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Purification and Characterization of Diacylglycerol Acyltransferase from the Lipid Body Fraction of an Oleaginous Fungus

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Diacylglycerol acyltransferase (DGAT) [EC 2.3.1.20] was purified to apparent homogeneity from the lipid body fraction of an oleaginous fungus, *Mortierella ramanniana* var. *angulispora*. The enzyme was solubilized from the lipid body fraction with 0.1% Triton X-100, and purified by subsequent column chromatography on Yellow 86 agarose, Superdex-200, Heparin-Sepharose, second Superdex-200, and second Yellow 86 agarose. The enzyme activity was finally enriched 4,802-fold over that of the starting 1,500×*g* supernatant. The apparent molecular mass of the enzyme was 53 kDa on SDS-polyacrylamide gel electrophoresis. The purified enzyme did not exhibit glycerol-3-phosphate acyltransferase, lysophosphatidic acid acyltransferase, lipase, transacylase, or acyl-CoA hydrolase activities, although 2-monoolein was acylated with about a half of the enzyme activity toward 1,2-diolein. The purified DGAT depended on exogenous *sn*-1,2-diolein and oleoyl-CoA, with the highest activity at about 200 and 20 μM, respectively. Purified DGAT utilized a broad range of molecular species of both diacylglycerol and acyl-CoA as substrates. The highest activity was observed with *sn*-1,2-diolein and lauroyl-CoA. Anionic phospholipids such as phosphatidic acid (PA) activated the purified enzyme, as found for the Triton X-100 extract. Sphingosine dose-dependently inhibited DGAT activity activated by PA and basal activity without PA. These results provide a basis for further studies on the molecular mechanism of triacylglycerol biosynthesis and lipid body formation, in which DGAT plays an important role.

Key words: acyl-CoA, diacylglycerol, diacylglycerol acyltransferase, fungi, phosphatidic acid.

Diacylglycerol acyltransferase (DGAT) [EC 2.3.1.20] catalyzes the final step in triacylglycerol (TG) biosynthesis, which utilizes diacylglycerol (DG) as a substrate in competition with phospholipid biosynthesis *via* the Kennedy pathway. TG and phospholipids thus formed have different physicochemical properties, which could affect intracellular membrane biogenesis. In addition, the fatty acid compositions of these lipids are well maintained, partly due perhaps to the substrate specificities of DGAT and DG-utilizing enzymes, as well as deacylation-reacylation of these lipids. TG is actively synthesized and accumulated in specific types of cells such as hepatocytes, adipocytes, plant seeds, and oleaginous fungi (*1-4*). DGAT is assumed to be involved in the specific increase in TG accumulation in these cells, and manipulation of DGAT could regulate the quality and quantity of TG, which may provide insights into TG disorders in mammalian cells, and allow the accumulation of designed oils in plant seeds and oleaginous fungi. In spite of the potential roles of DGAT, however, its molecular characteristics remain unclear. Only a few reports have described its purification (*5-7*), and the purified fraction contained several peptides, among which the catalytic subunit remains to be identified.

Mortierella ramanniana var. *angulispora* was found to rapidly accumulate lipids, especially TG. Since TG contains relatively large amounts of γ-linolenic acid, this fungus is efficient for producing γ-linolenic acid, an essential fatty acid, by fermentation (*8-10*). Such oleaginous fungi are expected to exhibit amplified expression of specific mechanisms for TG biosynthesis and TG accumulation in lipid bodies. Taking advantage of this oleaginicity, we have characterized DGAT in this fungus to elucidate the mechanisms of TG biosynthesis and accumulation. We found that much higher DGAT activity was detected in the lipid body fraction than in the membrane fraction, and that the properties of DGAT in the lipid body fraction and membrane fraction are apparently similar (*11, 12*). We also found that DGAT was activated by anionic phospholipids such as phosphatidic acid (PA) and phosphatidylserine (PS) (*13*), which provides new insight into DGAT regulation and into the requirements of a DGAT assay system after detergent solubilization. In the present study, we purified DGAT to apparent homogeneity from the lipid body fraction of the fungus and characterized the purified enzyme.

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Abbreviations: DG, diacylglycerol; DGAT, diacylglycerol acyltransferase; PA, phosphatidic acid; PAGE, polyacrylamide gel electrophoresis; PS, phosphatidylserine; TG, triacylglycerol.

