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Biosynthesis of Triacylglycerol Molecular Species in an Oleaginous Fungus, *Mortierella ramanniana* var. *angulispora*

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The incorporation of radiolabeled lipid precursors into triacylglycerol (TG) molecular species in *Mortierella ramanniana* var. *angulispora*, an oleaginous fungus, was studied to determine the biosynthetic pathways for TG molecular species. Radiolabeled TG molecular species were separated and quantified by reverse-phase high performance liquid chromatography with a radioisotope detector. The major TG molecular species labeled by [1-^{14}C]oleic acid at 30°C were OOP, OOO, and OPP (TG molecular species designations represent three constituent acyl groups. G, γ-linolenic acid; L, linoleic acid; O, oleic acid; S, stearic acid; P, palmitic acid), which were abundant TG molecular species in this fungus. The incorporation of [1-^{14}C]oleic acid at 15°C into these molecular species was the same, while that into most other species was decreased, suggesting that biosynthesis of major molecular species such as OOP, OOO, and OPP differs from that of other TG molecular species. [1-^{14}C]Linoleic acid incorporation indicated that the major labeled molecular species were LOP and LOO, which may be due to acylation of oleoyl, palmitoyl-glycerol, or dioleoyl-glycerol by exogenous linoleic acid. This is basically the same mechanism as for OOP and OOO biosynthesis from exogenous oleic acid. [^{14}C(*U*)]Glycerol incorporation suggested that TG molecular species containing palmitic acid such as OPP were more readily synthesized through the *de novo* pathway. Further experiments involving inhibitors such as sodium azide and cerulenin suggested that OOO biosynthesis included a mechanism differing from that in the cases of OOP and OPP. Trifluoperazine, which inhibits the conversion from phosphatidic acid to TG, decreased [1-^{14}C]oleic acid incorporation into all molecular species, suggesting that the incorporation into all molecular species included the *de novo* TG biosynthetic pathway *via* phosphatidic acid. These results revealed that the biosynthetic pathways for TG molecular species can be classified into several groups, which exhibit different sensitivities to low temperature and inhibitors of lipid metabolism. This implies that the composition of TG molecular species is regulated through different biosynthetic pathways responsible for specific TG molecular species, providing a new insight into the biosynthesis of TG molecular species.

Key words: molecular species, oleaginous fungus, reverse-phase HPLC, triacylglycerol, triacylglycerol biosynthesis.

Triacyglycerol (TG) is the major lipid component in oleaginous cells such as those of oleaginous microorganisms, plant oil seeds, and mammalian lipid storage tissues. TG is synthesized and assembled in organelles called lipid bodies, oil bodies, and lipid droplets. The TG structure is characterized by a combination of fatty acyl residues at the *sn*-1, *sn*-2, and *sn*-3 positions, which creates numerous molecular species. The distribution of TG molecular species has been determined in a variety of cells by high performance liquid chromatography (HPLC), or more recently HPLC mass spectrometry (*1, 2*), although very little is known about their biosynthetic pathways and regulation. The combination of enzymes for newly formed fatty acids and those for acylation/deacylation reactions yielding TG determines the formation of each TG molecular species. Fatty acids are generated by fatty acid synthase, elongases, desaturases, hydrolases, *etc.* (*3–5*), and acylation reactions yielding TG are catalyzed by diacylglycerol acyltransferase, transacylases, *etc.* (*6–9*). Although some of these enzymes have been characterized in detail, the combination of these enzymes for the synthesis of each TG molecular species is unknown. In plant seeds, phosphatidylcholine (PC) provides unsaturated fatty acids for TG molecular species (*10*), and phospholipid:diacylglycerol acyltransferase was recently found to play an important role in such a mechanism (*11*). In addition, some unusual fatty acids, mainly included in TG (*5*),

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Abbreviations: DG, diacylglycerol; HPLC, high performance liquid chromatography; PC, phosphatidylcholine; TCN, theoretical carbon number; TG, triacylglycerol.

may be distributed through channeling mechanisms. In spite of the growing evidence that TG molecular species may be differentially synthesized through specific enzyme pathways, TG molecular species have not been directly quantified.

