Subcellular Localization of Triacylglycerol Synthesis in Spinach Leaves

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ABSTRACT

The subcellular location of diacylglycerol acyltransferase (EC 2.3.1.20) in spinach leaves (*Spinacia oleracea* L. cv. Longstanding Bloomsdale) was determined after sucrose-gradient centrifugation of tissue homogenates. Enzyme activity was associated primarily with gradient fractions containing oil bodies and intact chloroplasts. Gradient fractions enriched with the endoplasmic reticulum contained insignificant levels of diacylglycerol acyltransferase activity. On the basis of chlorophyll, diacylglycerol acyltransferase activity in crude homogenates was not significantly different from that in intact chloroplasts after Percoll density-gradient centrifugation. Among membrane fractions isolated from hypotonically treated chloroplasts, envelopes contained the greatest diacylglycerol acyltransferase and galactosyltransferase activity. These data demonstrated that chloroplasts were a subcellular site for triacylglycerol biosynthesis in spinach leaves.
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INTRODUCTION

Triacylglycerol (TG) is an important form of storage lipid in many plant tissues (1). The pathway for TG biosynthesis appears to be similar in plant species; however, the subcellular location of diacylglycerol acyltransferase (DGAT), the enzyme that catalyses TG biosynthesis, is not well defined (2). Evidence from electron micrographs suggests that vessicles (sphereosomes) detached from terminal strands of the endoplasmic reticulum (ER) develop into oil bodies (3). The implication is that DGAT is associated with the ER and is transferred to oil bodies by the membrane-bounding sphereosomes. Although oil bodies isolated from developing castor beans (4) and crambe seed (5) may synthesize TG from [^{14}C]acyl-CoA or [^{14}C]glycerol-3-phosphate, no direct biochemical proof was found that oil bodies are formed from sphereosomes. In fact, Gurr concludes that sphereosomes do not develop into lipid bodies and that each entity has separate but distinct function (2). Ichihara (6) also contends that the origin of lipid bodies is independent of the ER in maturing safflower seed.

Given that sphereosomes are formed from the ER, the controversy over oleosome formation may abate with information on the subcellular location of DGAT activity. From previous reports, significant levels of TG synthetic activity are sedimented at 3,000 *g* from maturing safflower-seed homogenates (7) and at 3,000 *g* and 20,000 *g* from homogenates of spinach leaves (8). Although the differential centrifugation fractions could have been contaminated with ER, TG synthesis from [^{14}C]acetate is reported in pea plastids (9) and in purified spinach chloroplasts (10). Hence, in certain plant tissues, DGAT may be found in organelles other than the ER.

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The purpose of this study was to find direct evidence for associating DGAT activity with the ER and/or chloroplasts from spinach leaves. Spinach leaves contain relatively low levels of TG; however, that tissue source was used in the first report from our laboratory on isolating and characterizing DGAT (8). The DGAT assay developed in that work was used with sucrose and Percoll gradient centrifugation methods to fractionate cytoplasmic organelles. The findings of this investigation have revealed that DGAT activity is associated with chloroplast envelopes from spinach leaves.

MATERIALS AND METHODS

Preparing Tissue

Spinach (*Spinacia oleracea* L. cv. Longstanding Bloomsdale) plants were grown in a greenhouse. Fully expanded leaves were harvested and chilled to 4 C. Leaf tissue (10 g fresh weight) was chopped with an electric razor knife in 20 ml of buffered grinding medium. The grinding medium contained: 0.4 M sucrose; 50 mM Bicine [N,N-*bis*(2-hydroxy-ethyl glycine)], pH 7.6; 1 mM KCl; 0.1 mM $MgCl_2$; 1 mM NA-EDTA. The homogenate was squeezed through 6 layers of cheesecloth and one layer of Miracloth. A 7 ml aliquot of the filtrate was applied to a step gradient of 3 ml 2.3 M sucrose, 3 ml 1.9 M sucrose, 4 ml 1.8 M sucrose, 4 ml 1.75 M sucrose, 4 ml 1.7 M sucrose, 4 ml 1.5 M sucrose, 3 ml 1.3 M sucrose, 3 ml 1 M

sucrose, and 3 ml 0.83 M sucrose. Each gradient was replicated 8 times. All sucrose solutions were prepared in a medium containing 10 mM Bicine, pH 7.6; 1 mM NA-EDTA; 0.1 mM $MgCl_2$; 1 mM KCl.

