Plant Physiol. (1981) 67, 250–256
0032-0889/81/67/0250/07/$00.50/0

Synthesis of Long-Chain Acyl-CoA in Chloroplast Envelope Membranes[1]

Received for publication June 9, 1980 and in revised form September 5, 1980

JACQUES JOYARD[2] AND PAUL K. STUMPF
Department of Biochemistry and Biophysics, University of California, Davis, California 95616

ABSTRACT

The chloroplast envelope is the site of a very active long-chain acyl-coenzyme A (CoA) synthetase. Furthermore, we have recently shown that an acyl CoA thioesterase is also associated with envelope membrane (Joyard J, PK Stumpf 1980 Plant Physiol 65: 1039-1043). To clarify the interacting roles of both the acyl-CoA thioesterase and the acyl-CoA synthetase, the formation of acyl-CoA in envelope membranes was examined with different techniques which permitted the measurement of the actual rates of acyl-CoA formation. Using [^{14}C]ATP or [^{14}C]oleic acid as labeled substrates, it can be shown that the envelope acyl-CoA synthetase required both Mg^{2+} and dithiothreitol. Triton X-100 slightly stimulated the activity. The specificity of the acyl-CoA synthetase was determined either with [^{14}C]ATP or with [^{3}H]CoA as substrates. The results obtained in both cases were similar; that is, as substrates, the unsaturated fatty acids were more effective than saturated fatty acids, the velocity of the reaction increased from lauric acid to palmitic acid, and the maximum velocity was obtained with unsaturated C_{18} fatty acids.

The results obtained suggest that the acyl-CoA thioesterase associated with envelope membranes could be an ultimate control to prevent the transport (outside of the chloroplast) or the insertion (into chloroplast lipids) of fatty acids with chains shorter than C_{16}.

Some 20 years ago, evidence was presented that isolated leaf chloroplasts readily synthesized palmitic and oleic acid from ^{14}C-labeled acetate in the light (21, 33, 36). In 1965, Brooks and Stumpf (3) reported that fatty acid biosynthesis by stromal enzymes from disrupted chloroplasts was acyl carrier protein-dependent. Since then, many workers have confirmed and extended the role of chloroplasts in fatty acid synthesis (see ref. 35 for a review). In 1979, Ohlrogge *et al.* (25) published evidence that the chloroplast in spinach-leaf cells was indeed the only site of oleic and palmitic acid synthesis from the usual C_2 and C_3 precursors.

In the meantime, Douce's laboratory has assembled numerous data in support of the concept that the chloroplast envelope participated in the assembly of the different components of the principal polar lipids of the chloroplast, namely the galactolipids (see ref. 8 for a review).

Since both palmitic and oleic acids were synthesized inside the chloroplast, their further modification to polyunsaturated fatty acids or their incorporation into polar lipids required, first, their activation to a form suitable for further metabolism and, second, their transport across envelope membranes toward other cellular compartments where further modifications of the hydrocarbon chain may occur. Several investigators have demonstrated a long-chain acyl-CoA synthetase which is specifically localized in the envelope membrane (12, 14, 27). The acyl-CoA thus formed can be either directly incorporated into phosphatidic acid on envelope membranes (12) during the initial steps of galactolipid synthesis (8) or exported to the cytosol before being transferred into phospholipids on the ER (30, 32, 38).

However, we have shown recently (13) that the chloroplast envelope membranes also contained an acyl-CoA thioesterase. This enzyme has also been found in chromoplasts (17). To clarify the interacting roles of acyl-CoA thioesterase and acyl-CoA synthetase, both of which are localized on envelope membranes, we have studied the formation of acyl-CoA in envelope membranes with different techniques allowing us to measure the actual rate of acyl-CoA formations. Substrates specificity and other properties of the envelope acyl-CoA synthetase will also be reported here.

MATERIALS AND METHODS

Isolation of Chloroplasts and of Envelope Membranes

Chloroplasts were isolated from 1 to 2 kg spinach leaves obtained from the local markets. Deveined leaves were homogenized for 2 s in a 4-liter Waring Blendor in the following medium: 0.3 M sucrose, 30 mM Tricine-NaOH (pH 7.6), and 0.1% defatted BSA (volume, 2 l medium/kg leaves). A crude chloroplast fraction was obtained from the leaf homogenate as described by Douce *et al.* (10). In order to avoid "microsomes" and swollen grana lamellae in the chloroplast fraction, the intact chloroplasts were purified by the usual sucrose density gradient procedure or on Percoll (9). Envelope, stroma and thylakoids were prepared from intact purified chloroplasts as described by Douce *et al.* (10). From 1 kg spinach leaves, the yield of envelope membranes was 2 to 3 mg protein.

The essential absence of both NADH:Cyt *c* oxidoreductase and of phosphatidylethanolamine precluded significant contamination of the envelope fraction by extrachloroplastic membranes. Furthermore, inasmuch as the Chl content of the envelope fraction was less than 0.02 μg/mg protein, thylakoid fragments were negligible components.

Chemicals

[U-^{14}C]Adenosine 5'-triphosphate, tetrasodium salt (569 mCi/mmol), [G-^{3}H]CoA (1.2 Ci/mmol), [1-^{14}C]palmitoyl-CoA (53 mCi/mmol), and [1-^{14}C]oleoyl-CoA (43 mCi/mmol) were obtained from New England Nuclear. [1-^{14}C]Decanoyl-CoA (58 mCi/mmol), [1-^{14}C]lauroyl-CoA (58 mCi/mmol), [1-^{14}C]myristoyl-CoA (50 mCi/mmol), [1-^{14}C]stearoyl-CoA (57 mCi/mmol), and [1-^{14}C]acetic acid (sodium salt, 56.6 mCi/mmol) were purchased from Rosechem products. P-enolpyruvate (trisodium salt),

[1] This work was supported in part by Grant GM19213 from the United States Public Health Service.