EXPERIMENTAL PROCEDURES

Materials—[1-^{14}C]Oleoyl-CoA (58 mCi/mmol), [1-^{14}C]-palmitoyl-CoA (48 mCi/mmol), 1-palmitoyl, 2-[1-^{14}C]-oleoyl-phosphatidylcholine (58 mCi/mmol), and [1-^{14}C]-dioleoyl-phosphatidylcholine (114 mCi/mmol) were obtained from New England Nuclear (Boston, MA). [1-^{14}C]-Oleic acid (55 mCi/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). *sn*-1,2-[1-^{14}C]Diolein was prepared by incubation of [1-^{14}C]dioleoyl-phosphatidylcholine with phospholipase C (5 U) from *Bacillus cereus* for 30 min at 30°C. The product was purified by TLC and concentrated at 25°C. Acyl-CoAs, *sn*-1,2-diolein, 1,3-diolein, oleyl-alcohol, *sn*-PA (from egg lecithin), *sn*-glycerol-3-phosphate, *sn*-oleoyl-glycerol-3-phosphate (lysophosphatidic acid), D-sphingosine, trifluoperazine, phospholipase C (from *B. cereus*), and reactive Yellow 86 agarose were purchased from Sigma (St. Louis, MO). *sn*-1,2-Diacylglycerols, 2-monoolein, triolein, phosphatidylcholine (pig liver), phosphatidylethanolamine (pig liver), PS (beef brain), phosphatidylinositol (pig liver), cardiolipin (beef heart), lysophosphatidylcholine (pig liver), and lysophosphatidylserine (beef brain) were from Serdary Research Laboratories (Ontario, Canada). HiLoad 26/60 Superdex 200 pg and Heparin Sepharose CL-6B were obtained from Pharmacia (Uppsala, Sweden). Triton X-100 was from Nacalai Tesque (Kyoto). Silica gel G TLC plates were obtained from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

DGAT Activity Assay—DGAT activity was measured under two slightly different conditions. In subcellular fractions, the reaction mixture contained 10 mM phosphate buffer (pH 7.0), 0.15 M KCl, 3.4 μM (0.2 μCi/ml) [1-^{14}C]oleoyl-CoA, 1.5 mM *sn*-1,2-diolein, 0.1% Triton X-100, and enzyme source in a final volume of 100 μl as described elsewhere (*12*). In fractions after Triton X-100 solubilization, the reaction mixture contained 10 mM phosphate buffer (pH 7.0), 0.15 M KCl, 3.4 μM (0.2 μCi/ml) [1-^{14}C]oleoyl-CoA, 0.5 mM *sn*-1,2-diolein, 0.5 mM PA, 0.2% Triton X-100, and enzyme source in a final volume of 100 μl, which was optimized based on previous results (*13*). The reaction was carried out at 30°C for 5 min and DGAT activity was obtained from radioactivity incorporated in TG as previously described (*11–13*). In the experiment to study acyl-CoA specificity, 21.9 μM (about 85,000 dpm/2.19 nmol in 100 μl; specific activity was adjusted by adding unlabeled *sn*-1,2-diolein) *sn*-1,2-[1-^{14}C]diolein and 100 μM acyl-CoA were used under the same conditions as described above. Concentrations of *sn*-1,2-[1-^{14}C]diolein and acyl-CoA were determined to optimize ^{14}C incorporation into TG. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product/min.

DGAT Purification—All purification steps were conducted at 4°C. All column procedures were carried out using a Pharmacia FPLC system. *Mortierella ramanniana* var. *angulispora* (IFO 8187) was obtained and cultured as described elsewhere (*11*). Fungal cells cultured for 4 days were homogenized with a Braun homogenizer (Melsungen, Germany) and the lipid body fraction was prepared as described (*12*). The lipid body fraction was solubilized with 10 mM phosphate buffer (pH 7.0), 0.15 M KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 0.1% Triton X-100 for 1 h at 4°C, and the Triton X-100 extract was obtained by sucrose density gradient centrifugation as described (*13*). The solubilized fraction was filtered through a 0.22 μm filter unit.

The Triton X-100 concentration of the extract was then adjusted to 1.5% and the mixture was applied to a reactive Yellow 86 agarose column (1 × 15 cm) preequilibrated with buffer A [10 mM phosphate buffer (pH 7.0), 0.15 M KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10% ethyleneglycol, and 0.1% Triton X-100]. The column was washed at a flow rate of 0.3 ml/min with buffer A, then eluted with a linear gradient of 0.15–0.5 M KCl in buffer A. Fractions with enzyme activity were combined and concentrated in a Centriprep-10 (Amicon). The concentrate was applied to a HiLoad 26/60 Superdex 200 pg gel filtration column preequilibrated with buffer A, and eluted with the same buffer at a flow rate of 2 ml/min. Fractions with enzyme activity from the gel filtration column were applied to a Heparin Sepharose CL-6B column (0.7 cm × 5 cm) pre-equilibrated with buffer A. The column was washed with buffer A at a flow rate of 0.2 ml/min, then eluted with a linear gradient of 0.15–0.5 M KCl in buffer A. Fractions with enzyme activity were combined and concentrated in a Centriprep-10. The concentrate was applied to a HiLoad 26/60 Superdex 200 pg column preequilibrated with buffer A, and eluted with the same buffer at a flow rate of 2 ml/min. Finally, fractions with enzyme activity in the second gel filtration column were applied to a reactive Yellow 86 agarose column (1 × 15 cm) preequilibrated with buffer A. The column was washed with buffer A at a flow rate of 0.3 ml/min, then eluted with a linear gradient of 0.15–0.5 M KCl in buffer A. Enzyme preparations were always stored at −80°C between column procedures. Freeze-thawing of preparations appeared to have little effect on the activity. The final preparation was also stored at −80°C until use. Under this condition, the enzyme retained around 80% of the activity for three months.