Mortierella ramanniana var. *angulispora*, an oleaginous fungus, accumulates large amounts of lipids in lipid bodies (*12, 13*). Among lipid classes, TG constitutes more than 80% of total lipids (*14*), and hence the TG biosynthetic pathway in this fungus is very active, which may make this fungus suitable for studying the mechanisms of TG biosynthesis. We have already reported the purification and characterization of TG biosynthetic enzymes (*15–17*), and visualization of the lipid body biogenesis in this fungus (*13, 18*). We found that phospholipids provided unsaturated fatty acids for TG, which was distinct from the *de novo* TG biosynthetic pathway in this fungus (*18*). The present study focused on the biosynthesis of TG molecular species, using HPLC with a radioisotope detector. The pathways for the incorporation of radioactive precursors into TG molecular species in this fungus can be classified into several groups, which exhibit different sensitivities to low temperature and inhibitors of lipid metabolism.

MATERIALS AND METHODS

Materials—[1-^{14}C]Oleic acid (52 mCi/mmol), [1-^{14}C]linoleic acid (53 mCi/mmol), and [^{14}C(*U*)]glycerol (166 mCi/mmol) were obtained from NEN Life Science Products (Boston, MA, USA). Sodium azide was obtained from Nacalai Tesque (Kyoto). Cerulenin and trifluoperazine were purchased from Sigma (St. Louis, MO, USA). Triolein, dioleoyl palmitate, dipalmitoyl oleate, and oleoyl palmitoyl linoleate were obtained from Serdary Research Laboratory Inc. (Ontario, Canada). Silica gel 60 TLC plates were obtained from Merck (Darmstadt, Germany), and KC18 reverse phase TLC plates from Whatman (Maidstone, UK). All other reagents were of analytical grade.

Strains and Cultures—M. ramanniana var. *angulispora* (IFO 8187) was obtained from the culture collection of the Institute for Fermentation (Osaka). The liquid culture medium contained glucose, inorganic salts, and vitamins as described (*13, 14*). Cultures (300 ml in a 1 liter flask) were grown on a rotary shaker (180 rpm) at 30°C for the indicated times. Aliquots of cultures (1.5 ml of 42 h culture, about 10 mg of dry cell weight) were taken from a flask and incubated with 0.2 μCi [1-^{14}C]oleic acid (2.6 μM), [1-^{14}C]-linoleic acid (2.5 μM), or [^{14}C(*U*)]glycerol (0.8 μM) at 30 or 15°C for the indicated times on a rotary shaker for microtiter plates (MBSS-500; Marubishi Bioengineering, Tokyo). After incubation, fungal cells were put on ice, washed with 10 mM phosphate buffer (pH 7.0) containing 0.15 M KCl at 4°C, and then used for lipid analysis. In some experiments, incubation was conducted in the presence of 0.01% sodium azide, 100 μM cerulenin, or 50 μM trifluoperazine.

TG Separation—Lipids were extracted by the method of Bligh and Dyer (*19*). The extracted lipids were separated into neutral and polar lipids by hexane/methanol partitioning as described (*20*) with modification. Equal amounts (2.5 ml) of hexane and methanol, and three drops of water were added to the extracted lipids. After vigorous mixing and centrifugation, the two layers were separated. The hexane layer containing TG and diacylglycerol (DG) was evaporated under N_2 and then dissolved in chloroform for injection into the HPLC.

