Purification of an Acyl-CoA Hydrolase from Rat Intestinal Microsomes

A CANDIDATE ACYL-ENZYME INTERMEDIATE IN GLYCEROLIPID ACYLATION*

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We have purified to apparent homogeneity an acyl-CoA hydrolase activity from rat intestinal villus cell microsomes by hepaining and union exchange and affinity chromatography. The purified 54-kDa acyl-CoA hydrolase along with several mirosomal proteins form a covalent acyl-protein bond upon inection with an activated fatty acid (acyl-CoA). The acyl moiety of the acylated acyl-CoA hydrolase is stable to denaturation and extraction with organic solvents, but is displaced by neutral hydroxylamine or mercaptoethanol, indicating a labile high energy (thio)ester linkage. The enzyme is inhibited by thiol-directed reagents and activated by the presence of diithothreitol suggesting the presence of a cysteine residue(s) at or near the active site. Common serine-esterase inhibitors (NaF, phenylmethylsulfonyl fluoride) and activators (Mg2+, Ca2+) had no effect on the hydrolase activity. The enzyme hydrolyzed (transferred to water) 14-20 carbon acyl-CoA with similar efficiencies and did not utilize glycerophospholipids or mono- and diacylglycerols as potential acyl donors/acceptors. Phospholipids and mono- and diradylglycerols at concentrations below 100 µM or polyconal antibodies raised against the purified hydrolase did not inhibit the enzyme activity. However, the acyl-CoA hydrolase activity could be immunoprecipitated from solubilized microsomes or purified enzyme preparations with corresponding decrease of the hydrolase activity in the supernatant of the immunoprecipitate. Immuno blotting studies show cross-reactivity with a protein of an identical molecular mass in other rat or human tissues. It is concluded that the microsomal acyl-CoA hydrolase deserves consideration as a candidate acyl-enzyme intermediate in glycerolipid synthesis when associated with appropriate acyltransferases.

Long chain fatty acyl-CoAs are synthesized from endogenous and exogenous fatty acid pools by acyl-CoA ligase in the endoplasmic reticulum, which also contains several acyl-CoA-dependent enzymes catalyzing the transfer of the acyl moiety from acyl-CoA to suitable acceptors (acylglycerols, cholesterol, and proteins). A long chain acyl-CoA hydrolase activity was observed in particulate and soluble fractions of various cells (1-6), and several isozymes with distinct electrophoretic mobilities have been at least partially purified from rat liver (1, 2) and brain (5) and from bovine (3) and rabbit (6) hearts. The metabolic role of these enzymes has remained uncertain as their function as intermediates in glycerolipid biosynthesis has been compromised by their hydrolytic activity toward glyceryl esters.

We report purification of an acyl-CoA hydrolase activity from rat intestinal mucosa, which does not attack glyceryl esters and could serve as an intermediate in glycerolipid synthesis. The intestinal enzyme also possesses physicochemical properties different from those of the liver enzyme as judged by substrate specificities and inhibition studies. Direct attempts to demonstrate that the intestinal microsomal acyl-CoA hydrolase acts by forming a covalent high energy thioester acyl-protein as an intermediate during acylglycerol synthesis are in progress.

