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12-[(5-Iodo-4-azido-2-hydroxybenzoyl)amino]dodecanoic Acid: Biological Recognition by Cholesterol Esterase and Acyl-CoA:Cholesterol *O*-Acyltransferase†

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ABSTRACT: Potential probes of protein cholesterol and fatty acid binding sites, namely, 12-[(5-iodo-4-azido-2-hydroxybenzoyl)amino]dodecanoate (IFA) and its coenzyme A (IFA:CoA) and cholesteryl (IFA:CEA) esters, were synthesized. These radioactive, photoreactive lipid analogues were recognized as substrates and inhibitors of acyl-CoA:cholesterol *O*-acyltransferase (ACAT) and cholesterol esterase, neutral lipid binding enzymes which are key elements in the regulation of cellular cholesterol metabolism. In the dark, IFA reversibly inhibited cholesteryl [^{14}C]oleate hydrolysis by purified bovine pancreatic cholesterol esterase with an apparent K_i of 150 μM. Cholesterol esterase inhibition by IFA became irreversible after photolysis with UV light and oleic acid (1 mM) provided 50% protection against inactivation. Incubation of homogeneous bovine pancreatic cholesterol esterase with IFA:CEA resulted in its hydrolysis to IFA and cholesterol, indicating recognition of IFA:CEA as a substrate by cholesterol esterase. The coenzyme A ester, IFA:CoA, was a reversible inhibitor of microsomal ACAT activity under dark conditions (apparent K_i = 20 μM), and photolysis resulted in irreversible inhibition of enzyme activity with 87% efficiency. IFA:CoA was also recognized as a substrate by both liver and aortic microsomal ACATs, with resultant synthesis of ^{125}IFA:CEA. IFA and its derivatives, IFA:CEA and IFA:CoA, are thus inhibitors and substrates for cholesterol esterase and ACAT. Biological recognition of these photoaffinity lipid analogues will facilitate the identification and structural analysis of hitherto uncharacterized protein lipid binding sites.

Photoaffinity labeling using photoreactive lipid analogues has been of unique value in the structural study of hydrophobic proteins in their native states associated with the lipid bilayer. Lipid-soluble ligands have been used successfully for the identification and characterization of integral membrane proteins and receptors, many of which are easily denatured upon separation from cell membranes. For example, photoreactive phospholipid analogues incorporated into the surrounding membrane have been used to identify the transmembrane domains of glycophorin A and the membrane-anchoring groups of cytochrome b_5 (Ross et al., 1982; Takagaki et al., 1983).

Intracellular cholesterol metabolism involves many such highly hydrophobic, neutral lipid binding enzymes. For example, acyl-CoA:cholesterol *O*-acyltransferase (ACAT) catalyzes the intracellular synthesis of cholesteryl esters in vascular tissue, and large increases in ACAT activity are likely

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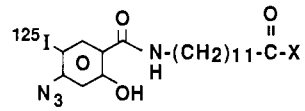

Compound	X
^{125}IFA	- OH
^{125}IFA:CEA	- O - Cholesterol
^{125}IFA:CoA	- S - CoA

FIGURE 1: Structure of 12-[(5-iodo-4-azido-2-hydroxybenzoyl)-amino]dodecanoate and derivatives.

responsible for cholesteryl ester accumulation in vascular tissue during atherogenesis (Kinnunen et al., 1988). However, ACAT has never been identified or characterized in detail, chiefly because it is tightly membrane bound and irreversibly denatured during purification attempts using detergents or organic solvents. Identification of this critical enzyme is essential prior to elucidation of the mechanism for its marked induction during atherogenesis and represents one potential application for lipid analogue photoaffinity labels.

A second important element of intracellular cholesterol metabolism is cholesterol esterase, which hydrolyzes intracellular cholesterol esters and thus together with ACAT constitutes an intracellular cholesterol ester cycle (Brown & Goldstein, 1975). This enzyme, although soluble, utilizes membrane-associated substrates and contains hydrophobic cholesterol and fatty acid binding sites. Localization as well as characterization of the structural features of these lipid binding sites has been lacking but could potentially be accomplished by using appropriate photoreactive lipid analogues.

As part of a strategy to understand the specific structure–function relationships of these cholesterol and fatty acid binding enzymes, and to develop a general methodology for the study of neutral lipid binding sites, we synthesized a unique photoaffinity analogue of a long-chain fatty acid, namely, 12-[(5-iodo-4-azido-2-hydroxybenzoyl)amino]dodecanoate (IFA, Figure 1). This compound contains an azido group attached to the 4-position of a phenyl ring and can be radioiodinated to high specific activity at the 5-position. This work describes the synthesis of this compound and its cholesteryl and coenzyme A ester derivatives and demonstrates that they are biologically recognized as inhibitors and substrate analogues for homogeneous bovine pancreatic cholesterol esterase and microsomal acyl-CoA:cholesterol *O*-acyltransferase (ACAT) at micromolar concentrations.

Materials and Methods

Reagents. All solvents used were of the highest commercially available grade and were used without further purification. Oxalyl chloride, *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, 14% boron trifluoride in methanol, coenzyme A, *p*-aminobenzoic acid, 12-aminododecanoic acid, dithiothreitol, ATP[1] (disodium salt), and sodium azide were purchased from Sigma. Fatty acid poor bovine serum albumin was obtained from Calbiochem. [1-^{14}C]Oleic acid (57 mCi/mmol) and Na^{125}I (carrier free) were purchased from Amersham. Chloramine T was obtained from Aldrich and silica gel 60 from EM Science.