[2] Permanent address: Laboratoire de Biologie Végétale, Department de Recherche Fondamentale, Centre d'Etudes Nucléaires et Université Scientifique at Medical de Grenoble, 85X38014 Grenoble, France.

adenosine 5'-triphosphate (tetrasodium salt), CoA (sodium salt), palmitoyl-CoA, and 5'-nucleotidase (from *Crotalus atrox* venom) were purchased from Sigma. Pyruvate kinase (solution in glycerol, from rabbit muscle) was obtained from Boehringer. Free fatty acids were obtained from Applied Science Laboratories or Sigma. Dowex 1-X8 was purchased from Bio-Rad Laboratories and Triton X-100 was from Rohm and Haas. All solvents used were reagent grade.

Protein Determination

Protein concentration was determined by the method of Bradford (2) with BSA as standard.

Assay of Acyl-CoA Synthetase

The envelope long-chain acyl-CoA synthetase catalyzes the following reaction:

$$\text{fatty acid} + \text{CoASH} + \text{ATP} \rightarrow \text{acyl-CoA} + \text{AMP} + \text{PPi} \quad (1)$$

The acyl-CoA thioesterase, associated with the chloroplast envelope (13) is responsible for the formation of free fatty acids and CoA from acyl-CoAs.

Since both the acyl-CoA thioesterase and the acyl-CoA synthetase were present in the envelope membranes, acyl-CoA synthetase activity was determined by three separate methods.

Method A. Using [^{14}C]ATP as labeled substrate, the reaction of the acyl-CoA synthetase (equation 1) was coupled to that of 5'-nucleotidase which converted the [^{14}C]AMP produced in reaction 1 to free [^{14}C]adenosine, according to the following reaction:

$$\text{AMP} \rightarrow \text{adenosine} + \text{Pi} \quad (2)$$

Adenosine can then be easily separated from the different nucleotides (AMP, ADP, and ATP) by use of a Dowex 1 formate column. The charged molecules were bound to the column, whereas the neutral adenosine was eluted with H_2O (5, 6). The activity of the acyl-CoA synthetase can be determined by measuring the production of [^{14}C]adenosine, as described by Cooper (5) for the long-chain acyl-CoA synthetase of castor bean endosperm.

The activity was routinely assayed at room temperature (20 to 23 C) in the following incubation mixture: 10 mM Tricine - NaOH (pH 8.0), [^{14}C]ATP, 5 mM (400 dpm/nmol), 0.5 mM CoASH, 5 mM P-enolpyruvate, 200 μg pyruvate kinase, 10 mM $MgCl_2$, 5 mM DTT, 200 μg 5'-nucleotidase; final volume was 600 μl. The reaction was initiated by addition of the different chloroplast fractions corresponding to 120 to 250 μg protein. At various times (from 5 to 30 min), 75-μl aliquots were taken for [^{14}C]adenosine analysis and determination of the initial velocity of the reaction. The reaction was terminated by addition of 500 μl ice-cold ethanol to the aliquots. The reaction mixture then was absorbed on a Dowex 1-formate column prepared from Dowex 1-X8 according to Canvin and Beevers (4) and Cooper (6). A large number of columns were prepared with 1.5 ml Dowex 1-formate in a 5-ml disposable pipette (Kimble) containing a glass wool plug. Ten columns were run simultaneously and [^{14}C]adenosine was eluted with 5 ml H_2O. In agreement with Cooper (5), we have verified that this procedure allows only the elution of [^{14}C]adenosine and that the other possible labeled products (AMP, ADP, and ATP) were strongly retained; only washing with formic acid and ammonium formate released these nucleotides from the column (6). The aqueous solution, containing [^{14}C]adenosine, was mixed with 10 ml PCS mixture (Amersham/Searle) and counted using a Beckman LS 230 liquid scintillation counter.

It has to be pointed out that two possible envelope enzymes, ATPase and adenylate kinase, can compete with the acyl-CoA synthetase for ATP and that the result of the coupling of these reactions is the formation of AMP. The Mg^{2+}-ATPase of the chloroplast envelope (10, 11) catalyzes the following reaction:

$$\text{ATP} \rightarrow \text{ADP} + \text{Pi} \quad (3)$$

The bound adenylate kinase, which could be associated with the chloroplast envelope (22) catalyzes the following reversible reaction:

$$2\ \text{ADP} \rightleftharpoons \text{ATP} + \text{AMP} \quad (4)$$

As Cooper (5) has pointed out, the addition of P-enolpyruvate and pyruvate kinase in large excess prevented the formation of ADP in the incubation mixture and the subsequent formation of AMP by a coupling of the reactions 3 and 4. The presence of large quantities of 5'-nucleotidase prevented the accumulation of AMP in the reaction mixture and the functioning of the adenylate kinase in the reverse direction. Thus, it is likely that the CoA-stimulated [^{14}C]adenosine measured was solely derived from AMP produced by the acyl-CoA synthetase.

Method B. The second assay method for acyl-CoA synthetase was based on the property of long-chain acyl-CoA to be acid precipitable (1, 31). The Millipore filter assay described by Polokoff and Bell (26) was employed. The activity was assayed in the following incubation mixture: 10 mM Tricine-NaOH (pH 8.0), 5 mM ATP, 0.2 mM [^{3}H]CoASH (7000 dpm/nmol), 5 mM P-enolpyruvate, 200 μg pyruvate kinase, 10 mM $MgCl_2$, 5 mM DTT, final volume was 600 μl. The reaction was initiated by the addition of the different chloroplast fractions corresponding to 120 to 250 μg protein. At various times (from 1 min to 30 min) 75 μl aliquots were taken for acyl-CoA analysis and determination of the initial velocity of the reaction. The reaction was terminated by addition of 20 μl 1 mg/ml BSA solution followed by addition of 1 ml ice-cold 0.3 M trichloroacetic acid. The tubes were stored on ice and the content of each tube was poured over a 25-mm, 0.45-μm, HAWP Millipore filter and filtered with a Millipore filtration apparatus (1225 Sampling Manifold). The tubes were washed three times with 2 ml ice-cold 0.3 M trichloroacetic acid and these washings were in turn poured over the filter. The filters were dissolved in 10 ml PCS/xylene (2:1, v/v) scintillation mixture (Amersham/Searle) and the radioactivity was determined as described previously (see above).