EXPERIMENTAL PROCEDURES

Materials—Oleoyl-CoA, palmitoyl-CoA, 1-oleoyl-rac-glycerol, diol en (mixed isomers), 1,2-dioleoyl-rac-[2-3H]glycerol, 1-triolein, 1,2-di oleoyl-[3H]glycerol, trioleoylglycerol, phosphatidylcholine (egg yolk), ATP, CoASH, CHAPS, bovine serum albumin (fatty acid-free), Trisma base, Cibacron blue 3G-A-agarose, and isoacacetamide were purchased from Sigma. [1-3H]-oleic acid (52 mCi/mmol), and 2-oleyl-sn-glycerol ethers were from Serdy Research Laboratories (London, Ontario, Canada). The 1- and 3-palmitoyl-sn-glycerols were gifts from Dr. D. Buchner (University of Toronto), Dithiothreitol, 2-mercaptoethanol, reagents, and molecular weight standards for SDS-polyacrylamide gel electrophoresis and Western blotting, Econo-Pac 9DG desalting columns, protein A-agarose, and Affi-Gel heparin were from Bio-Rad. Hydroxylamine hydrochloride was purchased from Fisher. Silica Gel H (Merck 60 H) and G (Merck 60 G) and Aquacide 100 were purchased from Terochem Laboratories Ltd. (Mississauga, Ontario, Canada). [2-3H]Glycerol triloleate (2 Ci/mmol), [1-14C]-oleic acid (52 mCi/ mmol), and CytoScint were from ICN Biochemicals Canada Ltd. (Montreal, Quebec). [9,10-3H]Oleic acid (8.9 Ci/mmol), [1,14C]-oleoyl-CoA (52 mCi/mmol), ENHANCE, and 2,5-diphenyloxazole were purchased from DuPont-NEN. Rainbow molecular weight markers for Western blotting were from Amersham Corp. All other chemicals and solvents were of reagent grade or better quality and were obtained from local suppliers.

2-oleoyl-sn-[2-3H]glycerol (5 mCi/mmol) was prepared by digestion of radiolabeled [2-3H]glycerol trioleate with porcine pancreatic lipase as was the non-labeled 1(3)- and 2-oleoyl-sn-glycerol from trioleoylglycerol (7). The 1,2-dioleoyl-rac-[2-3H]glycerol (specific activity, 0.25 mCi/mmol) and 1,2-dioleoylglycerol were prepared by Grignard degradation of [2-3H]glycerol trioleate and trioleoylglycerol, respectively (8).

Preparation of Microsomal Membranes—Male rats (Wistar, Charles River Canada Inc., La Salle, Que.) weighing 250-300 g were fed ad libitum with standard diet. They were anaesthetized with