Synthesis of [^{14}C]Oleoyl-CoA. [^{14}C]Oleoyl-CoA was synthesized via the acid chloride of [^{14}C]oleic acid as previously described (Bishop & Hajra, 1980) and then purified by column chromatography over DEAE-cellulose (Steinman & Hill, 1973).

Thin-Layer Chromatography. Thin-layer chromatography was performed by using glass plates coated with silica gel O (Analabs) developed at room temperature. Lipids were identified by exposure to iodine vapor or by exposure of parallel standards to iodine vapor when eluted lipids were to be used in subsequent reactions. Photoreactivity of lipids after TLC was assessed by direct exposure of the plate to UV light (Blak-Ray lamp) for 5 min.

Synthesis of 12-[(5-Iodo-4-azido-2-hydroxybenzoyl)-amino]dodecanoate (IFA). Unless otherwise described, all manipulations during synthesis of this photoreactive fatty acid analogue and its derivatives were performed under dark conditions. All reaction vessels were covered with aluminum foil, and products were stored in opaque or foil-covered vials.

(A) Synthesis of p-Azidosalicylic Acid. *p*-Azidosalicylic acid was synthesized by diazotization of *p*-aminosalicylic acid (Vanin & Ji, 1981). For example, 4 g of *p*-aminosalicylic acid was dissolved in 50 mL of 4 N H_2SO_4 on ice, and then 1.8 g of $NaNO_2$ in a minimal volume of water was added dropwise with stirring. After stirring for 10 min on ice, 1.8 g of sodium azide dissolved in a minimal volume of water was added dropwise with continued mixing. The reaction was allowed to stand on ice for 1 h and then extracted with ethyl acetate (200 mL) three times. The organic phase was then back-extracted with water and dried over anhydrous sodium sulfate, and the solvent was evaporated under vacuum. The reaction product was purified by chromatography over silica gel 60 (2.5 × 30 cm column eluted with 5/1 v/v chloroform/methanol) and then recrystallized from ethyl acetate. The reaction product was a photoreactive compound that migrated with an R_f of 0.5 in chloroform/methanol, 5/1, whereas the R_f of *p*-aminosalicylic acid, a nonphotoreactive lipid, was 0.2 in this solvent system. An IR spectrum of the reaction product (Nujol mull) showed absorption bands at 2100 and 1650 cm^{-1}, consistent with the presence of azide and carboxyl moieties (Kates, 1972). Chemical ionization mass spectroscopy of this product demonstrated a prominent peak at an *m/e* signal of 180, corresponding to the expected parent ion peak (M + H$^+$) of *p*-azidosalicylic acid ($C_7H_5O_3N_3$). Reaction yield was 30%.

(B) Synthesis of 12-[(4-Azido-2-hydroxybenzoyl)amino]-dodecanoic Acid. 12-[(4-Azido-2-hydroxybenzoyl)amino]-dodecanoic acid was synthesized from the *N*-hydroxysuccinimide ester of *p*-azidosalicylic acid. For example, dicyclohexylcarbodiimide (3.1 g) and *N*-hydroxysuccinimide (1.7 g) were added to 2.6 g of *p*-azidosalicylic acid dissolved in 20 mL of dry THF. The reaction was stirred overnight at room temperature and then filtered, and dry pyridine (2.4 mL) and 12-aminododecanoic acid (3.2 g) were added. After stirring at room temperature for 72 h, the mixture was filtered, concentrated HCl (2 mL) was added, and the mixture was evaporated to dryness under vacuum. Ethyl acetate (200 mL) was added, and the reaction mixture was extracted with pH 2 water. The organic phase was dried over sodium sulfate and then evaporated, and the reaction product was isolated by recrystallization from ethyl acetate/petroleum ether, 2/1, and further purified by column chromatography over silica gel (2.5 × 75 cm column eluted with 10/1 chloroform/methanol). The purified product was a photoreactive compound that migrated with an R_f of 0.6 on TLC in chloroform/methanol, 5/1. It migrated as a single spot on TLC in multiple solvent systems, and a chemical ionization mass spectrum indicated a peak at an *m/e* ratio of 377, corresponding to the parent ion (M + H$^+$) peak of the expected product, $C_{19}H_{28}O_4N_4$.

During several preparations the reaction product was found to be contaminated with another photoreactive lipid that closely

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine triphosphate; BSA, bovine serum albumin; DTT, dithiothreitol.

comigrated with the reaction product on TLC in multiple solvent systems. To identify this contaminant, mass spectroscopy was performed on the methyl ester of both the reaction product and the contaminant. The methyl ester of the desired reaction product, which migrated more slowly on TLC in petroleum ether/diethyl ether/acetic acid, 97/52/3, had a parent ion peak at an m/e ratio of 391, corresponding to (M + H^+) of methyl 12-[(4-azido-2-hydroxybenzoyl)amino]-dodecanoate ($C_{20}H_{30}O_4N_4$). Chemical ionization mass spectroscopy of the contaminant, which was faster migrating on TLC, showed a parent ion peak at an m/e ratio of 405 and contained sequential peaks 14 units apart consistent with fragmentation of a fatty acid moiety, indicating that this contaminant was likely methyl 12-[(4-azido-2-hydroxybenzoyl)amino]tridecanoate, probably due to contamination of the starting material 12-aminododecanoate with its 13-carbon analogue, 13-aminotridecanoic acid. When present, this contaminant was removed by column chromatography over silicic acid (2.5 × 75 cm column eluted with chloroform/methanol, 40/1).

