Control of Fatty Acid Incorporation into Chloroplast Lipids *in vitro*

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Z. Naturforsch. **39 c**, 593–599 (1984); received March 19, 1984

Glycerolipid Synthesis in Chloroplasts

1. In isolated chloroplasts which are provided with essential exogenous substrates for glycerolipid biosynthesis (sn-G3P and UDPgal) the incorporation of fatty acids into lipids shows the same pH dependence as the fatty acid synthesis itself with a stromal pH optimum close to 8.5.

2. Furthermore high rates of glycerolipid biosynthesis appear to be accompanied by a preferred oleate incorporation as compared to palmitate.

3. Reinvestigations of the sn-G3P requirement of plastid lysophosphatidic acid formation with rapidly prepared substrate-free chloroplast extracts under approximately physiological conditions reveal a lower specificity of the primary sn-G3P acylation for oleate, as recently found for the fatty acid transfer from purified acyl-ACP fractions on to sn-G3P, catalyzed by purified acyl transferase 1.

4. A comparison of calculated stromal sn-G3P levels under physiological conditions (0.1–0.3 mM) with those, required for half saturation of the primary acylation reaction either with oleate (K_m(sn-G3P) = 0.3 mM) or palmitate (K_m(sn-G3P) = 0.6 mM) in chloroplast extracts suggests, that both fatty acids to be involved in lysophosphatidic acid formation within chloroplasts, although oleate would be preferred.

5. The latter observation facilitates the understanding of a palmitate accumulation in chloroplast lipid fractions, induced by increasing sn-G3P concentrations in chloroplast suspensions.

6. Although stimulating fatty acid synthesis from acetate in intact chloroplasts, acyl-CoA-synthesizing-conditions (presence of CoA and ATP) in the applied chloroplast extracts appear to inhibit fatty acid incorporation into sn-G3P and thus to exert a regulatory function between the plastidary and extraplastidary glycerolipid biosynthesis.

Introduction

Recent reinvestigations on chloroplast fatty acid synthesis from acetate *in vitro* suggest that light induced changes in the stromal concentrations of ATP, Mg^{2+} and H^+ [1] which are already known essential factors of the key enzymes of acetate fixation too [2–6], will not only drive fatty acid synthesis but also affect chain elongation from palmitate to oleate [1], both of which represent the bulk of newly synthesized fatty acids [2–6]. The latter conclusion was drawn from the observation that as the pH in the stroma increased, the increasing capacity of intact chloroplasts to synthesize long-chain fatty acids from acetate was accompanied by a shift in the proportion of oleate to palmitate formation in favour of oleate [1]. But in order to promote the capacity of isolated chloroplasts to incorporate freshly synthesized fatty acids into polar lipids, the essential substrates, which are known to be provided from the cytosol *in vivo* (sn-G3P, UDPgal, CoA and ATP) must be provided in the incubation medium [7]. The enzyme activities catalysing these reactions are all but one localized on the envelope membranes [8–15].

$$\text{acyl-ACP} + \text{sn-G3P} \xrightarrow[\text{acyl transferase 1}]{} \text{lysophosphatidic acid} + \text{ACP (stroma)}$$

$$\text{acyl-ACP} + \text{lysophosphatidic acid} \xrightarrow[\text{acyl transferase 2}]{} \text{phosphatidic acid} + \text{ACP}$$

$$\text{phosphatidic acid} \xrightarrow[\text{phosphatidate phosphatase}]{} \text{diacylglycerol} + P_i$$

$$\text{diglyceride} + \text{UDPgal} \xrightarrow[\text{galactosyl transferase}]{} \text{MGDG} + \text{UDP}$$

$$\text{CoA} + \text{fatty acid} + \text{ATP} \xrightarrow[\text{acyl-CoA synthetase}]{} \text{acyl-CoA} + \text{AMP} + PP_i .$$

The rapid metabolization of newly synthesized fatty acids implies that changes in their composition

Abbreviations: ACP, acyl-carrier protein; CoA, coenzyme A; DAG, diacylglycerol; MGDG, monogalactosyl diglyceride; UDPgal, UDP galactose.