Other Analytical Methods—Electrophoresis was performed using 10–20% gradient gels (Bio Rad) based on the method of Laemmli (*14*), and protein bands were detected with a silver staining kit (Pharmacia). Protein was measured using the method of Bradford (*15*) in the presence of 0.05 N NaOH. Phospholipid concentrations were determined by phosphorus analysis as described (*16*).

RESULTS

DGAT Purification—Previous studies showed that DGAT activity in the lipid body fraction could be solubilized with Triton X-100 (*13*). We further examined the effect of Triton X-100 concentration on DGAT solubilization. We found that 0.1% Triton X-100 gave the highest specific DGAT activity in the extract (data not shown), so we chose this condition for further purification of DGAT. The extract was further centrifuged at 100,000 × *g* or filtered through a 0.22 μm filter unit. DGAT activity was not significantly decreased in the supernatant of centrifugation or the filtrate, confirming that DGAT activity in the extract was well solubilized.

The 0.1% Triton X-100 extract was further purified as detailed in "EXPERIMENTAL PROCEDURES." Column chromatography profiles for DGAT purification (Fig. 1, A–D)

showed that DGAT activity in the 0.1% Triton X-100 extract was bound to reactive Yellow 86 agarose when the Triton X-100 concentration of the extract was increased before application to the column (Fig. 1A). Unless Triton X-100 was added to the extract, most of the DGAT activity was not bound to the column. DGAT activity once bound to the column was not eluted in the presence of buffer A containing 0.1% Triton X-100. In contrast, DGAT activity after the second Superdex 200 chromatography was bound to Yellow 86 agarose in the presence of 0.1% Triton X-100 (Fig. 1D). The difference probably arose from the difference in constituents in the two samples; approximately 0.5 M sucrose was included in the 0.1% Triton X-100 extract instead of 10% ethylenglycol in buffer A. It should be noted that DGAT activity in the second Yellow 86 agarose chromatography reproducibly eluted at a slightly higher KCl concentration than in the first Yellow 86 agarose chromatography, possibly due to the absence of reagent(s) interfering with the interaction between DGAT and the column.

DGAT purification procedure is summarized in Table I. DGAT activity was purified 4,802-fold from the 1,500×*g* supernatant, with a yield of 0.92%. Although slightly different assay conditions for DGAT had to be used for samples before and after Triton X-100 solubilization, the above values based on DGAT activity in the 1,500×*g*