(C) Iodination of 12-[(4-Azido-2-hydroxybenzoyl)-amino]dodecanoic Acid. Iodination of 12-[(4-azido-2-hydroxybenzoyl)amino]dodecanoic acid was performed by using chloramine T (Ji & Ji, 1982). For example, 2.5 mg of fatty acid was dissolved in 300 μL of ethyl acetate/methanol (2/1 v/v). $Na^{125}I$ in 0.1 M NaOH and potassium iodide was then added to the desired specific radioactivity, followed by addition of 60 μL of chloramine T (10 mg/mL) in acetone. After incubation at room temperature for 2 h, the mixture was applied to a thin-layer chromatography plate which was developed with chloroform/methanol, 10/1. The photoreactive product (R_f 0.7 in this solvent system) was identified by exposure of a parallel standard to UV light. The area of the plate containing the product was then scraped, eluted with 2/1 chloroform/methanol, and stored in ethyl acetate at −20 °C until used. Yield after iodination was reproducibly greater than 90%.

Synthesis of 12-[(5-Iodo-4-azido-2-hydroxybenzoyl)-amino]dodecanoyl-CoA. Synthesis of the coenzyme A ester of this radioiodinated, photoreactive fatty acid was performed according to the method of Bishop and Hajra (1980). For example, 2.5 mg of IFA was incubated with 2 mL of oxalyl chloride under nitrogen for 2 h. The oxalyl chloride was then evaporated with N_2, 50 mg of coenzyme A dissolved in 3 mL of 2.2/1.0 tetrahydrofuran/150 mM sodium bicarbonate, pH 8.8, was added, and the mixture was incubated at room temperature for 1 h. The tetrahydrofuran was then evaporated with nitrogen, and the synthesized fatty acyl CoA was precipitated by cooling to 4 °C and addition of 300 μL of cold 10% perchloric acid. After removal of unreacted fatty acid by extraction with cold diethyl ether, the precipitate was pelleted by centrifugation at 40000g for 20 min, washed with cold 10% perchloric acid and acetone, and redissolved in 50 mM potassium phosphate, pH 6. The concentration of synthesized fatty acyl CoA was determined by assay with Ellman's reagent (Ellman, 1959) after hydrolysis with 0.1 N NaOH at 37 °C for 60 min, with coenzyme A as a standard.

Synthesis of Cholesteryl 12-[(5-Iodo-4-azido-2-hydroxybenzoyl)amino]dodecanoate (IFA:CEA). The acid chloride of IFA was first prepared by incubating IFA (3 mg) with oxalyl chloride (2 mL) for 2 h at room temperature under nitrogen. Excess oxalyl chloride was then removed by evaporation, followed by addition of cholesterol (20 mg) in dry ethyl acetate/pyridine, 10/1. The reaction was incubated at 22 °C overnight and then sequentially extracted with 1 N NaOH, 1 N HCl, and water. Thin-layer chromatography (petroleum ether/diethyl ether/acetic acid, 97/52/3) indicated the appearance of a new photoreactive, radioactive lipid which migrated near the solvent front (R_f of 0.9 compared to the R_fs of IFA and cholesterol, which were 0.2 and 0.4, respectively). Treatment of this product with base (0.1 N NaOH, 37 °C, 1 h), followed by TLC, resulted in the reappearance of the starting materials, as indicated by the reappearance of a photoreactive lipid with an R_f of 0.2 and a nonphotoreactive lipid with an R_f of 0.4. The cholesteryl ester was purified by preparative TLC in the same solvent system and stored in 2/1 chloroform/methanol at −20 °C. Chemical ionization mass spectroscopy was that of a typical cholesteryl ester with a peak at an m/e ratio of 369, consistent with the presence of a sterol skeleton (Wakeham & Frew, 1982). Yield was 4 mg of purified IFA:CEA (20% yield).

ACAT Assays. Microsomes were prepared from normal and cholesterol-fed rabbit aorta and liver by differential centrifugation as previously described (Kinnunen et al., 1988) and stored at −70 °C until used. ACAT assays included 0.8 mg/mL BSA, 5 mM ATP, 5 mM $MgCl_2$, 0.1 mM CoA, 1.3 mM DTT, 0–200 μg of microsomal protein, and 10 μM [^{14}C]oleoyl CoA (100 dpm/pmol) in a total volume of 0.5 mL of 0.1 M potassium phosphate, pH 7.4 (Brown et al., 1975). Assays were incubated for 30 min at 37 °C and then quenched and lipids extracted according to the method of Bligh and Dyer (1959), to which 1 μmol of cholesteryl oleate and 1 μmol of ethyl oleate (20000 dpm/μmol) were added as cold carrier and yield marker, respectively. Lipids were separated by TLC in petroleum ether/diethyl ether/acetic acid, 75/5/1, identified by exposure to iodine vapor which was removed by gentle heating, then scraped into scintillation vials, and counted with 10 mL of Aquasol (New England Nuclear). Yield was monitored by recovery of [^{3}H]ethyl oleate, and ACAT activity was calculated as picomoles of [^{14}C]cholesteryl oleate synthesized per minute per milligram of microsomal protein.