In agreement with Polokoff and Bell (26), we have verified that long-chain acyl-CoAs were completely retained on the Millipore filter; however, acyl-CoAs with shorter carbon chains (below C_{12}) were only partially retained.

Method C. The third method employed the conversion of [^{14}C]oleic acid to [^{14}C]oleoyl-CoA. The reaction was assayed in the following incubation medium: 10 mM Tricine-NaOH (pH 8.0) 5 mM ATP, 0.5 mM CoASH, 5 mM P-enolpyruvate, 200 μg pyruvate kinase, 10 mM $MgCl_2$, 5 mM DTT, 1 mM [^{14}C]oleic acid (8000 dpm/nmol), 0.5% (w/v) Triton X-100; final volume was 600 μl. The reaction was initiated by the addition of the different chloroplast fractions corresponding to 150 to 200 μg protein. The reaction was terminated, the free fatty acids were extracted, and the radioactivity was determined as described below for the assay of the acyl-CoA thioesterase activity.

Assay of Acyl-CoA Thioesterase

Activity was routinely assayed at room temperature using palmitoyl-CoA as substrate (13). The incubation mixture was as follows: 10 mM Tricine-NaOH (pH 9.0), 3 μM [1-^{14}C]palmitoyl-CoA (70,000 dpm/nmol); final volume was 400 μl. The reaction was initiated by addition of the different chloroplast fractions corresponding to 30 to 40 μg protein (for the envelope) or to 1 mg for the stroma and the thylakoids. At various times (from 15 s to 5 min), 60-μl aliquots were taken for lipid analysis and determination of the initial velocity of the reaction. The reaction was terminated by the addition of 1 ml isopropyl alcohol and 1 ml

water (containing 30 μl acetic acid) to the aliquots. Free fatty acids were extracted three times with 2 ml petroleum ether (bp 35 to 60 C) saturated with 50% isopropyl alcohol according to the procedure described by Mancha *et al.* (18). The radioactivity in the petroleum ether extract (containing the free fatty acids) and the aqueous phase (containing the acyl-CoA thioesters) was counted with 10 ml PCS/xylene (2:1, v/v) scintillation mixture (Amersham/Searle) as described above (see "Assay of Acyl-CoA Synthetase").

The reaction products were analyzed and identified as described in a previous paper (13).

ASSAY OF ACETYL-COA SYNTHETASE

This activity has been determined in the different chloroplast fractions by using the method described by Kuhn and Stumpf (16). The activity was assayed at room temperature in the following incubation mixture: 10 mM Tricine-NaOH, (pH 9.0), 5 mM ATP, 0.5 mM CoASH, 5 mM P-enolpyruvate, 200 μg pyruvate kinase, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM [^{14}C]acetate (6800 cpm/nmol); final volume was 600 μl. The reaction was initiated by addition of the different chloroplast fractions corresponding to 100 to 200 μg protein. At various times (from 5 to 30 min), 75-μl aliquots were taken for acetyl-CoA analysis and determination of the initial velocity of the reaction. The reaction was terminated by adding 100 μl of a suspension of activated charcoal (15 g/100 ml H_2O) and 100 μl glacial acetic acid. Acetyl-CoA in the mixture is strongly adsorbed to the charcoal. To remove the free acetate present in the mixture, the charcoal was washed by three successive centrifugations (2 min, Beckman microfuge) with 1 ml 1 M acetic acid - sodium acetate (pH 3.8). The [^{14}C]acetyl-CoA bound to the charcoal then was cleaved by incubation for 15 min at room temperature in presence of 0.5 ml NH_2OH-HCl (28%, w/v) and 0.5 ml NaOH (14%, w/v). This treatment quantitatively released into the supernatant the [^{14}C]acetyl component from [^{14}C]acetyl-CoA as [^{14}C]acetyl hydroxamate. The charcoal then was separated from the supernatant by centrifugation (2 min, Beckman microfuge) and the supernatant, containing [^{14}C]acetyl hydroxamate, was counted in 10 ml PCS:xylene (2:1, v/v) scintillation mixture as described above (see "Assay of Acyl-CoA Synthetase").

ASSAY OF ACYL-ACP[3] THIOESTERASE

Activity was assayed at room temperature using a mixture of 16:0 ACP (16%), 18:0 ACP (40%), and 18:1 ACP (44%) as substrate (19). The incubation mixture was as follows: 10 mM Tricine-NaOH (pH 9.0), 1 μM acyl-CoA (96,000 cpm/nmol); final volume was 400 μl. The reaction was initiated by addition of the different chloroplast fractions corresponding to 150 to 200 μg protein. At various times (from 2–15 min), 30-μl aliquots were taken for lipid analysis and determination of the initial velocity of the reaction. Termination of the reaction, extraction of free fatty acids, and determination of the radioactivity were as described above (see "Assay of Acyl-CoA Thioesterase").