Molecular Species Analysis of TG by HPLC—TG molecular species were separated by reverse-phase HPLC, using two solvent delivery pumps (Gilson, Middleton, WI, USA), as previously described (*21, 22*) with modification. An Inertosil ODS-2 column (5 μm, 250 mm × 6.0 mm *i.d.*, GL Sciences, Tokyo) was used for separation with a solvent system of acetonitrile/isopropanol (60:40, by volume) at a flow rate of 1.5 ml/min. Peaks were quantitatively analyzed with a light-scattering detector (Varex Co., Burtonsville, MD, USA), operated at a nebulizer nitrogen gas flow pressure of 55 psi and a drift tube temperature of 95°C. Some peaks were identified by comparison with TG standards and others assigned based on plots of retention volume vs the theoretical carbon number (TCN) (*21, 22*). Assignment was confirmed by the fatty acid composition of each peak, measured by gas chromatography as described elsewhere (*23*). For the separation of radiolabeled TG, peaks were monitored at 210 nm using a UV/VIS detector (Gilson, Middleton, WI, USA), and ^{14}C-label incorporation into TG molecular species was detected using a Ramona-90 radioisotope detector (Raytest, Strandenhardt, Germany). The radioactive peak area was converted to dpm by calculating the relationship between the total peak area and total dpm.

To investigate ^{14}C-labeled fatty acids in each TG molecular species, fractions corresponding to TG molecular species were collected after HPLC separation. Each TG molecular species was converted to fatty acid methyl esters, which were separated on KC18 reverse-phase TLC plates, and then radioactivity in the radiolabeled spots was quantified as described (*24*).

RESULTS

Separation and Quantification of TG Molecular Species—We determined the amounts of TG molecular species by reverse-phase HPLC with a light scattering detector. Although we had already measured the TG molecular species by reverse-phase HPLC with a refractive index detector, fungal cells in the stationary growth phase under different culture conditions were used (*22*). The present study involved fungal cells at the exponential phase, comparable to that for studying lipid body biogenesis (*12, 13, 16, 18*). We further optimized the molecular species separation by changing the mobile phase for HPLC, which made it possible to separate even minor molecular species.

In a typical elution pattern (Fig. 1), the major TG molecular species were OOP, OPP, and OOO, followed by several other molecular species such as GOP, LOP, GOO, and OSP. The designations OOP, OPP, *etc.* represent three constituent TG acyl groups (G, γ-linolenic acid; L, linoleic acid; O, oleic acid; S, stearic acid; P, palmitic acid; Po, palmitoleic acid), and do not mean one positional isomer, but a mixture of positional isomers. Since acyl group differences such as double bonds and chain length had little effect on the response of the light scattering detector and the response was mostly proportional to the amount of TG (*1, 2, 25, 26*), the relative proportion of TG molecular species of *M. ramanniana* var. *angulispora* cultured for 2 days (exponential growth phase) and 4 days (stationary growth phase) was calculated (Table I). The composition of OOP and OOO decreased with culture, whereas that of OPP remained

almost constant, and that of LOP and LOO increased.

Incorporation of ^{14}C-Fatty Acids and Glycerol into TG Molecular Species—To clarify the biosynthetic pathways for TG molecular species in *M. ramanniana* var. *angulispora*, we measured the incorporation of radiolabeled precursors into TG molecular species. [1-^{14}C]Oleic acid was incorporated into major TG molecular species, such as OOP, OOO, and OPP, at 30°C (Fig. 2). Significant incorporation into OOP and OOO was plausible since they were produced through oleic acid transfer to major DG molecular species, oleoylpalmitoyl glycerol (OP) and dioleoyl-glycerol (OO). Radiolabel incorporation into most TG molecular species similarly increased until 2 h, suggesting that most radiolabeled TG molecular species were not synthesized *via* other TG molecular species during the incubation time. Other molecular species containing linoleic acid and γ-linolenic acid were also labeled, and [1-^{14}C]oleic acid may be desaturated to linoleic acid and γ-linolenic acid. This was confirmed by analysis of the labeled fatty acids in each molecular species (data not shown). LOP and LOO contained ^{14}C-linoleic acid in addition to ^{14}C-oleic acid, and GOP and GOO contained ^{14}C-γ-linolenic acid in addition to ^{14}C-oleic acid. These results indicated that exogenous ^{14}C-oleic acid was desaturated to linoleic acid and γ-linolenic acid.