The gradients were centrifuged at 4 C in a Beckman L80 centrifuge equipped with a Beckman SW28 rotor. The centrifugation was conducted at 141,000 *g* for 4 hr. The w^2t value applied to all gradients was 1.22×10^{11}. An ISCO 185 density gradient fractionator was used to collect 2 ml fractions from the top of the gradients. All fractions were stored at 4 C before the enzyme assay.

Localizing Organelles

All gradient fractions were assayed for enzymes characteristic of specific organelles. Catalase activity, determined by the decrease in A at 240 nm (11), indicated the location of peroxisomes. Regions of the gradient containing ER were determined by measuring the NADH-dependent increase in A at 550 nm for antimycin A insensitive NADH-Cyt C reductase activity (12). Cyt C oxidase activity associated with mitochondrial inner membranes was determined by a decrease in A of reduced Cyt C at 550 nm (13). In the latter case, the fractions were incubated with 10 μl digitonin for 1 min before the addition of the buffer and substrate. RuBP carboxylase activity was determined by the incorporation of $^{14}CO_2$ into 3-phosphoglyceric acid (14). DGAT activity was determined by the incorporation of [^{14}C] 18:1-CoA into TG (8). Galactosyltransferase (GALT) activity associated with chloroplast envelopes was determined by incorporation of UDP-[^{14}C]galactose into glycolipids (15). Chlorophyll (chl) was determined by A at 663 and 645 nm (16); protein was determined by a modified Lowry procedure (17). All data represent mean values from all gradients performed.

Preparing Intact Chloroplasts

Spinach leaves (200 g fresh weight) were homogenized in 1 l grinding medium, as described earlier. Intact chloroplasts were isolated on a 10-80% (w/v) Percoll gradient (18). The chloroplasts were washed twice with a grinding medium, centrifuged at 2,000 *g* for 2 min and resuspended in the grinding medium. A fraction of those preparations was osmotically shocked in 50 mM Bicine, pH 7.6. Intact and osmotically shocked chloroplasts were adjusted to an equal concentration, 0.5 mg chl$(ml)^{-1}$, before incubation at 25 C for 30 min with 675 BAEE units of DPCC-treated trypsin (Sigma Chemical Co., St. Louis, MO) in a total volume of 0.25 ml. At the end of treatment, a 10-fold excess of trypsin inhibitor (soybean) was added to all treatments and controls.

Preparing Chloroplast Envelopes

Purified chloroplasts, prepared as described above, were resuspended in 28 ml swelling medium containing 10 mM Bicine, pH 7.6, and 4 mM $MgCl_2$. Equal portions of the preparation (14 ml) were loaded onto step gradients (19) containing: 6 ml 1.5 M sucrose, 6 ml 1.2 M sucrose, 6 ml 0.93 M sucrose and 6 ml 0.6 M sucrose. The gradients were centrifuged at 4 C in a Beckman L80 centrifuge equipped with a Beckman SW28 rotor for 1 hr at 141,000 *g*. Each set of gradients was replicated 4 times. four fractions were collected from the top of a gradient as before; the volume of fraction 1 was 14 ml and the volumes for fractions 2 to 4 were 6 ml each. Each fraction volume was increased to 38 ml with the swelling medium and centrifuged at 4 C for 1 hr at 141,000 *g*. The pellets were resuspended in 1 ml swelling buffer. Total chl, protein, and GALT, DGAT, Cyt C reductase, and Cyt C oxidase activities were determined as stated above.