RESULTS

Localization within Chloroplast of Different Activities Associated with Acyl Thioesters. The results presented in Table I show that a very active acyl-CoA synthetase activity was associated with envelope membranes. Regardless of the method employed to measure this activity, with oleic acid as the substrate, the specific activity of the acyl-CoA synthetase was in the range of 1.5 to 2.5 μmol oleoyl-CoA formed/h·mg protein. The activity found in the other chloroplast fractions (especially in the thylakoid fraction) could be entirely attributable to contamination by envelope fragments. This acyl-CoA synthetase activity was about 10 times higher than that of the acyl-CoA thioesterase, also localized in the envelope. In contrast, acetyl-CoA synthetase and acyl-ACP thioesterase activities were found in the soluble phase of the chloroplast (Table I), thus demonstrating that acyl-CoA synthetase and acetyl-CoA synthetase were distinct enzymes, as were acyl-CoA thioesterase and acyl-ACP thioesterase.

Requirements for Acyl-CoA Synthetase Activity. The complexity of measuring acyl-CoA synthetase activity when envelope membranes were incubated with [^{14}C]ATP is shown in Figure 1. A significant amount of [^{14}C]adenosine was formed in the assay system in the absence of CoA. Furthermore, this [^{14}C]adenosine formation in the absence of CoA was not increased by addition of oleic acid. This activity was not related to acyl-CoA synthetase activity but to ATP hydrolysis. Figure 1 also shows that ACP cannot replace CoA and that the acyl-CoA synthetase was specific for CoA. A significant increase in [^{14}C]adenosine production was observed on the addition of CoA alone. There is good evidence that some free fatty acids were present in the envelope preparation and/or were released during the incubation. This factor contributed to the extent of the magnitude of enhanced activity resulting from addition of oleic acid to different envelope preparations 3.3- and 2.3-fold in data of Table I and Fig. 1 respectively). The further addition of oleic acid resulted in the highest rate of [^{14}C]adenosine formation. These experiments, clearly demonstrate the need for proper controls in assaying for synthetase activity.

With [^{14}C]adenosine production as the assay system, the acyl-CoA synthetase required both Mg^{2+} and DTT; Triton X-100 slightly stimulated the activity in absence but mainly in presence of exogenous free fatty acids (Table II). These requirements were not observed with the acyl-[^{3}H]CoA assay system. Perhaps the coupling of the several enzymes used in the [^{14}C]adenosine assay required Mg^{2+} and DTT. However, for routine assay, these components were always added. As expected, the production of [^{14}C]adenosine was strongly dependent on the presence of 5′-nucleotidase in the incubation medium, whereas the formation of acyl-[^{3}H]CoA required the presence of ATP. In our experimental conditions, 0.5 mM CoA and 5 mM ATP were found to be saturating. Furthermore, the assay was linear during approximately 30 min, when [^{14}C]adenosine was measured, and only during 5 to 10 min, when acyl-[^{3}H]CoA was measured (Fig. 2). This difference was caused by the necessity to employ lower concentrations of CoA (0.2 mM) in the second assay system so that a suitable specific activity of the [^{3}H]CoA could be employed for counting purposes. The assay was also linear with the amount of envelope membranes in the incubation medium up to 500 μg protein/ml (results not shown). The envelope acyl-CoA synthetase was active in a wide range of pH from 7.0 to 9.5, with a maximum around 8.0 (results not shown). Since envelope preparations that was frozen at −20 C and then thawed showed appreciable loss of activity (72% decrease; Table II), all experiments were carried out promptly with freshly prepared membranes.

Effect of Fatty Acids on the Acyl-CoA Synthetase Activity. As already shown in Figure 1 and Table II, the addition of free oleic acid increased the activity of the envelope acyl-CoA synthetase. The maximum effect was obtained with free fatty acid (either oleic or palmitic acids) concentrations above 1 mM (Fig. 3). Significant activity occurred in the absence of added fatty acids (see also Table I). We suggest that the background activity related probably to low levels of endogenous fatty acid which could occur in all envelope preparations.

The specificity of the acyl-CoA synthetase for fatty acids as assayed with [^{14}C]ATP or with [^{3}H]CoASH as substrates is shown in Figure 4A. No significant differences were noted between the results obtained by the two procedures except in the absence of exogenous fatty acids or in the presence of lauric acid. The results show that unsaturated fatty acids were more effective than satu-

[3] Abbreviation: ACP, acyl carrier protein.

Table I. *Distribution of Acyl-CoA Synthetase and Thioesterase Activities in Spinach Chloroplasts*

The different chloroplast fractions were prepared as described under "Materials and Methods." Acyl-CoA and acetyl-CoA synthetases, acyl-CoA, and acyl-ACP thioesterases were assayed as described. When oleic acid was added to the incubation mixture, 0.5% (w/v) Triton X-100 was also added.

Chloroplast Fraction	Acyl-CoA Synthetase					Acetyl-CoA Synthetase	Acyl-CoA Thioesterase	Acyl-ACP Thioesterase
	[^{14}C]Adenosine		Acyl-[^{3}H]CoA					
	No FFA[a] added	+1 mM oleic acid	No FFA added	+1 mM oleic acid	[^{14}C] Oleoyl-CoA	[^{14}C] Acetyl-CoA	[^{14}C]Palmitic Acid	^{14}C-Free Fatty Acids
	nmol formed/h·mg protein							
Envelope	720	2370	240	1980	1650	14	218	0.7
Stroma	15	47	2	3	13	383	3	10.6
Thylakoids	60	148	18	62	74	12	10	0.3

[a] FFA, free fatty acid.