*The abbreviations used are: CHAPS, 3-[3-cholamidopropyldimethylammonio]-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.
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diethyl ether and exsugated via their abdominal aortae. The upper two thirds of the small intestine were removed and rinsed with 0.9% NaCl and 2 mM Hepes (pH 7.1). and the mucosal scrapings were obtained as described by Hoffman and Kuksis (9). Homogenization and low speed centrifugation procedures were adapted from a method by Pind and Kuksis (10). Tissue was homogenized in 5 ml of 0.9% NaCl and 2 mM Hepes (pH 7.1), and the mucosal scrapings from three dogs were suspended in approximately 200 ml of 300 mM mannitol, 5 mM EDTA, and 5 mM Hepes (pH 7.1) and homogenized in a Waring blender, set at low speed (power setting of 50 on a Powerstat) for 30 s. The homogenate was then gently filtered through a single layer of gauze (Nu Gauze, Johnson and Johnson Inc., Toronto, Ont.). Following a 10 min centrifugation at 10,000 g, the supernatant was further centrifuged at 106,000 g for 60 min to pellet microsomal membranes. Microsomes were washed by homogenization in 5 ml of 50 mM potassium phosphate (pH 7.4) containing 0.5 M KC1, 1 mM EDTA, 2 mM dithiothreitol, and 0.1% CHAPS with seven strokes of a motor-driven Potter-Elvehjem homogenizer. Following centrifugation at 106,000 X g for 60 min. All steps were carried out at 4°C. Washed microsomes were suspended in 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 2 mM dithiothreitol, 0.5 M KC1, and 10% glycerol to give a final concentration of protein between 6 and 7 mg/ml. Solubilization of Microsomal Membranes—Zwitterionic detergent CHAPS (1%; detergent/protein ratio, 2:1) was added to the microsomal membranes. The mixture was stirred on ice for 30 min. The solubilized crude enzyme extract was recovered with a Pasteur pipette after centrifugation at 106,000 X g for 60 min. The extract was desalted by a passage through a desalting column equilibrated with 50 mM potassium phosphate (pH 7.4), 10% glycerol, and 1% CHAPS. Purification of Acyl-CoA Hydrolase—All chromatographic procedures were carried out at 4°C except for HPLC, which was run at ambient temperature. Affi-Gel Heparin—The desalted solubilized microsomal extract was mixed for 1 h with 10 ml of Affi-Gel heparin preequilibrated with 50 mM potassium phosphate (pH 7.4), 10% glycerol, and 1% CHAPS (Buffer A). Subsequently, the mixture was poured into a column, washed with Buffer A, and eluted with Buffer A containing 0.25 and 1 M NaCl, respectively. HPLC-DEAE—Active fractions from Affi-Gel heparin were combined, dialyzed against 20 mM Tris-HCl, pH 7.4, 10% glycerol, 0.5% CHAPS (Buffer B), concentrated by Amosud III, and applied onto DEAE-5PW column. The column was eluted with a 0–1 M NaCl gradient. Cibacron Blue A 3GA-Agarose—Fractions eluted at 14–18 min from DEAE-HPLC were dialyzed against Buffer A and mixed for 50 min with Affi-Gel heparin-phosphate (pH 7.4), 10% glycerol, and 1% CHAPS (Buffer A). Subsequently, the mixture was poured into a column, washed with Buffer A, and eluted with Buffer B containing 1 ml NaCl. Polyacrylamide Gel Electrophoresis—10% SDS-polyacrylamide gels (11) were run under non-reducing conditions and were silver-stained by the method of Laemmli and others (12). Acyl-CoA Hydrolase Assay—Enzyme fractions (0–100 μg of protein) were incubated for 1 min at 37°C with 50 μM [1-4C]oleoyl-CoA (specific activity 2.5 mCi/mmol) in 0.2 ml of 50 mM potassium phosphate, pH 7.4. Incubations were stopped by addition of chloroform/methanol 2:1 (v/v). Following lipid extraction (13) and thinlayer chromatography using heptane/isopropyl ether/acidic acid (60:40:4, by volume) as the mobile phase, radioactivity in free fatty acids and other lipid components (phospholipid/monoacylglycerol, diacylglycerol, triacylglycerol, and cholesterol ester) were determined by scintillation counting. Effect of Inhibitors and Cofactors—Purified acyl-CoA hydrolase was preincubated with selected inhibitors and cofactors for 15 min at 37°C. Radiolabeled acyl-CoA was then added to the assay mixture and the acyl-CoA hydrolase activity was determined as indicated above. Specific activity is expressed as a percentage of the activity obtained with untreated enzyme (specific activity, 3–4 μmol/mg protein/min). Other Enzyme Assays—Monoacylglycerol hydrolase was assayed by monitoring 2-oleoyl[2-3H]glycerol (100 μM; specific activity, 5 mCi/mmol) in the absorption of 1 ml of Protein A-agarose. The mixture and assay conditions as well as the TLC solvent system were identical to those used for measuring acyl-CoA hydrolase activity. The monoacylglycerol was added in acetone (2.5% final concentration). After lipid extraction, residual monoacylglycerols were isolated by TLC and counted for radioactivity. Glycerophosphate, monoacylglycerol, diacylglycerol, and