Acyl–acyl carrier protein pool sizes during steady-state fatty acid synthesis by isolated spinach chloroplasts

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1. INTRODUCTION

Although long-chain acyl-ACPs are substrates for acyl-chain elongation [1], desaturation [2], hydrolysis [3] and acyl transfer [4], there have been no reported measurements of their endogenous concentrations during steady-state fatty acid synthesis by isolated chloroplasts. Since each of the major long-chain acyl-ACPs will participate in 2 or 3 alternative reactions, it has become important to establish the physiological concentrations of the different acyl-ACPs so that quantitative correlations may be made between results obtained with purified enzymes and those obtained with intact organelles. Total [ACP] in spinach chloroplasts has been reported as 8 μM [5] and the number of possible ACP esters is 32 so that an individual ACP ester could be as low as 0.25 μM (11–12 pmol · mg chl^{-1}). Chloroplasts isolated from leaves of hydroponically-grown spinach plants synthesized 3–4 nmol long-chain fatty acids · min^{-1} · mg chl^{-1} [6–10] at linear rates [7,8,10] and apparently exclusively from exogenous acetate [6]. The pool size of ACP therefore, is equivalent to 5 s of fatty acid synthesis and the specific radioactivity of acyl carbons within the acyl-ACP fraction should quickly equilibrate with the specific radioactivity of the supplied [^{14}C]acetate. Concentrations of individual ACP esters may then be calculated from radioactivities recovered in the separated acyl chains.

Here, we have utilized highly-active chloroplast preparations and high specific radioactivity [1-^{14}C]acetate to measure acyl-ACP pool sizes during steady-state fatty acid synthesis.

2. MATERIALS AND METHODS

Sodium [1-^{14}C]acetate came from Amersham International (Buchs), Triton X-100 from BDH Chemicals (Poole) and G3P and CoA from Sigma Chemicals (St Louis MO). Sodium methoxide was 0.5 M in methanol/dimethoxypropane (95:5, v/v). *Spinacia oleracea* plants (hybrid 102) were grown and chloroplasts were isolated as in [7]. The basal incubation medium contained 0.33 M sorbitol, 10 mM $KHCO_3$, 0.2 mM [1-^{14}C]acetate (5 μCi) and 25 mM [4-(2-hydroxyethyl)-1-piperazine ethane–sulphonic acid/NaOH to give a final pH of 7.9. Chloroplasts were added to illuminated media to start reactions which were stopped at the times indicated by adding an equal volume of 2.5% glacial acetic acid in propan-2-ol [11]. The acyl-ACP fraction was recovered according to [11] and the washed precipitates were resuspended in 2 ml 0.5 M $NaOCH_3$ for 20 min at room temperature. Labelled fatty acid methyl esters, along with 40 μg each of unlabelled methyl palmitate, stearate,

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Abbreviations: ACP, acyl carrier protein; G3P, *sn*-glycerol 3-phosphate

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oleate, linoleate and linolenate, added as carriers, were then extracted into petroleum ether immediately following the addition of 2 ml H_2O to the methylation mixture. Portions (5–10%) of the combined petroleum ether extracts were taken for scintillation counting to give a measure of 'total acyl-ACP' radioactivity whereas the balance of the extracts were dried under N_2 and long-chain fatty acid methyl esters were separated by argentation TLC. Following a light spray with dichlorofluorescein, radioactivity in the separated bands was measured by scintillation counting. In some cases, the saturated and monoenoic bands were eluted from the adsorbent into diethylether and were re-chromatographed by reverse-phase TLC. Separated bands were detected by autoradiography and radioactivity measured by scintillation counting. The petroleum–ether extracts and chloroform/methanol supernatants from the procedure in [11] were combined, concentrated under reduced pressure and resolved into unesterified fatty acids, 1,2-diacylglycerols and polar lipids as in [7].

3. RESULTS

When the acyl-ACP precipitate was treated with KOH as in [11], the recovered long-chain fatty acids accumulated [1-^{14}C]acetate linearly for 15 min. However, if the acyl esters in the precipitate were specifically converted to methyl esters using $NaOCH_3$ and any unesterified fatty acids subsequently removed by partitioning into aqueous NaOH, then the fatty acid methyl esters thus recovered ceased accumulating radioactivity after 1–2 min (fig.1). Therefore, although the acyl-ACP fraction as prepared was apparently contaminated with labelled unesterified fatty acids, this contamination could be removed from the analysis by employing transmethylation as described.