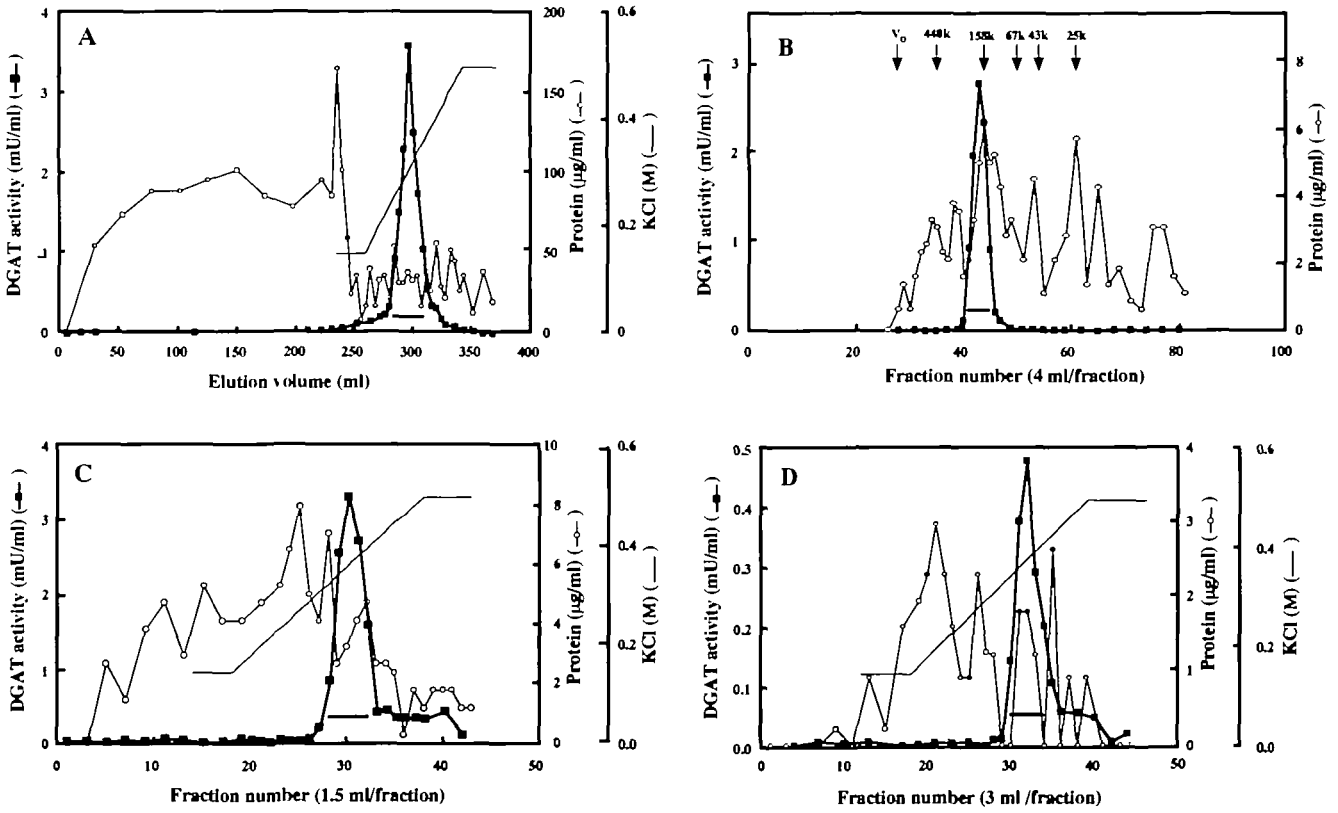

Fig. 1. **Purification of solubilized DGAT by column chromatography.** A, Yellow 86 agarose; B, Superdex-200; C, Heparin-Sepharose; D, second Yellow 86 agarose. Experimental details are given under "EXPERIMENTAL PROCEDURES." DGAT activity (■) and protein concentration (○) were determined as described under "EXPERIMENTAL PROCEDURES." The solid lines represent the concentration of KCl. The fractions indicated by the bars were used for further purification (A-C), or as the final preparation (D). Arrows in B indicate the elution positions of blue dextran (V_o), ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa).

TABLE I. **DGAT purification from the lipid body fraction of *Mortierella ramanniana* var. *angulispora*.**

Purification step	Protein (mg)	Total activity* (milliunits)	Specific activity (milliunits/mg)	Yield (%)	Purification (fold)
1,500×*g* supernatant	1,148	793	0.69	100	1
Lipid body fraction	81	651	8.04	82.1	12
Triton X-100 extract	16.5	221	13.4	27.9	19
Yellow 86 agarose	0.42	62.3	148.3	7.86	215
Superdex 200	0.098	26.4	269.4	3.33	390
Heparin-Sepharose	0.035	16.0	457.1	2.02	662
Second Superdex 200	0.012	8.97	747.5	1.13	1,083
Second Yellow 86 agarose	0.0022	7.29	3,313.6	0.92	4,802

*DGAT activities in the 1,500×*g* supernatant and lipid body fraction were measured with 1,500 μM 1,2-diolein in the presence of 0.1% Triton X-100, whereas DGAT activities in other fractions were measured with 500 μM 1,2-diolein in the presence of 500 μM phosphatidic acid and 0.2% Triton X-100.

supernatant roughly represent the degree of purification. The polypeptide profile of DGAT fractions at different steps of purification was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2). Silver staining showed that the final preparation contained a single protein of 53 kDa, which had been concentrated during the purification procedure. Nevertheless, several attempts to detect DGAT activity associated with the 53-kDa peptide after SDS-PAGE separation have been unsuccessful, probably because DGAT was irreversibly denatured by SDS treatment. The native molecular mass of DGAT in Triton X-100 micelles as judged from Superdex 200 gel filtration was 174 kDa [Fig. 1B; DGAT was also eluted at the same fraction in the second Superdex 200 column (data not shown)], whereas excess Triton X-100 micelles had an apparent molecular mass of 139 kDa under the same elution conditions. Simple subtraction of the molecular mass of DGAT from that of Triton X-100 micelles gave 35 kDa, which might be the native molecular mass of DGAT itself.

Characterization of Purified DGAT—It was confirmed by gel filtration that Triton X-100, *sn*-1,2-diolein, and PA in the DGAT assay for the purified enzyme formed uniform Triton X-100 mixed micelles (data not shown). The optimum pH of purified DGAT was 7.0, the same as that of the crude enzyme. Purified DGAT activity was unstable to elevated temperature; preincubation of the enzyme preparation at 40°C for 30 min completely abolished the activity. Purified DGAT activity depended on exogenous *sn*-1,2-diolein and oleoyl-CoA, with the highest activity at about 200 and 20 μM, respectively (Fig. 3). Concentration dependence curves of the substrates did not fit well with either Michaelis-Menten or cooperative kinetics. Since DG up to 500 μM formed uniform mixed micelles with Triton X-100 and PA in the reaction mixture, the unusual substrate dependence curves were not due to the formation of undefined DG structure. Higher concentrations of both substrates inhibited DGAT activity.