Lipid Analysis

Whole spinach leaves (10 g fresh weight) were homogenized consecutively with a Brinkman Polytron and a Ten-Broeck tissue grinder in 40 ml chloroform/methanol (2:1 v/v). The homogenate was filtered with an additional 20 ml chloroform/methanol (2:1 v/v) and 30 ml methanol. After filtration, 50 ml of deionized water was added to the filtrate. The mixture was shaken and centrifuged at 1,000 *g* for 20 min to form a biphasic solution. The phase containing lipids was dried in vacuo. Lipids were extracted from a 1 ml portion of 4 separate, intact chloroplast preparations by adding 4 ml chloroform/methanol (1:1, v/v). The mixture was vortexed and centrifuged at 1,000 *g* for 10 min. The phase containing lipids was dried under N_2. Fat layers (oil bodies) obtained from the 4 sucrose gradients were extracted by the method described for intact chloroplasts. All lipid extracts were redissolved in chloroform/methanol (2:1, v/v). Total polar lipid (TPL), diacylglycerol (DG), and TG were separated from the total lipid extract by TLC on precoated Absorbosil-Plus 5 plates (Applied Science Lab., State College, PA) with petroleum ether/diethyl ether/glacial acetic acid (80:20:0.8, v/v/v) as the developing solvent. Lipid classes were identified by parallel chromatography with authentic phosphatidylcholine, DG, and TG standards (Sigma Chemical Co.,

St. Louis, MO). Lipid classes were quantitatively analyzed by GC as described previously (20). Radioactive TG from DGAT reactions was isolated by cochromatography with a mixture of TG, sterylester, and free fatty acid standards (8).

RESULTS AND DISCUSSION

The activities of marker enzymes for subcellular organelles from spinach leaves, chl, protein, and DGAT activity within sucrose-gradient fractions were calculated on a volume basis (Figs. 1-2). The ER, identified by antimycin A insensitive NADH-Cyt C reductase activity, banded at a density of ca. 1.13 $g(cc)^{-1}$, fractions 4-6; mitochondria, located by Cyt C oxidase activity, had a density of ca. 1.18 $g(cc)^{-1}$, fractions 7-9; intact chloroplasts, indicated by RuBP-carboxylase activity, banded at 1.22 $g(cc)^{-1}$, fractions 10 to 11; and peroxisomes, distinguished by catalase activity, had density of ca. 1.24 $g(cc)^{-1}$, fractions 15 to 16. The supernatant of the gradient (fractions 1 to 3) contained significant levels of RuBP-carboxylase and catalase activity because the disruption of intact organelles could not be prevented. The major chlorophyll bands also indicated the location of broken chloroplasts (fraction 7) and intact chloroplasts (fractions 10 and 11).

The major peaks for DGAT activity were found at sucrose-gradient densities of ca. 1.08 $g(cc)^{-1}$ and 1.22 $g(cc)^{-1}$. These activities did not coincide with regions of the gradient enriched with ER. Hence, these data cast further doubt on the speculation that oil bodies were formed from the ER. DGAT activity was coincident with gradient fractions containing intact chloroplasts, yet insignificant levels of DGAT activity were found in fractions containing broken chloroplasts (presumably thylakoids or grana). The work of Shine et al. (21) clearly demonstrated that spinach grana did not contain DGAT activity. In addition, low levels of DGAT activity in the supernatant fraction containing RuBP-carboxylase activity suggested that chloroplastic DGAT was not a stromal enzyme. By deduction, one might then presume that DGAT in intact chloroplasts was associated with the chloroplast envelopes. The density of chloroplast envelopes (22) was reported to be about 1.08 $g(cc)^{-1}$, the density at which oil bodies were found in our gradients. We have not been able to devise a definitive means for distinguishing whether the DGAT activity found in the fat layer was attributable to the oil bodies per se or to the presence of chloroplast envelopes. We could, however, attempt to prove that the proposed association between DGAT and chloroplast envelopes was valid.

