Biosynthesis of Triacylglycerols Containing Very Long Chain Monounsaturated Acyl Moieties in Developing Seeds

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ABSTRACT

Particulate (15,000g) fractions from developing seeds of honesty (Lunaria annua L.) and mustard (Sinapis alba L.) synthesize radioactive very long chain monounsaturated fatty acids (gadoleic, erucic, and nervonic) from [1-14C]oleoyl-CoA and malonyl-CoA or from oleoyl-CoA and [2-14C]malonyl-CoA. The very long chain monounsaturated fatty acids are rapidly channeled to triacylglycerols and other acyl lipids without intermediate accumulation of their CoA thioesters. When [1-14C]oleoyl-CoA is used as the radioactive substrate, phosphatidylcholines and other phospholipids are most extensively radioabeled by oleoyl moieties rather than by very long chain monounsaturated acyl moieties. When [2-14C]malonyl-CoA is used as the radioactive substrate, no radioactive oleic acid is formed and the newly synthesized very long chain monounsaturated fatty acids are extensively incorporated into phosphatidylcholines and other phospholipids as well as triacylglycerols. The pattern of labeling of the key intermediates of the Kennedy pathway, e.g., lysophosphatidic acids, phosphatidic acids, and diacylglycerols by the newly synthesized very long chain monounsaturated fatty acids is consistent with the operation of this pathway in the biosynthesis of triacylglycerols.

Seed oils of many Cruciferae are rich in TG containing VLMFA, such as 20:1, 22:1, and 24:1(2). Formation of VLMFA by chain elongations of 18:1 has been shown from compositional changes or radiolabeling studies in developing seeds of Brassica napus(5), Crambe abyssinica(3,7), Tropaeolum majus(19), Sinapis alba(12,14), and Lunaria annua(15). More recently, the biosynthesis of VLMFA via condensation of 18:1-CoA with malonyl-CoA has been demonstrated in particulate fractions of developing seeds, such as the 15,000g fraction from seeds of B. juncea(1), L. annua, T. majus, and S. alba(16,17).

It is generally accepted that in higher plants TG containing C16 and C18 acyl moieties are formed via the G3P pathway according to Kennedy (10) which consists of sequential acylations of G3P and LPA by acyl-CoAs followed by dephosphorylation and acylation of the resulting DG(20). Several studies indicate that the G3P pathway is also involved in the formation of TG containing VLMFA in seeds of C. abyssinica(8) and S. alba(11,13). However, the incorporation of VLMFA from their CoA thioesters into TG via the key intermediates of the G3P pathway, e.g. LPA, PA, and DG, could not be successfully demonstrated so far(6,11,13,21).

We report here the mode of channeling of VLMFA, synthesized via condensation of 18:1-CoA with malonyl-CoA by the 15,000g particulate fractions from developing seeds of L. annua and S. alba, for the synthesis of TG and other acyl lipids. The pattern of incorporation of VLMFA into TG and the key intermediates of the G3P pathway are found to be consistent with the operation of this pathway.

MATERIALS AND METHODS

Plant Materials

Plants of honesty (Lunaria annua L., garden variety) and mustard (Sinapis alba L., cv Albatros) were grown outdoors and the developing seeds, collected at 6 weeks after flowering, were used. The seed coats were removed and the 15,000g particulate fractions were prepared from the cotyledons (750 mg L. annua or 200 mg S. alba) as described earlier (16).

Incubations

The particulate fractions were suspended in a solution composed of 80 mM Hepes buffer (pH 7.2), 320 mM sucrose, and 10 mM ß-mercaptoethanol. Aliquots (50 µL) of the 15,000g particulate fractions containing 100 to 150 µg protein were used. The incubation mixture (total volume 100 µL) contained as final concentrations 1 mM ATP, 1 mM CoASH, 0.5 mM NADPH, 0.5 mM NADH, 2 mM MgCl2 in 80 mM Hepes (pH 7.2). Radiactive substrates (New England Nuclear, Inc and Amersham) used were either 7.4 to 18.5 KBq [1-14C]18:1-CoA (40-100 µM) and 1 µM malonyl-CoA or 3.7 to 18.5 KBq [2-14C]malonyl-CoA (20-100 µM) and 100 µM

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Figure 1. Distribution of radioactivity in total lipids and aqueous phase (acyl-CoA) recovered from 15,000g particulate fractions from developing seeds of *L. annua* upon incubation with the following substrates. A, [1-14C]18:1-CoA (18.5 KBq, 100 µM) and malonyl-CoA (1 mM); B, [2-14C]malonyl-CoA (18.5 KBq, 100 µM) and 18:1-CoA (100 µM).