cholesterol acyltransferases were assayed essentially by the procedure used to assess acyl-CoA hydrolase activity except that sn-glycerol-3-phosphate (300 μM), 2-oleoylacylglycerol (60 μM), 1,2-dioleoyl-rac-glycerol (250 μM), and cholesterol (300 μM) were added in the presence of protein A-agarose and were incubated for 1 h. Following incubation, the mixture was filtered through a single layer of gauze, and the agarose fraction was recovered with 1 ml of 4 mM MgCl2, 1 mM dithiothreitol, and 2 mg/ml bovine serum albumin. Hydrolysis of non-radioabeled acyl-ester substrates was detected by a mass assay using gas-liquid chromatography. Incubations and lipid extractions were carried out essentially as outlined above except that the reaction volume was 1 ml. TLC was used to visualize no other lipid carriers were added during the lipid extraction. The lipid extract was dried under nitrogen, converted to their corresponding trimethylsilyl ethers/esters by reaction with Sylon BFT (Supelco, Canada Ltd. Oakville, Ontario) in pyridine (11: v/v) and 1/10th of the sample was subjected to gas-liquid chromatography analysis on a polar capillary column with a 170–350°C temperature program (14). The amount of fatty acid released by the enzyme during incubations and of non-hydrolyzed substrates was calculated after correction for differences in a flame ionization detector area response (15) and subtraction of control runs (substrates minus enzyme). Fatty Acid Labeling of Proteins—50–100 μg of microsomal protein or less than 1 μg of Cibacron blue-agarose-purified Acyl-CoA hydrolase were incubated in 37°C with 250 μM [1-4C]oleic acid (100 μCi/mmol) or [1-4C]acidic acid (5 mCi/mmol), 20 mM ATP, 2.5 mM CoA, or with 50 μM [1-4C]oleoyl-CoA (5 mCi/mmol) in Tris-HCl or phosphate (pH 7.4), 10% glycerol, and 1% CHAPS. Purification of Acyl-CoA Hydrolase—All chromatographic procedures were carried out at 4°C except for HPLC, which was run at ambient temperature. Western Blotting—Proteins separated on 10% SDS-PAGE were electroblotted onto an Immobilon (polyvinylidene difluoride) membrane in ice-cold 25 mM Tris, 192 mM glycine, pH 8.3, transfer buffer at 350 mA constant current for 1 h. Following transfer, the membrane was blocked with 5% skim milk in TBS (Blotto) at room temperature for 1 h and then incubated for 1 h with a 1:4,000 dilution of rabbit anti-rabbit IgG (H + L), diluted 1:1,000 with TBS containing 1% skim milk. The membrane was then washed twice in 0.5% Tween 20 (TTBS) for 15 min and three times in TBS for 10 min. The bound antibody was detected by incubation solution containing a 100:1:1 mixture of 0.1 mM sodium carbonate, 0.1 mM magnesium chloride, pH 9.5, 1.5% 5-brano-4-chloroindoxyl phosphate, 0.1% CHAPS (1%; detergent/protein ratio, 2:1) was added to the incubation mixture and the acyl-CoA hydrolase activity was determined as indicated above. The specificity of the acyl-protein bond toward a reducing agent was evaluated by reaction with mercaptoethanol. Also, the acyl-proteins were extracted with chloroform/methanol 2:1 (v/v), and the recovered proteins were analyzed by SDS-PAGE and fluorography. Antibody Preparation—Purified acyl-CoA hydrolase (approximately 100 μg) in 0.5 ml of 10 mM Tris-HCl (pH 7.0), 150 mM NaCl (Tris-buffered saline, TBS), was mixed with 0.5 ml of complete adjuvant, and the thick emulsion was injected subcutaneously into two rabbits (10–12 weeks old). Booster intradermal injections of 25 μg of antigen in Freund’s incomplete adjuvant were given 4 and 8 weeks later. Rabbits were bled from the marginal ear vein 1 week prior to (preimmune) and 9 weeks after (immune) the initial immunization. Serum was prepared and stored at -70°C. Western Blotting—Proteins separated on 10% SDS-PAGE were electroblotted onto an Immobilon (polyvinylidene difluoride) membrane in ice-cold 25 mM Tris, 192 mM glycine, pH 8.3, transfer buffer at 350 mA constant current for 1 h. Following transfer, the membrane was blocked with 5% skim milk in TBS (Blotto) at room temperature for 1 h and then incubated for 1 h with a 1:4,000 dilution of rabbit serum in TBS containing 1% skim milk. The membrane was subsequently washed twice with Blotto for 15 min and incubated for 1 h with the second antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG (H + L), diluted 1:1,000 with TBS containing 1% skim milk. The membrane was then washed twice in 0.5% Tween 20 (TTBS) for 15 min and three times in TBS for 10 min. The bound antibody was detected by incubation solution containing a 100:1:1 mixture of 0.1 mM sodium carbonate, 0.1 mM magnesium chloride, pH 9.5, 1.5% 5-brano-4-chloroindoxyl phosphate, 0.1% CHAPS, and 3% nitro blue tetrazolium in 70% N,N-dimethylformamide. After color development, the membrane was washed with water and air-dried.
M Tris, pH 8.0. The purified antibodies were dialyzed for 16 h against TBS with one change after 8 h.