Table II shows the acyl-acceptor specificity of purified DGAT. 1,3-Diolein and *rac*-1,2-diolein were utilized similarly to *sn*-1,2-diolein. Since isomerization of 1,2-diolein did not occur during the DGAT assay, 1,3-diolein could be directly used by DGAT. DGAT also transferred an acyl-moiety to 2-monoolein. DGAT did not use oleyl-alcohol, *sn*-glycerol-3-phosphate, or lysophosphatidic acid as a

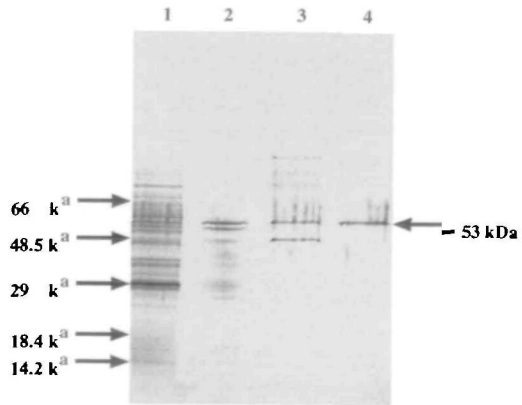

Fig. 2. **SDS-PAGE at various steps of DGAT purification.** Samples from different steps of DGAT purification were subjected to SDS-PAGE under reducing conditions using a 10-20% gradient gel, followed by silver staining. Molecular mass standards (Sigma) are indicated by arrows at the left. Lane 1, 0.1% Triton X-100 extract (0.1 μg protein); lane 2, DGAT fraction of Yellow 86 agarose (0.1 μg protein); lane 3, DGAT fraction of Heparin-Sepharose (0.1 μg protein); lane 4, purified DGAT obtained by second Yellow 86 agarose (0.05 μg protein).

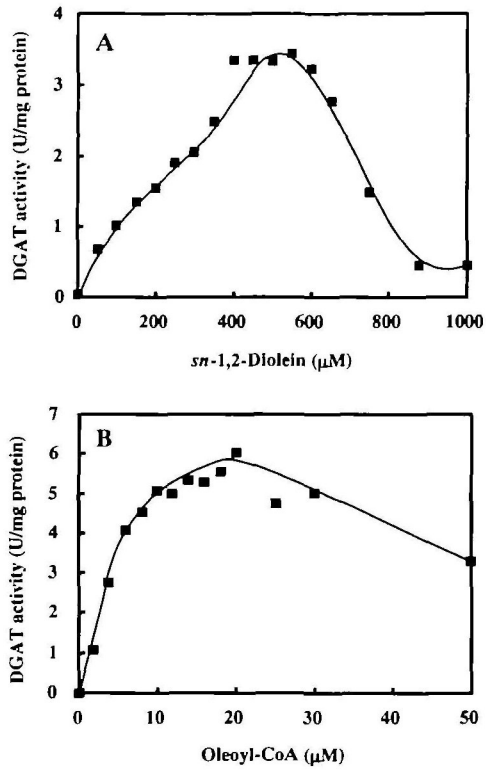

Fig. 3. **Dependence of purified DGAT on *sn*-1,2-diolein and oleoyl-CoA concentrations.** Purified DGAT (1.2 ng protein) was assayed with 3.4 μM [1-^{14}C]oleoyl-CoA at different *sn*-1,2-diolein concentrations (A) or with 500 μM *sn*-1,2-diolein at different [1-^{14}C]-oleoyl-CoA concentrations (B). Assay conditions are described under "EXPERIMENTAL PROCEDURES." Data are presented as means of duplicates.

TABLE II. **Acyl-acceptor specificity of purified DGAT.** Purified DGAT (1.2 ng protein) was assayed with 3.4 μM [1-^{14}C]oleoyl-CoA and 500 μM acyl acceptor. Assay conditions are described under "EXPERIMENTAL PROCEDURES." The enzyme activity using *sn*-1,2-diolein as a substrate was used as the control. Data are presented as means of triplicates ± SD.

Acyl acceptor (500 μM)	Acyl transfer from [^{14}C]oleoyl-CoA[a] (% of control)
sn-1,2-Diolein	100 ± 4.8
rac-1,2-Diolein	116 ±10
1,3-Diolein	98.7± 6.6
2-Monoolein	45.8± 2.9
Oleyl alcohol	0
sn-Glycerol-3-phosphate	0
Lysophosphatidic acid	0
Lysophosphatidylcholine	4.2± 1.2
H_2O (acyl hydrolase activity)[b]	0

[a]Incorporations of [^{14}C]oleoyl moiety into diacylglycerol and triacylglycerol from 2-monoolein, lysophosphatidic acid and phosphatidic acid from glycerol-3-phosphate, phosphatidic acid from lysophosphatidic acid, phosphatidylcholine from lysophosphatidylcholine, and free fatty acid from H_2O were measured. [b]No acceptor lipids were added to the assay mixture.

substrate, indicating that the enzyme is distinct from esterases and the other two acyltransferases for *de novo* TG biosynthesis. Finally, the enzyme did not produce oleic acid from oleoyl-CoA, with or without acyl-acceptors, which meant that acyl-CoA hydrolysis was not separable from acyl group transfer under assay conditions. The purified DGAT showed specificity toward *sn*-1,2-DG; unsaturated or shorter chain fatty acids were more readily utilized (Fig. 4). Its behavior was quite similar whether oleoyl-CoA or palmitoyl-CoA was used as an acyl donor.