Vesicle Preparation for Cholesterol Esterase Assay. Vesicles were prepared by sonication for 10 min under nitrogen at 4 °C using a Branson probe tip sonicator, followed by centrifugation at 100000g for 60 min to isolate unilamellar vesicles (Haley et al., 1980). Vesicles used to assay cholesteryl oleate hydrolysis contained cholesteryl [^{14}C]oleate (127 μM, 7 dpm/pmol) and egg phosphatidylcholine (13 mM) in 10 mM Tris-HCl, pH 7.

Cholesterol Esterase Assay. Bovine pancreatic cholesterol esterase (MW 67000) was purchased from Sigma and purified to homogeneity by heparin affinity chromatography before use (Cox et al., 1990). Standard cholesterol esterase assays included 0–10 μg of cholesterol esterase, cholesteryl [^{14}C]-oleate–phosphatidylcholine vesicles (final cholesteryl [^{14}C]-oleate concentration 32 μM, 7 dpm/pmol), and 8 mM sodium taurocholate in a final volume of 300 μL of 150 mM Tris, pH 7.2. Assays were incubated at 37 °C for 10 min and then quenched and extracted by addition of 3 mL of benzene/chloroform/methanol, 1/0.5/1.2, and 0.6 mL of 0.3 M NaOH (Belfrage & Vaughn, 1969). Cleavage of cholesteryl [^{14}C]-oleate was measured by quantitating [^{14}C]oleate released and extracted into the alkaline aqueous phase, determined by liquid scintillation counting of an aliquot of the aqueous phase. Background was determined in blank assays containing no enzyme. Efficiency of extraction was determined by extracting vesicles containing a known quantity of [^{14}C]oleic acid.

Photolysis. Photolysis was performed for 5–10-min periods using a hand-held UV lamp (Blak-Ray). Samples to be photolyzed were placed in borosilicate glass test tubes 0.5 cm

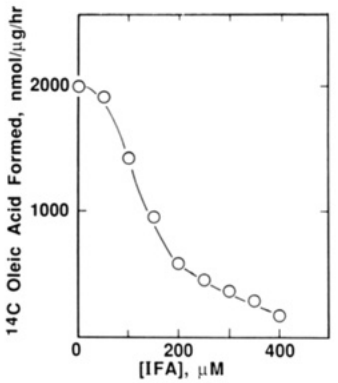

FIGURE 2: Nonphotolytic inhibition of cholesterol esterase by IFA. Homogeneous bovine pancreatic cholesterol esterase (0.02 μg) was incubated in the dark with 32 μM cholesteryl [^{14}C]oleate (7 dpm/pmol) incorporated into phosphatidylcholine vesicles, 8 mM sodium taurocholate, and 0–400 μM IFA in a total volume of 300 μL of 150 mM Tris, pH 7.2. Assays were incubated at 37 °C for 10 min in the dark and quenched with an alkaline extraction medium, and cholesterol esterase activity was determined as described under Materials and Methods. Results shown represent the average of duplicate assays, which varied less than 10%.

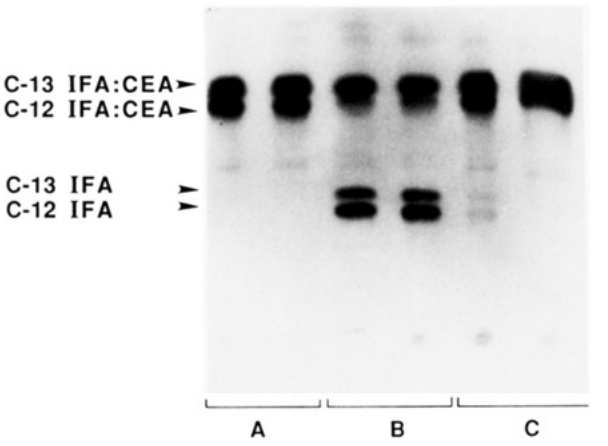

FIGURE 3: Hydrolysis of IFA:CEA by pancreatic cholesterol esterase. Purified bovine pancreatic cholesterol esterase (200 μg) was incubated in the dark in a total volume of 0.3 mL of 10 mM Tris, pH 7.4, containing 7 μM ^{125}IFA:CEA (2000 dpm/pmol) incorporated into phosphatidylcholine vesicles (1.1 mM PC in assay) and 2 mM sodium taurocholate (B). Parallel incubations were performed containing no enzyme (A) and enzyme boiled for 5 min prior to incubation (C). After incubation at 37 °C for 2 h, the reactions were quenched and lipids extracted with a Bligh and Dyer extraction. Extracted lipids were separated by TLC in petroleum ether/diethyl ether/acetic acid, 90/52/3. An autoradiogram of the TLC plate is shown, with the positions of C-12 and C-13 IFA and IFA:CEA standards indicated.

from the light source and agitated gently during UV exposure.

Infrared Spectroscopy. IR spectra were determined in carbon tetrachloride or Nujol mull on a Perkin-Elmer 237b grating IR spectrometer.

