Relationship between fatty-acyl composition of diacylgalactosylglycerol and turnover of chloroplast phosphatidate

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Chloroplasts from plants that contain different proportions of the 2-hexadecatrienoyl-1-linolenoyl molecular species in total diacylgalactosylglycerol were examined for their ability to dephosphorylate phosphatidate that had been labelled *in situ* with [1-¹⁴C]acetate. The turnover rate of chloroplast phosphatidate may be related to the accumulation of a prokaryotic diacylgalactosylglycerol, suggesting that chloroplast phosphatidate phosphatase controls the fatty acyl composition of the diacylgalactosylglycerol synthesized in the intact plant.

Whereas the chloroplast diacylgalactosylglycerol of most higher plants contains predominantly or exclusively linolenate (C_{18:3}), that of some plants contains significant amounts of hexadecacis-7,cis-10,cis-13-trienoate (C_{16:3}) (Jamieson & Reid, 1971; Heinz, 1977). C₁₆ fatty acids occur in relatively high proportions in the diacylgalactosylglycerol of blue-green (Zepke *et al.*, 1978; Sato *et al.*, 1979) and green algae (Safford & Nichols, 1970) and the presence of C_{16:3} in diacylgalactosylglycerol of some higher plants may indicate a residual prokaryotic form of lipid metabolism. The C₁₆ fatty acids are located almost exclusively at the *sn*-2 position of diacylgalactosylglycerol (Heinz, 1977). Diacylgalactosylglycerol from *Spinacia oleracea* can be resolved into two major molecular species, galactosyl-1,2-dilinenoyl- and galactosyl-2-hexadecatrienoyl-1-linolenoyl-glycerol (Siebertz *et al.*, 1980).

Attempts to explain the simultaneous occurrence of these two types of diacylgalactosylglycerol within some plants have concentrated on the likelihood of there being two separate pathways of glycerolipid synthesis in plant cells (Roughan *et al.*, 1980; Roughan & Slack, 1980). Chloroplasts isolated from *Spinacia oleracea* synthesized diacylglycerol, which is the precursor of galactosyl-1-oleoyl-2-palmitoylglycerol (McKee & Hawke, 1979; Roughan *et al.*, 1979b, 1980), *de novo* and subsequently desaturated the constituent fatty acids (Roughan *et al.*, 1979b; Willemot *et al.*, 1982), but the galactosyl-2-hexadecatrienoyl-1-linolenoylglycerol thus formed constitutes only 50% of the

diacylgalactosylglycerol synthesized in the intact *Spinacia oleracea* plant (Gardiner *et al.*, 1982). In recent studies virtually no acyl-labelled diacylgalactosylglycerol was synthesized by chloroplasts or by chloroplast enzymes isolated from C_{18:3}-plants (Heinz & Roughan, 1982; Frentzen *et al.*, 1982). It has been proposed that in these plants all the diacylglycerol for diacylgalactosylglycerol synthesis is derived from microsomal lipid metabolism, whereas in C_{16:3}-plants the diacylglycerol precursors are both microsomal (1,2-dioleoyl-) and chloroplast (1-oleoyl-2-palmitoyl-). This then gives rise to a mixture of galactosyl-1,2-dilinenoylglycerol and galactosyl-2-hexadecatrienoyl-1-linolenoylglycerol in the intact plant (Roughan *et al.*, 1980; Roughan & Slack, 1980).

Chloroplasts isolated from C_{18:3}-plants accumulate phosphatidate rather than diacylglycerol (Heinz & Roughan, 1982) and it has been suggested that activity of phosphatidate phosphatase located in the chloroplast membrane (Joyard & Douce, 1979) plays a key role in determining availability of the diacylglycerol species required for synthesis of galactosyl-2-hexadecatrienoyl-1-linolenoylglycerol in the chloroplast (Joyard & Douce, 1979; Heinz & Roughan, 1982; Frentzen *et al.*, 1982). Very low activity of this enzyme would hence lead to the absence of the above molecular species of diacylgalactosylglycerol in C_{18:3}-plants and higher activities to its varying proportions in total diacylgalactosylglycerol of C_{16:3}-plants.

