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Cholinephosphotransferase and Diacylglycerol Acyltransferase[1]

Substrate Specificities at a Key Branch Point in Seed Lipid Metabolism

Guido Vogel and John Browse*

Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164–6340

Many oilseed plants accumulate triacylglycerols that contain unusual fatty acyl structures rather than the common 16- and 18-carbon fatty acids found in membrane lipids of these plants. In vitro experiments demonstrate that triacylglycerols are synthesized via diacylglycerols in microsomal preparations and that this same subcellular fraction is the site for the synthesis of phosphatidylcholine, which in seeds is synthesized from diacylglycerol by CDP-choline: diacylglycerol cholinephosphotransferase. In microsomes from *Cuphea lanceolata*, a plant that accumulates fatty acids with 10 carbons and no double bonds (10:0) in its oil, the diacylglycerol acyltransferase exhibited 4-fold higher activity with 10:0/10:0 molecular species of diacylglycerol than with molecular species containing 18-carbon fatty acids. In castor bean (*Ricinus communis*), which accumulates oil containing ricinoleic acid, diricinoleoyldiacylglycerol was the favored substrate for triacylglycerol synthesis. In contrast to these modest specificities of the diacylglycerol acyltransferases, the cholinephosphotransferases from these plants and from safflower (*Carthamus tinctorius*) and rapeseed (*Brassica napus*) showed little or no specificity across a range of different diacylglycerol substrates. Consideration of these results and other data suggests that the targeting of unusual fatty acids to triacylglycerol synthesis and their exclusion from membrane lipids are not achieved on the basis of the diacylglycerol substrate specificities of the enzymes involved and may instead require the spatial separation of two different diacylglycerol pools.

Plant seed oils from species such as soybean, sunflower, and canola are the major source of essential polyunsaturated fatty acids in human diets. Other plants produce oils with unusual fatty acid structures that meet the requirements of a range of industrial applications. These unusual fatty acid structures include medium chain lengths (C_8–C_{12}), very long chain lengths (C_{20}–C_{24}), or substitutions such as hydroxyl, epoxy, and cyclopropane groups (Gunstone et al., 1986; Somerville and Browse, 1991; Ohlrogge, 1994). Such fatty acids are incompatible with membrane lipid structure and function, and plants that accumulate them in seed oils appear to have developed mechanisms to specifically target such unusual structures to storage lipid synthesis.

Triacylglycerols accumulate in the ground cells of the developing cotyledon, which after germination of the seed will differentiate into photosynthetically competent mesophyll cells. The biochemistry of triacylglycerol synthesis in developing cotyledons can follow the typical Kennedy pathway (Fig. 1) (Kennedy, 1961), with DAG being converted to TAG by DAGAT (EC 2.3.1.20). However, the cellular DAG pool must also provide substrate for synthesis of phospholipids that are required for membrane biogenesis. A striking feature of lipid synthesis in oilseeds (and in all plant tissues) is the fact that the major structural lipid of the ER, PC, is also the main substrate for 18:1 desaturation to 18:2 and 18:3 (Fig. 1), and in many seeds PC is rapidly turned over as an intermediate in TAG synthesis (Browse and Somerville, 1991). The enzyme CPT (EC 2.7.8.2) is a key enzyme in oilseed metabolism because it catalyzes both the synthesis of PC from DAG and the reverse reaction, which is one route by which polyunsaturated fatty acids are made available for incorporation into TAG (Slack et al., 1985; Browse and Somerville, 1991). (The second route involves the synthesis of polyunsaturated acyl-CoAs by action of a reversible acyl-CoA:*lyso*-phosphatidylcholine acyltransferase followed by reactions of the Kennedy pathway [Stymne and Stobart, 1987]). Thus, the first question to be asked about CPT in oilseeds concerns, its importance relative to alternative reactions in transferring polyunsaturated fatty acids and glycerol into the DAG pool for TAG synthesis. This question is relevant primarily to those oilseeds that synthesize oils high in 18:2 and 18:3 (Slack et al., 1985; Browse and Somerville, 1991). A quite different question arises for those oilseed species that contain short-chain, very-long-chain, or unusual fatty acids. In these plants the uncommon fatty acids are largely or completely excluded from the membrane lipids of the seed

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* Corresponding author; e-mail jab@wsu.edu; fax 1–509–335–7643.

Abbreviations: ACP, acyl carrier protein; CPT, cholinephosphotransferase (CDP-choline:diacylgylcerol cholinephosphotransferase); DAG, diacylglycerol; DAGAT, diacylglycerol acyltransferase (acyl-CoA:diacylglycerol acyltransferase); LPC, *lyso*-phosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; TAG, triacylglycerol; X:Y, a fatty acyl group containing X carbon atoms and Y *cis* double bonds.

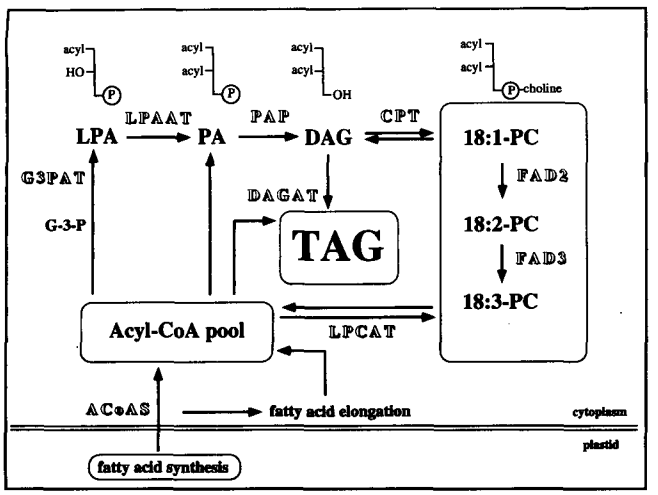

Figure 1. Simplified scheme of the reactions involved in TAG biosynthesis in developing cotyledons of oilseed plants. ACoAS, Acyl-CoA-synthetase; FAD2, oleoyl desaturase; FAD3, linoleoyl desaturase; G-3-P, glycerol-3-phosphate; G3PAT, glycerol-3-phosphate acyltransferase; LPA, *lyso*-phosphatidic acid; LPAAT, *lyso*-phosphatidic acid acyltransferase; LPCAT, *lyso*-phosphatidylcholine acyl transferase; PAP, PA phosphatase.

tissue and appear to be efficiently channeled into TAGs. For these plant species, one attractive hypothesis is that CPT plays a role in excluding DAG molecular species with membrane-incompatible fatty acids from being used for the synthesis of PC and that DAGAT has a preference for these same precursors (Bafor et al., 1990).

