Coenzyme A-independent Monoacylglycerol Acyltransferase from Rat Intestinal Mucosa*

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**Rat intestinal mucosa contains high diacylglycerol-synthesizing activity (monoacylglycerol acyltransferase (MGAT) activity) due to monoacylglycerol and fatty acid, independently of coenzyme A and ATP. MGAT activity was purified from rat intestinal mucosa by successive chromatography separations on DEAE-cellulose, CM-Sephadex, and anti-IgG-Sepharose against rat pancreatic lipase. The enzyme was electrophoretically homogeneous, and its molecular weight was 49,000, which is identical with that of rat pancreatic lipase. Immunoblotting analysis with antibody against rat pancreatic lipase showed one immunoreactive protein with an estimated molecular weight of 49,000. The activity of the purified enzyme was completely inhibited by addition of the antibody. Using immunocytochemical techniques, it was found that immunoreactive protein against rat pancreatic lipase was uniformly distributed within the absorptive cells of the intestine but was absent from the microvillar membrane. The MGAT activity of intestinal mucosal homogenate was inhibited by about 65% by addition of antibody against rat pancreatic lipase. Trioleoylglycerol- and dioleoylglycerol-hydrolyzing activities of the purified enzyme and pancreatic lipase were inhibited by addition of intestinal mucosa extract.

These results suggest that pancreatic lipase is present in intestinal absorptive cells and that it may contribute to resynthesis of diacylglycerol from monoacylglycerol and fatty acids in these cells.

**EXPERIMENTAL PROCEDURES**

Materials—The sources of the enzyme substrates and reagents used were as follows: 1-[1-14C]oleic acid (2.1 GBq/mmol), [9,10-3H]tripalmitoylglycerol (991.6 GBq/mmol), 1-[14C]trioleoylglycerol (3.95 GBq/mmol), and [9,10-3H]dipalmitoylphosphatidylcholine (1554 GBq/mmol) were from DuPont NEN. [3H]dipalmitoylglycerol was prepared from [3H]dipalmitoylphosphatidylcholine by phospholipase C treatment as described previously (12). [14C]Monooctadecanoylglycerol was prepared from [14C]trioleoylglycerol by pancreatic lipase treatment. After a 2-h incubation, lipids were extracted by the method of Bligh and Dyer (13), and 2-monooctadecanoylglycerol was isolated by preparative thin layer chromatography. Trioleoylglycerol and sodium cholate were from Sigma. Oleic acid and dioleoylglycerol were from NuChek Prep., Inc. (Elysian, MN). Monoacylglycerols and monooleoylglycerol ethers were from Serdary Research Laboratories (London, Ontario, Canada). Peroxidase anti-rabbit IgG (heavy and light) was from Daiichi Kayaku (Tokyo, Japan). Bovine serum albumin was from Wako Pure Chemical Industries (Osaka, Japan) and was extracted by the method of Chen (14) to remove free fatty acid.

Purification of MGAT—Wistar strain male rats, weighing 150–180 g, were killed, and their intestines (the first 15 cm) were excised and rinsed with 0.9% NaCl. Intestinal mucosa was obtained by scraping the luminal surface and homogenized in a Polytron homogenizer (Kinematica, Switzerland) with 9 volumes of 20 mM Tris-HCl, pH 8.0, containing 2 mM benzamidine (buffer 1). The homogenate was centrifuged for 20 min at 100,000 × g. Powdered ammonium sulfate was then added to the supernatant, and the active protein, precipitated with 40% saturation of ammonium sulfate, was dissolved in buffer 1. This enzyme solution was dialyzed against the same buffer and centrifuged for 1 h at 100,000 × g.