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0341-0382/84/0600-0593 $ 01.30/0

will be manifested in their lipid incorporation. Furthermore increasing levels of sn-G3P have been previously found to elevate the palmitate/oleate ratio in chloroplast suspensions [12, 16] as well as in the tissue of spinach [17]. Because this increase of the cellular concentrations of sn-G3P was simultaneously accompanied by an accumulation of typical plastid (prokaryotic) at the expense of extraplastid (eukaryotic) lipids it has been interpreted in terms of a two-pathway hypothesis for leaf glycerolipid biosynthesis, giving an explanation for the typical fatty acid patterns in several plant species [12, 17, 18]. But the suggestion that the sn-G3P induced accumulation of palmitate in chloroplast lipids is due to an initial acylation of sn-G3P by palmitoyl-ACP [16, 17, 19] is not easy to reconcile with information about the pool sizes and transport rates of sn-G3P determined for intact chloroplasts [20] on one hand and the sn-G3P levels which would be required to compensate the recently found fatty acid selectivity of the primary sn-G3P acylation (at the C_1-position) for oleate in favour of palmitate [15] on the other.

Thus, in order to demonstrate that factors, like the stromal pH which affect the rate of chloroplast fatty acid synthesis [1], are also affecting incorporation of fatty acids into lipids [19] and to elucidate further above mentioned discrepancies referring to the sn-G3P effect on fatty acid selectivity of the acyl transferase 1, the following studies have been done:

1. The effect of pH on incorporation of fatty acids into lipids has been investigated by following [1-^{14}C]acetate incorporation into lipid bound fatty acid species of intact chloroplasts, incubated in a glycolipid synthesizing mode [7, 18].

2. The kinetics of the primary sn-G3P acylation have been reinvestigated in extracts from chloroplasts, which had been pulse labelled in their fatty acid fraction from [1-^{14}C]acetate in the light before they were lysed in Triton X-100 and freed of their substrates by elution over a short Sephadex G-25 column according to Laing and Roughan [21].

Materials and Methods

Spinach was grown in hydroponic culture and intact chloroplasts were prepared as in [1]. The standard medium for chloroplast incubation contained 0.33 M sorbitol, 50 mM Tricine/KOH (pH 8.0), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM K_2HPO_4, 10 mM $NaHCO_3$, 0.15 mM [1-^{14}C]acetate (specific activity: 49 mCi · mmol^{-1}) and 40 µg · ml^{-1} catalase from bovine liver (Boehringer, Mannheim). Further additions for glycerolipid biosynthesis and acyl-CoA synthesizing conditions are noted in the legends of the tables. Extraction, separation and investigation of the labelled lipid fractions has been described earlier [19].

Preparation of leaf extracts for determination of sn-G3P levels in intact leaf tissue modified according to Stitt er al. [22, 23]

At different time points during a light-dark cycle two leaf discs (diameter 3 cm) per sample were rapidly cut from attached spinach leaves and immediately frozen in liquid N_2. As the liquid N_2 evaporated, the samples were homogenized to a powder and suspended for 30 min at −5 °C in 10% (v/v) $HClO_4$, before they were centrifuged (2 min, Eppendorf microfuge) to remove precipitated protein, neutralized with 5 N KOH, 1 N triethanolamine, and again centrifuged to remove the $KClO_4$. Parallel samples of leaf discs were homogenized in 80% (v/v) acetone for chlorophyll determination.

Preparation of chloroplast extracts for determination of stromal sn-G3P levels

Chloroplasts were prepared from the same leaf material (4 leaves of the same size), taken for the leaf discs, as in [1]. Ice-cold chloroplast suspensions immediately after isolation were centrifuged through a layer of silicone oil into 20 µl 1 M $HClO_4$ using the method of Heldt *et al.* [24].

The sn-G3P concentration in the neutralized leaf- and chloroplast extracts (bottom layer after silicone oil centrifugation) was assayed spectrophotometrically via glycerophosphate dehydrogenase [20].