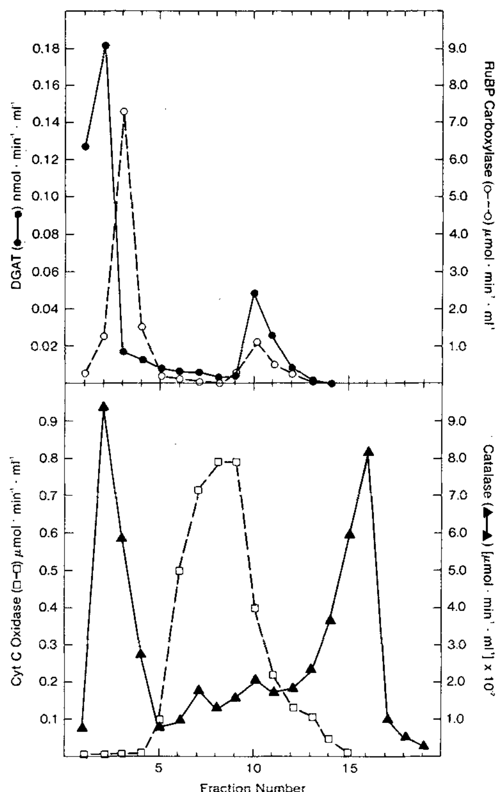

FIG. 1. Distribution of organelle marker enzymes in sucrose density gradients. RuBP carboxylase (○—○), Cyt C oxidase (□—□), catalase (▲—▲), and DGAT (●—●) from spinach leaf homogenates applied directly to a sucrose step gradient and centrifuged as described in *Methods*. Enzyme activities were expressed on a volume basis. All fractions were 2 ml.

Intact chloroplasts, isolated on Percoll density gradients and washed twice with a grinding medium, contained similar levels of DGAT or GALT activity on a chl basis, as did crude extracts (Table 1). These chloroplast preparations contained insignificant levels of Cyt C oxidase or Cyt C reductase activity. Although DGAT or GALT activity was lower when intact chloroplasts were lysed, tryptic digestion did not significantly affect the activity of either enzyme. These data suggested that both enzymes, or at least their active sites, were embedded within the chloroplast membranes. To show the proposed association of DGAT with chloroplast envelopes, purified intact chloroplast preparations were hypotonically disrupted (19) and fractionated by discontinuous sucrose-gradient centrifugation (23). Fractions enriched with stromal enzymes, envelopes, plastoglobuli, and thylakoids were characterized as described (23). The activity

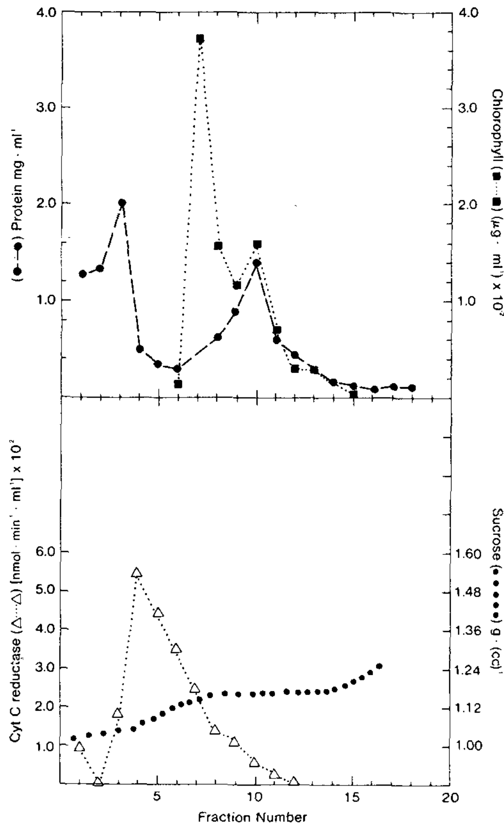

FIG. 2. Distribution of organelle marker enzymes in sucrose density gradients. Sucrose density (........), protein (●—●), Cyt C reductase (△—△), and chlorophyll (■—■). Enzyme activity was expressed on a volume basis. All fractions were 2 ml.