18:1-CoA. Incubations were carried out by gyratory shaking (120 gyrations·min⁻¹) at 30°C for various periods.

In several experiments, acyl acceptors, such as G3P, sn-1-oleoylphosphatidic acid or sn-1,2-(2,3)-dioleoylglycerols, 0.5 mM each, were added to the incubation mixture. LPA and DG were added in chloroform to the empty tube, the solvent was removed, and, after adding all the constituents of the incubation mixture, the sample was sonicated with a Branson sonifier.

**Extraction and Analysis of Lipids**

Incubations were halted by adding 5 mL of a mixture of chloroform:methanol (2:1, v/v) to yield a monophasic solution. Subsequently 1 mL of 0.7% (w/v) NaCl in 0.1% (v/v) acetic acid was added to separate the chloroform-soluble lipids from the aqueous phase containing the acyl-CoA and malonyl-CoA thioesters.

The chloroform-soluble lipids were fractionated by TLC on Silica Gel H. The chromatograms were developed twice with diethyl ether to a height of 2 cm to separate the neutral lipids and unesterified fatty acids from PL that remained at the origin. Subsequently, TG, unesterified fatty acids, DG, and MG were separated by development with hexane:diethyl ether:acetic acid (70:30:1, v/v). The PL were fractionated on Silica Gel H using chloroform:acetonewater (10:4:2:2:1, v/v) as the developing solvent. The lipid fractions were stained with iodine vapor, scraped from the TLC plate, and radioactivity was measured in a LKB 1214 liquid scintillation spectrometer using toluene scintillator (PPO 5 g/L, POPOP 0.1 g/L) (Packard Instruments Inc). Alternatively, the lipid fractions were made visible under UV light by spraying lightly with 0.7% (w/v) ethanolic 2,7-dichlorofluorescein solution and scraped from the TLC plate. Lipids contained in the scrapings were converted to methyl esters and analyzed by radio-GLC as described earlier (16).

Radioactivity in aliquots of the aqueous phase remaining after separation of the chloroform-soluble lipids was measured using Aquasol-2 (New England Nuclear, Inc). Composition of the radioactive products present in the aqueous phase was checked by TLC on Silica Gel H using 1-butanol:acetic acid:water (5:2:3, v/v) as solvent and [1-14C]stearoyl-CoA as a standard. The radioactive products present in the aqueous phase were almost entirely composed of malonyl-CoA and/or acyl-CoA thioesters. Acyl-CoA thioesters contained in the aqueous phase were hydrolyzed, the resulting fatty acids treated with diazemethane and the methyl esters analyzed by radio GLC as described earlier (16).

**RESULTS AND DISCUSSION**

Incubation of the 15,000g fraction from the developing seeds of *Lunaria annua* with [1-14C]18:1-CoA together with malonyl-CoA or [2-14C]malonyl-CoA together with 18:1-CoA led to rapid incorporation of radioactivity into the chloroform-soluble lipids and simultaneous reduction of radioactivity in the CoA thioesters, as shown in Figure 1.

The distribution of radioactivity in the constituent acyl moieties of the lipids of the 15,000g particulate fractions from *L. annua* seeds upon incubation with [1-14C]18:1-CoA for different periods is shown in Figure 2.

The time course of decrease of radioactive 18:1 upon incubation with [1-14C]18:1-CoA and concomitant increase of
particulate fraction from *L. annua* seeds with [1-14C]18:1-CoA or [2-14C]malonyl-CoA is shown in Figure 3.

Upon incubation with [1-14C]18:1-CoA, the only radioactive product of elongation present in the acyl-CoA pool was found to be 20:1 (Fig. 3A), although the corresponding chloroform-soluble lipids contained both radioactive 22:1 and 24:1 in addition to 20:1 (Fig. 2). It appears, therefore, that 22:1-CoA and 24:1-CoA formed by elongation are more rapidly metabolized to chloroform-soluble lipids than 20:1-CoA.

Upon incubation with [2-14C]malonyl-CoA both radioactive 20:1 and 22:1 were detected in the acyl-CoA pool (Fig. 3B). Decrease of radioactive 20:1-CoA with time and concomitant increase of radioactive 22:1-CoA (Fig. 3B), taken together with initial decrease of radioactive 20:1 in chloroform-soluble lipids, are in agreement with successive elongations of 18:1 at the level of the CoA thioesters.