The incorporation of [1-^{14}C]oleic acid into TG molecular species at 15°C was also examined to determine whether not the incorporation was affected by low temperature (Fig. 2). Radiolabel incorporation at 15°C was similarly high into major TG molecular species such as OOP, OOO, and OPP, whereas that into other molecular species such as LOP, LOO, GOP, and GOO was decreased at 15°C compared to that at 30°C. These results suggest a biosynthetic pathway to OOP, OOO, and OOP derived from oleic acid, which is resistant to low temperature.

The major TG molecular species labeled by [1-^{14}C]linoleic acid were LOP and LOO at 30°C (Fig. 3), which may be similarly produced through linoleic acid transfer to major

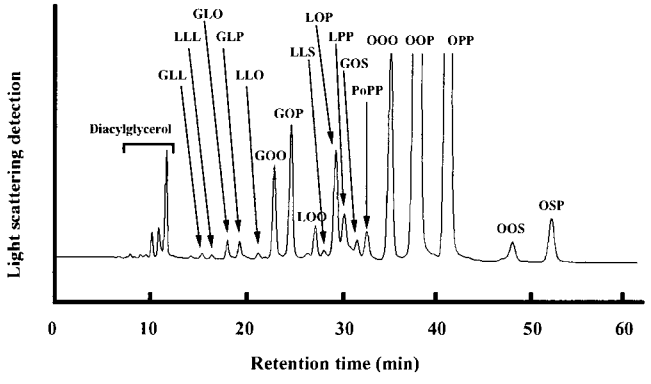

Fig. 1. **Reverse-phase HPLC profile of TG molecular species.** A neutral lipid fraction prepared from fungal cells cultured for 2 days was subjected to reverse-phase HPLC and peaks were detected with a light scattering detector as described under "MATERIALS AND METHODS." The designation of TG molecular species, such as OOP, is explained in the text. Peaks corresponding to GLL, LLL, and PoPP were assigned on the basis of TCN values and were not confirmed by gas chromatography.

TABLE I. **TG molecular species composition of *Mortierella ramanniana* var. *angulispora*.** Neutral lipids extracted from fungal cells cultured for 2 days or 4 days were subjected to reverse-phase HPLC, and TG molecular species were quantified with a laser scattering detector. Values represent means ± SD of triplicate determinations.

TG molecular species (% of total)	2 day culture	4 day culture
GLL	0.30 ± 0.06	0.57 ± 0.02
LLL	0.10 ± 0.03	not detected
GLO	0.60 ± 0.09	0.65 ± 0.07
GLP	0.60 ± 0.05	0.64 ± 0.08
LLO	0.10 ± 0.01	0.48 ± 0.02
GOO	2.50 ± 0.44	2.96 ± 0.08
GOP	4.80 ± 0.31	4.17 ± 0.09
LOO	0.90 ± 0.15	2.08 ± 0.11
LLS	0.70 ± 0.17	0.55 ± 0.04
LOP	2.90 ± 0.57	7.13 ± 0.32
LPP	0.60 ± 0.03	0.32 ± 0.01
GOS	0.43 ± 0.02	0.24 ± 0.01
PoPP	0.65 ± 0.04	1.18 ± 0.06
OOO	11.0 ± 1.5	7.25 ± 0.50
OOP	48.1 ± 1.4	36.4 ± 0.4
OPP	21.3 ± 1.8	21.1 ± 0.6
OOS	0.80 ± 0.09	0.67 ± 0.01
OSP	1.50 ± 0.06	1.35 ± 0.13