These considerations indicate that DAG is a key branch-point intermediate in all oilseeds. Subcellular fractionation studies demonstrate that both DAGAT and CPT activities are localized to the microsomal fraction, and the enzymes are believed to be integral membrane proteins of the ER. To facilitate an investigation of the substrate specificities of these enzymes, we have synthesized a series of [^{14}C]DAG substrates (Vogel and Browse, 1995) and here we apply them to measurements of DAGAT and CPT from four different oilseed species. The model systems we chose were safflower (*Carthamus tinctorius*), a plant storing only normal fatty acids in its TAGs; castor bean (*Ricinus communis*), which contains predominantly ricinoleate in its oil; *Cuphea lanceolata*, which synthesizes TAGs containing medium-chain fatty acids; and rapeseed (*Brassica napus*), which introduces very-long-chain fatty acids at the *sn-1* and *sn-3* positions of TAG.

MATERIALS AND METHODS

Chemicals and Reagents

[U-^{14}C]glycerol (147 mCi/mmol) was obtained from DuPont-New England Nuclear. All solvents were purchased from J.T. Baker in their HPLC grade. TLC was performed on Merck (Darmstadt, Germany) Kieselgel 60 TLC plates or Aldrich cellulose TLC plates. The scintillation fluid BCS from Amersham was used after mixing with 10% water.

Labeled Diacylglycerols

Sn-1,2-, 18:1/18:1-, 18:2/18:2-, 10:0/10:0-, and 22:1/22:1-DAGs were synthesized according to the method of Vogel and Browse (1995). The lipids 16:0/18:2-, 18:1/18:2-, 20:1/18:1-, 20:1/18:2-, 22:1/18:1-, and 22:1/18:2-DAG were isolated from the lipid extract of 2 g of developing rapeseed (*Brassica napus*) seeds incubated for 70 min at 25°C with 20 μCi of [U-^{14}C]glycerol (147 mCi/mmol) in 3 mL of 50 mM Mes/NaOH buffer at pH 5 containing 0.5 mM sodium acetate. The DAGs were first separated from the rest of the lipids by preparative silica gel TLC using chloroform:acetone (100:5, v/v) as the mobile phase. This mixture of DAGs was then fractionated according to the number of double bonds per molecule on silica gel plates containing $AgNO_3$. The solvent system for these separations was chloroform:ethanol (100:6, v/v). Bands containing any of the above-listed DAGs were then refractionated by reversed-phase HPLC using a SpectraPhysics (San Jose, CA) SP 8800 ternary pump, a 4 mm × 250 mm Nucleosil (Alltech Associates, San Jose, CA) 100 C_{18} column with UV detection at 210 nm. The separation was achieved by an isocratic elution with acetonitrile:2-propanol (85:15, v/v) at a flow rate of 1.5 mL/min. *Sn*-1,2-DAGs containing either one or two ricinoleic acids (18:1-OH) were obtained from the lipid extract of 3 g of very young developing castor bean (*Ricinus communis*) endosperm incubated for 7 h with 35 μCi of [U-^{14}C]glycerol (147 mCi/mmol). These two DAG molecular species were isolated by preparative TLC on silica gel plates using chloroform:acetone (10:1, v/v) as the solvent system.

The specific radioactivities of all of these ^{14}C-labeled *sn*-1,2-DAGs were determined by quantifying the fatty acids by GLC using 17:0 as an internal standard and measuring the radioactivity in an aliquot of their chloroform solution (Vogel and Browse, 1995).

Plant Material

Safflower (*Carthamus tinctorius*, Federal Plant Introduction No. PI 30688), *Cuphea lanceolata* (high capric acid), castor bean (USDA germ plasma program No. PI 265504), and rapeseed (cv Indore) were grown under a 16-h light/8-h dark cycle in a greenhouse with a supplementary light source. Developing seeds were collected between 15 and 21 d, 8 and 12 d, 30 and 40 d, and 10 and 18 d after flowering, respectively, and separated from their seed coats. The microsomes from developing cotyledons were prepared as described by Slack et al. (1985), divided into aliquots in 8-mL screw-capped glass tubes, snap-frozen in liquid nitrogen, and stored at −70°C. Protein was measured by the method of Bradford (1976) using Bio-Rad reagent with BSA as the standard.

Assay Procedures

CPT was assayed using the method of Vogel and Browse (1995). Combined assays of CPT and DAGAT were performed as follows. Aliquots of microsomal suspensions (100–300 μg of microsomal protein) were lyophilized in 8-mL screw-capped glass tubes and saturated with an equivalent of 900 nmol of radioactively labeled DAG per