Figure 4 shows the distribution of radioactivity in the total PL, MG, DG, and TG upon incubation of the 15,000g particulate fraction from *L. annua* seeds with the two radioactive CoA thioester substrates. Extensive initial labeling of PL occurred from both [1-14C]18:1-CoA and [2-14C]malonyl-CoA, which was followed by decrease in the labeling of PL and concomitant increase of radioactivity in TG with simultaneous labeling of DG and MG (Fig. 4). These findings are consistent with the operation of the G3P pathway (10). Formation of radioactive unesterified fatty acids (not shown) is

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**Figure 3.** Distribution of radioactivity in acyl moieties of acyl-CoA fraction recovered from 15,000g particulate fractions from developing seeds of *L. annua* upon incubation with 14C-labeled substrates as given in Figure 1 for indicated times. A, [1-14C]18:1-CoA; B, [2-14C]malonyl-CoA.

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radioactive 22:1 and 24:1 (Fig. 2) is consistent with successive elongations from 18:1-CoA via condensations with malonyl-CoA, as observed earlier (16, 17). Accumulation of the intermediate 20:1 was not observed.

Incubation of the 15,000g particulate fraction from *L. annua* with [2-14C]malonyl-CoA yielded exclusively radioactive VLMFA (20:1 and 22:1), obviously from 18:1-CoA by successive condensations, but formation of radioactive 16:0 or 18:1 intermediates by de novo synthesis was not observed (data not shown). After 0.5 h of reaction time the relative proportion of radioactive 20:1 and 22:1 in the lipids was about 70:30, after 1.5 h the proportion was 50:50 and it remained essentially unchanged with longer incubation periods.

The composition of the radioactive acyl moieties of the acyl-CoA fraction resulting from incubation of the 15,000g particulate fraction from *L. annua* seeds with [1-14C]18:1-CoA or [2-14C]malonyl-CoA is shown in Figure 3.

Upon incubation with [1-14C]18:1-CoA, the only radioactive product of elongation present in the acyl-CoA pool was found to be 20:1 (Fig. 3A), although the corresponding chloroform-soluble lipids contained both radioactive 22:1 and 24:1 in addition to 20:1 (Fig. 2). It appears, therefore, that 22:1-CoA and 24:1-CoA formed by elongation are more rapidly metabolized to chloroform-soluble lipids than 20:1-CoA.

Upon incubation with [2-14C]malonyl-CoA both radioactive 20:1 and 22:1 were detected in the acyl-CoA pool (Fig. 3B). Decrease of radioactive 20:1-CoA with time and concomitant increase of radioactive 22:1-CoA (Fig. 3B), taken together with initial decrease of radioactive 20:1 in chloroform-soluble lipids, are in agreement with successive elongations of 18:1 at the level of the CoA thioesters.

Figure 4 shows the distribution of radioactivity in the total PL, MG, DG, and TG upon incubation of the 15,000g particulate fraction from *L. annua* seeds with the two radioactive CoA thioester substrates. Extensive initial labeling of PL occurred from both [1-14C]18:1-CoA and [2-14C]malonyl-CoA, which was followed by decrease in the labeling of PL and concomitant increase of radioactivity in TG with simultaneous labeling of DG and MG (Fig. 4). These findings are consistent with the operation of the G3P pathway (10). Formation of radioactive unesterified fatty acids (not shown) is
attributed to hydrolysis of the 14C-labeled acyl-CoA thioesters and/or acyl lipids during the incubations.

The data presented in Figure 5A show that the major class of polar lipids that became radioactively labeled upon incubation with [1-14C]18:1-CoA was PC. Extensive radiolabeling of LPA and LPC, in addition to PC, occurred when [2-14C]-malonyl-CoA was used as the substrate (Fig. 5B).

Figure 6 and Figure 7 show the distribution of radioactivity in the acyl moieties of the major classes of PL, e.g. PC, LPC, PA, and LPA, as well as in TG upon incubation of the 15,000g particulate fraction from L. annua seeds with the two 14C-labeled CoA thioester substrates.

