction then was carried out in the presence of [^{14}C]glycine ethyl ester in order to trap any electrophilic intermediates that were generated by DCCD. The modified protein was treated with trypsin, and the resulting peptides were separated by HPLC. Two major radioactive peptides were isolated as well as one minor peptide. MgATP protected all three peptides from covalent modification. The two major peaks contained the same modified carboxyl group, which corresponded to Asp-184. The minor peak contained a modified glutamic acid, Glu-91. Both of these acidic residues are conserved in all protein kinases, which is consistent with their playing essential roles. The positions of Asp-184 and Glu-91 have been correlated with the overall domain structure of the molecule. Asp-184 may participate as a general base catalyst at the active site. A third carboxyl group, Glu-230, also was identified. This carboxyl group was protected by an inhibitor peptide and MgATP, but not by MgATP alone.

Protein kinases represent a very large and diverse family of enzymes (Krebs, 1985). In spite of this diversity, these enzymes share a homologous catalytic core, implying not only that they have evolved from a common precursor but also that they share many conserved features of secondary and tertiary structure (Hunter, 1987; Taylor et al., 1988). The catalytic (C) subunit of cAMP-dependent protein kinase represents one of the simplest of the protein kinases primarily because the major regulatory elements are part of a distinct regulatory subunit, which dissociates in the presence of the activating ligand cAMP. The C subunit also is one of the best understood protein kinases biochemically. For example, many features of the active site have been defined for the C subunit. Lysine-72 has been shown by affinity labeling to be an essential component of the MgATP binding site (Zoller et al., 1981; Zoller & Taylor, 1979), and Cys-199 has been shown to be in close proximity to the β- and/or γ-phosphates of ATP (Bhatnagar et al., 1984). Cys-199 also was targeted by affinity labeling with a substrate analogue (Bramson et al., 1982).

In addition to affinity labeling, group-specific labeling has proven to be a successful approach for identifying specific functional residues. In the case of the C subunit, there are several potential roles that might be played by carboxyl groups. For example, several lines of evidence suggest that the mechanism utilized by the C subunit involves a general base catalyst (Yoon & Cook, 1987; Bramson et al., 1984). In addition, carboxyl groups are frequently associated with metal binding sites in other kinases that act on small molecules. Thus, we chose to explore the effects of the hydrophobic carbodiimide dicyclohexylcarbodiimide (DCCD) on the C subunit. The particular intention for choosing a hydrophobic reagent was to identify carboxyl groups that may lie in close proximity to a hyrophobic environment such as the ATP binding site.

DCCD previously was found to be a very good inhibitor of the C subunit. Although a unique peptide was consistently modified as a consequence of treatment with DCCD, it was not possible to identify a specific carboxyl group that was modified by DCCD. The conclusion from those initial studies

† This research was supported in part by U.S. Public Health Service Grant GM 19301.
‡ Supported by USPHS Training Grant AM07233.
§ Member of the Center for Molecular Genetics, University of California, San Diego.

0006-2960/88/0427-7356$01.50/0