mg of microsomal protein dissolved in benzene (216 nmol/100 μL). This benzene suspension was sonicated in an ice-chilled sonicating water bath (Fisher, model F3911) for 1 min and immediately frozen by immersion in liquid nitrogen. After the solvent was removed by evaporation under reduced pressure, 500 μL of assay buffer containing 50 mM Hepes/KOH, pH 7.5, 1 mM EDTA, 0.5 mM EGTA, 12 mM Suc, 5 mM DTT, 0.5 mM CDP-choline, 20 μM oleoyl-CoA, and 6 mM $MgCl_2$ was added to each tube with mixtures of microsomes and DAGs. The assay mixture was again sonicated for 1 min in an ice-chilled sonicating water bath and then shaken for 15 min in a shaking water bath at 30°C. The enzyme reactions were stopped by adding 3 mL of chloroform:methanol (1:1, v/v) followed by vigorous mixing. After addition of 1 mL of chloroform and 1 mL of 0.2 M H_2PO_4/1 M KCl solution (Hajra, 1974), the extracts were separated into organic and aqueous phases by low-speed centrifugation. After removing the organic phase, the aqueous phase was washed three times with 1.5 mL of chloroform. The combined organic phases were dried under a stream of nitrogen, and the lipid residue was redissolved in 2 mL of chloroform. Two 50-μL aliquots of this lipid solution were used for the determination of the total radioactivity by liquid scintillation counting. Half of the remaining lipids were separated by one-dimensional TLC (Merck, Kieselgel 60) using chloroform:methanol:water (65:30:4, v/v/v) as the solvent system to resolve the polar lipids. The second half of the lipids was separated by one-dimensional TLC (Merck, Kieselgel 60) using chloroform:acetone (100:5, v/v) as the solvent system for the separation of TAGs. The lipids were identified after staining with iodine vapor or after spraying the plates with a solution of 0.001% primuline in 80% acetone, followed by visualization under UV light. Radioactive substrate and products were located by autoradiography and transferred to 5 mL of scintillation fluid for the quantitative determination of radioactivity. The recovery of radioactivity for different incubation experiments ranged from 92 to 97%.

The identity of the products of CPT assays using 18:1/18:1-, 18:2/18:2-, 10:0/10:0-, or 22:1/22:1-DAG as substrate was determined after mild deacylation of the lipids by the method of Kates (1986) and separation of the water-soluble deacylated lipids on 20 × 20 cm cellulose TLC plates. The TLC plates were developed using a solvent system containing *n*-butanol:acetic acid:water (3:1:2, v/v/v). The deacylated, radioactively labeled substrate (glycerol) and product (glycerol-3-phosphorylcholine) were visualized by autoradiography and identified on the basis of their comigration with authentic standards.

The experiments reported were repeated two to five times using different preparations of microsomes. The absolute rates of enzyme activities with a particular substrate, such as 18:1/18:1-DAG, varied approximately 2-fold between different preparations. However, the relative rates of CPT or DAGAT activities with different DAG substrates and the relative rates of CPT to DAGAT with a given substrate were both very reproducible and the data presented are representative of replicate experiments in that context.

RESULTS

Simultaneous Assay of CPT and DAGAT

Previously described methods for measuring CPT activity mostly followed the transfer of ^{14}C- or ^{32}P-labeled phosphorylcholine from CDP-choline to DAG (Slack et al., 1985; Hjelmstad and Bell, 1991). DAGAT assays, on the other hand, were performed using [^{14}C]acyl-CoAs as the traceable substrate (Cao and Huang, 1987; Wiberg et al., 1994). The water-insoluble substrate for these enzyme reactions, DAG, was used in only two reports in the radioactively labeled form to follow its conversion to PC (Slack et al., 1985; Vogel and Browse, 1995). To simultaneously analyze the influence of the fatty acid structure on the CPT and DAGAT activity, we decided to measure the conversion of DAGs to PCs and TAGs. The label was placed on the glycerol backbone to prevent problems resulting from acyl-exchange reactions (Vogel and Browse, 1995). The naturally occurring isomers, *sn*-1,2-DAGs, were either synthesized chemically as described in an earlier report (Vogel and Browse, 1995) or isolated from seed lipid extracts after incubation with [U-^{14}C]glycerol.

A suitable method was established and optimized using microsomes from developing cotyledons of safflower and 18:1/18:1-DAG as the labeled substrate. The delivery of the water-insoluble DAGs to the microsomal CPT and DAGAT was achieved by adding a benzene solution of this substrate to lyophilized membranes and slowly evaporating the solvent under reduced pressure (Slack et al., 1985; Vogel and Browse, 1995). The enzyme reaction was then started with the addition of aqueous buffer containing CDP-choline and oleoyl-CoA. The rates of CPT and DAGAT activity reported here are at least as high as those measured previously (Slack et al., 1985; Cao and Huang, 1987; Wiberg et al., 1994). In our hands, attempts to provide exogenous DAGs dissolved in detergent were substantially unsuccessful, with incorporation rates of label into both PC and TAG being less than 10% of those obtained using substrates dissolved in benzene.

Throughout this study, we refer to the conversion of labeled DAG to TAG as being the result of DAGAT activity. Recently, evidence has been presented for a DAG dismutase activity in microsomal membranes from castor bean seeds (Mancha et al., 1995), and it is very possible that such a dismutase might contribute to the [^{14}C]TAG synthesis in our experiments with castor and the other oilseed plants. However, this possibility does not affect our conclusions concerning substrate specificities or the partitioning of DAG between PC and TAG synthesis except that the measured rates of TAG synthesis may reflect a combination of both DAGAT and DAG dismutase activities.

Initial experiments using 150 nmol of DAGs per milligram of microsomal protein always showed a sharp decline in the reaction rates of CPT and DAGAT after 4 to 5 min. Similar results were obtained by Slack et al. (1985) and Vogel and Browse (1995) when assaying CPT alone. The linear range of safflower DAGAT activity was also described to be rather short (10 min) in the report of Ichihara et al. (1988). One possibility for the decline in reaction rate

was that DAGs were rapidly becoming rate limiting during the enzyme assays. Indeed, 40 to 50% of the initial substrate was consumed in some of these experiments (Slack et al., 1985; Vogel and Browse, 1995). Therefore, in a series of experiments we increased the amounts of 18:1/18:1-DAG added as a benzene solution to dried microsomes. The extent of conversion of DAG to PC or to TAG in 15-min assays at 30°C leveled off at approximately 700 and 500 nmol of DAG per milligram of microsomal protein, respectively. The time course of in vitro PC and TAG synthesis using 900 nmol of 18:1/18:1-DAGs per milligram of microsomal protein is shown in Figure 2. In contrast with previous experiments, the initial CPT and DAGAT reaction rates continued for at least 20 min. Standard assays for this enzyme were therefore conducted at 30°C for 15 min using 900 nmol of radioactively labeled DAGs per milligram of microsomal protein.