Preparation of Immunoaffinity Columns—Covalently linked antibody-protein A-agarose columns were prepared essentially as described by Schneider et al. (17). Antibodies were bound to protein A beads as described above, the beads were washed twice with 10 column volumes of 0.2 M sodium borate (pH 9.0) and were cross-linked to the protein A using 20 mM dimethylpimelimidate in 10 volumes of 0.2 M sodium borate (pH 9.0). The cross-linking reaction was stopped after 30 min by incubating the beads with 10-column volumes of 0.2 M ethanamine (pH 8.0) followed by extensive washing with TBS.

Other Methods—Protein concentrations were determined after precipitation with deoxycholate and trichloroacetic acid (18) by bicinchoninic acid assay (Pierce Chemical Co.).

Unilamellar egg yolk phosphatidylcholine vesicles were prepared by sonication (Branson Sonic Power Co., 50% duty cycle, output control setting 5) of 10 mg phosphatidylcholine/ml suspended in 25 mM Tris, pH 7.8, 150 mM NaCl for 30 min on ice followed by ultracentrifugation at 42,000 rpm (TI 70 rotor) for 3 h. The supernatant was recovered with a Pasteur pipette, and the concentration of phosphatidylcholine was determined by gas-liquid chromatography after phospholipase C treatment using tridecanoylglycerol as an internal standard (15).

RESULTS

Purification of Acyl-CoA Hydrolase—Microsomal membranes isolated from 8–10 rats were depleted of their luminal content and peripheral proteins by treatment with a low concentration of detergent (0.1% CHAPS) and 0.5 M KCl, respectively. The membrane-bound enzyme was then solubilized by 1% CHAPS. CHAPS and other bile salt conjugates were superior to non-ionic detergents (Triton and Zwittergent 3-10N) in keeping the activity bound to the affinity medium was recovered from the Cibacron blue A-agarose column. The acyl-CoA hydrolase activity starting from the detergent solubilized extract showed a polypeptide of apparent molecular weight of 54 kDa (Fig. 2). The overall purification of the acyl-CoA hydrolase with radiolabeled oleoyl-CoA was estimated at 450-fold with 24% recovery of the original solubilized extract (Table I).

Characterization of the Purified Acyl-CoA Hydrolase—The purified enzyme displayed closely similar activities toward a variety of acyl-CoA esters (Table II). The highest activity was obtained with arachidonoyl-CoA and the lowest with stearoyl-CoA. It did not hydrolyze long chain monoaeylglycerols, short and long chain diacylglycerols, or glycerophospholipids. Furthermore, various isomers of long chain monoacylglycerols or short and long chain diradylglycerols had little or no effect on the acyl-CoA hydrolase activity up to 150 μM concentrations (Table III). Higher concentrations of dioleoyl and dioctanoylglycerols (1 mM) resulted in approximately 50% decrease of the hydrolase activity. It is important to note that the activity was not affected by preincubation with sodium fluoride or phenylmethylsulfonyl fluoride, potent inhibitors of monoacylglycerol lipase and other carboxyesterases and by divalent cations (Ca2+, Mg2+) which are necessary cofactors for optimal activities of most lipases. The enzyme was activated almost 2-fold by inclusion of diethiothreitol (5 mM) in the assay suggesting the presence of a critical cysteine residue at or near the active site. In agreement with this observation the enzyme binds to Affi-Gel 501 (organomercurial agarose, Bio-Rad) specific for sulfhydryl containing proteins (result not shown). Furthermore, the activity can be inhibited by sulfhydryl-directed reagents such as iodoacetamide (Table III).

The solubilized and purified enzyme did not exhibit phospholipid dependence as indicated by incubations with increasing concentrations of unilamellar phospholipid vesicles (Fig. 3). In fact, at concentrations in excess of 200 μg of phosphatidylcholine/ml, a substantial decrease of acyl-CoA hydrolysis (transfer) was observed.