Measurement of sn-G3P acylation in crude extracts, prepared from [1-^{14}C]acetate pulse labelled spinach chloroplasts

During pulse labelling in the standard medium (15 min saturating white light; $T = 25$ °C) with [1-^{14}C]acetate (0.5 mM; specific activity: 49 mCi · mmol^{-1}) for dense chloroplast suspensions (1 mg Chl · ml^{-1}) and thus for relative low rates of acetate

incorporation has been taken allowance, in order to keep the chlorophyll dilution during the following procedure in limits. Subsequently the chloroplasts were lysed by addition of 0.2% (w/v) Triton X-100 (for 1 min in the light) and rapidly freed of their substrates by elution over a short Sephadex G25 column according to Laing and Roughan [21]. The whole procedure did not last longer than 30 min. For sn-G3P acylation measurements the column eluate was incubated for 15 min in a medium containing 50 mM Tricine/KOH (pH 8.5), 0.5 mM NADH, 0.5 mM NADPH, 2 mM $NaHCO_3$, 1 mM DTT, 1 mM $MgCl_2$, 1 mM ATP and changing amounts of sn-G3P at a final chlorophyll concentration of 0.2 mg Chl · ml^{-1}. The CoA effect was measured in the simultaneous presence of varying CoA concentrations. For controls aliquots of the chloroplast suspension directly after pulse labelling with [1-^{14}C]acetate were analyzed for lipid incorporation.

Results and Discussion

1. By varying the substrate mixtures and incubation conditions during feeding of [1-^{14}C]acetate to spinach chloroplast suspensions [7, 18], the usual flux of newly synthesized fatty acids into different lipid products is observed (Table I). Thus, presence of [1-^{14}C]acetate alone leads to an accumulation of newly synthesized free oleic acid (making up about 90% of total free C_{18}-acids) at the expense of C_{16}-acids, while addition of sn-G3P drives diacylglycerol synthesis (DAG mode; [7, 18]) via lysophosphatidic- and phosphatidic acids, which is further followed by monogalactosyl diglyceride formation (MGDG mode, [7, 18]), when exogenous UDPgal is provided. The data in Table I furthermore support the notion, that the sn-G3P induced glycerolipid synthesis is accompanied by an accumulation of palmitic acid (making up more than 90% of total C_{16}-fatty acids) as compared to C_{18}-fatty acids in all plastidary lipid fractions [12, 16, 25]. The simultaneous presence of CoA and ATP during the incubation (Table I) appears to accelerate fatty acid turnover by acyl-CoA-thioesterformation for extraplastidary lipid biosynthesis [7, 26] in the outer envelope membrane [11, 14] and to stimulate in this way not only synthesis and lipid incorporation of fatty acids from acetate but also to influence the fatty acid pattern in the lipid fractions [16, 20, 26].

During subsequent "dark metabolism" of lipid intermediates, accumulated by chloroplasts from [1-^{14}C]acetate in the light, a significant short-term lipid breakdown as possible consequence of the well known inhibition of fatty acid synthesis in the dark [2–6] under appropriate conditions (Table Ib) seems to occur, but only under acyl-CoA synthesizing conditions (presence of CoA and ATP), when newly synthesized fatty acids are withdrawn from the reaction centre [7, 26]. Besides leading to a reduced rate of total lipid incorporation of [1-^{14}C]-acetate, this lipid breakdown is also indicated by decreasing label in the fractions of diacylglycerols and free fatty acids as already shown by other authors [7].

Table I. Dependence of the lipid incorporation of fatty acids, freshly synthesized from [1-^{14}C]acetate by intact spinach chloroplasts, on the incubation mode. The latter was manipulated by addition of sn-G3P (0.5 mM), UDPgal (0.1 mM), CoA (0.5 mM) and ATP (2 mM) to the standard medium at pH 8.0. Acetate incorporation was followed for 15 min (25 °C) under saturating white light (a) or for a further 10 min in the dark (b) following this illumination period. Fatty acid composition in chloroplast lipids is demonstrated in brackets by the distribution of label between C_{18}- and C_{16}-fatty acids ($^{14}C_{18}/^{14}C_{16}$-ratio). Only the predominantly labelled lipid fractions are depicted.