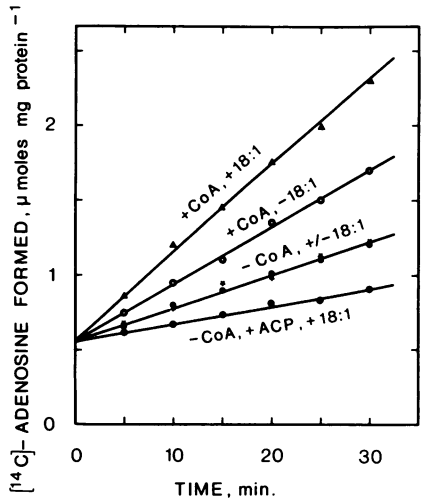

FIG. 1. Formation of [^{14}C]adenosine by chloroplast envelope membranes incubated in presence of [^{14}C]ATP and 5'-nucleotidase. The basic incubation mixture and the assay system were as described under "Assay of Acyl-CoA Synthetase." Where indicated, 1 mM oleic acid was added to the incubation mixture, in this case, 0.5% (w/v) Triton X-100 was also added. The different experiments presented in this figure were made either in presence (two upper curves) or in absence (two lower curves) of CoA; in the lowest curve, CoA was replaced by 100 μg ACP (from *Escherichia coli*).

rated fatty acids; the velocity of the reaction increased from lauric acid to palmitic acid and the maximum velocity was obtained with unsaturated C_{18} fatty acids. As already shown in Table I, the envelope acyl-CoA synthetase cannot use short carbon chains, such as acetate, as a substrate. The comparison of these results with those obtained with the acyl-CoA thioesterase (Fig. 4B) is interesting since the acyl-CoA thioesterase was more active with medium-chain acyl-CoA than with long-chain acyl-CoA.

Effect of Substrates of Acyl-CoA Synthetase on Acyl-CoA Thioesterase Activity. Fatty acids, and especially unsaturated fatty acids, inhibited the acyl-CoA thioesterase (13). When the fatty acid concentration in the incubation medium reached the value where the stimulation of the acyl-CoA synthetase was approximately maximum (1 mM; see Fig. 3), the inhibition of the acyl-CoA thioesterase activity was even more significant (Fig. 4C): only 10% of the acyl-CoA thioesterase activity (with palmitoyl-CoA as substrate) remained in presence of 1 mM of unsaturated fatty acids (from 16:1 to 18:3), but approximately 60% remained when the fatty acids used were saturated (from 12:0 to 18:0).

Although either ATP or CoA alone did not inhibit the acyl-CoA thioesterase, the addition of ATP, CoA, and Mg^{2+} (required for the function of the acyl-CoA synthetase) to the reaction medium resulted in an apparent decrease in thioesterase activity (around 70% of the release of palmitic acid from palmitoyl-CoA (Table III). Finally, addition of Triton X-100, up to 1% (w/v), was without effect on the envelope acyl-CoA thioesterase activity.

Table II. *Requirements for Acyl-CoA Synthetase Activity*

Envelope membranes were prepared as described under "Materials and Methods" and assayed directly or following overnight storage at −20 C. Assays were performed as described under "Assay of Acyl-CoA Synthetase." Additions or omissions to the incubation mixture were made as indicated.

Incubation Medium	Acyl-CoA Synthetase	
	[^{14}C] Adenosine	Acyl-[^{3}H]-CoA
	nmol formed/h·mg protein	
Complete (with fresh envelope)	634	218
− Envelope	0	0
− Mg^{2+}	207	205
− DTT	320	260
+ Triton (0.5%)	721	288
+ Triton (1%)	730	290
+ 18:1 (1 mM)	1393	1680
+ 18:1 (1 mM) + Triton (0.5%)	1805	1980
− 5'-Nucleotidase	0	
− ATP (5 mM)		2
Complete (with frozen and thawed envelope)	179	

DISCUSSION

Chloroplast envelope membranes were able to synthesize long-chain acyl-CoAs at a high rate from free fatty acids. Assuming that envelope membranes represent 1% of the total chloroplast proteins (7) and that, in intact chloroplasts, the ratio of protein to Chl is approximately 20, the formation of 1.5 to 2.5 μmol acyl-CoA/h·mg envelope protein correspond to 0.3 to 0.5 μmol acyl-CoA formed/h·mg Chl. This value matches those recently obtained for fatty acid formation in isolated chloroplasts. For instance, Roughan *et al.* (29) have reported rates of [^{14}C]acetate incorporation into long-chain fatty acids up to 1.5 μmol/h·mg Chl, which correspond approximately to the formation of 0.2 μmol fatty acids/h·mg Chl. Thus, the envelope acyl-CoA synthetase can readily match the high rates of fatty acid synthesis in the chloroplast. Furthermore, the acyl-CoA synthetase and the acetyl-

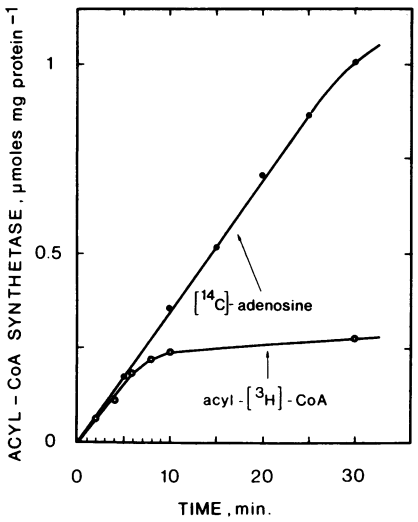

FIG. 2. Acyl-CoA synthetase activity of chloroplast envelope membranes. The activity was measured as described under "Assay of Acyl-CoA Synthetase," except that 1 mM oleic acid and 0.5% (w/v) Triton X-100 were added. Two different procedures were used: (*a*) the CoA-stimulated [^{14}C]adenosine formation by envelope membranes incubated in presence of [^{14}C]ATP and 5'-nucleotidase (see Fig. 1) (CoA concentration, 0.5 mM); (*b*) the ATP-stimulated acyl-[^{3}H]CoA formation by envelope membranes incubated in presence of [^{3}H]CoA (CoA concentration, 0.2 mM). The initial velocity of the reaction was the same in both cases. The reaction was linear during a longer time when [^{14}C]adenosine was measured than when acyl-[^{3}H]CoA was measured. This difference is only caused by the necessity to employ lower concentrations of CoA (0.2 instead of 0.5 mM) in the second case so that a suitable specific radioactivity of the labeled substrate could be used for counting purposes.