Acyl-CoA derivatives were the only acyl donors for the enzyme as far as we examined; the enzyme did not acylate *sn*-1,2-DG with 1-palmitoyl, 2-oleoyl-phosphatidylcholine, 1,2-diolein, or oleic acid as an acyl donor (data not shown). This indicates that the enzyme is distinct from transacylases or lipases. The enzyme showed a broad specificity toward acyl-CoA derivatives; medium-chain acyl-CoA such as lauroyl-CoA and long-chain acyl-CoA were actively used, although short-chain acyl-CoA such as acetyl-CoA and butyryl-CoA were not incorporated by the enzyme (Fig. 5).

The purified DGAT was activated by PA (Fig. 6), in a similar manner to the crude preparations of DGAT as described before (*13*). It should be noted that small basal DGAT activity was seen reproducibly without exogenous PA. It was not clear whether the basal DGAT activity was due to trace amounts of PA bound to the purified DGAT or DGAT activity independent of PA. The activation by PA was dose-dependently antagonized by sphingosine. Sphingosine at 500 μM completely inhibited DGAT activity at lower concentrations of PA, including the basal activity independent of PA. The effect of other lipids on purified DGAT was basically the same as that on crude preparations

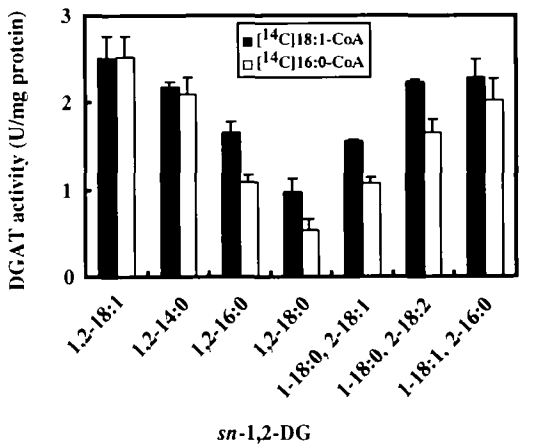

Fig. 4. **Dependence of purified DGAT activity on *sn*-1,2-DG molecular species.** Different *sn*-1,2-DGs (500 μM) were added to the assay mixture for purified DGAT (1.2 ng protein) with 3.4 μM [1-^{14}C]oleoyl-CoA or 3.4 μM [1-^{14}C]palmitoyl-CoA. Assay conditions are described under "EXPERIMENTAL PROCEDURES." Data are presented as means of triplicates ± SD.

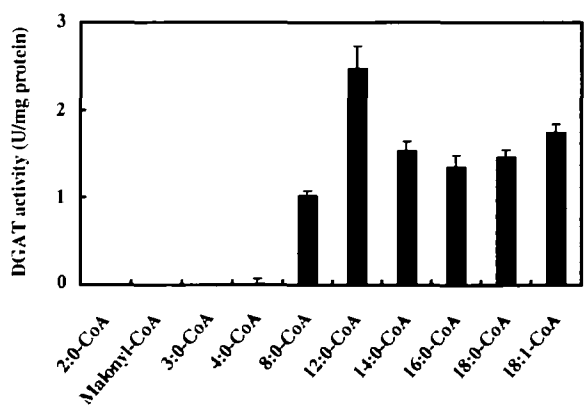

Fig. 5. **Dependence of purified DGAT activity on acyl-CoAs.** Different acyl-CoAs (100 μM) were added to the assay mixture for purified DGAT (2.4 ng protein) with 21.9 μM *sn*-1,2-[1-^{14}C]diolein. Assay conditions are described under "EXPERIMENTAL PROCEDURES." Data are presented as means of triplicates ± SD.

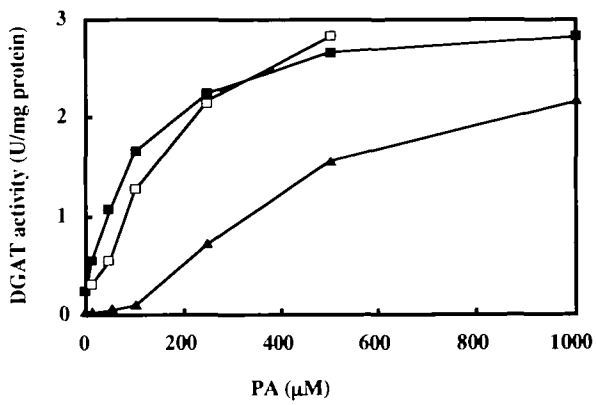

Fig. 6. **Effects of PA and sphingosine on purified DGAT activity.** Purified DGAT activity (1.2 ng protein) was assayed with 3.4 μM [1-^{14}C]oleoyl-CoA and 500 μM *sn*-1,2-diolein at different PA concentrations in the absence of sphingosine (■) or in the presence of 100 μM (□) or 500 μM (▲) sphingosine. Assay conditions are described under "EXPERIMENTAL PROCEDURES." Data are presented as means of duplicates.