Cold additions to basal medium	Incorporation of [1-^{14}C]acetate (nmol · mg^{-1} Chl · h^{-1}) into					
	pH	Phospholipids [a]	Monogalactosyl diglycerides	Diglycerides	Free fatty acid	Total lipid incorp.
a. none	8.0	7 (1.2)	–	17 (1.0)	72 (6.1)	101
sn-G3P	8.0	37 (0.7)	3 (0.6)	69 (0.6)	33 (1.4)	160
sn-G3P, UDPgal	8.0	52 (0.8)	113 (0.7)	14 (1.0)	73 (2.1)	261
sn-G3P, UDPgal, CoA, ATP	8.0	92 (1.9)	301 (1.1)	38 (1.0)	169 (3.5)	635
b. sn-G3P, UDPgal	8.0	63 (0.7)	212 (0.6)	9 (2.1)	61 (1.9)	369
sn-G3P, UDPgal, CoA, ATP	8.0	55 (2.0)	193 (1.0)	10 (1.7)	71 (2.5)	350

[a] The phospholipids are predominantly represented by lysophosphatidic- and phosphatidic acids.

Table II. Effect of the pH on lipid incorporation of fatty acids freshly synthesized from [1-^{14}C]acetate by intact spinach chloroplasts. Lipid synthesizing conditions during incubation were manipulated by addition of either sn-G3P (0.5 mM) and UDPgal (0.1 mM) alone (a) or together with CoA (0.5 mM) and ATP (2 mM) (b) to the basal medium. Acetate incorporation was stopped after 15 min under saturating white light (25 °C). The distribution of label between C_{18}- and C_{16}-fatty acids in the plastidary lipid fractions is demonstrated by the $^{14}C_{18}/^{14}C_{16}$-ratio in brackets.

Cold additions to basal medium	Incorporation of [1-^{14}C]acetate (nmol · mg^{-1} Chl · h^{-1}) into					
	pH	Phospholipids [a]	Monogalactosyl diglycerides	Monoglycerides	Diglycerides	Free Fatty Acids
a. sn-G3P, UDPgal	7.0	17 (0.6)	15 (0.8)	6 (0.7)	3 (0.8)	20 (2.8)
	7.5	42 (0.7)	54 (0.6)	4 (1.3)	4 (1.4)	24 (2.3)
	8.0	52 (0.8)	113 (0.7)	9 (2.3)	14 (1.0)	73 (2.1)
b. sn-G3P, UDPgal, CoA, ATP	7.0	59 (6.0)	16 (0.7)	22 (3.9)	47 (0.8)	29 (1.5)
	7.5	54 (4.0)	82 (0.7)	29 (2.6)	122 (0.7)	72 (3.6)
	8.0	92 (1.9)	301 (1.1)	35 (2.1)	38 (1.0)	169 (3.5)
	8.5	68 (1.8)	183 (1.1)	21 (2.2)	21 (1.4)	64 (3.9)

[a] The phospholipids are predominantly represented by lysophosphatidic- and phosphatidic acids.

The optimal stroma pH for long-chain fatty acid synthesis in isolated spinach chloroplasts from acetate has been recently found to be close to 8.5, which corresponds to about 8.0 in the incubation medium [1]. Consequently, if exogenous substrates for further glycerolipid biosynthesis are made available (Table II), fatty acids accumulating in the stroma under these alkaline stroma conditions are rapidly incorporated into chloroplast lipids. The latter notion is documented by maxima in glycerolipid biosynthesis at pH 8, which in this case, however, are not always accompanied by maximal rates of oleate incorporation (Table II) although this would be expected from the oleate/palmitate ratios of freshly synthesized fatty acids in media free of substrates ([1, 25], see also Table I). The latter observation suggests that under physiological conditions other criteria, like the substrate levels and pH conditions required for the different steps of polar lipid biosynthesis, may determine further lipid incorporation of fatty acids and not just the availability of the different fatty acid species. This possibility was further investigated.