In all assays, the main polar lipid produced from radioactive DAGs was PC. It usually contained more than 94% of the radioactivity recovered in the polar lipids. Small amounts of label were also recovered in the bands containing LPC (typically less than 3% of polar lipids) and PA (typically less then 3% of polar lipids), but as described below LPC was an important product in assays using 10:0/10:0 and 18:1-OH/18:1-OH DAGs as substrates.

TAGs (R_F 0.9) were well separated from DAGs (R_F 0.45 for 18:1/18:1-DAG) and polar lipids (R_F 0.0) after separation by TLC using chloroform:acetone (100:5, v/v) as the solvent system. The DAGAT activity was determined by analyzing the relative incorporation of label into all of the TAGs.

Characterization of the Safflower Enzymes

Safflower was used as a model plant because it does not accumulate unusual fatty acids in its TAGs. Results from a typical experiment are shown in Figure 3. Data from a total of five experiments gave substantially the same results in terms of the relative activities with different DAG substrates, even though, as discussed in "Materials and Methods," absolute rates varied to some extent among different microsomal preparations. The 18:1/18:1- and 18:2/18:2-DAGs (which are natural substrates for CPT in safflower) as well as the 10:0/10:0 and 22:1/22:1 molecular species not produced by these oilseeds were successfully converted to PC at comparable initial rates. Justin et al. (1985) provided data that suggested that CPT in microsomes from pea leaf and germinating soybean showed a preference for the endogenous 16:0/18:2 molecular species of DAG. Therefore, we isolated radioactive 16:0/18:2 DAG from [^{14}C]glycerol-labeled seeds and tested the safflower activity with this substrate. In these experiments, 16:0/18:2 DAG was incorporated into PC at rates very similar to those measured for 18:1/18:1 DAG using the same microsomal preparation (data not shown). Overall, these results indicate that CPT in safflower seeds is unable to distinguish between DAGs with drastically different fatty acid patterns.

In all of the experiments with safflower microsomes, the DAGAT showed the highest activity toward 10:0/10:0-DAGs and the lowest toward 22:1/22:1-DAGs (Fig. 3). The difference in activity between these two substrates was approximately 2-fold.

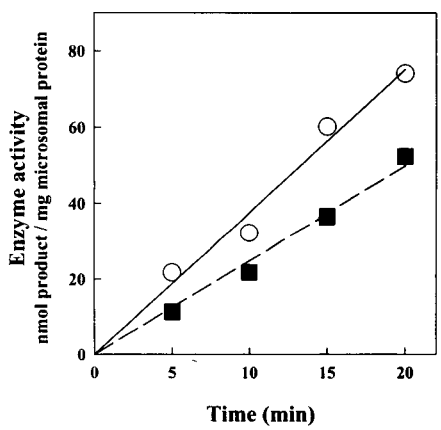

Figure 2. Time course of CPT (○) and DAGAT (■) activities in microsomes from developing cotyledons of safflower. Microsomes from developing cotyledons of safflower were supplied with 18:1/18:1-[^{14}C]DAG, CDP-choline, and 18:1-CoA as described in "Materials and Methods." Samples were incubated at 30°C for the times indicated before assays were terminated by the addition of $CHCl_3$:CH_3OH (1:1, v/v). Extracted lipids were separated by silica-gel TLC as described in "Materials and Methods," and the radioactivity in PC and TAG was determined by scintillation counting.

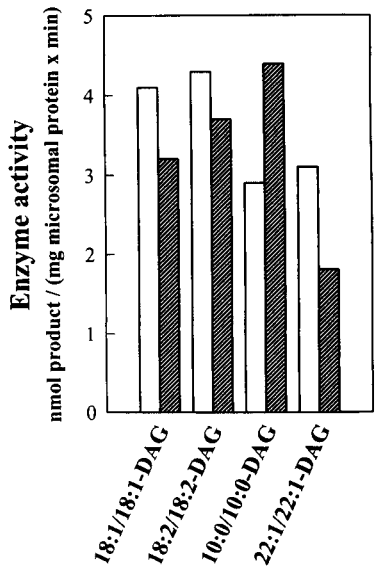

Figure 3. Rates of CPT (open bars) and DAGAT (hatched bars) activities in microsomes from developing cotyledons of safflower. Batches of microsomes (144 μg of protein each) were supplied with one of the four [^{14}C]DAG substrates indicated, CDP-choline, and 18:1-CoA as described in "Materials and Methods." After incubation for 15 min at 30°C, assays were terminated by addition of $CHCl_3$:CH_3OH (1:1, v/v). Enzyme activities were determined as the radioactivity incorporated into the PC and TAG products.

Substrate Specificities of CPT and DAGAT in *Cuphea* Microsomes

C. lanceolata accumulates high proportions (up to 90%) of medium-chain fatty acids (C_8–C_{12}) in its TAGs, but these

same fatty acids are almost completely excluded from membrane lipids of the oleaginous seed tissue (Bafor et al., 1990). In preliminary experiments with *Cuphea* microsomes, we observed lower rates of [^{14}C]PC accumulation when 10:0/10:0-DAG was the exogenous substrate than when 18:1–18:1-DAG was the precursor provided. In addition, the two-phase extraction of the 10:0/10:0-DAG assays resulted in partitioning of a significant proportion of the label into the aqueous phase. Very little radioactivity was in the aqueous phases of either time-0 assays with 10:0/10:0-DAG or 20-min assays with 18:1/18:1-DAG as substrate. To identify the water-soluble compound derived from 10:0/10:0-DAG, the aqueous phase was lyophilized and the residue was re-extracted with $CHCl_3/CH_3OH$ (1:1, v/v). Separation of this extract by silica gel TLC identified a single radioactive product that co-migrated with authentic LPC. The polarity of 10:0-LPC is sufficiently high to ensure that this compound partitions almost quantitatively into the aqueous phase of our two-phase extraction system (Stahl et al., 1995).