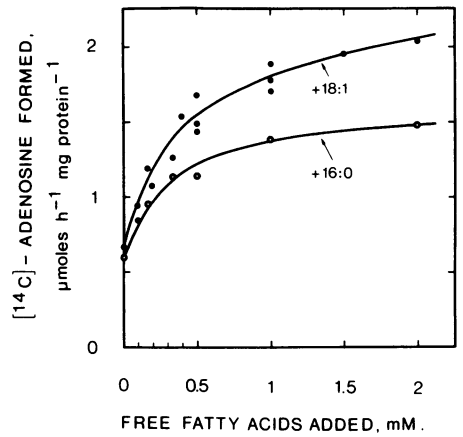

FIG. 3. Stimulation by palmitic acid and oleic acid of the [^{14}C]adenosine formation by envelope membranes incubated in presence of [^{14}C]ATP and 5'-nucleotidase. The assay conditions were as described under "Assay of Acyl-CoA Synthetase," except that increasing concentrations of palmitic or oleic acids and 0.5% (w/v) Triton X-100 were added to the basic incubation medium. Similar results were obtained when acyl-[^{3}H]CoA was measured instead of [^{14}C]adenosine.

CoA synthetase have similar rates of activity: the acetyl-CoA synthetase, localized in the soluble phase of the chloroplast, is able to form 0.4 μmol acetyl-CoA/h·mg protein. Assuming that the stromal proteins represent 50% of the chloroplast proteins, this activity corresponds to the formation of 4 μmol acetyl-CoA/h·mg Chl which allows the synthesis of 0.4 to 0.5 μmol fatty acid/h·mg Chl, thus suggesting that the two enzymes involved in the first step of fatty acid synthesis (acetyl-CoA synthetase) and the final step in the utilization of fatty acids (acyl-CoA synthetase) are not

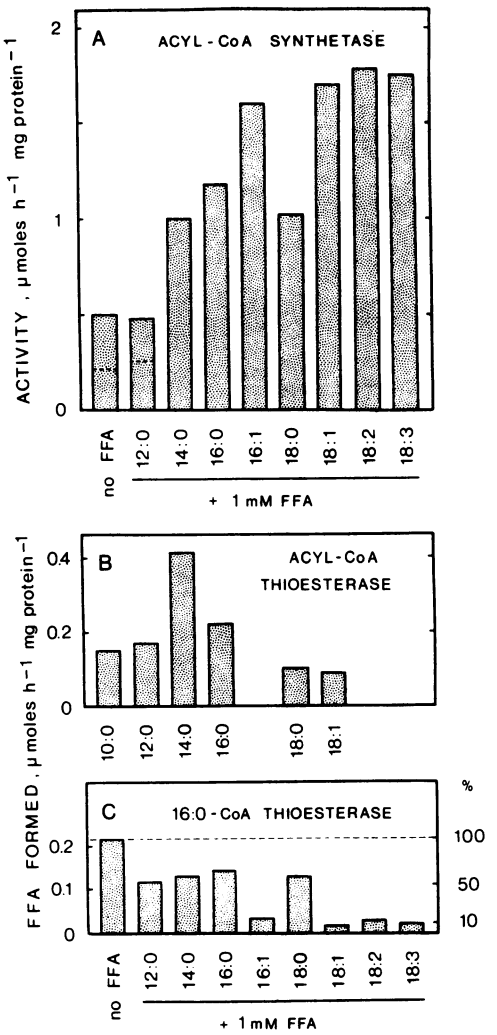

FIG. 4. Substrate specificities of the acyl-CoA synthetase and thioesterase associated with chloroplast envelope membranes. Envelope membranes were prepared from intact and purified spinach chloroplasts as described by Douce *et al.* (10). A, substrate specificities of the envelope acyl-CoA synthetase. The results presented were obtained by the two different procedures described under "Assay of Acyl-CoA Synthetase" (formation of [^{14}C]adenosine or of acyl-[^{3}H]CoA; see also Fig. 2). The incubation medium was as described under "Assay of Acyl-CoA Synthetase," except that, where indicated, 1 mM free fatty acids (FFA) and 0.5% (w/v) Triton X-100 were added. No significant differences were noted between the results obtained by the two procedures, except in absence of exogenous fatty acids or in presence of lauric acid in the incubation mixture (- - -, activities measured in this case with acyl-[^{3}H]CoA). B, substrate specificities of the envelope acyl-CoA thioesterase. The incubation mixture was as described under "Assay of Acyl-CoA Thioesterase," except that palmitoyl-CoA was not the only substrate used: the other acylCoAs used were as indicated on the figure by the fatty acid moiety of the molecules. C, inhibition of the envelope palmitoyl-CoA thioesterase by exogenous free fatty acids. The incubation mixture was as described under "Assay of Acyl-CoA Thioesterase," except that where indicated, 1 mM free fatty acids were added.

limiting factors for [^{14}C]acetate incorporation into fatty acids.

As shown in Figure 1 and Table II, the formation of acyl-CoA was dependent on ATP and CoA; ACP could not replace CoA. However, a rather high synthetase activity was observed in absence of exogenous fatty acids (Fig. 1; Table II). This activity was expected inasmuch as it has been demonstrated that envelope membranes (in presence of stroma) can acylate *sn*-glycerol 3-P

Table III. *Effect of Substrates of Acyl-CoA Synthetase and of Triton X-100 on Acyl-CoA Thioesterase Activity*

Envelope membranes were prepared from intact and purified spinach chloroplasts as described by Douce *et al.* (10). The assay conditions were as described under "Assay of Acyl-CoA Thioesterase." Additions or omissions to the basic incubation mixture were made as indicated.