2. One parameter, which has been recently found to influence the fatty acid composition in plant lipids in a definite manner, appears to be the cellular sn-G3P level [12, 17]. As demonstrated in Fig. 1 in spinach leaves the latter oscillates between 10–12 nmol · mg^{-1} Chl during the day and 6–7 nmol · mg^{-1} Chl in the night, which is in good agreement with values of other authors (11 nmol · mg^{-1} Chl, [17]). It is simultaneously shown that the plastidary (stromal) portion of the sn-G3P level in spinach leaf cells makes up 25% (tantamount to 3 nmol · mg^{-1} Chl) during the day and 60% (tantamount to 4 nmol · mg^{-1} Chl) in the night. As-

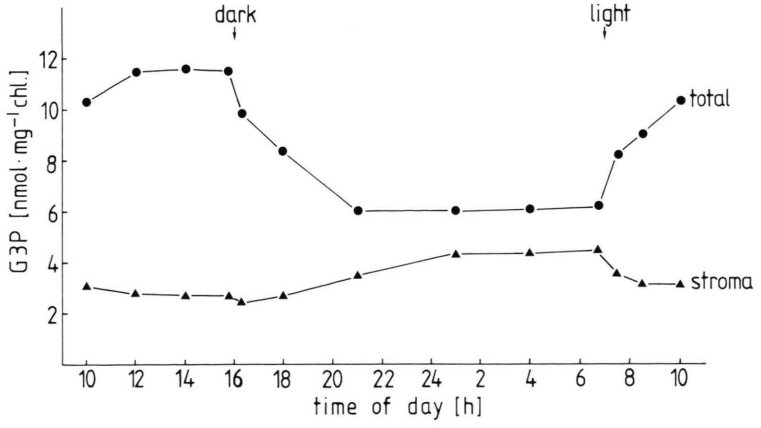

Fig. 1. Comparison of the diurnal changes of the sn-G3P concentration in total leaves and in the chloroplast stroma from spinach.

suming a stromal volume of about 20 µl · mg^{-1} Chl from these values stromal concentrations of 0.14 mM (light) and 0.22 mM (dark) have been calculated for spinach leaves [20]. The stromal sn-G3P level in all experiments, even with chloroplasts isolated from wheat leaf protoplasts (data not shown), did not exceed 0.3 mM [25]. The stromal sn-G3P pool is adjusted by a transport of sn-G3P from the cytoplasm, the site of its synthesis, into the chloroplast, mediated by the phosphate translocator in the inner envelope membrane [24]. Under lipid synthesizing conditions (presence of 0.15 mM acetate and 0.3 mM K_2HPO_4, saturating white light, $T = 25\,°C$) in the incubation medium the sn-G3P uptake by intact spinach chloroplasts showed similar K_m-values (0.5–1 mM; 20) as under standard conditions (in media free of inorganic phosphate and acetate at 4 °C in the dark), usually applied for transport measurements [24]. Against that, only 10% of the freshly uptaken sn-G3P was incorporated into plastidary glycerolipids [20]. This new synthesis of glycerolipids increased linearly up to 0.5 mM sn-G3P in the medium and reached a maximum at 1–2 mM sn-G3P. Under latter conditions rates of sn-G3P uptake of 18–22 nmol · mg^{-1} Chl were measured, which, a stromal volume of 20 µl · mg^{-1} Chl provided, corresponds to a sn-G3P uptake of 0.9–1.1 mM. Higher sn-G3P concentrations apparently did not increase glycerolipid synthesis any longer [20, 25]. Thus, in contrast to physiological conditions when the stromal concentration did not exceed 0.3 mM, the stromal sn-G3P uptake in isolated chloroplasts [16] appears to allow for higher stromal sn-G3P concentrations (of about 1 mM; [20, 25]). Based on the above calculations of the stromal pool sizes of sn-G3P the recently found differences in the K_m-values for sn-G3P of the palmitic-(K_m (sn-G3P) = 3.2 mM) and oleic acid transfer (K_m (sn-G3P) = 0.03 mM) from purified palmitoyl- and oleoyl-ACP fractions on to sn-G3P by purified plastidary acyl transferase 1 [15] appear to contradict the conception of an initial acylation of sn-G3P by palmitoyl-ACP as explanation for the sn-G3P induced accumulation of palmitate in chloroplast lipids [16, 17, 19].

