Long-Chain Acyl-Coenzyme A Synthetase Activity of Spinach Chloroplasts is Concentrated in the Envelope

By P. GRATTAN ROUGHAN and C. ROGER SLACK

Plant Physiology Division, Department of Scientific and Industrial Research,
Private Bag, Palmerston North, New Zealand

(Received 29 November 1976)

Purified chloroplasts were disrupted and then fractionated by discontinuous sucrose-density-gradient centrifugation. Envelopes contained long-chain acyl-CoA synthetase at a specific activity 80 times the activity in the lamellae or the stroma. Acetyl-CoA synthetase was concentrated in the stroma, and chlorophyll was confined to the lamellae membranes. Phospholipase D was not detected in any fraction.

The chloroplast envelope has come under increasingly close scrutiny as a possible major site of lipid metabolism in spinach leaf cells. The envelope has been found to be the site of monogalactosyl diglyceride biosynthesis (Douce, 1974; Van Hummel *et al.*, 1975), to be rich in the polar lipid precursor 1,2-diacylglycerol (Joyard & Douce, 1976a) and to have a high lipid:protein mass ratio (Douce *et al.*, 1973; Poincelot, 1973). In addition, our previous observation (Roughan *et al.*, 1976) of ATP-dependent long-chain [^{14}C]acyl-CoA synthesis from [^{14}C]acetate by isolated intact chloroplasts was interpreted as indicating that the synthesis could be taking place in the chloroplast envelope.

On the basis of [^{14}C]acetate incorporation into leaf lipids *in vivo*, we have hypothesized (Slack & Roughan, 1975) that oleic acid, synthesized in the chloroplasts, is transferred to phosphatidylcholine of the endoplasmic reticulum, where further desaturation to linoleic acid occurs (Slack *et al.*, 1976). This movement of a long-chain fatty acid out of the chloroplast would presumably be facilitated by the formation of fatty acyl-CoA at the plastid/cytosol interface and subsequent release of the derivative into the aqueous milieu. Synthesis of endoplasmic-reticular phosphatidylcholine from this acyl-CoA would be consistent with the observation that *in vitro* the oleate moiety is rapidly transferred from oleoyl-CoA to microsomal phosphatidylcholine (Baker & Lynen, 1971; Vijay & Stumpf, 1971; Slack *et al.*, 1976).

The present paper reports on the distribution of long- and short-chain acyl-CoA synthetases in chloroplast fractions isolated by discontinuous sucrose-density-gradient centrifugation.

Materials and Methods

Spinach plants (*Spinacea oleracea*, cultivar Hybrid 102) were grown as described previously (Slack &

Roughan, 1975). CoA and ATP were from Sigma Chemical Co., St. Louis, MO, U.S.A., and [^{14}C]oleic acid (54 mCi/mmol), [^{14}C]palmitic acid (56 mCi/mmol) and sodium [^{14}C]acetate (58 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Chloroplasts for incubation with ammonium [^{14}C]oleate or ammonium [^{14}C]palmitate were isolated as described previously (Slack & Roughan, 1975). For the separation of chloroplast components, chloroplasts were isolated, purified and fractionated by the method of Joyard & Douce (1976b), except that the discontinuous sucrose-density-gradient centrifugation for the separation of envelope and lamellae membranes was carried out at 25000 g for 60 min in the HB-4 rotor of a Sorvall RC5 centrifuge. Envelope and lamellae pellets were resuspended in 25 mM-Hepes*/NaOH, pH 7.9, containing 5 mM-MgCl₂, 2 mM-EDTA and 1 mM-dithiothreitol. A microsomal fraction, prepared from spinach leaf homogenates by magnesium precipitation (Diesperger *et al.*, 1974), was resuspended in the same buffer.

Long-chain acyl-CoA synthetase activity of intact chloroplasts was measured in a system identical with that used previously for [^{14}C]acetate incorporation (Roughan *et al.*, 1976) except that the ammonium salt of ^{14}C -labelled fatty acid (18.5 μM , 0.25 μCi) replaced acetate, and 0.5 mM-CoA with or without 2 mM-ATP were included in a reaction volume of 0.25 ml. Reactions normally contained chloroplasts equivalent to 25–30 μg of chlorophyll. Formation of acyl-CoA was followed either by the filter-paper-disc method (see below) or by partitioning the [^{14}C]acyl-CoA into acidified aqueous propan-2-ol and the ^{14}C -labelled fatty acid into light petroleum (b.p. 40–60°C) (Mancha *et al.*, 1975). When the same reaction mixture was analysed by the two methods the observed incorporation of ammonium oleate into oleoyl-

* Abbreviation: Hepes, 4 - (2 - hydroxyethyl) - 1 - piperazine-ethanesulphonic acid.