Mass Spectroscopy. Direct chemical ionization mass spectroscopy (methane) was performed by using a Finnigan 3300 mass spectrometer at the Research Resource for Mass Spectroscopy at Washington University School of Medicine.

RESULTS

To assess the utility of this photoreactive, radioiodinated fatty acid analogue and its derivatives in the study of cellular cholesterol and fatty acid metabolism, we examined whether these fatty acid derivatives were biologically recognized by enzymes involved in these metabolic pathways. We employed bovine pancreatic cholesterol esterase (MW 67 000), purified to homogeneity by heparin–agarose affinity chromatography (Cox et al., 1990), and liver and aortic microsomal ACAT activities as representative of the two classes of this transferase (Kinnunen et al., 1988).

Interactions of IFA and CEA with Bovine Pancreatic Cholesterol Esterase. (A) As an Inhibitor. We have previously found that oleic acid inhibits aortic cholesterol esterase hydrolytic activity with an apparent K_i of 50 μM (data not shown). Thus, we first determined whether IFA inhibited aortic cholesterol esterase under dark conditions by incubating homogeneous bovine pancreatic cholesterol esterase with cholesteryl [^{14}C]oleate (32 μM) incorporated into phospholipid vesicles in the presence of varying concentrations (0–400 μM) of IFA. Cholesteryl oleate hydrolysis was inhibited in a concentration-dependent manner by IFA with an apparent K_i of 150 μM (Figure 2). In similar experiments using rabbit aortic high-speed supernatant, concentration-dependent inhibition of acid cholesterol esterase activity was also seen, with a calculated K_i of 100 μM (data not shown).

To determine whether photolytic activation of IFA led to the covalent modification of cholesterol esterase, a mixture of bovine pancreatic cholesterol esterase (15 μg/mL) and IFA (400 μM) at pH 7.2 was photolyzed for 10 min and diluted 20-fold, and 25-μL aliquots were added to standard cholesterol esterase assays. After photolysis and dilution, cholesterol esterase activity was 90% inhibited compared to control enzyme. In contrast, there was no inhibition of cholesterol esterase activity in a parallel sample incubated with IFA but not photolyzed prior to dilution. Enzyme activity was also not inhibited by photolysis in the absence of IFA. Thus, inhibition of cholesterol esterase by IFA was fully reversible under dark conditions but irreversible after photolysis. Additionally, inclusion of oleic acid (0–2 mM) in the photolysis mixture reduced the amount of photolytic inhibition by IFA in a concentration-dependent manner. Photolytic inhibition by IFA (400 μM) was reduced from 90% (in the absence of oleic acid) to 50% after photolysis in the presence of 1 mM oleic acid and to 15% in the presence of 2 mM oleic acid. These results suggested the presence of an inhibitory fatty acid binding site on cholesterol esterase which reversibly binds both oleic acid and IFA under dark conditions and can be covalently modified by photolysis in the presence of IFA.

(B) As a Substrate. To further assess the specific biological recognition of IFA as a fatty acid analogue by cholesterol esterase, the cholesteryl ester derivative of IFA, IFA:CEA, was tested as a substrate. Thus, ^{125}IFA:CEA (7 μM) incorporated into phosphatidylcholine vesicles was incubated with bovine pancreatic cholesterol esterase at pH 7.4, followed by lipid extraction and thin-layer chromatography. Autoradiography of the TLC plate indicated the appearance of IFA (Figure 3, C-12 IFA), whereas parallel incubations containing no enzyme or boiled enzyme showed no hydrolysis of IFA:CEA. Thus, IFA:CEA was recognized as a substrate by bovine pancreatic cholesterol esterase, implying that this cholesteryl ester analogue binds to the enzyme active site.

Also present in these incubations was cholesteryl 13-[(5-iodo-4-azido-2-hydroxybenzoyl)amino]tridecanoate (C-13 IFA:CEA; see Materials and Methods), the cholesteryl ester of a 13-carbon contaminant of IFA. This compound was also recognized as a substrate by purified pancreatic cholesterol esterase, as indicated by the appearance of the corresponding fatty acid (C-13 IFA) in the incubations containing active enzyme. However, utilization of the 12-carbon IFA:CEA under these conditions appeared more efficient than utilization of the 13-carbon IFA:CEA, as indicated by the relative intensities of the C-12 and C-13 IFA spots. Thus, both IFA:CEA and cholesteryl 13-[(5-iodo-4-azido-2-hydroxy-

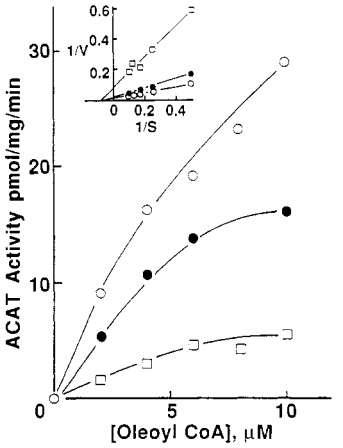

FIGURE 4: Aortic ACAT inhibition by IFA:CoA. Standard ACAT assay mixtures containing aortic microsomes (100 μg of protein) prepared from rabbits fed 2% cholesterol chow for 8 weeks were preincubated in the dark at 37 °C for 30 min, followed by the addition of 0 (○), 4 (●), or 8 (□) μM (final concentration) ^{127}IFA:CoA and 0–10 μM [^{14}C]oleoyl-CoA (107 dpm/pmol). Assays were then incubated for 30 min at 37 °C in the dark and quenched, and lipids were extracted and separated by TLC as described under Materials and Methods. ACAT activity is expressed as pmol of cholesteryl [^{14}C]oleate formed/(min·mg of microsomal protein).

benzoyl)amino]tridecanoate are recognized as substrates by purified pancreatic cholesterol esterase.