The supernatant was applied to a column of DEAE-cellulose (1.9 × 23 cm) equilibrated with buffer 1. Most enzyme activity was recovered in the nonadsorbed fraction. The active fractions were pooled, dialyzed against 40 mM potassium phosphate buffer, pH 6.1, and applied to a
CM-Sephadex C-50 column (1.9 × 20 cm) equilibrated with the same buffer. The protein was eluted from the column with a linear gradient of phosphate buffer (40 to 400 mm), pH 6.1. The active fractions were pooled, dialyzed against 150 mM potassium phosphate buffer, pH 7.2, containing 100 mM NaCl, and loaded on to a column of anti-pancreatic lipase IgG equilibrated with the same buffer. The protein and the gel were incubated at 4°C overnight under gentle agitation. Then, the column was washed successively with equilibrated phosphate buffer, Triton X-100 buffer (150 mM phosphate buffer, 100 mM NaCl, and 0.2% Triton X-100, pH 7.2) and then again with the original buffer. The active protein was eluted with a 200 mM glycine-Cl buffer, pH 2.8.

Each fraction was immediately neutralized with 0.1 mM NaOH. The active fractions were pooled, dialyzed against 10 mM potassium phosphate buffer, pH 7.0, and stored at −80°C.

Purification of Rat Pancreatic Lipase—Pancreatic lipase was purified from rat pancreas by the procedure of Gidez (15) with some modifications. The purified enzyme gave a single band on SDS-polyacrylamide gel electrophoresis from which its molecular weight was estimated to be 49,000.

Enzyme Assay—MGAT activity was determined at 37°C in a total volume of 0.1 ml. The assay system contained the following components: 40 nmol of [14C]oleic acid (240,000 dpm), 50 nmol of 2-monooctanoylglycerol in acetone (2.5 μl), 10 μmol of potassium phosphate, and 50 μl of enzyme solution. Incubation was carried out at pH 7.0 for 10 min. The reaction was stopped by the addition of 1.5 ml of a mixture of 2-propanol, heptane, and water (80:20:20, v/v) containing [3H]diacylglycerol (0.3 μmol, 100,000 dpm). The mixture was shaken for 15 s, then 1.0 ml of heptane and 0.5 ml of water were added, and the mixture was shaken for an additional 15 s. The upper phase was washed twice with 2 ml of a mixture of ethanol, water, and 0.5% NaOH (50:50:10, v/v). The radioactivity of the upper heptane phase (0.7 ml) was counted in 8 ml of ACS-II (Amersham Japan, Tokyo, Japan). Values were adjusted for yield, determined from the recovery of [3H].

In studies on the effect of fatty acid chain length, the activity was also determined using [14C]monoleoylglycerol. Incubations were carried out at pH 7.0 for 30 min, and diacylglycerols were extracted by the method described above. The upper heptane phase (1 ml) was dried with a stream of nitrogen, solubilized in 50 μl of a mixture of hexane, and separated by thin layer chromatography (Whatman K-5 silica gel plates) with a hexane:diethyl ether:acetic acid (60:40:1, v/v). Lipids were located with iodine vapor, and spots of diacylglycerol were scraped off for measurement of their radioactivity.

Trioleoylglycerol- and dioleoylglycerol-hydrolyzing activities were determined by measuring the rate of release of [3H]oleic acid. The assay mixture consisted of 0.2 ml of 100 mM potassium phosphate buffer, pH 7.0, containing 0.3 μmol of [3H]triacylglycerol (120,000 dpm) or 0.3 μmol of [3H]diacylglycerol (80,000 dpm) and 1 mg of gum arabic. Incubations were carried out at 37°C for 30 min, and the free oleic acid was extracted and determined by the method of Belgrade and Vaughan (16).

Preparation of Anti-IgG Column against Rat Pancreatic Lipase—Antiserum against purified rat pancreatic lipase was raised in rabbits. Anti-IgG was purified by the procedure described previously (9). Anti-IgG (25 mg) was coupled to 10 ml of swollen CNBr-activated Sepharose 6B gel as described previously (7). Under these conditions, more than 60% of the initial protein was covalently coupled to the gel.