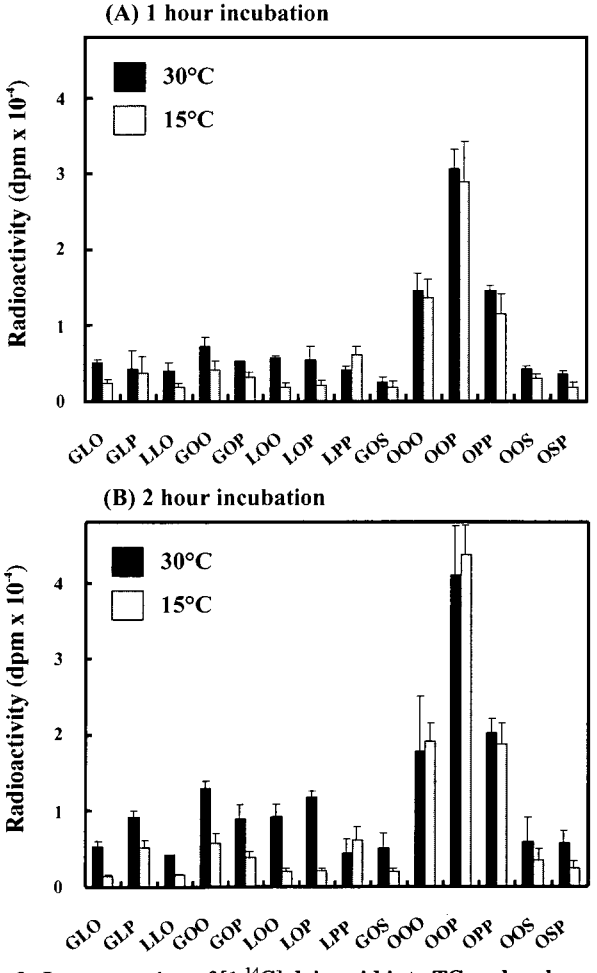

Fig. 2. **Incorporation of [1-^{14}C]oleic acid into TG molecular species.** Fungal cells cultured for 2 days were incubated with 0.2 μCi [1-^{14}C]oleic acid (2.6 μM) at 30°C or 15°C for 1 h (A) and 2 h (B). Radioactivity incorporated into TG molecular species from fungal cells under these conditions was measured by reverse-phase HPLC with a radioisotope detector as described under "MATERIALS AND METHODS." Values represent means ± SD of triplicate determinations.

DG molecular species, OP and OO. Incorporation into other TG molecular species containing linoleic acid and γ-linolenic acid was also found, although the labeling of these molecular species was not so specific for [1-^{14}C]linoleic acid. The incorporation of [1-^{14}C]linoleic acid into peaks near OOP, OPP, and OSP was observed, but it was unreasonable to label these molecular species with [1-^{14}C]linoleic acid. Judging from TCN values, these probably corresponded to minor molecular species such as LOS, LSP, and LSS. Although incubation at 15°C decreased the total [1-^{14}C]linoleic acid incorporation into TG, the decrease of that into LOP and LOO was not so prominent (Fig. 3) compared to that into LOP and LOO from [1-^{14}C]oleic acid.

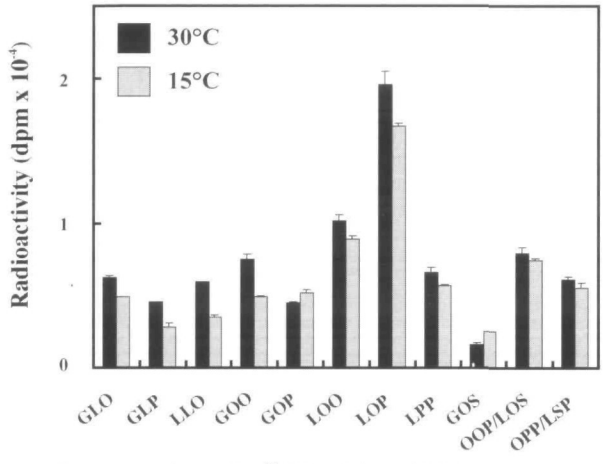

Fig. 3. **Incorporation of [1-^{14}C]linoleic acid into TG molecular species.** Fungal cells cultured for 2 days were incubated with 0.2 μCi [1-^{14}C]linoleic acid (2.5 μM) at 30°C or 15°C for 2 h. Radioactivity incorporated into TG molecular species from fungal cells under these conditions was measured by reverse-phase HPLC with a radioisotope detector as described under "MATERIALS AND METHODS." Values represent means ± SD of triplicate determinations.