Interactions of IFA:CoA with Acyl-CoA:Cholesterol O-Acyltransferase (ACAT). (*A*) *As an Inhibitor.* To aid in the identification of ACAT, a tightly membrane-bound transferase catalyzing the intracellular synthesis of cholesteryl esters, we synthesized the coenzyme A ester of IFA for use as a photoaffinity label. To assess its biological recognition by ACAT as a long-chain fatty acyl CoA, we tested IFA:CoA as a potential inhibitor of aortic microsomal ACAT activity. Thus, IFA:CoA (0–8 μM) was added to standard ACAT assays containing [^{14}C]oleoyl-CoA (0–10 μM) under dark conditions, which resulted in the concentration-dependent inhibition of ACAT activity in cholesterol-fed rabbit aortic microsomes (Figure 4). Lineweaver-Burk analysis of cholesterol-fed aortic ACAT inhibition by IFA:CoA showed a common x-axis intercept with a calculated K_i of 20 μM (Figure 4, insert). Identical results were found for normal rabbit aortic microsomes (data not shown). Thus, IFA:CoA inhibited both normal and cholesterol-fed aortic microsomal ACAT activities under dark conditions, with an apparent K_i of 20 μM.

Similarly to cholesterol esterase inhibition by IFA, ACAT inhibition by IFA:CoA was reversible under dark conditions but became irreversible after photolysis with UV light (Figure 5A). Addition of 40 μM IFA:CoA to a standard ACAT assay under dark conditions inhibited aortic microsomal ACAT activity more than 90% compared to control ACAT activity measured in the absence of inhibitor. However, ACAT activity in the same microsomes after 25-fold dilution was only 10% inhibited compared to control, indicating that ACAT inhibition by IFA:CoA under dark conditions was fully reversible by dilution. In contrast, photolysis of the reaction mixture containing IFA:CoA led to recovery of only 10% of ACAT activity after dilution, indicating irreversible inhibition of ACAT activity. These results are consistent with photolysis-dependent, covalent modification of ACAT by IFA:CoA.

Determination of the concentration dependence of photolytic inhibition of ACAT by IFA:CoA was next performed (Figure 5B). Cholesterol-fed aortic microsomes were photolyzed with IFA:CoA (0–60 μM) and then diluted 20-fold into standard ACAT assay mixtures. Compared to unphotolyzed samples, there was concentration-dependent ACAT inhibition by IFA:CoA up to 15 μM, with little further increase in inhibition above this concentration. Maximum ACAT inhibition was 87% after photolysis of microsomes with 60 μM IFA:CoA. The irreversible photolytic inhibition of ACAT by IFA:CoA was thus concentration dependent and efficient.

(*B*) *As a Substrate.* To confirm that IFA:CoA was binding to the active site of ACAT rather than to an essential cofactor or nonspecifically to other membrane components, its recognition as a substrate was assessed. Liver microsomes, used as a source of ACAT activity, were incubated with 20 μM IFA:CoA under dark conditions for 0, 1, or 2 h. After lipid extraction and thin-layer chromatography, autoradiography of the TLC plate demonstrated the time-dependent synthesis of an ^{125}I-labeled lipid with an R_f of 0.48 (Figure 6). This lipid was photoreactive and comigrated with the chemically synthesized cholesteryl ester of IFA, IFA:CEA. Chemical ionization mass spectroscopy demonstrated a peak at m/e = 369, consistent with a sterol nucleus derived from a cholesteryl ester (Wakeham & Frew, 1982) and identical with that ob-