Incubation Medium	Acyl-CoA Thioesterase	
	nmol[^{14}C] Palmitic acid formed/h·mg protein	%
Complete	281	100
− Envelope	0	0
+ ATP (5 mM)	269	95.7
+ CoA (0.5 mM)	280	99.6
+ ATP (10 mM) + CoA (0.5 mM) + $MgCl_2$ (10 mM)	95	33.8
+ Triton (0.5%)	290	103
+ Triton (1%)	280	99.6

without addition of exogenous free fatty acids when ATP and CoA were added to the incubation mixture (12). It is clear that endogenous fatty acids (probably released during the incubation) can serve as substrate for the acyl-CoA synthetase. However, addition of free fatty acids to the incubation medium further increased the acyl-CoA synthetase activity (Table II; Fig. 4). Addition of Triton X-100 increased the activity in presence of free fatty acids, whereas, in the absence of free fatty acids, only a modest stimulation was noted (Table II). These observations suggest that Triton X-100 allows the substrate to have better access to the membrane enzyme. This was particularly true when the substrate was saturated; for instance, acyl-CoA synthetase activity measured in presence of 1 mM palmitic acid was stimulated 50% when Triton X-100 was present. Higher concentrations of detergent (up to 2%) did not produce a stronger stimulation (results not shown). These observations could partly explain previous observations on the stimulation of [^{14}C]acetate incorporation into fatty acids (28, 29, 37): the stimulation of acyl-CoA formation by Triton X-100 could facilitate the transport of fatty acids to the outside of the chloroplast, thus stimulating fatty acid synthesis.

The envelope acyl-CoA thioesterase has been characterized previously (13). The additional data presented here have confirmed that the acyl-CoA thioesterase differs from the acyl-ACP thioesterase since both enzymes are located in two different compartments (Table I) and, of course, both enzymes have different substrate specificities. The acyl-CoA thioesterase was not inhibited by Triton X-100 (Table III). The presence of the acyl-CoA thioesterase on the envelope membranes could reduce the formation of acyl-CoAs. From our results, this possibility seems to be unlikely for the following reasons. (*a*) the activity of the acyl-CoA synthetase as measured by [^{14}C]adenosine formation (thioesterase independent) and by acyl-[^{3}H]CoA synthesis (thioesterase activity would lower accumulation of acyl-CoA) was similar in both cases. The differences between the results obtained with both assays were not significant (Table II; Figs. 2 and 4A). (*b*) The activity of the acyl-CoA thioesterase for long-chain acyl-CoAs was at least 10 times lower than the rate of their formation in the envelope (Table I; Fig. 4, A and B). (*c*) When ATP and CoA were added to the incubation medium, the formation of free fatty acids from acyl-CoA was strongly reduced (Table III). This observation is in agreement with the suggestion of Kleinig and Liedvogel (15) that the high levels of ATP (and CoA) required for the incorporation of fatty acid into chloroplast polar lipids facilitate the acyl-CoA synthetase and diminish the thioesterase effect. (*d*) Furthermore, the acyl-CoA thioesterase was strongly inhibited by unsaturated fatty acids (Fig. 4C) which are the main species formed in isolated chloroplasts (23, 28).

Under these conditions, when fatty acid synthesis was active in chloroplasts, the acyl-CoA thioesterase was probably not active and free oleic acid formed in the chloroplast via the oleoyl-ACP pathway (34) was transformed very quickly by the envelope acyl-CoA synthetase into oleoyl-CoA which is the form used for further metabolism.

A possible role for the acyl-CoA thioesterase is suggested by its fatty acid specificity: the envelope acyl-CoA thioesterase is more active with medium-chain acyl-CoAs than with long-chain acyl-CoAs (Fig. 4B) (13). Conversely, all the enzymes involved in the last steps of fatty acid synthesis are highly specific for C_{18} fatty acids: the desaturation of saturated fatty acids is specific for stearoyl-ACP (20); the acyl-ACP thioesterase has a 5- to 10-fold preference for oleoyl-ACP (18, 24); the acyl-CoA synthetase is more active with unsaturated C_{18} fatty acids (Fig. 4A). Under these conditions, the envelope acyl-CoA thioesterase could be an ultimate control factor to prevent the transport (outside of the chloroplast) or the insertion (into chloroplast lipids) of fatty acids with carbon chains shorter than C_{16}. A more precise localization of the acyl-CoA synthetase and thioesterase on the inner or outer envelope membrane would greatly improve the understanding of the interacting role of these two enzymes. Unfortunately, as already stated (13), such a study is not yet possible.

Acknowledgments—One of us (J. J.) is indebted to Professor Roland Douce for his constant advice and interest. We would also like to thank our colleagues in the laboratory, especially Dr. Tom McKeon for providing us with acyl-ACP, Ken Hilt for providing us with E. *coli* ACP, David Kuhn for his help in measuring acetyl-CoA synthetase, and Mrs. Billie Gabriel for her help in preparing the manuscript.