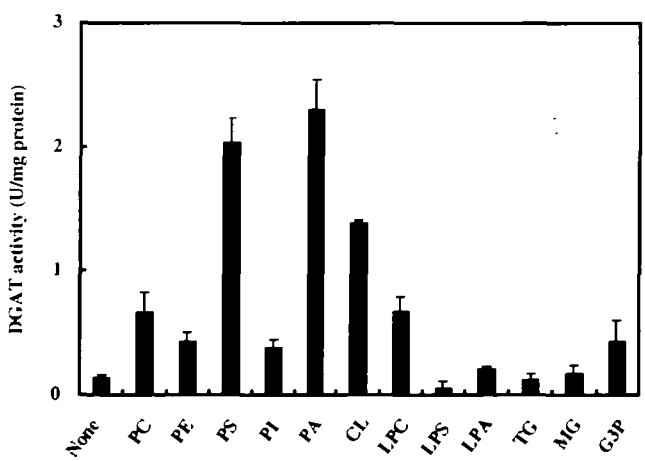

Fig. 7. **Effects of various lipids on purified DGAT activity.** Different lipids (500 μM) were added to the assay mixture for purified DGAT (1.2 ng protein) with 3.4 μM [1-^{14}C]oleoyl-CoA and 500 μM *sn*-1,2-diolein. Assay conditions are described under "EXPERIMENTAL PROCEDURES." Data are presented as means of triplicates ± SD. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; CL, cardiolipin; LPC, lysophosphatidylcholine; LPS, lysophosphatidylserine; LPA, lysophosphatidic acid; TG, triacylglycerol; MG, monoacylglycerol; G3P, glycerol-3-phosphate.

TABLE III. **Effect of reagents on purified DGAT.** Purified DGAT (1.2 ng protein) was assayed with 3.4 μM [1-^{14}C]oleoyl-CoA and 500 μM *sn*-1,2-diolein in the presence of various reagents. Assay conditions are described under "EXPERIMENTAL PROCEDURES." Data are presented as means of triplicates ± SD.

Addition	DGAT activity (% of control)
$MgCl_2$ (5 mM)	47±1
$CaCl_2$ (2 mM)	33±7
KCl (500 mM)	40±3
EDTA (5 mM)	100±7
N-Ethylmaleimide (1 mM)	102±4
Dithiothreitol (1 mM)	113±6
NaF (50 mM)	106±4
Trifluoperazine (500 μM)	115±4
Control (in the presence of 100 μM PA)	70±10
Trifluoperazine (500 μM) (in the presence of 100 μM PA)	37±4

of DGAT as described elsewhere (*13*). PS and cardiolipin activated DGAT more highly, whereas phosphatidylcholine and phosphatidylethanolamine caused a moderate increase in DGAT activity (Fig. 7). The activation of purified DGAT by most lipids was higher than in crude preparations. PA was the most potent activator, and activated DGAT around 16-fold.

Metal ions such as Mg^{2+} and Ca^{2+} inhibited purified DGAT activity (Table III), whereas these metal ions did not affect DGAT activity in the lipid body fraction (*12*). This may be due to different assay conditions; PA was added in the assay for purified DGAT, but not in the assay for DGAT in the lipid body fraction. In addition, KCl at 500 mM inhibited purified DGAT. Other reagents had little effect on purified DGAT, as in the case of crude DGAT. Trifluoperazine, structurally and functionally similar to sphingosine (*17*), did not inhibit the enzyme at the optimal concentration of PA, but inhibited it at the suboptimal concentration of PA (Table III). Thus, the effect of trifluoperazine on the enzyme seems to be similar to that of sphingosine, although trifluoperazine was less potent.

DISCUSSION

DGAT was purified to apparent homogeneity from the lipid body fraction of an oleaginous fungus, *Mortierella ramanniana* var. *angulispora*. SDS-PAGE of the purified preparation revealed a single band of 53 kDa. As far as we know, this is the first time that a polypeptide having DGAT activity has been described. It is also in contrast to previous reports suggesting a DGAT subunit structure (*5-7*). None of the peptides reported previously as subunits for DGAT (*5-7*) resemble the present one in molecular mass. Thus, fungal DGAT might be a different type from those in mammalian tissues and higher plants. Photoaffinity labeling studies showed a 54 kDa peptide and 3 other peptides as acyl CoA binding proteins in mammalian microsomes (*18*), and 53 and 69 kDa peptides were suggested as DGAT components in adipose cells from studies of ATP-dependent inactivation of DGAT (*19*). The peptides whose molecular mass was about 53 kDa may correspond to DGAT in the present study. The native molecular mass of DGAT was calculated as 35 kDa on the basis of gel filtration chromatography, lower than the value of 53 kDa determined by SDS-PAGE. We speculate that the native molecular mass may have been underestimated due to embedding of the enzyme in Triton X-100 micelles.