In the present paper we demonstrate an inverse relationship between the presence of galactosyl-2-hexadecatrienoyl-1-linolenoylglycerol and the half-life of breakdown of phosphatidate to diacylglycerol in five plant species.

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

Experimental

Materials

Sodium [$1\text{-}^{14}\text{C}$]acetate (sp. radioactivity 61.6 Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and *sn*-glycerol 3-phosphate from Sigma, St. Louis, MO, U.S.A. Seeds of *Spinacia oleracea* (hybrid 102) and *Pisum sativum* (cultivar Victory Freezer) were obtained from Arthur Yates, Auckland, New Zealand. *Carthamus tinctorius* seeds were a gift from Dr. J. M. McEwan and other seeds were collected from the wild.

Methods

Spinacea oleracea (hybrid 102), *Carthamus tinctorius* (cultivar 0-22), *Solanum nodiflorum* and *Chenopodium quinoa* were grown in a controlled environment in aerated nutrient solution as described previously (Slack & Roughan, 1975). *Pisum sativum* seeds were grown in a glasshouse in pumice/peat watered with the above nutrient solution. Chloroplasts were isolated from expanding leaves of 11-day-old *Pisum sativum* shoots by the method of Nakatani & Barber (1977) modified by the addition of 0.25% bovine serum albumin to the homogenizing buffer and 0.1% bovine serum albumin to the wash buffer for all plants except *Spinacia oleracea*.

Chloroplasts were incubated with shaking at 25°C in 0.25 ml of a medium contain 0.33 M-sorbitol, 25 mM-Hepes/NaOH (pH 7.9), 10 mM- NaHCO_3 , 2 mM-EDTA, 1 mM- MgCl_2 , 1 mM- MnCl_2 , 0.5 mM- K_2HPO_4 , 0.4 mM-*sn*-glycerol 3-phosphate and 0.13 mM- $[1\text{-}^{14}\text{C}]$ acetate (sp. radioactivity 61.6 Ci/mol) for 5 min under illumination ($200\text{ }\mu\text{E}/\text{m}^2$ per s of photosynthetically active radiation) and then in darkness for the time indicated. Chlorophyll ranged from 15 to 70 $\mu\text{g}/\text{assay}$ (Arnon, 1949). Reactions were stopped at the times indicated by adding 2.5 ml of chloroform/methanol (1:1, v/v) and total lipids were recovered from the chloroform layer after partitioning against 0.875 ml of 1 M-KCl/0.2 M- H_3PO_4 (Hajra, 1974). Radioactive phosphatidate was co-purified with carrier phosphatidate, prepared by the action of phospholipase D on egg-yolk phosphatidylcholine, by two-dimensional t.l.c. on silica-gel H containing 0.3% (w/w) EGTA with the solvent systems described previously (Roughan *et al.*, 1978) and detected by light staining in I_2 vapour. The phosphatidate was scraped up into 8 ml of 3.3% (w/v) *p*-terphenyl in xylene/Triton X-100/water (20:10:3, by vol.) for scintillation counting. Corrections for quenching were made by using the channels-ratio method. Rates of total lipid synthesis were estimated after t.l.c. of further portions of the extracts on silica-gel G containing 5% (w/w) H_3BO_3 (Roughan *et al.*, 1979a). Relative linolenate and hexadecacatrienoate content of diacylgalactosyl-

glycerol isolated from total leaf lipids by t.l.c. was determined by g.l.c. (Gardiner *et al.*, 1982).