CoA differed by less than 10%. Oleoyl-CoA synthetase and palmitoyl-CoA synthetase activities of separated chloroplast fractions were assayed at 25°C in the resuspension buffer (see above) containing 37 μ M-ammonium [1-¹⁴C]oleate or -ammonium [1-¹⁴C]palmitate (0.1 μ Ci), 0.5 mM-CoA, 2.0 mM-ATP, 5 mM-dithiothreitol and 1–3 μ g of protein in 50 μ l total volume. Assays were performed in plastic centrifuge tubes since chloroplast envelopes seemed to adhere to glass surfaces. Blank incubations contained no CoA or ATP. Portions (20 μ l) of the reaction mixtures were removed at 2 and 4 min and spotted on to 2.5 cm-diam. discs of Whatman no. 1 filter paper. The moist discs were immersed for 5 min in 5 ml of 0.25% (w/v) trichloroacetic acid in ethanol/diethyl ether (1:4, v/v), then the solvent was decanted. This washing was repeated, and after a final wash in 5 ml of diethyl ether the discs were air-dried, transferred to 8 ml of 0.5% (w/v) *p*-terphenyl in xylene, and the residual radioactivity was measured by scintillation counting. This is similar to the method described by Galton & Frazer (1969), but is greatly simplified and is suitable for use with oleic acid. Acetyl-CoA synthetase was measured in exactly the same way except that 160 μ M-sodium [1-¹⁴C]acetate (176000 d.p.m.) replaced the long-chain fatty-acyl substrate. Phospholipase D was measured by choline release (Dawson & Hemington, 1967) from phosphatidylcholine isolated from chloroform/methanol extracts of cauliflower florets by a combination of column chromatography on silicic acid (Rouser *et al.*, 1967) and DEAE-cellulose (Nichols & James, 1964). Chlorophyll was determined by the method of Arnon (1949), and protein was measured by a modified Lowry procedure (Schacterle & Pollack, 1973) with bovine serum albumin as the reference. Standards and unknowns were suspended in 1% (w/v) sodium dodecyl sulphate before the addition of the copper reagent.

Results and Discussion

Oleoyl-CoA formation from ammonium [1-¹⁴C]-oleate and CoA by illuminated photosynthetically active chloroplasts was stimulated 10-fold from about 20 to 200 nmol/h per mg of chlorophyll, by the presence of ATP in the media surrounding the chloroplasts. Reaction mixtures contained ammonium oleate at 180 nmol/mg of chlorophyll, and at this concentration HCO₃⁻-dependent O₂ evolution did not differ from that in the absence of ammonium oleate. However, with 3 times the concentration of ammonium oleate O₂ evolution was completely inhibited. Since the chloroplasts were capable of high rates of CO₂ fixation, we argued that the marked stimulation of oleoyl-CoA synthetase activity by added ATP might indicate that the enzyme was localized at a site that was not accessible to the ATP

pool within the chloroplast. It has been shown that ATP cannot penetrate the chloroplast envelope (Stokes & Walker, 1971), and therefore one possible location for the enzyme was in the envelope.

Such a localization of the enzyme could also be inferred from the finding that rates of oleoyl-CoA synthesis were not enhanced in chloroplasts incubated with ammonium [1-¹⁴C]oleate, CoA and ATP in 0.03 M-compared with 0.33 M-sorbitol, i.e. in broken compared with intact organelles. This suggests that the internal membranes of the chloroplast probably did not contribute to the synthesis.

To examine further the role of envelopes in acyl-CoA synthesis, we separated envelope, lamellae and stroma fractions from purified chloroplasts by the method of Joyard & Douce (1976b). In our preparations the fraction banding at the 0.6 M/0.93 M-sucrose interface, which has been identified by electron microscopy as being composed chiefly of envelopes (Joyard & Douce, 1976b), contained no chlorophyll, but had long-chain acyl-CoA synthetase in high specific activity. In contrast, the stromal fraction, which remained at the top of the gradient, contained acetyl-CoA synthetase in high specific activity, but had a low specific activity for long-chain acyl-CoA synthetase (Table 1). The fraction banding at the 1.2 M/1.5 M-sucrose interface, and identified as chloroplast lamellae (Joyard & Douce, 1976b), contained all of the chlorophyll from the original purified chloroplasts, but contained negligible acyl-CoA synthetase activity. In our preparations this lamellae fraction contained 55–65% of the total chloroplast protein, the envelopes contained 1–2% of the total protein and the stromal protein made up the balance.

Since oleic acid was the predominant free fatty acid synthesized by isolated chloroplasts (Roughan *et al.*, 1976), it was expected that the long-chain acyl-CoA synthetase of chloroplasts might be relatively specific for oleate. Whole chloroplasts synthesized oleoyl-CoA at 3–4 times the rate for palmitoyl-CoA in the present study, and isolated envelopes, at high protein concentrations (300–400 μ g of protein/ml) in the assay, showed a similar preference for oleoyl-CoA synthesis. However, in the region where synthetase activity was proportional to protein concentration (10–50 μ g of protein/ml), ammonium palmitate was esterified at about 66% of the rate for ammonium oleate (Table 1).