Immunohistochemistry—Male rats (Wistar strain; 150–180 g) were killed under anesthesia, and their intestines were excised. At a distance of about 7 cm below the common duct entry, a 1.0-cm length of intestine was excised and embedded in paraffin wax by standard procedure. Cross-sections (4 μm thick) were prepared on silanized slides, deparaffinized, and hydrated by standard procedure. After a rinse in 0.05 M Tris-Cl buffer (pH 7.6) containing 0.05% Triton X-100, the sections were incubated with 3% H2O2 for 10 min at 37°C, for inhibition of internal peroxidase activity. The sections were incubated for 15 min with 10% normal goat serum and then incubated with anti-IgG overnight at 4°C. The specimens were treated by the linked streptavidin-biotin method employing a DAKO LSAB-2 kit (DAKO Japan, Kyoto, Japan). The immunoglobulin reaction was visualized by 0.02% diaminobenzidine tetrahydrochloride containing 0.01% H2O2. After rinsing with water, sections were stained with Myer’s hematoxylin.

Other Procedures—SDS-gel electrophoresis was performed in 10% gel under reducing conditions as described by Laemmli (17). For immunoblotting analysis, proteins were transferred electrophoretically to nitrocellulose and stained using an antibody against rat pancreatic lipase as described previously (7). Relative protein concentrations were determined with a Bio-Rad protein assay kit with bovine serum albumin as a standard.

RESULTS

MGAT activity was determined in the absence of coenzyme A and ATP. The tissue distribution of MGAT activity in the rat was measured by homogenizing and centrifuging the tissue at 1,000 × g and assaying the supernatant (Table I). The highest MGAT activity was observed in the intestinal mucosa, 34–120-fold that found in other tissues. The MGAT activity of intestinal mucosa extract was proportional to the amount of protein and the incubation time (data not shown). The enzyme concentration dependence was not changed by addition of coenzyme A and ATP (data not shown). Over a 15-min incubation period, the activity decreased slightly with time when coenzyme A and ATP were added.

Rat intestinal mucosa (78 g) were homogenized, extracted, fractionated by ammonium sulfate, and applied to a DEAE-cellulose column. The enzyme did not adsorb to the column, and the active wash fraction was pooled and applied to a CM-Sephadex C-50 column (Fig. 1A). The MGAT activity was completely bound and eluted a single peak, developed with a linearly increasing gradient of phosphate concentration. Active fractions (tubes 11–17) were pooled, dialyzed, and applied to an anti-IgG Sepharose column. The enzyme was completely bound and eluted with glyvne buffer, pH 2.8 (Fig. 1B).

The enzyme purification steps and the enzyme yield at each step are summarized in Table I. At the final step of purification, the specific MGAT activities were 3,210 nmol/mg of protein/min. Purified enzyme hydrolyzed trioleoylglycerol and dioleoylglycerol, whose specific activities for trioleoylglycerol and dioleoylglycerol were 35,900 and 43,500 nmol/mg of protein/min, respectively. The purified enzyme gave a single band on SDS-polyacrylamide gel electrophoresis from which its molecular weight was estimated to be 49,000 (Fig. 2). Pancreatic lipase purified from rat pancreas also gave a single band, having the same molecular weight. Immunoblotting analysis with antibody against rat pancreatic lipase showed one immunoreactive protein with an estimated molecular weight of 49,000 (Fig. 2). The purified enzyme had an isoelectric point of pH 6.8, which was identical with that found for rat pancreatic lipase (data not shown).

Purified enzyme was mixed with various amounts of anti-IgG prepared with rat pancreatic lipase, left to stand overnight at 4°C, centrifuged to remove insoluble material, and the residual enzyme activities in the supernatant measured. MGAT activity in the supernatant decreased as the amount of anti-IgG increased (Fig. 3). Similar profiles were observed for the inhibition of trioleoylglycerol- and dioleoylglycerol-hydrolyzing activities (data not shown). All three activities were unaffected by nonimmune IgG, which was used as a control (data not shown).