Results

To measure the rate of conversion of phosphatidate into diacylglycerol in isolated chloroplasts, the chloroplasts were first incubated for 5 min in the light to incorporate $[1\text{-}^{14}\text{C}]$ acetate into phosphatidate and then transferred to darkness, where loss of radioactivity from phosphatidate was observed. The time of preincubation in the light and media composition were chosen to give optimal accumulation of radioactive phosphatidate without unduly aging the chloroplasts, which were to be incubated up to 30 min longer in darkness. Of the radioactive fatty acids synthesized 15–23% were found in phosphatidate under these conditions (Table 1), but the labelling of phosphatidate fatty acids had not necessarily equilibrated with the supplied $[1\text{-}^{14}\text{C}]$ acetate within the 5 min period (S. E. Gardiner, unpublished work). Incorporation rates of $[1\text{-}^{14}\text{C}]$ acetate into fatty acids measured during the preincubation were: *Spinacea oleracea*, 690–1200; *Solanum nodiflorum*, 450–470; *Carthamus tinctorius*, 160–270; *Chenopodium quinoa*, 1120–1220; and *Pisum sativum*, 240–1270 nmol of acetate/mg of chlorophyll per h. These are conservative measurements, as an initial lag in incorporation is observed in some species (P. G. Roughan, unpublished work). Labelled fatty acids synthesized from the $[1\text{-}^{14}\text{C}]$ acetate were found principally in diacylglycerol, non-esterified fatty acids and phos-

Table 1. Distribution of radioactivity from $[1\text{-}^{14}\text{C}]$ acetate in lipids synthesized by isolated chloroplasts after 5 min incubation in the light

Chloroplasts isolated from five different plant species were incubated under illumination with shaking for 5 min in the presence of $[1\text{-}^{14}\text{C}]$ acetate (sp. radioactivity 61.6 Ci/mol) at 25°C in the medium outlined under 'Methods'. Reactions were stopped, lipids extracted, separated by t.l.c. and radioactivity determined as outlined under 'Methods'. Results are expressed as percentages of total incorporation. Minor components comprising lysophosphatidic acid, acyl-CoA and other polar lipids are omitted from the Table.

Plant	Proportion of total lipid radioactivity (%)		
	Phosphatidate	Non-esterified fatty acids	Diacylglycerol
<i>Spinacia oleracea</i>	23	26	31
<i>Solanum nodiflorum</i>	15	23	47
<i>Chenopodium quinoa</i>	17	57	6
<i>Carthamus tinctorius</i>	22	58	2
<i>Pisum sativum</i>	16	62	3

phatidate. In agreement with Heinz & Roughan (1982), we found that less radioactivity was accumulated in diacylglycerol of chloroplasts from the $C_{18:3}$ -plants *Carthamus tinctorius* and *Pisum sativum* than the $C_{16:3}$ -plants *Spinacia oleracea* and *Solanum nodiflorum* (Table 1).

Fatty acid synthesis from $[1-^{14}C]$ acetate ceased when chloroplasts were darkened (results not shown), but the conversion of phosphatidate into diacylglycerol continued. The decrease in labelling of

phosphatidate was not exactly matched by increase in labelling of diacylglycerol. However, there was no evidence that the phosphatidate was metabolized by any mechanism other than dephosphorylation, whereas it is likely that a slow deacylation of diacylglycerol occurred, as the labelling of non-esterified fatty acids slowly increased during incubation (Roughan *et al.*, 1980; S. F. Gardiner, unpublished work). Therefore disappearance of phosphatidate was chosen to indicate phosphatidate phosphatase activity. The amounts of accumulated radioactive phosphatidate decreased comparatively rapidly in *Solanum nodiflorum* and *Spinacia oleracea*, more slowly in *Chenopodium quinoa* and slowest in *Carthamus tinctorius* and *Pisum sativum* (Fig. 1). Loss of radioactivity from phosphatidate appeared to be first order and linear regressions of log (% radioactivity in phosphatidate) versus time were used to obtain rate constants and half-life values for phosphatidate (Table 2). Phosphatidate half-lives were shortest in *Spinacia oleracea* and *Solanum nodiflorum* (1.3 and 1.7 min), of intermediate length in *Chenopodium quinoa* (5.8 min) and longest in *Pisum sativum* and *Carthamus tinctorius* (39 min). There was no significant difference between phosphatidate half-lives for *Pisum sativum* and *Carthamus tinctorius*, or between *Solanum nodiflorum* and *Spinacia oleracea* but the slopes of the phosphatidate decay curves for the two groups were significantly different from each other and from that for *Chenopodium quinoa* at the $P < 0.001$ level. Content of galactosyl-2-hexadecatrienoyl-1-linolenoylglycerol was high in *Solanum nodiflorum* and *Spinacia oleracea* and lower in *Chenopodium quinoa*. *Carthamus tinctorius* and *Pisum sativum* contained little or none of this molecular species of diacylgalactosylglycerol.