The conversion of 10:0/10:0-PC to LPC in microsomes from developing cotyledons of *Cuphea* was further characterized by incubating these membranes with 150 nmol of either 18:1/18:1- or 10:0/10:0-DAGs per milligram of microsomal protein and analyzing the production of PC and LPC during a 30-min assay at 30°C (Fig. 4). In this experiment, 18:1/18:1-DAGs were rapidly converted to 18:1/18:1-PC by the action of CPT and no significant amount of LPC was produced. Although 10:0/10:0-DAGs were also transformed to 10:0/10:0-PC by the same enzyme, the amount accumulating during the 30-min incubation period was significantly lower. Instead, after a very short time lag, radioactive LPC was generated from the initial 10:0/10:0-PC product and quickly accounted for 50% or more of the total polar lipid products synthesized. A 10:0-PC acyl hydrolase activity in *Cuphea* microsomes has recently been described (Stahl et al., 1995). The results shown in Figure 4 demonstrate that this activity very rapidly degrades newly synthesized 10:0/10:0-PC. Stahl et al. (1995) reported hydrolysis of 10:0 at both the *sn-1* and *sn-2* positions of PC, but in our assays LPC was the major product and neither [^{14}C]glycerophosphorylcholine nor [^{14}C]glycerol accumulated to any extent during 15- to 30-min assays (data not shown). For assays in which PC hydrolysis was confirmed, the rate of CPT activity was calculated by summing the radioactivity in PC and LPC.

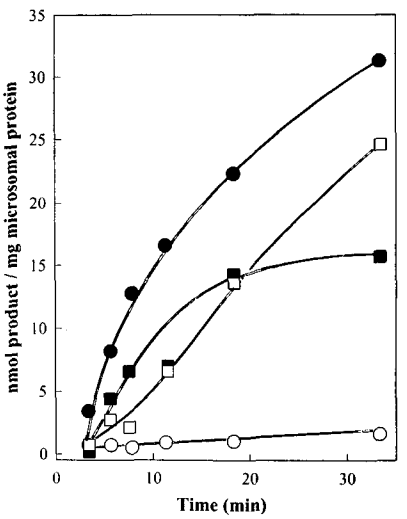

Figure 4. Time course of LPC production in CPT assays with microsomes from *C. lanceolata*. Two batches of microsomes (886 μg of protein each) were supplied with either 18:1/18:1- or 10:0/10:0-[^{14}C]DAG and CDP-choline using the method of Vogel and Browse (1995). Aliquots of the reaction mixtures were removed at intervals, and the lipids were extracted in $CHCl_3$:CH_3OH (1:1, v/v). Products of the assay were analyzed as described in "Materials and Methods." ●, PC from 18:1/18:1-DAG; ○, LPC from 18:1/18:1-DAG; ■, PC from 10:0/10:0-DAG; □, LPC from 10:0/10:0-DAG.

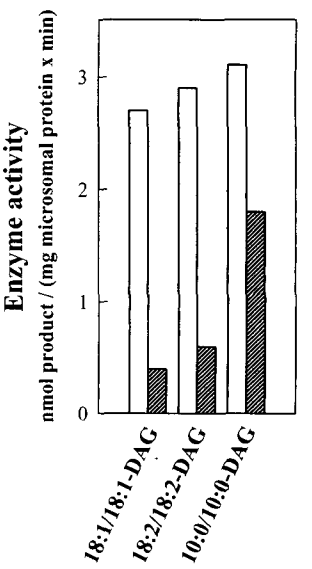

Figure 5. Rates of CPT (open bars) and DAGAT (hatched bars) activities in microsomes from developing cotyledons of *C. lanceolata*. Batches of microsomes (220 μg of protein each) were supplied with one of the three [^{14}C]DAG substrates indicated, CDP-choline, and 18:1-CoA as described in "Materials and Methods." After incubation for 15 min at 30°C, assays were terminated by addition of $CHCl_3$:CH_3OH (1:1, v/v). Enzyme activities were determined as the radioactivity incorporated into the PC (plus LPC) and TAG products.

The activities of CPT and DAGAT in microsomal preparations from developing cotyledons of *C. lanceolata* were assayed with either 18:1/18:1-, 18:2/18:2-, or 10:0/10:0-DAGs. All three substrates were transformed to PC at comparable rates (Fig. 5). In contrast, DAGAT activity in these same assays showed much higher rates when 10:0/10:0-DAG was the substrate (Fig. 5). Compared to 18:1/18:1-DAG, the substrate with medium-chain fatty acids was converted to TAG at least 4 times faster, indicating a distinct preference for this precursor.

Experiments with Castor Bean Microsomes

Castor bean (*R. communis*) stores mostly ricinoleic (18:1-OH) acid in its seed TAGs. CPT and DAGAT from developing castor bean endosperm were tested for their ability to discriminate between 18:1/18:1-, 18:2/18:2-, 10:0/10:0-, 22:1/22:1-, 18:1-OH/18:1-OH-, and 18:1-OH/18:X-DAGs

(Fig. 6). Similar to the enzymes from safflower and *Cuphea*, the CPT in castor microsomes converted each of these DAG molecular species to PC and showed no preference for any of the substrates presented. Thus, in vitro at least, the CPT is unable to exclude DAGs with ricinoleic acid from being used for the synthesis of PC. The range of CPT activity measured when using different microsomal preparations with 18:1/18:1-DAG as the substrate was between 4.1 and 8.5 nmol of PC produced per milligram of microsomal protein and per minute and in any given preparation, the rate for 18:1-OH containing DAGs was within 20% of the rate observed with the di-18:1 substrates. However, in the same assays, DAGAT clearly demonstrated a preference for 18:1-OH/18:1-OH-DAGs, which were converted to TAG at rates 3- to 4-fold higher than the rate of TAG synthesis from 18:1/18:1-DAG (Fig. 6). It is interesting that the DAGAT exhibited low activity toward DAGs with only one ricinoleic acid residue (five to six times lower compared to assays with DAGs containing two ricinoleic acids). The lowest transformation rate of DAG to TAG was found with 22:1/22:1-DAG as the substrate, and this is similar to the result obtained with microsomes from developing safflower cotyledons.