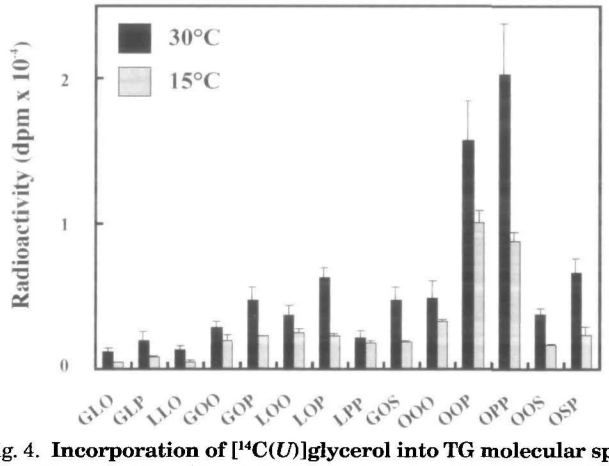

Fig. 4. **Incorporation of [^{14}C(*U*)]glycerol into TG molecular species.** Fungal cells cultured for 2 days were incubated with 0.2 μCi [^{14}C(*U*)]glycerol (0.8 μM) at 30°C or 15°C for 10 h. Radioactivity incorporated into TG molecular species from fungal cells under these conditions was measured by reverse-phase HPLC with a radioisotope detector as described under "MATERIALS AND METHODS." Values represent means ± SD of triplicate determinations.

To investigate the *de novo* synthesis of TG molecular species, we incubated fungal cells with [^{14}C(*U*)]glycerol at 30°C. The incorporation profile of [^{14}C(*U*)]glycerol (Fig. 4) differed from that of [1-^{14}C]oleic acid incorporation (Fig. 2) and the composition of TG molecular species (Table I). The highest labeling of [^{14}C(*U*)]glycerol incorporated was observed for OPP, followed by OOP. Radiolabel incorporation into OOO was lower than expected from its abundance. These results suggest that *de novo* TG synthesis provides TG molecular species containing palmitic acid more readily than ones containing other fatty acids. This [14C(*U*)]glycerol incorporation into TG molecular species decreased dramatically at 15°C (Fig. 4).

Effects of Inhibitors on Incorporation of ^{14}C-Fatty Acids and Glycerol—We studied the effects of several inhibitors on the incorporation of [1-^{14}C]oleic acid into TG molecular species to determine their biosynthetic pathways (Table II). Sodium azide, an energy poison, inhibited the incorporation of [1-^{14}C]oleic acid into most TG molecular species including OOP and OPP at 30°C, whereas it did not decrease radiolabel incorporation into OOO, LOO, and GOO. Cerulenin, an inhibitor of fatty acid synthase, had a similar effect to sodium azide; the incorporation into most molecular species decreased, but that into OOO, LOO, and GOO was almost unchanged. These results suggest that the biosynthetic pathway for OOO, LOO, and GOO, monitored with [1-^{14}C]oleic acid, is due to the same mechanism and differs from those for other TG molecular species. Trifluoperazine, which was found to inhibit [1-^{14}C]oleic acid incorporation into TG in this fungus, probably due to inhibition of conversion from phosphatidic acid to TG (27), inhibited the incorporation into all TG molecular species. The effect of trifluoperazine was more prominent on molecular species containing palmitic acid such as OPP, LOP, and GOP than on OOO, OOS, and LOO.

Sodium azide and cerulenin decreased the incorporation of [1-^{14}C]linoleic acid into most molecular species (data not shown), unlike [1-^{14}C]oleic acid incorporation. Sodium azide and cerulenin greatly decreased the incorporation of [^{14}C-(*U*)]glycerol into TG (94% inhibition by sodium azide and 83% inhibition by cerulenin), suggesting that *de novo* TG synthesis requires newly synthesized fatty acids, provided by cerulenin-sensitive fatty acid synthase. The results sug-

TABLE II. **Effects of inhibitors on incorporation of [1-^{14}C]oleic acid into TG molecular species.** A fungal cell suspension (1.5 ml) of a 2-day culture was incubated with 0.2 μCi [1-^{14}C]oleic acid (2.6 μM) at 30°C for 2 h in the presence of 0.01% sodium azide, 100 μM cerulenin, or 50 μM trifluoperazine. Radioactivity incorporated into TG molecular species from fungal cells under these conditions was measured by reverse-phase HPLC with a radioisotope detector as described under "MATERIALS AND METHODS." Values represent means ± SD of triplicate determinations.