When excess unlabelled acetate was added to chloroplasts of *Spinacia oleracea* incubating in the light, essentially preventing further incorporation of radioactivity, a similar value for phosphatidate

Fig. 1. *Phosphatidate decay in darkened chloroplasts* Illuminated chloroplasts were pre-incubated with shaking for 5 min in the presence of $[1-^{14}C]$ acetate (sp. radioactivity 61.6 Ci/mol) at 25°C in the medium outlined under 'Methods'. Incubation was then continued in the dark and reactions were stopped at the times indicated. Lipids were extracted and radioactivity in phosphatidate was determined as outlined under 'Methods'. Results are expressed as percentages of radioactivity in phosphatidate at the start of dark incubation. ●, *Pisum sativum*; □, *Carthamus tinctorius*; △, *Chenopodium quinoa*; ○, *Spinacia oleracea*; ■, *Solanum nodiflorum*.

Table 2. *Galactosyl-2-hexadecatrienoyl-1-linolenoylglycerol content and chloroplast phosphatidate half-life in five plant species*

Percentage of the 2-hexadecatrienoyl-1-linolenoyl molecular species in total diacylgalactosylglycerol was determined from the relative levels of linolenate and hexadecatrienoate in this lipid (see under 'Methods'). Linear regressions of log [radioactivity in phosphatidate (%)] versus time were employed to obtain rate constants and half-life values for phosphatidate using the data from Fig. 1. Values with different superscripts differ significantly ($P < 0.001$). k = apparent first-order rate constant. Results for k are means $\pm$ S.D.

Plant	2-Hexadecatrienoyl-1-linolenoyl species of diacylgalactosylglycerol (% of total diacylgalactosylglycerol)	k (min ⁻¹)	Half-life of phosphatidate (min)
<i>Pisum sativum</i>	0	0.018 $\pm$ 0.002 ^a	38.5
<i>Carthamus tinctorius</i>	1	0.018 $\pm$ 0.002 ^a	38.5
<i>Chenopodium quinoa</i>	8	0.120 $\pm$ 0.009 ^b	5.8
<i>Solanum nodiflorum</i>	40	0.520 $\pm$ 0.060 ^c	1.3
<i>Spinacia oleracea</i>	50	0.416 $\pm$ 0.019 ^c	1.7

half-life was observed as in a parallel experiment in which the chloroplasts were darkened, indicating that the phosphatidate decay in the dark was equivalent to phosphatidate decay in the light.

Discussion

The low capacity of chloroplasts isolated from $C_{18:3}$ -plants to accumulate diacylglycerol is shown by the data in Table 1. The differences between $C_{18:3}$ - and $C_{16:3}$ -plants are even more marked than in the recent report of Heinz & Roughan (1982), probably due to the shorter incubation time used in the present study. As the proportions of labelled phosphatidate were similar in the two groups of plants, it seemed possible that the $C_{18:3}$ -plants were deficient in phosphatidate phosphatase activity. Our investigation of this point demonstrated that the half-life of radioactive phosphatidate synthesized by isolated chloroplasts varied considerably between chloroplasts isolated from plants containing different proportions of the two diacylgalactosylglycerol species. The turnover of plastid phosphatidate was 23–30-fold faster in the $C_{16:3}$ -plants *Spinacia oleracea* and *Solanum nodiflorum* and 7-fold faster in the 'transitional- $C_{16:3}$ -plant' *Chenopodium quinoa* than it was in the $C_{18:3}$ -plants *Pisum sativum* and *Carthamus tinctorius*. No significant differences were found between phosphatidate turnover rates in plastids from *Solanum nodiflorum* and *Spinacia oleracea* or from *Pisum sativum* and *Carthamus tinctorius*. The activity of chloroplast phosphatidate phosphatase was therefore related to $C_{16:3}$ content of diacylgalactosylglycerol in the plants studied, and we suggest that this enzyme plays a major role in fixing the proportions of the two forms of diacylgalactosylglycerol found in any particular plant by determining the molecular species of diacylglycerol that will be available for diacylgalactosylglycerol synthesis.