Therefore we have tried to reinvestigate the primary acylation of sn-G3P in chloroplasts under more physiological conditions by using rapidly prepared extracts from spinach chloroplasts, which had been pulse labelled in their fatty acid fraction from [1-^{14}C]acetate in the light, before they were lysed in Triton X-100 and freed of their substrates

Table III. Primary and secondary acylation of sn-G3P in extracts from spinach chloroplasts, which had been pulse-labelled in their fatty acid fraction from [1-^{14}C]acetate in the light (I), before they were lysed in Triton X-100 and freed of their substrates by elution over a short Sephadex G25 column. In the following chase (15 min) the effect of sn-G3P on the transfer of labelled fatty acids on to lysophosphatidic- and phosphatidic acids in these extracts (II) in the absence (a) and presence of CoA (b) or CoA and ATP (acyl-CoA synthesizing conditions; c) has been compared.

					Incorporation of [1-^{14}C]acetate (nmol · mg^{-1} Chl · h^{-1}) into the fatty acid fractions of								
					pH	Total lipids	Lysophosphatidic acid				Phosphatidic acid			
							C_{16}-acids	C_{18}-acids	$C_{16:0}$	$C_{18:1}$	C_{16}-acids	C_{18}-acids	$C_{16:0}$	$C_{18:0}$
I. Chloroplasts (after ^{14}C-pulse):					8.0	17.0	0.23	0.33	0.18	0.19	0.60	0.29	0.57	0.17
II. Chloroplast extracts: additions to chase medium (mM)														
	Mg^{2+}	sn-G3P	ATP	CoA										
a.	1	0.25	1	–	8.5	13.9	0.35	1.78	0.29	1.39	1.14	1.28	1.07	0.97
	1	0.50	1	–	8.5	14.3	0.56	2.40	0.44	1.93	1.36	1.75	1.24	1.22
	1	1.00	1	–	8.5	16.6	0.76	2.93	0.63	2.31	1.34	2.25	1.27	1.85
	1	1.50	1	–	8.5	17.2	0.87	3.26	0.68	2.56	2.08	3.72	1.92	2.71
b.[a]	1	0.25-1	–	0.5	8.5	14.5	0.33	1.05	0.25	0.69	0.57	0.74	0.50	0.49
c.	1	0.25	1	0.5	8.5	9.5	0.25	1.04	0.20	0.83	0.96	1.09	0.88	0.80
	1	0.50	1	0.5	8.5	11.0	0.38	1.58	0.31	1.31	0.71	1.31	0.64	1.04
	1	1.00	1	0.5	8.5	13.4	0.52	2.22	0.44	1.69	0.85	1.49	0.78	1.15

[a] In the presence of CoA alone, the inhibition of fatty acid incorporation did not differ considerably with sn-G3P concentration. Therefore an average is represented.