Formation and Characterization of Acyl-proteins in Microsomal Fractions and Purified Acyl-CoA Hydrolase—We observed selective incorporation of fatty acyl into microsomal proteins in the presence of ATP and CoA (Fig. 4). The protein acylation was time-dependent with significant levels of labeling observed after 5-min incubations (Fig. 5). Incubation of the purified acyl-CoA hydrolase with radiolabeled oleoyl-CoA also resulted in a time-dependent acyl-protein formation (Fig. 6). The inclusion of ATP and CoA was necessary but not sufficient for acyl-protein formation as it also depended on the presence of acyl-CoA ligase. No acyl-enzyme was obtained upon incubation of the purified hydrolase with radiolabeled oleic acid in the presence of ATP and/or CoA indicating that activated fatty acid was necessary to form the acyl-protein and that the enzyme did not possess acyl-CoA ligase activity (result not shown). A significant amount of the radioactive acyl moiety was lost from the acyl-enzyme upon subsequent incubation with methanolic KOH and neutral hydroxylamine (Fig. 7) or with β-mercaptoethanol (Fig. 8). A 10-fold excess of free CoASH did not prevent protein acylation (Fig. 4). However, a futile cycle of protein acylation and regeneration of acyl-CoA in the absence of ATP by trans-thioesterification could not be eliminated. Extraction of the labeled protein with chloroform/methanol 2:1 (v/v) did not remove the label thus confirming presence of a covalent acyl-enzyme linkage (result not shown). Similar results were obtained with the labeled microsomal proteins (not shown).

Immunocharacterization of Acyl-CoA Hydrolase—The purified enzyme was used to prepare polyclonal anti-acyl-CoA hydrolase antibodies in rabbits. Western blot analysis indicated that the antibody reacted with purified acyl-CoA hydrolase and did not show any apparent cross-reactivity with other rat intestinal proteins (Fig. 9). The antibody interacted with a protein of an apparently identical molecular mass of 54 kDa in rat liver, kidney, and heart total homogenates (Fig. 9) as well as in human cerebellum (Fig. 10). The colonic cancer cell line CaCo-2 displayed heterogenous cross-reactivity (Fig. 10) as did various other established cell lines (human melanoma 74-36 cells, colon carcinoma T-84 cells, rat hepatoma and rat glioma C-6 cells, not shown). Antibodies purified on protein A beads did not appear to inhibit the enzyme activity when added to the assay mixture containing purified enzyme or microsomes at antibody/enzyme weight ratio of 1:1 indicating that the polyclonal antibodies are not directed against the
FIG. 1. Elution profiles of acyl-CoA hydrolase on (A) Affi-Gel heparin, (B) DEAE-5PW, and (C) Cibacron blue 3GA-agarose. Protein was monitored at 280 nm (---, DEAE; ○--○, Affi-Gel heparin and Cibacron blue A). Fractions were assayed for acyl-CoA hydrolase activity (●—●). Changes in NaCl concentrations are indicated by arrows (A and C) or by (--) in B.
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Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of active acyl-CoA hydrolase fractions at different stages of purification. A, Affi-Gel heparin; B, DEAE-HPLC; C, Cibacron blue A. 0.1-1 μg of protein was electrophoresed in a 10% SDS-polyacrylamide gel under non-reducing conditions (minus dithiothreitol or mercaptoethanol). Protein was visualized by silver staining. Molecular mass standards are indicated in the left margin.

Table I

Purification of acyl-CoA hydrolase from rat intestinal mucosa

| Step                | Protein | Specific activity | Purification Recovery |
|---------------------|---------|------------------|-----------------------|
| CHAPS extract       | 45.0    | 8                | 80 %                  |
| Affi-Gel-heparin    | 3.9     | 65               | 80 %                  |
| DEAE-HPLC           | 0.13    | 960              | 70 %                  |
| Cibacron blue 3GA   | 0.03    | 3600             | 24 %                  |

Table II

Substrate specificity of purified rat intestinal acyl-CoA hydrolase

Enzyme activity was determined by gas-liquid chromatography analyses of released fatty acids as described under “Experimental Procedures.” The standard assay medium contained, in 0.5 ml, 0.5 μg of purified enzyme, 100 μM non-radiolabeled substrates indicated below, and 50 mM potassium phosphate, pH 7.4. The glycerol esters were added to the incubation mixture in acetone (final concentration of acetone 2.5%). The values represent averages of two independent experiments performed in duplicate.