The incorporation of 2-monoleoylglycerol and oleic acid into dioleoylglycerol were determined. With a constant 2-monoleoylglycerol and oleic acid.
oleoylglycerol level of 0.5 mM, dioleoylglycerol formation increased with the concentration of oleic acid (Fig. 4A). With oleic acid constant at 0.4 mM, the enzyme activity increased with the concentration of 2-monooleoylglycerol up to 0.5 mM and then decreased (Fig. 4B). Table III shows the effect of fatty acid chain length on diacylglycerol synthesis by the purified enzyme. With the saturated fatty acid tested, its activity was maximal with myristic acid and fell with decreasing fatty acid chain length. The MGAT activity for caproic acid was not detected. The activity for oleic acid was 8-fold that for stearic acid. The enzyme activity for 2-monoacylglycerol was about 3 to 6 times that for 1-monoacylglycerol (Table IV). With ether analogs of monoacylglycerol as substrates, the MGAT activity for glycerol ether substituted at position 2 was essentially identical with that for glycerol ether substituted at position 1.

Photomicrography revealed a brown reaction on the anti-pancreatic lipase IgG-treated sections of small intestine. The reaction product was consistently visualized within adsorptive cells (Fig. 5A). The submucosa of these sections were also stained strongly by 3,3-diaminobenzidine tetrahydrochloride (data not shown). On adjacent sections treated with nonimmune IgG, little or no reaction was observed with absorptive cells (data not shown). Anti-IgG against rat pancreatic lipase blocked the MGAT activity of rat intestinal mucosa extract in a dose-dependent manner; about 65% activity was inhibited by addition of the antibody (data not shown). Control nonimmune IgG did not affect MGAT activity in the intestinal mucosa (data not shown). Trioleoylglycerol-hydrolyzing activity of the purified enzyme and pancreatic lipase was inhibited in a dose-dependent fashion by addition of intestinal mucosa extract (data not shown). Dioleoylglycerol-hydrolyzing activity was also inhibited by the addition of intestinal mucosa extract (data not shown).

Table II

| Purification step       | Total protein mg | Total activity nmol/min | Specific activity nmol/mg/min |
|-------------------------|-----------------|-------------------------|-----------------------------|
| 10,000 x g supernatant | 4360            | 5720                    | 1.31                        |
| DEAE-cellulose          | 132             | 1110                    | 8.45                        |
| CM-Sephadex             | 28.2            | 1010                    | 35.8                        |
| IgG-Sepharose           | 0.17            | 546                     | 3210                        |

Fig. 1. A, ion exchange chromatography of rat intestinal MGAT on a CM-Sephadex C-50 column. The nonadsorbed enzyme fractions from DEAE-cellulose were applied to the column. The column was washed with buffer and developed with a linear gradient of phosphate buffer (40 to 400 mM) (arrow). B, immunoaffinity chromatography of rat intestinal MGAT on anti-IgG-Sepharose gel. The active proteins from the CM-Sephadex C-50 column were incubated with anti-IgG-Sepharose against rat pancreatic lipase. The gel was washed with phosphate buffer, Triton X-100 buffer, and then again with the phosphate buffer. The active proteins were eluted with 200 mM glycine-HCl buffer, pH 2.8 (arrow). A280 (○) and MGAT activity (●) were measured.

Fig. 2. SDS-polyacrylamide gel electrophoresis of MGAT from rat intestinal mucosa protein obtained from CM-Sephadex (approximately 37 µg) (lanes 1 and 4) and anti-IgG Sepharose (approximately 3.8 µg) (lanes 2 and 3). The gel was stained with Coomassie Brilliant Blue R-250 (lanes 1, 2, and 3) and analyzed by immunoblotting with anti-IgG against rat pancreatic lipase (lanes 4 and 5).

Fig. 3. Effect of the rat pancreatic lipase antibody on the MGAT activity of purified rat intestinal MGAT. The purified MGAT (1.2 µg) was mixed with various amounts of anti-IgG against rat pancreatic lipase. After overnight incubation at 4 °C, the mixture was centrifuged to remove insoluble material. Residual MGAT activity was then measured.
DISCUSSION

In this paper, we purified MGAT from rat intestinal mucosa and found that its activity did not depend on coenzyme A and ATP. The purified enzyme might be identical with rat pancreatic lipase, as concluded from the following observations: (a) the purified enzyme had MGAT activity and triacylglycerol- and diacylglycerol-hydrolyzing activities; (b) antibody against rat pancreatic lipase inhibited the activity of MGAT prepared from rat intestinal mucosa (Fig. 3); (c) the band of purified enzyme on SDS-polyacrylamide gel electrophoresis was identical with that of rat pancreatic lipase, and it was detected by

![Image](https://example.com/image1.png)

**Fig. 4.** Effect of substrate concentration on MGAT activities. Purified rat intestinal MGAT was incubated with various amounts of oleic acid and 2-monoooleoylglycerol. A and B show plots of reaction rate versus substrate concentration with substrate of oleic acid and monoooleoylglycerol, respectively. In A, the monoooleoylglycerol concentration was 0.5 mM, and, in B, the oleic acid concentration was 0.4 mM.