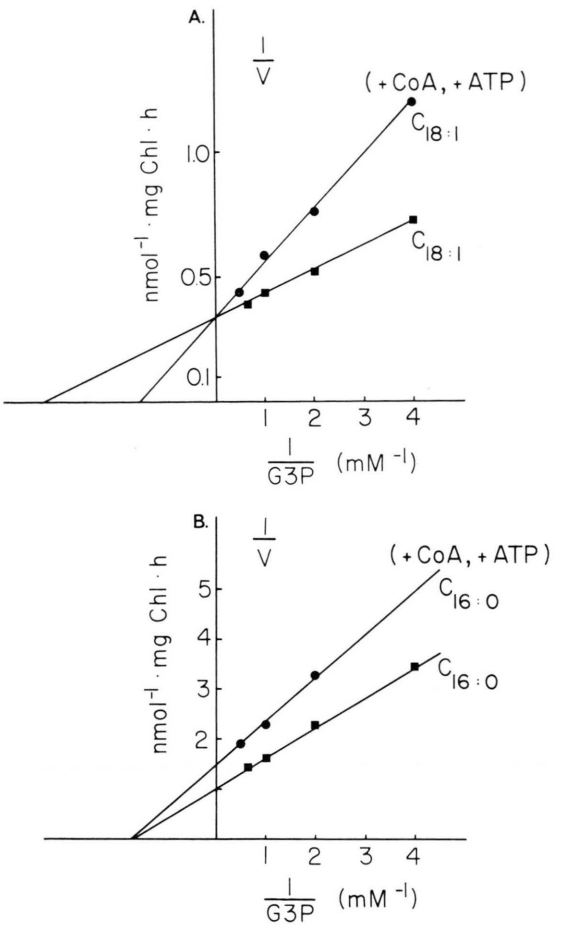

Fig. 2. Dependence of the sn-G3P concentration on the oleic-($C_{18:1}$; A) and palmitic acid ($C_{16:0}$; B) incorporation into lysophosphatidic acid by substrate free chloroplast extracts in the absence and presence of acyl-CoA synthesizing conditions (presence of CoA and ATP) in the reaction mixture. Chloroplast extracts were prepared by induction of fatty acid synthesis from [1-^{14}C]acetate in illuminated chloroplasts and their subsequent disruption (by Triton X-100) and elution over a short Sephadex G25 column. For details see Table III and Methods. K_m values derived from this figure are given in Table IV.

by elution over a short Sephadex G25 column [21]. Under these experimental conditions the sn-G3P dependent incorporation of palmitic acid into lysophosphatidic acid (K_m (sn-G3P) = 0.6 mM) showed only twice the K_m of oleic acid (K_m (sn-G3P) = 0.3 mM) (Table IV, Fig. 2). With regard to above cited physiological and unphysiological stroma concentrations of sn-G3P, these K_m values indeed support the recently found preference of the primary acylation reaction of sn-G3P for oleic acid [15] but reveal simultaneously the possibility of a palmitic acid incorporation into lysophosphatidic acids [16, 17]. The presumably disperse occurrence of envelope fragments (site of phosphatidic acid synthesis) in the chloroplast extracts we used apparently allowed for a small phosphatidic acid formation only (Table III), which is distinguished however by the typical selective palmitic acid transfer on to lysophosphatidic acids [15]. Another interesting phenomenon, observed in these measurements, appears to be the inhibition of fatty acid incorporation into lysophosphatidic acids (and consequently into phosphatidic acids) in the presence of CoA alone (Table III b) and together with ATP (acyl-CoA synthesizing conditions, Table III c) in the incubation medium. The inhibition of the primary acylation of sn-G3P under acyl-CoA synthesizing conditions in the reaction mixture appears competitively with respect to oleic acid (Fig. 2 A) and not competitively with respect to plamitic acid incorporation (Fig. 2 B). Latter observation is in contrast to above mentioned stimulation of fatty acid synthesis in intact chloroplasts under same conditions [7, 26] and may exert therefore a regulatory function between glycerolipid biosynthesis in- and outside the chloroplast [12, 17, 18].

Table IV. Comparison of the sn-G3P dependence for the transfer of palmitic- and oleic acid on to sn-G3P by acyl transferase 1 in spinach chloroplast extracts (A) with that, recently found by use of the purified enzyme and purified acyl-ACP fractions as acyl donors (B; 15). For details see Table III and Methods.

Transferred fatty acid species	K_m sn-G3P [mM]	
	A.	B.
oleic acid	0.29	0.03
palmitic acid	0.59	3.15

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft. The authors are grateful to Dr. M. Stitt for corrections.

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