TG molecular species	Sodium azide	Cerulenin	Trifluoperazine
	(% of control ^{14}C-incorporation)		
GOO	106 ± 60	101 ± 1	41 ± 3
GOP	53 ± 32	56 ± 8	23 ± 1
LOO	98 ± 54	99 ± 3	50 ± 9
LOP	46 ± 10	29 ± 3	25 ± 5
OOO	98 ± 6	113 ± 4	58 ± 7
OOP	55 ± 30	54 ± 11	47 ± 8
OPP	20 ± 11	14 ± 15	29 ± 6
OOS	52 ± 2	47 ± 9	61 ± 4
OSP	26 ± 7	84 ± 7	43 ± 1

gest that [^{14}C(*U*)]glycerol incorporation into TG is energy-dependent.

DISCUSSION

The present paper described the incorporation of radiolabeled precursors into TG molecular species, which shed new light on the biosynthesis of TG molecular species in an oleaginous fungus, *M. ramanniana* var. *angulispora*. Although TG biosynthesis in general has been extensively studied in various organisms, little is known about the biosynthetic pathways for TG molecular species. Stereospecific analysis of TG in various organisms has shown that fatty acids are not randomly distributed at the *sn*-1,2,3 positions (*28*), suggesting mechanisms distributing appropriate fatty acids to each position. The slow progress in this field is mainly due to difficulties in analyzing TG molecular species, since no single technique can separate all TG molecular species including stereospecific isomers. Reverse-phase HPLC is widely used to analyze TG molecular species, the separation being based on the chain length and double bonds of constituent fatty acids (*1, 2*). The fatty acid composition of *M. ramanniana* var. *angulispora* is relatively simple for HPLC separation, but diverse enough to study the distribution of polyunsaturated fatty acids in TG (*24*). In spite of the technical progress as to HPLC separation of TG molecular species, very few reports have described the incorporation of radiolabeled precursors into TG molecular species. The incorporation of radiolabeled precursors into TG molecular species in microsomes was recently reported (*29, 30*), in which PC molecular species for the biosynthesis of major TG molecular species were proposed.

The results obtained for this fungus can be explained by several mechanisms for incorporating exogenous radiolabeled fatty acids into TG, such as (i) direct acylation of DG, (ii) acylation of DG that is already radiolabeled through de novo phosphatidic acid biosynthesis, (iii) DG acylation by acyl transfer from radiolabeled PC or other phospholipids, and (iv) transacylation of DG, *etc.* (*6–10*). The incorporation into each molecular species may involve a combination of some of these mechanisms. We previously found some of the above mechanisms were operating in this fungus. TG biosynthesis was very active, endogenous DG being used by DG acyltransferase in the membrane and lipid body fractions (*16*). The addition of trifluoperazine caused the accumulation of radiolabeled phosphatidic acid and DG, and a concomitant decrease in radiolabeled TG in fungal cells incubated with radiolabeled fatty acids, indicating active TG biosynthesis through the *de novo* pathway (*27*). Finally, unsaturated fatty acids in PC were transferred to TG through a temperature-sensitive mechanism (*18*). In addition to these observations, we assume that in most cases exogenous radiolabeled fatty acid incorporation into each TG molecular species did not occur *via* other TG molecular species during the incubation time (2 h), since [1-^{14}C]oleic acid incorporation into most TG molecular species was similarly increased (Fig. 2), and TG labeled by exogenous fatty acids was not readily metabolized (*24*). Fatty acid modification such as desaturation, hydroxylation, *etc.* in TG has not been reported. Based on the above information, we deduced several biosynthetic pathways for TG molecular species in this fungus.