**TABLE III**

| Fatty acid  | Activity (nmol/mg/min) |
|------------|------------------------|
| Oleic acid | 2720 ± 53              |
| Stearic acid | 334 ± 9.0             |
| Palmitic acid | 337 ± 5.0          |
| Myristic acid | 789 ± 23             |
| Lauric acid | 273 ± 13              |
| Capric acid | 166 ± 12              |
| Caprylic acid | 206 ± 13            |
| Caproic acid | 0.00 ± 0.0           |

**TABLE IV**

| Monoacylglycerol | MGAT activity (nmol/mg/min) |
|------------------|-----------------------------|
| 1-Monooleoylglycerol | 816 ± 2.0                   |
| 2-Monooleoylglycerol | 2870 ± 20                  |
| 1-Monopalmitoylglycerol | 216 ± 2.3                 |
| 2-Monopalmitoylglycerol | 1200 ± 14                 |
| 1-Monooleoylglycerol ether | 479 ± 13                |
| 2-Monooleoylglycerol ether | 453 ± 20                 |

**FIG. 5.** Photomicrograph of immunohistochemically stained section prepared using anti-pancreatic lipase IgG in intestinal absorptive cells of rats treated with antibody against rat pancreatic lipase (A) and control IgG (B). Magnification, × 780.
immunoblotting analysis using antibody against rat pancreatic lipase (Fig. 2); (d) immunoreactive protein against rat pancreatic lipase was distributed within intestinal cells (Fig. 5).

We have previously reported that lipase(s) and esterase(s) synthesize fatty acid esters. Carbonylrease from rat adipose tissue can synthesize fatty acid ethyl esters from free fatty acid and ethanol (7). Lipoprotein lipase and carboxyl ester lipase also synthesize their esters from fatty acid and ethanol (8, 9). The mechanism of fatty acid ethyl ester formation by the enzyme is as follows. When the enzyme was incubated with fatty acid and ethanol, an acyl-enzyme intermediate was formed and fatty acid ethyl ester was synthesized though nucleophilic displacement by ethanol. Exchange of fatty acid carbonyl oxygens with water oxygens was demonstrated by Muderhwa et al. (18) using 14O water. They suggested that fatty acids are good "substrates" for lipase(s). Mechanistic studies indicated that reactions catalyzed by the enzyme proceed via an acyl-enzyme intermediate. The primary structure of these enzymes contains Gly-Xaa-Ser-Xaa-Gly, which is the common active site sequence of a serine enzyme (19). The catalytic mechanism of these enzymes resembles that of serine proteinase catalysis, which proceeds via an acyl-enzyme intermediate. Pancreatic lipase is also a serine enzyme (20). It catalyzes the synthesis of fatty acid ethyl ester from fatty acid and ethanol (21). In this paper, we demonstrated that pancreatic lipase catalyzed the synthesis of diacylglycerol from free fatty acid and 2-monoacylglycerol. In this reaction, it was supposed that diacylglycerol was also synthesized through nucleophilic displacement by monoacylglycerol via an acyl-enzyme intermediate.