| Substrate           | Fatty acid released (nmol) |
|---------------------|----------------------------|
| Myristoyl-CoA (14:0)| 19.4                       |
| Palmitoyl-CoA (16:0)| 19.8                       |
| Stearoyl-CoA (18:0) | 16.4                       |
| Oleoyl-CoA (18:1)   | 18.2                       |
| Elaidoyl-CoA (18:1trans) | 17.7                  |
| Arachidonoyl-CoA (20:4) | 24.0                  |
| 2-Oleoylglycerol    | <1                        |
| 1(3)-Oleoylglycerol | <1                        |
| 1,2-Dioleoyl-rac-glycerol | 0                   |
| 1,2-Dioctanoyl-rac-glycerol | 0                  |
| Phosphatidylcholine | 0                         |
| 1-Palmitoyl-2-lysophosphocholine | 0             |

Table III

Effect of cofactors and selected glycerol esters on rat intestinal acyl-CoA hydrolase activity

Enzyme activity was determined as described under “Experimental Procedures.” The standard assay medium contained, in 0.2 ml, 0.05 μg of purified enzyme, 50 μM radiolabeled oleoyl-CoA, and 50 mM potassium phosphate, pH 7.4. The standard assay gave the specific activity of 3.5-3.8 nmol/mg of protein/min. The values represent averages of two separate experiments.

| Inhibitor              | Activity % control |
|------------------------|--------------------|
| Standard               | 100                |
| Dithiothreitol (5 mM)  | 180                |
| Iodoacetamide (0.5 mM)| 70                 |
| NaF (20 mM)            | 95                 |
| Phenylmethylsulfonyl fluoride (1 mM) | 98           |
| MgCl₂ (4 mM)           | 105                |
| CaCl₂ (4 mM)           | 102                |
| 2-Oleoylglycerol (60 μM) | 120        |
| 2-Oleoylglycerol ether (60 μM) | 117        |
| 1(3)-Oleoylglycerol (60 μM) | 110        |
| 2-Octanoylglycerol (60 μM) | 103        |
| 1,2-Dioleoyl-rac-glycerol (150 μM) | 90         |
| 1,2-Dioleoyl-rac-glycerol (1 μM) | 46          |
| 1,2-Dioctanoyl-rac-glycerol (150 μM) | 93        |
| 1,2-Dioctanoyl-rac-glycerol (1 mM) | 54          |

Fig. 3. Inactivation of the acyl-CoA hydrolase activity by phospholipid. The purified enzyme was incubated with increasing amounts of unilamellar phosphatidylcholine vesicles for 30 min on ice. Radiolabeled acyl-CoA was then added to the mixture, and the assay was performed as described under “Experimental Procedures.” Data are representative of three independent experiments performed in duplicates.

Discussion

In this study a homogeneous preparation of acyl-CoA hydrolase has been obtained from solubilized rat intestinal microsomes by chromatography on Affi-Gel heparin, anion exchange, and Cibacron blue-agarose. Previous work has established the presence of the acyl-CoA hydrolases in tissues of several animal species (1-6) indicating an ubiquitous need for such activity in cells. Despite extensive studies, the role of the enzyme in intracellular processes and its regulation has not yet been elucidated. It appears that different tissues express different isozymes with various substrate specificities.
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*Fig. 4. Fatty acid labeling of proteins from rat intestinal microsomes.* 100 μg of microsomal protein were incubated for 20 min at 37 °C with: A, [3H]oleic acid; B, [3H]oleic acid + 20 mM ATP; and C, [3H]oleic acid + 20 mM ATP + 2.5 mM CoASH. Reactions were stopped by addition of electrophoresis sample buffer and the incorporation of radioactivity into proteins was analyzed by SDS-PAGE and fluorography as described under “Experimental Procedures.”