LITERATURE CITED

1. Banis RJ, SB Tove 1974 Solubilization of a long-chain acyl-CoA synthetase from chicken adipose tissue microsomes. Biochim Biophys Acta 348: 210–220
2. Bradford MM 1976 A rapid method for the quantitation of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
3. Brooks JL, PK Stumpf 1965 A soluble fatty acid synthesizing system from lettuce chloroplasts. Biochim Biophys Acta 98: 213–216
4. Canvin DT, H Beevers 1961 Sucrose synthesis from acetate in the germinating castor bean. Kinetics and pathway. J Biol Chem 236: 988–995
5. Cooper TG 1971 The activation of fatty acids in castor bean endosperm. J Biol Chem 246: 3451–3455
6. Cooper TG 1977 Ion exchange. *In* The Tools of Biochemistry, John Wiley & Sons, New York, pp 157–165
7. Douce R, J Joyard 1979 Structure and function of the plasmid envelope. Adv Bot Res 7: 1–16
8. Douce R, J Joyard 1980 Plant galactolipids. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Academic Press, New York, pp 321–362
9. Douce R, J Joyard 1981 Purification of the chloroplast envelope. *In* Mallick, NH Chua, M Edelman, eds, Methods in Chloroplast Molecular Biology, Academic Press, New York In press
10. Douce R, RB Holtz, AA Benson 1973 Isolation and purification of the envelope of spinach chloroplasts. J Biol Chem 248: 7215–7222
11. Joyard J, R Douce 1975 Mn-dependent ATPase of the envelope of spinach chloroplasts. FEBS Lett 51: 335–340
12. Joyard J, R Douce 1977 Site of synthesis of phosphatidic acid and diacylglycerol in spinach chloroplasts. Biochim Biophys Acta 486: 273–285
13. Joyard J, PK Stumpf 1980 Characterization of an acyl-CoA thioesterase activity associated with the envelope of spinach chloroplasts. Plant Physiol 65: 1039–1043
14. Kleinig H, B Liedvogel 1978 Fatty acid synthesis by isolated chromoplasts from the daffodil. [^{14}C]Acetate incorporation and distribution of the labelled acids. Eur J Biochem 83: 499–505
15. Kleinig H, B Liedvogel 1979 On the energy requirements of fatty acid synthesis in spinach chloroplasts in the light and in the dark. FEBS Lett 101: 339–342
16. Kuhn DN, PK Stumpf 1980 Subcellular localization of acetyl-CoA synthetase in protoplasts of *Spinacea oleracea*. Plant Physiol 65: 5–145
17. Liedvogel B, H Kleinig, JA Thompson, H Falk 1978 Chromoplasts of *Tropaeolum majus* L.: lipid synthesis in whole organelles and subfractions. Planta 141: 303–309
18. Mancha M, GB Stokes, PK Stumpf 1975 Fat metabolism in higher plants. The determination of acyl-acyl carrier protein and acyl coenzyme A in a complex lipid mixture. Anal Biochem 68: 600–608
19. McKeon TA, PK Stumpf 1980 Acyl-ACP thioesterase from safflower. Methods Enzymol 71: In press

20. McKeon TA, PK Stumpf 1980 Stearoyl-ACP desaturase from safflower. Methods Enzymol 71: In press
21. Mudd JB, TT McManus 1962 Metabolism of acetate by cell-free preparations from spinach leaves. J Biol Chem 237: 2057–2063
22. Murakami S, H Strotmann 1978 Adenylate kinase bound to the envelope membranes of spinach chloroplasts. Arch Biochem Biophys 185: 30–38
23. Murphy DJ, RM Leech 1978 Lipid biosynthesis from [^{14}C]bicarbonate, [2-^{14}C]pyruvate and [^{14}C]acetate during photosynthesis by isolated spinach chloroplasts. FEBS Lett 88: 192–196
24. Ohlrogge JB, WE Shine, PK Stumpf 1978 Fat metabolism in higher plants. Characterization of plant acyl-ACP and acyl-CoA hydrolases. Arch Biochem Biophys 189: 382–391
25. Ohlrogge JB, DN Kuhn, PK Stumpf 1979 Subcellular localization of acyl carrier protein in leaf protoplasts of *Spinacia oleracea*. Proc Natl Acad Sci USA 76: 1194–1198
26. Polokoff MA, RM Bell 1975 Millipore filter assay for long-chain fatty acid: CoASH ligase activity using ^{3}H-labelled coenzyme A. J Lipid Res 16: 397–402
27. Roughan PG, CR Slack 1977 Long-chain acyl-coenzyme A synthetase activity of spinach chloroplast is concentrated in the envelope. Biochem J 162: 457–459
28. Roughan PG, CR Slack, R Holland 1976 High rates of [1-^{14}C]acetate incorporation into the lipids of isolated spinach chloroplasts. Biochem J 158: 593–601
29. Roughan PG, CR Slack, R Holland 1979 On the control of long-chain fatty acid synthesis in isolated spinach (*Spinacia oleracea*) chloroplasts. Biochem. J 184: 193–202
30. Roughan PG, R Holland, CR Slack 1980 The role of chloroplasts and microsomal fractions in polar-lipid synthesis from [1-^{14}C]acetate by cell-free preparations from spinach (*Spinacia oleracea*) leaves. Biochem J 188: 17–24
31. Seubert W 1960 *S*-Palmitoyl coenzyme A. Biochem Prep 7: 80–83
32. Slack CR, PG Roughan, N Balasingham 1977 Labelling studies *in vivo* on the metabolism of the acyl and glycerol moieties of the glycerolipids in the developing maize leaf. Biochem J 162: 289–296
33. Smirnov BP 1960 The biosynthesis of higher plant fatty acids from acetate in isolated chloroplasts of *Spinacia oleracea* leaves. Biokhimiya 24: 545–555
34. Stumpf PK 1975 Biosynthesis of fatty acids in spinach chloroplasts. *In* T Galliard, EI Mercer, eds, Recent Advances in the Chemistry and Biochemistry of Plant Lipids, Academic Press, New York, pp 95–113
35. Stumpf PK 1980 Biosynthesis of saturated and unsaturated fatty acids. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 4, Academic Press, New York, pp 177–204
36. Stumpf PK, AT James 1963 The biosynthesis of long-chain fatty acids by lettuce chloroplast preparations. Biochim Biophys Acta 70: 20–32
37. Stumpf PK, NK Boardman 1970 Fat metabolism in higher plants. Effect of adenosine triphosphate and triton X-100 on lipid synthesis by isolated spinach chloroplasts. J Biol Chem 245: 2579–2587
38. Vijay IK, PK Stumpf 1971 Fat metabolism in higher plants. XLVI. Nature of the substrate and the product of oleoyl coenzyme A desaturase from *Carthamus tinctorius*. J Biol Chem 246: 2910–2917