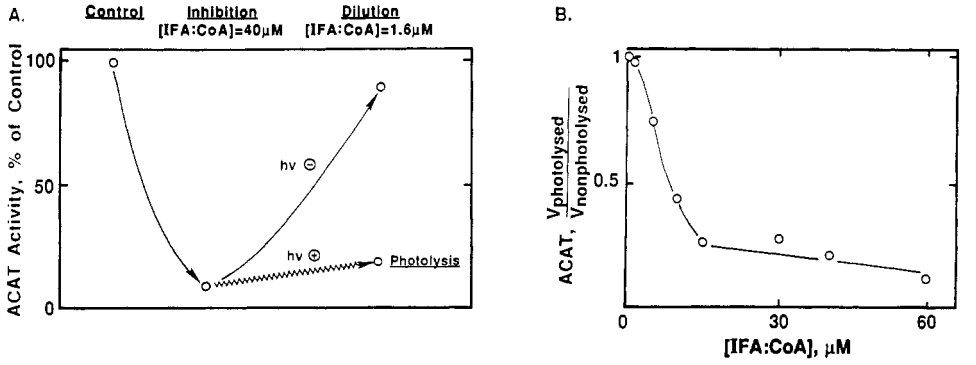

FIGURE 5: Irreversible ACAT inhibition by IFA:CoA after photolysis. (A) Aortic microsomes (3.8 mg/mL) prepared from rabbits fed 2% cholesterol chow for 30 days were incubated at 4 °C for 1 min with 40 μM ^{127}IFA:CoA and then at room temperature for 5 min and diluted 25-fold into a standard ACAT assay. A parallel incubation mixture was photolyzed for 5 min with UV light and then diluted into a standard ACAT assay. Control activity was measured in microsomes photolyzed for 5 min in the absence of IFA:CoA and then diluted into ACAT assays. All assays and extractions were performed under dark conditions. Each point represents the mean of duplicate assays, which varied less than 10%. (B) Concentration dependence. Aortic microsomes (2 mg/mL) prepared from rabbits fed 2% cholesterol chow for 30 days were incubated at 4 °C for 1 min with 0–60 μM ^{127}IFA:CoA and then photolyzed with UV light for 5 min at a path length of 0.5 cm. Aliquots were then diluted 20-fold into standard ACAT assay mixtures, and ACAT assays were carried out under dark conditions. Parallel incubations were performed without photolysis prior to dilution, to correct for inhibitory effects of the small quantities of inhibitor present in the assay mixtures after dilution. Results are expressed as ACAT activity in the photolyzed samples divided by activity in the nonphotolyzed samples at the same inhibitor concentrations. Each point represents the mean of duplicate assays.

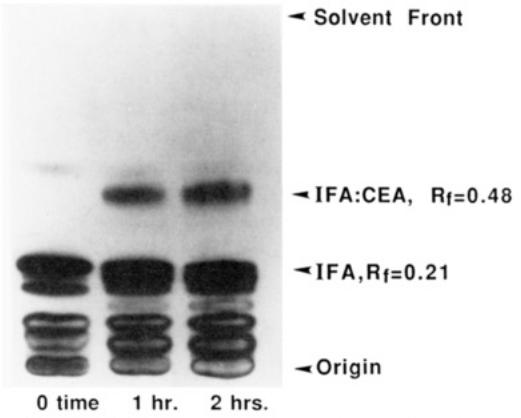

FIGURE 6: IFA:CoA is recognized as a substrate by ACAT. Liver microsomes (2.5 mg/mL) were incubated with 10 μM ^{125}IFA:CoA (1000 dpm/pmol) in a total volume of 2 mL containing 0.8 mg/mL BSA, 5 mM ATP, 5 mM $MgCl_2$, 0.1 mM CoA, and 1.3 mM DTT at 37 °C for 0, 1, or 2 h, followed by lipid extraction, TLC (petroleum ether/diethyl ether/acetic acid, 97/75/1), and autoradiography of the TLC plate (autoradiogram shown). Incubations and extractions were performed under dark conditions. The R_fs of chemically synthesized IFA and IFA:CEA standards run in parallel are indicated (0.21 and 0.48, respectively).

served for cholesterol oleate and chemically synthesized IFA:CEA. The identity of this photoreactive, radioiodinated reaction product was therefore assigned as the cholesteryl ester of IFA on the basis of TLC behavior and chemical structural data. Similar experiments utilizing both cholesterol-fed and normal aortic microsomes as the source of ACAT activity also resulted in the time-dependent synthesis of a photoreactive, ^{125}I-labeled lipid with an R_f of 0.48, consistent with the synthesis of ^{125}IFA:CEA by aortic microsomes (data not shown). Thus, IFA:CoA was recognized as a substrate by liver and aortic ACATs, indicating that it binds specifically to the ACAT active site.

Discussion

We have synthesized a radioiodinatable, photoreactive long-chain fatty acid analogue that can be derivatized to the corresponding cholesteryl and coenzyme A esters. These lipid analogues are inhibitors and substrates of purified pancreatic cholesterol esterase and ACAT, indicating their biological recognition by the active sites of these enzymes. In both cases, enzyme inhibition was reversible under dark conditions and photolysis of the enzyme in the presence of the appropriate lipid analogue resulted in irreversible inhibition of enzyme activity, data most consistent with covalent modification of these enzymes at or near their active sites. Although inactivation could occur due to covalent modification at sites distant from the active sites, binding to the active site is indicated by their recognition as substrates. Thus, IFA, IFA:CEA, and IFA:CoA are active site directed photoaffinity labels for cholesterol esterase and ACAT, although final determination of the actual sites of covalent modification and mechanisms for photolytic irreversible enzyme inhibition will require further detailed structural studies.

One important use for the CoA ester of this photoreactive fatty acid analogue relates to the identification of ACAT. IFA:CoA is both an inhibitor and a substrate analogue for ACAT, and the apparent K_i of 20 μM for reversible ACAT inhibition indicates an apparent binding affinity similar to that of the preferred substrate, oleoyl-CoA (K_m = 7 μM). As noted above, photolytically mediated covalent modification of ACAT by IFA:CoA occurs at or near the enzyme active site. Thus, IFA:CoA should be useful in differential labeling experiments to identify ACAT, and subsequently to characterize the active site of this very hydrophobic protein.