*Fig. 5. Time course of protein acylation.* 100 μg of microsomal protein were incubated for 0.5–20 min with [1-14C]oleic acid, 20 mM ATP, and 2.5 mM CoASH. Reactions were stopped by addition of electrophoresis sample buffer and the incorporation of radioactivity into proteins was analyzed by SDS-PAGE and fluorography as described under “Experimental Procedures.”

For instance, the 57–60-kDa liver enzyme catalyzes hydrolysis of palmitoyl-CoA but exhibits only a marginal activity toward other long chain CoA esters (2, 19). In addition, it hydrolyzes monoacylglycerols with similar efficiency (19). The 41-kDa bovine heart microsomal acyl-CoA hydrolase showed marked preference for arachidonoyl-CoA, but other medium to long chain saturated and polyunsaturated fatty acyl-CoAs were also suitable substrates (3). The enzyme was 80% inhibited by 1 μM lysophosphatidylcholine or lysophosphatidylglycerol but was not affected by up to 5 μM concentrations of lysophosphatidylethanolamine (3). Cytosolic and mitochondrial acyl-CoA hydrolase activities in various tissues with differences in specificities have also been reported (5, 20–22). The purified enzyme from intestinal microsomes appears to be a form of the enzyme different from those reported earlier. The apparent molecular mass of 54 kDa as estimated by denaturing gel electrophoresis is lower than that found for the rat liver enzyme (57–59 kDa). A polyclonal antibody raised against the purified hydrolase cross-reacts with a 54-kDa protein present in total cell homogenates of rat liver, heart, and kidney, as well as human cerebellum. More important, the enzyme does not hydrolyze glyceryl esters and their presence did not significantly activate or inhibit the activity. However, high concentrations of diacylglycerols and phospholipids in the assay mixture interfere with the hydrolysis reaction. This could be perhaps attributed to the interference of the phospholipid and diacylglycerol with the substrate availability. Inactivation of the activity due to asymmetrical incorporation of the enzyme into the phospholipid bilayer with 50/50 outside/inside active site orientation may also be considered but it is unlikely as the lipid/protein...
molar ratio required to obtain 50% inactivation is high. The acyl-CoA hydrolase was not dependent on the presence of cations and was not inactivated by known lipase inhibitors. The enzyme was sensitive to thiol-directed reagents, suggesting that it contains important cysteine residues in or near its active site.

Several microsomal proteins including acyl-CoA hydrolase were found to form covalent acyl-proteins upon incubation with activated fatty acid. The rate of incorporation of the radioactive acyl groups into the proteins corresponded closely to neutral hydroxylamine mediated hydrolysis (23). The acyl-protein formation may represent an intermediate step in the lipid biosynthetic pathway, where the enzyme acylation would precede acyl transfer to acyl acceptors. It has been recently suggested (24) that a common acyl-CoA-binding subunit (acyl-CoA hydrolase) may be a member of a hetero-oligomeric complex containing acyl acceptor subunits. Hence if substrates for the acyltransferases or the acyltransferase subunits are not present, only the hydrolysis (transfer to water) of acyl-CoA would occur. There exists some evidence for involvement of acylated cysteine residues in fatty acyl transfer/ hydrolysis. The reaction proceeding through a covalent cysteine-linked acyl-enzyme intermediate has been demonstrated for fatty acid synthetase (25), myristoyl-CoA:protein N-myristoyltransferase (26), and acyl-protein synthetase and acyl-CoA reductase of the fatty acid reductase complex from Photobacterium phosphoreum (27). A group of proteins which served as acceptors for fatty acids in cell-free extracts from mouse heart, kidney, and liver has also been recently identified (28). We are presently investigating the role of acyl-CoA hydrolase in intestinal triacylglycerol biosynthesis. We have purified by affinity (29) or hydrophobic interaction (30) chromatography a triacylglycerol synthetase complex from rat intestinal mucosa containing monoaoylglycerol and diacylglycerol acyltransferases as well as acyl-CoA hydrolase and ligase. We have obtained evidence that acyl-CoA hydrolase inhibitors also inhibit triacylglycerol synthesis from monoaoylglycerol and acyl-CoA substrates and prevent the formation of the covalent acyl enzyme.²

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