When 18:1-OH/18:1-OH-DAGs were used as the substrate in assays with castor microsomes, approximately 60% of the product of CPT activity were recovered as LPC after the standard 15-min assay at 30°C. As with the assays

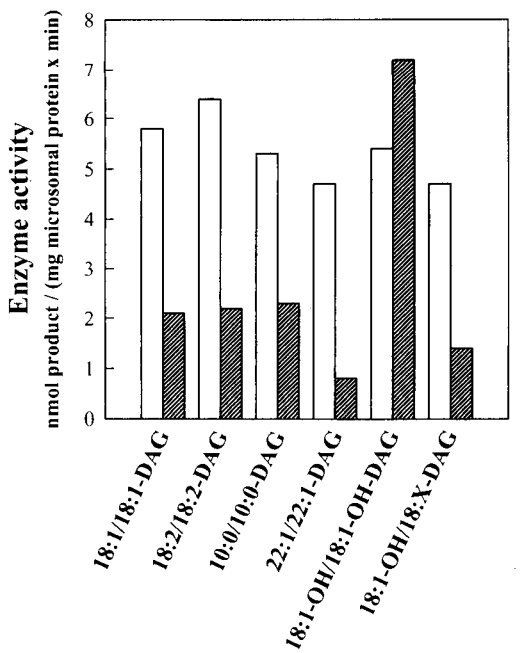

Figure 6. Rates of CPT (open bars) and DAGAT (hatched bars) activities in microsomes from developing endosperm of castor bean. Batches of microsomes (179 μg of protein each) were supplied with one of the six [^{14}C]DAG substrates indicated, CDP-choline, and 18:1-CoA as described in "Materials and Methods." After incubation for 15 min at 30°C, assays were terminated by addition of $CHCl_3$:CH_3OH (1:1, v/v). Enzyme activities were determined as the radioactivity incorporated into the PC (plus LPC) and TAG products.

of *Cuphea* microsomes, no significant further degradation of the labeled 18:1-OH (or 10:0-LPC) to [^{14}C]glycerophosphorylcholine or [^{14}C]glycerol was observed in the 30-min assays. The existence of acylhydrolase specifically removing hydroxylated fatty acids from PC had been reported by Bafor et al. (1991) and Stahl et al. (1995). This activity was first identified during experiments on the synthesis of ricinoleate. It was shown to rapidly remove 18:1-OH from PC following its formation by the hydroxylation of oleate (18:1) at the Δ12 position. It is interesting that 10:0/10:0-PC produced during assays with 10:0/10:0-DAG was equally well hydrolyzed to LPC in castor microsomes (data not shown).

Assays with Microsomes from Rapeseed

Developing rapeseed cotyledons synthesize very-long-chain fatty acids (predominantly 20:1 and 22:1) that accumulate to approximately 55% of the total fatty acids in TAGs of mature seeds. In our first assays of rapeseed microsomal preparations, the initial rates of PC synthesis from 18:1/18:1-DAG were in the range of 0.3 to 0.6 nmol mg^{-1} protein min^{-1}, which is 10-fold lower than the rates observed with microsomes from the other oilseed species studied. To test whether the rapeseed tissue contained compounds that were inhibiting the CPT activity, we homogenized developing safflower and rapeseed cotyledons together and prepared a mixed microsomal preparation. The rate of CPT activity in this microsomal preparation was approximately half of the rate observed for safflower microsomes in the same experiment. This result is consistent with the rapeseed material diluting the safflower activity without causing any inhibition of the safflower CPT and indicates that rapeseed microsomes contain an inherently lower CPT activity than the other oilseeds studied.

Comparisons of CPT activity in rapeseed microsomes against 18:1/18:1-DAG and 22:1/22:1-DAG consistently showed a 60% lower rate of conversion for the very-long-chain DAG substrate (Fig. 7). However, 22:1/22:1-DAG is not a significant intermediate of the Kennedy pathway in rapeseed because the acyl-CoA:*lyso*-PA acyltransferase does not recognize 22:1-CoA as a substrate (Sun et al., 1988). Typically, DAGs in rapeseed contain 20:1 and 22:1 only at the *sn-1* position. To determine whether the CPT of rapeseed microsomes could discriminate against such DAG species, we assayed the activity using 20:1/18:1-, 20:1/18:2-, 22:1/18:1-, and 22:1/18:2-DAGs isolated from rapeseed cotyledons that had been incubated with [^{14}C]-glycerol. The rates of PC synthesis observed with all of the naturally occurring DAG substrates containing very-long-chain fatty acids were between 0.11 and 0.14 nmol mg^{-1} protein min^{-1} in assays in which PC synthesis from 18:1/18:1-DAGs proceeded at a rate approaching 0.30 nmol mg^{-1} protein min^{-1} (Fig. 7). Although the fact that CPT in rapeseed microsomes discriminates in favor of 18:1/18:1- and 18:2/18:2-DAGs is consistent with the need to exclude very-long-chain fatty acids from the membrane lipids, the 2- to 3-fold difference in rates is relatively small.