Incubation with [1-14C]18:1-CoA led to extensive incorporation of 18:1 and minor incorporation (15-20%) of 20:1 into PC, LPA, and PA (Fig. 6A). However, 22:1 and 24:1, synthesized from 14C-18:1-CoA, which were readily incorporated into TG (Fig. 7A), were not found in PC, LPC, PA, or LPA (Fig. 6A). Thus, in the presence of a large excess of 18:1-CoA in the incubation mixture, 18:1 moieties rather than their elongation products are channeled to PC, LPC, PA, and LPA. Apparently, 18:1-CoA is the preferred substrate of the acyltransferases involved in the formation of PC, LPC, PA, and LPA.

Upon incubation with [2-14C]malonyl-CoA, however, no labeled 18:1-CoA was detected (Fig. 3B), but large proportions of labeled 20:1 and 22:1 were synthesized which were readily incorporated into PC, LPC, PA, and LPA (Fig. 6B) as well as TG (Fig. 7B). Thus, when VLMFA-CoA thioesters are offered to the acyltransferases in a large excess over the preferred substrate 18:1-CoA, the former are also extensively utilized for the synthesis of PC and LPC as well as PA and LPA, that are key intermediates of the G3P pathway, as well as TG.

With [1-14C]18:1-CoA as substrate, the virtual absence of 14C-labeled 22:1 and 24:1 in LPA and PA (Fig. 6A), but abundance of the VLMFA in TG (Fig. 7A) indicates that under these conditions the acylation of the sn-3 position of DG by VLMFA might be the major step in the biosynthesis of TG as proposed earlier (11, 13). The pattern of labeling observed upon incubation of the 15,000g particulate fraction from L. annua seeds with [1-14C]18:1-CoA was also found upon incubation of the whole seeds with [1-14C]acetate (6). Thus, predominantly radioactive 18:1 but very little newly synthesized VLMFA were incorporated into PA and DG, although the TG were extensively labeled by VLMFA almost exclusively at the sn-1,3 positions.
The patterns of labeling of acyl lipids by 18:1 and VLMFA upon incubations of the 15,000g particulate fraction from the *S. alba* seeds with [1-^14^C]18:1-CoA or [2-^14^C]malonyl-CoA were found to be quite similar (Figs. 8–10) to those observed for the corresponding fraction from *L. annua* seeds (Figs. 1–7).

Incubation of the 15,000g particulate fraction from *S. alba* with [1-^14^C]18:1-CoA and malonyl-CoA yielded radioactive VLMFA (20:1 and 22:1) (Fig. 8A; and refs. 14, 15). Most of the radioactive acyl moieties were transferred from the acyl-CoA pool to the chloroform-soluble lipids within 3 h (not shown). Considerable radiolabeling of PC occurred, and the time course of labeling of PA, DG, and TG by 18:1 and its elongation products was found to be consistent with the operation of the Kennedy pathway (Fig. 8B).

Figure 9 shows the time course of incorporation of 18:1 and its elongation products into PA, DG, and TG upon incubation of the 15,000g particulate fraction from *S. alba* with [1-^14^C]18:1-CoA. PC, as opposed to the other lipid classes, was labeled overwhelmingly with 18:1 (80–90%), to a minor extent with 20:1 (10–20%), but with no 22:1 at all (not shown). This is consistent with the data presented for *L. annua* in Figures 5, 6, and 7. TG as well as the key intermediates of the Kennedy pathway, PA and DG, became radiolabeled with substantial proportions of 20:1 and 22:1 in addition to 18:1 (Fig. 9). The time course of labeling of PA, DG, and TG with 18:1 and its elongation products is in good agreement with the involvement of the Kennedy pathway in the biosynthesis of TG containing VLMFA.

Incubation of the 15,000g particulate fraction from *S. alba* for 1.5 h with 18:1-CoA and [2-^14^C]malonyl-CoA yielded radioactive VLMFA (5–15% 20:1 and 85–95% 22:1) but no radioactive 18:1 (not shown).