Acyl-CoA acyltransferase was recently purified and its role as a reservoir for the TG synthetase complex in rat intestinal mucosa has been proposed (*20, 21*). Although the molecular mass of 54 kDa is similar, the purified DGAT preparation did not have acyl-CoA hydrolase activity in the absence of acyl acceptors (Table II), indicating that DGAT in this fungus does not work as an acyl-CoA reservoir for other acyltransferases. Furthermore, the purified DGAT did not have lipase or transacylase activity, which could also contribute to TG biosynthesis (*22*). On the other hand, purified DGAT acylated monoacylglycerol to form DG and TG. Whether purified DGAT is involved in the monoacylglycerol pathway to TG biosynthesis as well as the Kennedy pathway is of interest. It was suggested that one DGAT in rat intestine used DG derived from both PA and monoacylglycerol and was separable from monoacylglycerol acyltransferase (*23*).

Substrate specificities of DGAT have been studied to elucidate the mechanism of fatty acid distribution of TG and to provide a strategy for TG manipulation, especially in plant oilseeds (*24*). Purified DGAT in the present study had broad specificities toward both DG and acyl-CoA. The results are consistent with a broad DG specificity of DGAT in mammalian cells (*25, 26*) or higher plants (*27, 28*), and a broad acyl-CoA specificity of DGAT in mammalian cells (*25, 26, 29, 30*) or higher plants (*31, 32*). The present study also indicates that purified DGAT favors fluid DG containing more unsaturated, shorter fatty acids among tested DGs. Although it was pointed out that dipalmitin and distearin solubility affected DGAT activity (*33*), these DGs were at least visibly dissolved in the assay mixture containing 0.2% Triton X-100, and the results were quite reproducible. Another interesting point is that the purified DGAT had the highest activity toward lauroyl-CoA in spite of the absence of lauric acid in this fungus. The preference for medium-chain fatty acyl-CoA was also observed in mammalian cells (*26, 30*) or higher plants (*34*) and the specificity of DGAT was not reflected in the *sn*-3 position of TG either. The specificity of DGAT for medium-chain fatty acyl-CoA in this fungus, however, may offer potential to produce medium-chain fatty acids by genetic manipulation of this fungus. Some authors have reported DGAT activity selective for specific DGs (*35, 36*) and specific acyl-CoA (*35-38*). Mutation affecting DGAT activity could change the fatty acid composition in *Arabidopsis* (*39*). Thus, there is a possibility that DGAT in this fungus has selectivity against DG and acyl-CoA containing γ-linolenic acid, whose derivatives are not easily available and not tested yet. Further studies including more detailed enzyme kinetics such as surface dilution kinetics (*40, 41*) are needed.

Purified DGAT was activated by anionic phospholipids such as PA and PS (Figs. 6 and 7) as described for a crude DGAT preparation (*13*). This confirms that these anionic phospholipids directly influence the enzyme or the enzyme-substrate interaction. DGAT activation by anionic phospholipids was dose-dependently repressed by sphingosine (Fig. 6). Sphingosine is also known to inhibit monoacylglycerol acyltransferase (*42, 43*), PA phosphatase (*44*), protein kinase C (*45*), PS synthase (*46*), and CTP:phosphocholine cytidylyltransferase (*47*), all of which are activated by anionic phospholipids, although sphingosine activated

one DG kinase isozyme and inhibited another isozyme that was activated by PS (*48*). It would be interesting to evaluate further the putative recognition between enzymes and lipid substrates/cofactors involved in the signaling mechanism for TG biosynthesis or lipid body formation.

Purified DGAT was inhibited by divalent cations such as Mg^{2+} and Ca^{2+} (Table III), whereas these cations had little effect on DGAT in the lipid body fraction (*12*). The difference may be due to the presence of PA in the assay mixture (this was not included in the assay for crude DGAT). Ionic interactions between DGAT and PA may be interfered with by these divalent cations. The ionic interaction between DGAT and PA may also explain the inhibition of the enzyme by 0.5 M KCl. However, the interaction may be not merely ionic, but specific to PA, because the effects of different anionic phospholipids on DGAT differed (Fig. 7). It was also reported that the activation of monoacylglycerol acyltransferase by PA was not based only on electrostatic interactions, since the enzyme was inhibited by high salt or Ca^{2+} both in the presence and absence of PA (*43*).

DGAT activity was detected in the membrane fraction and in the lipid body fraction of this fungus (*12*). The relationship between DGAT activity in these fractions is of interest from the viewpoint of regulation of TG molecular species and lipid body formation. Since the substrate specificities of DGAT itself are not likely to determine the fatty acid composition of TG, the availability of specific DG and acyl-CoA pools to this DGAT activity might be important. The difference between DGAT activity in the two fractions is sensitivity to *N*-ethylmaleimide and dithiothreitol; DGAT activity in the membrane fraction was significantly inhibited by these reagents (*11*), whereas that in the lipid body fraction was not (*12*). The purified DGAT from the lipid body fraction was not affected by these reagents, either, indicating that the insensitivity to these reagents is inherent and not due to the enzyme environment. Further studies on the purification and biochemical characterization of DGAT from the membrane fraction and gene cloning of these DGAT(s) should reveal the structural and functional relationships of DGAT activity in the lipid body and membrane fractions. Knowledge of the structure and function of DGAT(s) would be very helpful in clarifying the mechanism of biogenesis of lipid bodies and in the manipulation of the accumulated TG.

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