Unexpectedly, the DAGAT activity in these same assays showed a similar bias against DAG species with one or two

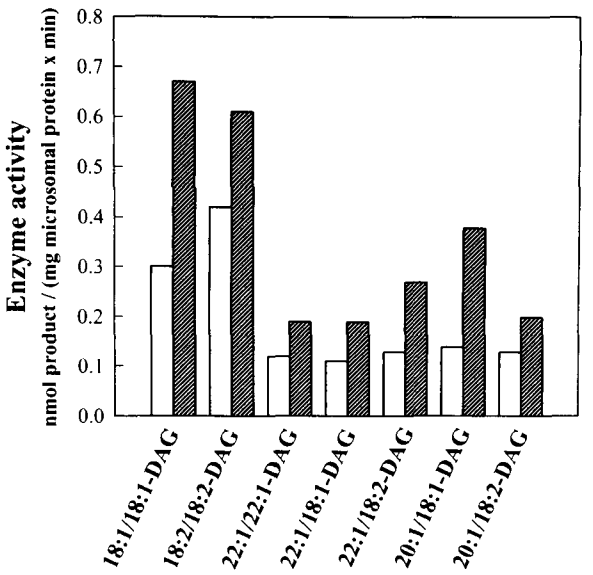

Figure 7. Rates of CPT (open bars) and DAGAT (hatched bars) activities in microsomes from developing cotyledons of rapeseed. Batches of microsomes (150 μg of protein each) were supplied with one of the seven [^{14}C]DAG substrates indicated, CDP-choline, and 18:1-CoA as described in "Materials and Methods." After incubation for 15 min at 30°C, assays were terminated by addition of $CHCl_3$:CH_3OH (1:1, v/v). Enzyme activities were determined as the radioactivity incorporated into the PC and TAG products.

very-long-chain fatty acids and in favor of 18:1/18:1- or 18:2/18:2-DAGs (Fig. 7). In principle, the similar levels of discrimination of CPT and DAGAT against DAGs that contain 20:1 or 22:1 fatty acids could result from a failure of these molecular species to enter the microsomal bilayer and become available as substrates. However, microsomes from safflower and castor bean both exhibited CPT activities with 22:1/22:1-DAG that were 75 to 80% of those with 18:1/18:1-DAG (Figs. 3 and 5), indicating that transfer of 20:1- and 22:1-containing DAGs to the rapeseed membranes is unlikely to be limiting.

DISCUSSION

The goal of this study was to directly investigate competition between CPT and DAGAT activities at the branch point between membrane lipid synthesis and storage lipid synthesis in microsomes from developing oilseed tissues. There is considerable evidence that both of these enzymes are present in microsomes prepared from oilseeds and that, in vivo, both are localized to the ER (Slack et al., 1985; Stymne and Stobart, 1985, 1987; Browse and Somerville, 1991), which is also the site of DAG synthesis from glycerol-3-phosphate and acyl-CoA (Griffiths et al., 1985; Stymme and Stobart, 1987). For those plants that accumulate unusual fatty acids in their oils, it has been hypothesized that the CPT should prevent incorporation of unusual fatty acids into PC by discriminating against DAG species that contain such fatty acids (Bafor et al., 1990). The importance of preventing the accumulation of significant levels of 10:0, 18:0-OH, 22:1, or other unusual acyl groups in membrane phospholipids lies in the fact that these fatty acid structures are incompatible with the maintenance of a functional lipid bilayer. Equally, the predominance of unusual fatty acids in TAGs of the plants we have studied requires that the corresponding DAG species must be the major substrate utilized by DAGAT.

Contrary to expectations, none of the four oilseed plants investigated possessed a CPT activity that showed any substantial ability to discriminate against DAG molecular species containing unusual fatty acids. In *Cuphea*, the rate of PC synthesis from 10:0/10:0-DAG was slightly higher than the rates from 18:1/18:1- or 18:2/18:2-DAGs. Only the enzyme in microsomes from developing rapeseed cotyledons used 18-carbon DAGs at higher rates than molecular species containing either one or two very-long-chain acyl groups. However, a 2- to 3-fold reduction in CPT activity toward 20:1- and 22:1-DAG does not appear to be sufficient discrimination to explain the substantial absence of 20:1- and 22:1 from seed PC. Thus, in *Cuphea*, castor bean, and rapeseed it is very unlikely that the CPT enzyme itself plays a major role in excluding membrane-incompatible fatty acids from PC.

In *Cuphea* microsomes, newly synthesized 10:0/10:0-PC was rapidly degraded to LPC, presumably by a fatty-acid-specific phospholipid acyl hydrolase activity (Stahl et al., 1995). In castor bean microsomes, PCs containing either 18:1-OH or 10:0 fatty acids were hydrolyzed in a similar manner. The breakdown of PC was extremely rapid so that more than 60% of the 10:0/10:0-PC synthesized by *Cuphea* microsomes during a 35-min assay were hydrolyzed (Fig. 4). In our assays, we did not observe any further breakdown of LPC similar to that recorded by Stahl et al. (1995) in 120-min assays. In castor bean endosperm, 18:1-OH is synthesized on PC and an 18:1-OH-specific phospholipase is an important component of TAG synthesis (Bafor et al., 1991). Nevertheless, it is clear that fatty-acid-specific phospholipases could also usefully degrade inappropriate molecular species of PC made by an undiscriminating CPT enzyme. However, substantial reliance on such a mechanism would set up a futile cycle. For this reason, we consider it likely that the fatty-acid-specific phospholipases in microsomes would have only a secondary role in supporting a primary mechanism that targets unusual fatty acids for TAG synthesis and excludes them from membrane phospholipids. It is noteworthy that transgenic Arabidopsis and rapeseed that contain a 12:0-specific acyl-ACP thioesterase do accumulate 12:0 in their oils (Voelker et al., 1992). Preliminary reports indicate that 12:0 is present in membrane lipids from developing seeds of these transgenic plants, although this fatty acid largely disappears from the membrane lipids as the seeds approach maturity (Wiberg et al. 1995). These data suggest that rapeseed may lack at least some of the mechanisms that act in *Cuphea* to prevent accumulation of 12:0 in membrane lipids of the seeds.