The time course of distribution of radioactivity in the products of incubation shows that the decrease of radioactivity in malonyl-CoA was accompanied by rapid radiolabeling of the chloroform-soluble lipids, but the level of labeled VLMFA-CoA thioesters remained low and essentially constant throughout the incubation (Fig. 10A). It appears, therefore, that 'metabolite channeling' is involved in rapid transfer of the newly synthesized VLMFA from the acyl-CoA pool to the acyl lipids.
BIOSYNTHESIS OF VERY LONG CHAIN TRIACYLGlycerols

In all the experiments described so far no acyl acceptor, such as G3P, LPA, or DG, was included in the incubation mixture. It is most likely that the endogenous acyl acceptors were responsible for the observed patterns of radiolabeling of the glycerolipids from 18:1-CoA and VLMFA-CoA. To obtain further conclusive evidence on the operation of the Kennedy pathway in the biosynthesis of TG containing VLMFA, the 15,000g particulate fractions from L. annua were incubated with 18:1-CoA and [2-14C]malonyl-CoA in the presence of G3P, LPA, or DG. The effects of acyl acceptors are shown in Figures 11 and 12.

Figure 11 shows that the incorporation of radioactive VLMFA, newly synthesized from [14C]18:1-CoA, into total lipids of the 15,000g particulate fraction was barely affected, as compared to the controls, by the addition of either G3P or DG. However, distinctly lower proportions of the newly synthesized VLMFA were incorporated into the lipids when LPA was included in the incubation medium. This might be attributed to inhibitory action of LPA on the elongase system catalyzing the synthesis of VLMFA and/or on the acyltransferases involved in the synthesis of glycerolipids.

The data presented in Figure 10B also show radiolabeling of PC by VLMFA derived from [2-14C]malonyl-CoA, although to a lower extent than the radiolabeling of PC by 18:1 derived from [1-14C]18:1-CoA (Fig. 8B). This is again in agreement with 18:1-CoA rather than VLMFA-CoA being the preferred substrate for the acyltransferases involved in the synthesis of PC (Figs. 4, 5, and 6). The time course of radiolabeling of PA, DG, and TG by VLMFA (Fig. 10B) is consistent with the operation of the G3P pathway (10) in the biosynthesis of TG.

The data presented in Figure 10B also show radiolabeling of PC by VLMFA derived from [2-14C]malonyl-CoA, although to a lower extent than the radiolabeling of PC by 18:1 derived from [1-14C]18:1-CoA (Fig. 8B). This is again in agreement with 18:1-CoA rather than VLMFA-CoA being the preferred substrate for the acyltransferases involved in the synthesis of PC (Figs. 4, 5, and 6). The time course of radiolabeling of PA, DG, and TG by VLMFA (Fig. 10B) is consistent with the operation of the G3P pathway (10) in the biosynthesis of TG.

Figure 9. Distribution of radioactivity in individual acyl moiety classes recovered from 15,000g particulate fractions from developing seeds of S. alba upon incubation with [1-14C]18:1-CoA as given in Figure 8 for various periods.

Figure 10. Distribution of radioactivity in lipids recovered from 15,000g particulate fractions from developing seeds of S. alba upon incubation with [2-14C]malonyl-CoA (3.7 KBq, 20 µM) and 18:1-CoA (100 µM) for various periods. A. Malonyl-CoA, acyl-CoA, and total chloroform-soluble lipids; B. various classes of chloroform-soluble lipids.

Figure 11. Incorporation of radioactivity into lipids recovered from 15,000g particulate fractions from developing seeds of L. annua upon incubation with [2-14C]malonyl-CoA (7.4 KBq, 36 µM) and 18:1-CoA (100 µM) for various periods using different acyl acceptors. The control is marked by an asterisk (*).
to physical state of the exogenous DG emulsion system that was added to the reaction mixture, which may have affected the accessibility of the exogenous DG to the DG acyltransferase as has been found by other authors (4, 9). Alternatively, it is possible that endogenous DG is the greatly preferred substrate for the DG acyltransferase, possibly due to metabolite channeling as was found previously for the oleate desaturase system (18).

In summary, the results reported here suggest that VLMFA are formed initially as thioesters by chain elongation of 18:1-CoA, but that VLMFA thioesters are then rapidly channeled toward TG via the Kennedy pathway. The synthesis of VLMFA and their subsequent transfer to glycerol backbone probably proceeds via a concerted mechanism in vivo with little accumulation of intermediates. This may explain why previous attempts to observe the incorporation of exogenous VLMFA into TG and the key intermediates of the Kennedy pathway have been of limited success (6, 11, 13, 21).

**Figure 12.** Incorporation of radioactivity into individual lipid classes recovered from 15,000g particulate fractions from developing seeds of *L. annua* upon incubation with [2-14C]malonyl-CoA as given in Figure 11 for various periods using different acyl acceptors. A, Polar lipids; B, neutral lipids.

**LITERATURE CITED**

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