Direct acylation of DG by DG acyltransferase should highly contribute to the formation of radiolabeled OOP and OOO from [1-^{14}C]oleic acid, or radiolabeled LOP and LOO from [1-^{14}C]linoleic acid, since OP and OO were prominent DG molecular species (*22*). However, the incorporation into all TG molecular species was decreased by trifluoperazine, with concomitant accumulation of radiolabeled phosphatidic acid and DG (*27*). The decrease caused by trifluoperazine was more prominent for molecular species containing palmitic acid (Table II). This observation suggests that *de novo* TG biosynthesis *via* phosphatidic acid formation also contributes to the formation of radiolabeled TG molecular species and that the *de novo* pathway more readily incorporates palmitic acid. The latter was confirmed by the observation that [^{14}C(*U*)]glycerol incorporation into OOP and OPP was much more notable than that into OOO. Furthermore, the biosynthesis of OOP and OPP requires palmitic acid newly synthesized by fatty acid synthase, which is suggested by the effect of cerulenin. The TG biosynthetic pathway from PC, which was temperature- and energy-dependent (*18*), corresponded to [1-^{14}C]oleic acid incorporation into LOP, GOP, and other minor molecular species containing linoleic acid and γ–linolenic acid, except LOO and GOO.

In addition to the above pathways, some other pathways for TG molecular species were indicatd by our observations. The pathway of incorporation of [1-^{14}C]oleic acid into OOO, and that into OOP and OPP, which were resistant to low temperature, differed in the sensitivity to sodium azide and cerulenin. Since OOO and OOP were assumed to be synthesized through direct acylation of DG and *de novo* TG biosynthesis, the difference may be explained by another mechanism specific to OOO biosynthesis. We speculate that TG acyl-turnover, a combination of lipase and DG acyltransferase or DG transacylase, may operate for specific incorporation of oleic acid into OOO. The acyl-turnover has been reported to rearrange TG molecular species without ATP in plant seeds (*31, 32*). DG transacylase has also been reported in mammalian and plant cells (*33, 34*).

Cerulenin did not inhibit [1-^{14}C]oleic acid incorporation into OOO, LOO, and GOO, whereas it decreased the incorporation of [^{14}C(*U*)]glycerol and [1-^{14}C]linoleic acid into all TG molecular species, especially ones containing palmitic acid. The results suggest that an intracellular pool of activated fatty acids involved in TG biosynthesis is rich in oleic acid, but less so in palmitic acid and linoleic acid.

Stereospecific analysis of fatty acids in plant oils indicated that saturated fatty acids such as palmitic acid and stearic acid are distributed to the *sn*-1 and 3 positions, and oleic acid, linoleic acid, and linolenic acid are mainly distributed to the *sn*-2 position (*28*), which was similar to in oleaginous fungi and yeasts (*35*). *Mucor circinelloides*, an oleaginous fungus that has a similar TG fatty acid composition to *M. ramanniana* var. *angulispora*, exhibited the typical stereospecific distribution of fatty acids in TG, except that γ-linolenic acid was mainly present at the *sn*-3 position (*36*). The non-random distribution of fatty acids in TG also suggests the existence of different incorporation pathways for each fatty acid, such as those described in this study.

In conclusion, we found that the TG molecular species in *M. ramanniana* var. *angulispora* were not equally synthesized, but can be classified into several groups based on their biosynthetic pathways with different sensitivities to low temperature and inhibitors of lipid metabolism. The

separated groups included the major molecular species OOP, TG molecular species containing palmitic acid, TG molecular species containing two oleic acids, and TG molecular species containing linoleic acid and γ-linolenic acid. These results imply that the composition of TG molecular species is regulated through different biosynthetic pathways responsible for specific molecular species. Future studies are necessary to clarify the enzymatic systems responsive to these pathways for TG molecular species. The insights we have given will provide a basis for designing TG molecular species through genetic engineering.

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