For comparison we have included an analysis of the acyl-CoA synthetase activities and phospholipase D activity of a spinach leaf microsomal fraction (Table 1). Both oleoyl-CoA synthetase and palmitoyl-CoA synthetase activities were present at about 0.5 times the specific activity of that measured in chloroplast envelopes, but the acetyl-CoA synthetase had 15-fold higher specific activity in the microsomal fraction than in envelopes. Phospholipase D activity was readily detected in the microsomal fraction, but

Table 1. *Distribution of acyl-activating enzymes, phospholipase D, chlorophyll and protein among chloroplast fractions and a microsomal fraction isolated from spinach leaf*

Chloroplasts were purified and fractionated by the method of Joyard & Douce (1976b) and the results shown are from a typical experiment. A microsomal fraction was prepared by magnesium precipitation by the method of Diesperger *et al.* (1974) and the results shown are mean values from two experiments. Units of synthetase activities are nmol of CoA ester formed/min per mg of protein, and phospholipase D are μ mol of choline released/min per mg of protein.

	Chloroplast fraction			Microsomal fraction
	Envelope	Lamellae	Stroma	
Acetyl-CoA synthetase	0.1	0.05	7.5	1.5
Oleoyl-CoA synthetase	12.5	0.15	0.07	6.7
Palmitoyl-CoA synthetase	9.1	0.07	0.05	3.5
Phospholipase D	0	0	0	0.5
Chlorophyll (μ g/mg of protein)	0	90	0	0.01
Protein (mg)	2.5	68	50	—

was apparently absent from each of the chloroplast fractions. These findings preclude the possibility of significant contamination of the envelope fraction by the microsomal fraction.

Knowing (a) the contribution of the protein in the separate fractions to the total chloroplast protein (Table 1) and (b) the protein:chlorophyll ratio (22:1, w/w) in our isolated intact chloroplasts, we have calculated that the oleoyl-CoA synthetase activity of the chloroplast envelope was 2 times that required to cope with maximum rates of oleic acid synthesis by chloroplasts *in vitro* (Roughan *et al.*, 1976). Similarly, the acetyl-CoA synthetase activity of the chloroplast stroma was 5 times that necessary to account for maximum observed rates of [14 C]acetate incorporation into the lipids of isolated chloroplasts (Roughan *et al.*, 1976).

We thank Mr. Ross Holland for performing the phospholipase D assays.

References

- Arnon, D. I. (1949) *Plant Physiol.* **24**, 1–15
 Baker, N. & Lynen, F. (1971) *Eur. J. Biochem.* **19**, 200–210
 Dawson, R. M. C. & Hemington, N. (1967) *Biochem. J.* **102**, 76–86
 Diesperger, H., Muller, C. R. & Sanderman, H. Jr., (1974) *FEBS Lett.* **43**, 155–158

- Douce, R. (1974) *Science* **183**, 852–853
 Douce, R., Holtz, R. B. & Benson, A. A. (1973) *J. Biol. Chem.* **248**, 7215–7222
 Galton, D. J. & Frazer, T. R. (1969) *Anal. Biochem.* **28**, 59–64
 Joyard, J. & Douce, R. (1976a) *Biochim. Biophys. Acta* **424**, 125–131
 Joyard, J. & Douce, R. (1976b) *Physiol. Vég.* **14**, 31–48
 Mancha, M., Stokes, G. B. & Stumpf, P. K. (1975) *Anal. Biochem.* **68**, 600–608
 Nichols, B. W. & James, A. T. (1964) *Fette Seifen Anstrichm.* **66**, 1003–1006
 Poincelot, R. P. (1973) *Arch. Biochem. Biophys.* **159**, 134–142
 Roughan, P. G., Slack, C. R. & Holland, R. (1976) *Biochem. J.* **158**, 593–601
 Rouser, G., Kritchevsky, G., Simon, G. & Nelson, G. J. (1967) *Lipids* **2**, 37–40
 Schacterle, G. R. & Pollack, R. C. (1974) *Anal. Biochem.* **51**, 654–655
 Slack, C. R. & Roughan, P. G. (1975) *Biochem. J.* **152**, 217–228
 Slack, C. R., Roughan, P. G. & Terpstra, J. (1976) *Biochem. J.* **155**, 71–80
 Stokes, D. M. & Walker, D. A. (1971) in *Photosynthesis and Photorespiration* (Hatch, M. D., Osmond, C. B. & Slatyer, R. O., eds.), pp. 226–231, Wiley-Interscience, New York
 Van Hummel, H. C., Hulsebos, Th. J. M. & Wintermans, J. F. G. M. (1975) *Biochim. Biophys. Acta* **380**, 219–226
 Vijay, I. K. & Stumpf, P. K. (1971) *J. Biol. Chem.* **246**, 2910–2917