Heinz & Roughan (1982) recently proposed three possible control points for chloroplast diacylgalactosylglycerol synthesis, after noting that chloroplasts from $C_{18:3}$ -plants had a lower capacity to synthesize glycerolipids, a lower phosphatidate phosphatase activity and a reduced ability to galactosylate endogenously synthesized diacylglycerol compared with chloroplasts from $C_{16:3}$ -plants. The present study confirms their finding about glycerolipid synthesis and suggests that differences in phosphatidate phosphatase activity are even greater than indicated by their study. Data comparing phosphatidate half-lives in five plant

species, together with information on content of galactosyl-2-hexadecatrienoyl-1-linolenoylglycerol in the same plants suggests a regulatory role of chloroplast phosphatidate phosphatase in plant diacylgalactosylglycerol synthesis. Plants studied included all those from which it is currently possible to isolate chloroplasts with a high capacity to incorporate [$1-^{14}C$]acetate into lipids.

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References

- Arnon, D. I. (1949) *Plant Physiol.* **24**, 1–15
- Frentzen, M., Heinz, E., McKeon, J. & Stumpf, P. K. (1982) *Proc. Int. Symp. Plant Lipids 5th* in the press
- Gardiner, S. E., Roughan, P. G. & Slack, C. R. (1982) *Plant Physiol.* in the press
- Hajra, A. K. (1974) *Lipids* **9**, 502–505
- Heinz, E. (1977) in *Lipids and Lipid Polymers in Higher Plants* (Telvini, M. & Lichtenthaler, H. K., eds.), pp. 102–120, Springer-Verlag, Berlin, Heidelberg and New York
- Heinz, E. & Roughan, P. G. (1982) *Proc. Int. Symp. Plant Lipids 5th* in the press
- Jamieson, G. R. & Reid, E. H. (1971) *Phytochemistry* **10**, 1837–1843
- Joyard, J. & Douce, R. (1979) *FEBS Lett.* **102**, 147–150
- McKee, J. W. A. & Hawke, J. C. (1979) *Arch. Biochem. Biophys.* **197**, 322–332
- Nakatani, H. Y. & Barber, J. (1977) *Biochim. Biophys. Acta* **461**, 510–512
- Roughan, P. G. & Slack, C. R. (1980) in *Biogenesis and Function of Plant Lipids* (Mazliak, P., Benveniste, P., Costes, C. & Douce, R., eds.), pp. 11–17, Elsevier/North-Holland, Amsterdam
- Roughan, P. G., Slack, C. R. & Holland, R. (1978) *Lipids* **13**, 497–503
- Roughan, P. G., Holland, R. & Slack, C. R. (1979a) *Biochem. J.* **184**, 193–202
- Roughan, P. G., Mudd, J. B. & McManus, T. T. (1979b) *Biochem. J.* **184**, 571–574
- Roughan, P. G., Holland, R. & Slack, C. R. (1980) *Biochem. J.* **188**, 17–24
- Safford, R. & Nichols, B. W. (1970) *Biochim. Biophys. Acta* **210**, 57–64
- Sato, N., Murata, N., Miura, Y. & Ueta, N. (1979) *Biochim. Biophys. Acta* **572**, 19–28
- Siebertz, H. P., Heinz, E., Joyard, J. & Douce, R. (1980) *Eur. J. Biochem.* **108**, 177–185
- Slack, C. R. & Roughan, P. G. (1975) *Biochem. J.* **152**, 217–228
- Willemot, C., Slack, C. R., Browse, J. & Roughan, P. G. (1982) *Plant Physiol.* **70**, 78–81
- Zepke, H. D., Heinz, E., Radunz, A. & Pesch, R. (1978) *Arch. Microbiol.* **119**, 157–162