The other enzyme involved in the utilization of DAGs is DAGAT. In plants storing unusual fatty acids in their seed TAGs, a very large flux of DAGs is channeled through this enzymatic step during the period of active TAG accumulation (Slack et al., 1985). In our experiments, DAGAT from

C. lanceolata and castor bean both showed a distinct preference for the molecular species of DAG containing either two medium-chain (10:0) or two hydroxylated fatty acids, respectively. Wiberg et al. (1994) were able to show similar properties of DAGAT using a different experimental approach. Provided with a mixture of 10:0/10:0- and 18:1/18:1-DAG, DAGAT from *Cuphea procumbens* strongly selected (14.5-fold) the DAG with medium-chain fatty acids when [^{14}C]18:1-CoA was used as the acyl donor. With [^{14}C]10:0-CoA, the preference of the 10:0/10:0-DAG was even stronger (44-fold), indicating a possible influence of the acyl-CoA pool composition on the selectivity of DAGAT in oleaginous tissue of this plant. In comparable experiments with microsomes from developing endosperm of castor bean, DAGAT showed a 1.7- to 2.6-fold preference for DAGs with two ricinoleoyl groups compared to 18:1/18:1-DAG. These results are similar to our findings, although the absolute rates of enzyme activity were about 5-fold lower in the experiments reported by Wiberg et al. (1994), who supplied exogenous DAGs dissolved in detergents. Of the three species studied that accumulate unusual fatty acids, only the DAGAT activity in rapeseed microsomes showed a preference of 18:1/18:1- and 18:2/18:2-DAGs over the DAG molecular species containing unusual fatty acids, which are the predominant molecular species utilized in vivo.

The high activities of DAGAT enzymes of *Cuphea* and castor bean toward 10:0/10:0-DAG or 18:1-OH/18:1-OH-DAG, respectively, suggest that one means to restrict the entry of unusual fatty acids into PC and other membrane phospholipids might be to rapidly convert these DAG molecular species into TAG as they are formed. The levels of 10:0-DAG in *Cuphea* seeds and 18:1-OH-DAG in castor bean endosperm are significant, accounting for 20 to 30% of the total DAG molecular species (Bafor et al., 1990; G. Vogel, unpublished data). These data are based on whole-tissue measurements and it is not possible to accurately assess levels in the ER in vivo. However, comparisons of labeling experiments in which microsomes from safflower, *Cuphea*, and castor bean were supplied with [^{14}C]glycerol-3-phosphate and either 18:1-, 10:0-, or 18:1-OH-CoA, respectively (Griffiths et al., 1985; Bafor et al., 1990, 1991), suggest that the derived [^{14}C]DAG pool is of approximately the same size in each case. Such data are indirect evidence against the possibility that rapid utilization or substrate channeling provides for a pool of unusual DAG that is so small that it contributes very little (relative to the more abundant normal DAG species) to PC synthesis by CPT.

Assuming that our in vitro assays substantially reflect the in vivo DAG specificities of the CPT and DAGAT enzymes, there appears to be no enzymatic mechanism to account for the very substantial segregation of metabolism between unusual fatty acids targeted to TAG synthesis and normal 16- and 18-carbon fatty acids utilized for membrane lipid synthesis. Certainly, if DAGs containing unusual fatty acids are present in a common pool, then it is very likely that any of the CPT enzymes in the species we have studied would convert them to PC.

There may be multiple mechanisms to prevent the synthesis and accumulation of membrane lipids containing unusual fatty acids. The substrate-specific phospholipid acyl hydrolase activities (Stahl et al., 1995; Fig. 4) and the molecular species specificities of DAGAT (Figs. 5 and 6; Wiberg et al., 1994) will certainly contribute. A third possibility is that membrane lipid synthesis and TAG synthesis, in vivo, are carried out in substantially separate domains of the ER. It is now well established that the ER is a complex organelle in which spatial separation of biochemical functions is common (Palade, 1975; Shore and Tata, 1977; Li et al., 1993). It is likely that the TAG storage organelles, the oil bodies, arise from the ER, perhaps by accumulation of TAG between the two leaflets of the bilayer (Schwarzenbach, 1971; Herman, 1995). Available evidence suggests that the developing oil bodies may be separated from cisternal ER membranes and arise at the distal ends of specific structures, the tubular ER (Herman, 1995). If such a model is correct, then spatial compartmentation of biosynthetic enzymes—with DAGAT, but not CPT, being associated with tubular ER domains—would provide a satisfactory means of separating membrane and storage lipid syntheses in plants that accumulate unusual fatty acids.

Under this hypothesis, the DAGAT compartment would contain isozymes of glycerol-3-phosphate acyltransferase and *lyso*-phosphatidic acid acyltransferase that were specific for the production of unusual DAGs, whereas the CPT compartment would contain distinct isozymes favoring the synthesis of DAGs containing fatty acids commonly found in the membrane lipids. In principle, the degree of substrate selectivity of the enzymes and transfer between the compartments may allow some normal fatty acids to be incorporated into TAGs. As discussed above, the corresponding incorporation of unusual fatty acids into PC would be resolved by the action of substrate-specific acyl hydrolases.

Although spatial separation of CPT and DAGAT is an attractive hypothesis for oilseeds that synthesize TAGs containing uncommon fatty acyl structures, it is not an obviously good model for plants, such as safflower, that accumulate polyunsaturated 18-carbon fatty acids. In these plants, CPT might be expected to be closely associated with DAGAT because PC is the major substrate for 18:2 and 18:3 synthesis, and CPT is known to provide polyunsaturated molecular species of DAG used in TAG synthesis (Slack et al., 1985; Browse and Somerville, 1991). However, it is possible to envision these plants as representing a situation in which there is extensive transfer between the ER compartments or in which a single compartment has been created by appropriately altered targeting of the enzymes involved. Given the limited resolution of subcellular fractionation techniques, empirical testing of these possibilities will require antibodies to the CPT and DAGAT proteins, which can then be used in ultrastructural immunolocalization experiments.

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