of marker enzymes for the ER and mitochondria were negligible among these chloroplast membrane fractions. The distribution of DGAT activity among the fractions verified that DGAT was not a stromal enzyme and revealed that the greatest activity was associated with chloroplast envelopes (Table 2). Because the distribution of GALT activity paralleled DGAT activity among the fractions, the DGAT activity found with plastoglobuli and thylakoids was attributed to contamination by chloroplast envelopes. Hence, these data suggest that chloroplastic DGAT could be exclusively associated with chloroplast envelopes.

Intact chloroplasts, after Percoll gradient centrifugation, contained 67.8 ± 1.2 μmol TG $(mg\ chl)^{-1}$; in addition, the fatty acid composition of chloroplastic TG was not statistically different from cytoplasmic TG (Table 3). The association of DGAT activity with chloroplasts, plus the evidence that chloroplasts also have enzymes necessary for acyl-CoA and DG formation (24-27), have provided both a means and a method for TG biosynthesis in chloroplasts. Chloroplastic TG could then be a constituent of the osmiophilic bodies often observed within chloroplasts from higher plants (28). The biological relevence of these findings will be tested in studies with plastids from developing oilseeds.

TABLE 1

Effect of Trypsin upon DGAT and GALT Activity

Preparation[a]	Trypsin[b]	DGAT	GALT
		-nmol $(min)^{-1}$	$(mg\ ch)^{-1}$ -
Intact chloroplasts	+	0.336	0.525
	–	0.386	0.615
Lysed chloroplasts	+	0.257	0.265
	–	0.290	0.355
Crude extract	–	0.039	0.640
LSD 0.05		0.079	0.120

[a]Chloroplasts were isolated on Percoll density gradients; a portion of the preparation was lysed by osmotic shock. All preparations were adjusted to a final concentration of 0.5 mg $chl(ml)^{-1}$ before trypsin treatment.

[b]675 BAEE units of DPCC-treated trypsin was added to each preparation as indicated. After 30 min at 25 C, a 10-fold excess of trypsin inhibitor was added to arrest trypsin activity. Trypsin inhibitor per se had no effect upon DGAT or GALT activity.

TABLE 2

Distribution of DGAT and GALT Activity Among Membrane Fractions from Hypotonically Disrupted Chloroplasts

Fraction	Protein	Chlorophyll	GALT	DGAT
	$mg(ml)^{-1}$		nmol $(min)^{-1}$	$(ml)^{-1}$
Stroma	1.4	ND[a]	0.05	0.09
Envelopes	1.6	ND[a]	2.29	1.14
Plastoglobuli	1.8	0.028	1.04	0.83
Thylakoids	40.0	6.693	0.95	0.37
LSD 0.05			0.39	0.20

[a]Not detectable.

TABLE 3

Triacylglycerol Composition of Preparations from Spinach Leaves

Sample	Fatty acid						Total
	16:0	16:1	18:0	18:1	18:2	18:3	
	mol %						nmol TG (mg chl)$^{-1}$
Whole leaf[a]	21.5	2.8	6.0	16.5	22.5	30.7	246.3 ± 4.1
Intact chloroplast[b]	23.1	2.2	7.1	17.1	20.8	29.7	67.8 ± 1.2
Fat layer[c]	20.6	2.9	5.8	16.2	23.5	31.0	138.6 ± 3.0
LSD 0.05	3.0	0.9	1.7	1.1	3.2	1.6	

[a]Spinach leaves contained 1.4 mg chl(g fresh wt)$^{-1}$.

[b]From Percoll density gradient centrifugation.

[c]TG content of the fat layer was calculated on a fresh weight basis; nmol TG (mg chl)$^{-1}$ was extrapolated from the mg chl(g fresh weight)$^{-1}$ (leaf)$^{-1}$.

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