Another potential use for IFA and its cholesteryl ester derivative is the characterization of cholesterol esterase, which is important extracellularly in mediating intestinal cholesterol absorption through localization and hydrolysis of ingested cholesteryl esters at the intestinal brush border prior to the absorption of their component cholesterol and free fatty acid moieties (Bosner et al., 1988). Although pancreatic cholesterol esterase has been purified to homogeneity (Cox et al., 1990), identification and characterization of its hydrophobic substrate binding site(s) have not been reported in detail. Further studies utilizing these analogues to photolytically label homogeneous pancreatic cholesterol esterase should yield specific information about the enzyme active site and any additional cholesterol and fatty acid binding sites.

The identification and characterization of vascular intracellular cholesterol esterase will also be facilitated through the use of this photolabel. The postulated role of this enzyme is the hydrolysis of internalized lipoprotein-derived cholesteryl esters prior to their reesterification by ACAT (Brown & Goldstein, 1986). However, this enzyme has never been purified to homogeneity or characterized in detail, and so its role in intracellular cholesteryl ester accumulation during atherogenesis has not been clearly delineated. In addition to the studies outlined above, IFA and IFA:CEA should prove useful in the identification and characterization of this enzyme.

In summary, we have synthesized a photoreactive long-chain fatty acid analogue and demonstrated it to be recognized by the active sites of two major types of lipid-metabolizing enzymes, an acyltransferase (ACAT) and a lipase (cholesterol esterase). Unique features of this molecule include its radioiodinatability to high specific radioactivity and the proximity of the radiolabel to the photoreactive azide group, which provides optimal stability of photolabeling. Further studies using this photoreactive lipid and its derivatives as specific, active site directed photoaffinity labels will be useful in the study of intracellular cholesterol metabolism as well as the study of other lipid binding enzymes and proteins.

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Expression of the Amino-Terminal Half-Molecule of Human Serum Transferrin in Cultured Cells and Characterization of the Recombinant Protein†

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ABSTRACT: A human liver cDNA library was screened with a synthetic oligonucleotide, complementary to the 5′ region of human transferrin mRNA, as a hybridization probe. The full-length human cDNA clone isolated from this screen contained part of the 5′ untranslated region, the complete coding region for the signal peptide and the two lobes of transferrin, the 3′ untranslated region, and a poly(A) tail. By use of oligonucleotide-directed mutagenesis in vitro, two translational stop codons and a *Hin*dIII site were introduced after the codon for Asp-337. This fragment was inserted into two different expression vectors that were then introduced into *Escherichia coli*. As judged by NaDodSO$_4$–polyacrylamide gel electrophoresis and Western blot analysis, however, recombinant hTF/2N was undetectable in bacteria transformed by these plasmids. Concurrently, we developed a plasmid vector for the expression of recombinant hTF/2N in eukaryotic cells. In this case, a DNA fragment coding for the natural signal sequence, the hTF/2N lobe, and the two stop codons was cloned into the expression vector pNUT, such that the expression of hTF/2N was controlled by the mouse metallothionein promoter and the human growth hormone termination sequences. Baby hamster kidney cells containing this hTF/2N-pNUT plasmid secreted up to 20 mg of recombinant hTF/2N per liter of tissue culture medium. Recombinant hTF/2N was purified from the medium by successive chromatography steps on DEAE-Sephacel, Sephadex G-75, and FPLC on Polyanion SI. The purified protein was characterized by NaDodSO$_4$–PAGE, urea–PAGE, amino-terminal sequence analysis, UV–visible spectroscopy, iron-binding titration, and proton NMR. By these criteria, the recombinant hTF/2N appeared to behave identically with the proteolytically derived half-molecule, but to show a higher degree of monodispersity than the latter protein. Addition of *m*-fluorotyrosine to the culture medium resulted in random incorporation of this amino acid into cellular protein in lieu of tyrosine. Purified recombinant ^{19}F-Tyr hTF/2N gave four well-resolved ^{19}F NMR resonances of 20–40 Hz line width, two with suggestions of shoulders.

The iron-binding pseudoglobulins collectively called transferrins or siderophilins comprise a class of proteins with strikingly similar features. X-ray crystallographic analyses of human lactoferrin (Anderson et al., 1987) and rabbit serum transferrin (Bailey et al., 1988) reveal that these proteins consist of two similar lobes connected by a short bridging peptide and that each lobe contains two domains defining a deep cleft containing the binding site for a metal ion and a synergistic anion. To date, the amino acid sequences for five transferrins have been reported (Jeltsch & Chambon, 1982; MacGillivray et al., 1983; Metz-Boutigue et al., 1984; Rose et al., 1986; Baldwin & Weinstock, 1988), and comparisons reveal 35–75% sequence identity among the proteins and between the two lobes of a given transferrin. Highly conserved residues have been implicated previously in the binding of metal ions and synergistic anions. Most previous physicochemical studies are consistent with the structures determined by X-ray crystallography; however, some aspects of the proposed iron-binding sites require further clarification. For instance, nuclear magnetic resonance (NMR)[1] titration studies

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[1] Abbreviations: hTF, human serum transferrin; hTF/2N, amino-terminal half-molecule of hTF; hGH, human growth hormone; BHK, cultured baby hamster kidney cells; DHFR, dihydrofolate reductase; DMEM, Dulbecco's modified essential medium; FPLC, fast protein liquid chromatography; MTX, methotrexate; NMR, nuclear magnetic resonance; NTA, nitrilotriacetate; PAGE, polyacrylamide gel electrophoresis.

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