The lipase(s) is reported to synthesize some esters in organic or alcohol media. Porcine pancreatic lipase catalyzes transesterification between tributylglycerol and various primary and secondary alcohols in a 99% organic medium (22). Microbial lipase(s) also synthesize oleo-glycerols from oleic acid and glycerol in an 80% glycerol medium (23). Proteases also catalyze the synthesis of peptide bonds in organic media (24). These serine enzymes are used industrially for ester or peptide synthesis in organic media. In an organic medium, which contains a very low concentration of water, the enzyme also forms an acyl-enzyme intermediate, and the esters are easily synthesized by nucleophilic displacement by alcohols instead of water. In other word, "alcoholysis" rather than "hydrolisis" of the acyl-enzyme intermediate takes place. Usually, the reaction site of lipase is the lipid-water interface, such as the plasma membrane or lipoprotein surface in an aqueous medium. Alcohols such as ethanol or monoacylglycerol might more easily approach the lipid-water interface than water because alcohols are more hydrophobic than water. Therefore, lipase(s) might be able to synthesize the esters in an aqueous medium at the lipid-water interface. In this study, we demonstrated that pancreatic lipase catalyzes diacylglycerol synthesis in an aqueous medium.

Dietary fat (triacylglycerol) is hydrolyzed by pancreatic lipase to 2-monoacylglycerols and free fatty acids prior to its absorption by the intestinal villus cells. In enterocytes, these products enter the monoacylglycerol pathway, the predominant route for acylglycerol biosynthesis in the absorptive cells. The sequential intracellular re-esterification of 2-monoacylglycerol to triacylglycerol is catalyzed by a triacylglycerol synthetase complex (25, 26), which distributes intestinal microsomes and consists of MGAT, diacylglycerol acyltransferase, and acyl-coenzyme A synthetase. MGAT (monoglyceride acyltransferase, Enzyme Nomenclature EC 2.3.1.22) catalyzes the synthesis of 1,2-diacylglycerols from 2 monoacylglycerols and long chain fatty acid coenzymes A. High levels of activity were observed in intestinal mucosa and liver microsomes of suckling rats (4, 5). In the present study, we demonstrated that pancreatic lipase might be distributed within intestinal cells and that it had MGAT activity independently of coenzyme A and ATP. The specific MGAT activity (3.21 μmol/mg of protein/min) of pancreatic lipase was about three times that of purified MGAT from rat liver (1.22 μmol/mg of protein/min) (27). The presence of ester-synthesizing activity as a reverse catalytic activity of lipase is well known. The specific activity of the forward reaction (diacylglycerol hydrolysis, 43.5 μmol/mg of protein/min) was about 14 times that of the reverse reaction (MGAT activity). However, the diacylglycerol-hydroyzing activity of the lipase was inhibited by addition of intestinal mucosa extract. Indeed, when free fatty acid and 2-monooleoylglycerol were incubated with rat intestinal mucosa extract, diacylglycerols were formed to an extent depending on dose and time. These activities were not activated by the addition of ATP and coenzyme A. Furthermore, these activities were inhibited in intestinal mucosa extract by addition of the antibody against rat pancreatic lipase.

Bosner et al. (28) demonstrated that human pancreatic lipase bound to intestinal cell membranes and modulated the uptake of fatty acid derived from triacylglycerols cleaved by the lipase. They also suggested that the binding site was composed of a heparin which is a component of the brush-border membrane. However, MGAT activity in the intestinal mucosa was not changed by washing it with heparin (100 units/ml) or high concentrations of NaCl (2 M) (data not shown). Furthermore, in an immunocytochemical study using antibody against rat pancreatic lipase, immunoreactive product was not observed on the microvillar surface (Fig. 5), although it was observed within absorptive cells. Similar distribution was observed for another pancreatic enzyme, cholesterol esterase. Gallo et al. (29, 30) demonstrated that "intestinal" cholesterol esterase was of pancreatic origin and was localized within the absorptive cells. They proposed two roles for pancreatic cholesterol esterase in the process of adsorption of dietary cholesterol in the intestine: catalysis of the hydrolysis of dietary cholesterol ester and maybe the catalysis of cholesterol ester re-esterification after being taken up by the villus cell. In this study, we propose two roles for pancreatic lipase in the process of dietary fat adsorption, like a cholesterol esterase; the digestion of dietary fats to 2-monoacylglycerols and fatty acids in the intestinal lumen, and possibly the catalysis of diacylglycerol resynthesis from 2-monoacylglycerol and free fatty acid in the intestinal absorptive cells.

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