It seems highly probable that acyl-ACP alone contributed labelled fatty acid methyl esters to the analysis for the following reasons:

(1) The rapid rise to constant labelling, particularly upon re-illumination (fig.1), of the acyl-ACPs whilst total fatty acids continued to accumulate radioactivity is characteristic of a metabolic intermediate with a high turnover rate;

(2) The complete loss of long-chain acyl-ACP in the dark indicated that interference from accumulating unesterified fatty acids and non-thiol acyl esters was minimal;

(3) The distribution of radioactivity among the different long-chain fatty acids of the acyl-ACP fraction (table 1) was quite different from that within the unesterified fatty acids or glycerides accumulated by isolated spinach chloroplasts [9].

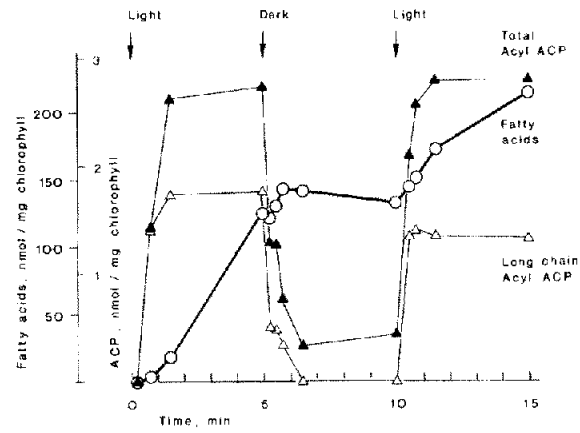

Fig.1. Light synthesis and dark catabolism of long chain and total acyl-ACP in spinach chloroplasts. Reactions (0.25 ml, 45 μg chl) were started by adding chloroplasts to illuminated media, were transferred to total darkness at 5 min and reilluminated at 10 min. Long-chain fatty acyl methyl esters derived from the acyl-ACP fraction were purified by TLC on Silica gel G and total fatty acids were also purified by TLC [7], following saponification of total lipids recovered as in section 2. Results are expressed as nmol [1-^{14}C]acetate incorporated into: total long-chain fatty acids (○—○); long-chain acyl-ACPs (△—△); total acyl-ACPs (▲ ▲).

The complete decay of long-chain acyl-ACP radioactivity within 90 s of transferring reactions to the dark (fig.1) indicated a very small pool size and reflected the almost instantaneous cessation of long-chain fatty acid accumulation by isolated spinach chloroplasts when darkened [10].

When total fatty acid methyl esters from the acyl-ACP fraction were chromatographed on unmodified silica gel G, ≥80% of the radioactivity on the chromatogram was associated with a single band. Remaining labelled material had relatively low chromatographic mobility and may have been

Table 1

Influence of Triton X-100 and exogenous CoA on the incorporation of [1-^{14}C]acetate into long-chain acyl-ACPs and acyl lipids of isolated spinach chloroplasts

Labelled products	Additions to incubation media			
	None	CoA	Triton	Triton + CoA
Saturated acyl-ACPs	0.54[a]	0.79	0.91	1.10
Oleoyl-ACP	0.21	0.35	0.49	0.46
Unesterified fatty acids	124.7	208.9	148.8	179.9
1,2-diacylglycerols	22.7	42.7	144.5	169.9
Polar lipids	23.5	31.4	53.3	54.2
Overall rate	0.88[b]	1.41	1.73	2.01

[a]nmol acetate . mg chl^{-1}; [b]μmol acetate . h^{-1} . mg chl^{-1}

Reactions (0.5 ml, 85 μg chl) were incubated with illumination for 12 min. CoA was 0.5 mM and Triton X-100 was 0.13 mM. No radioactivity was detected in linoleate associated with ACP

Table 2

Influence of *sn*-glycerol 3-phosphate and Triton X-100 on [1-^{14}C]acetate incorporation into long-chain acyl-ACPs and acyl lipids of spinach chloroplasts

Labelled products	Additions to incubation media			
	None	G3P	Triton	Triton + G3P
$C_{14:0}$-ACP	0.10[a]	0.12	0.11	0.10
$C_{16:0}$-ACP	0.38	0.14	0.60	0.43
$C_{18:0}$-ACP	0.10	0.05	0.42	0.18
$C_{18:1}$-ACP	0.35	0.14	0.88	0.38
Unesterified fatty acids	65.2	21.8	60.3	20.6
1,2-diacylglycerols	22.7	38.6	47.3	52.1
Polar lipids	17.2	29.4	30.0	34.9
Overall rate	1.27[b]	1.08	1.68	1.31

[a]nmol acetate . mg chl^{-1}; [b]μmol acetate . h^{-1} . mg chl^{-1}

Reactions (0.5 ml, 120 μg chl) were incubated with illumination for 5 min. *sn*-Glycerol 3-phosphate (G3P) was 0.5 mM and Triton X-100 was 0.13 mM

3-hydroxy- or 3-keto-acyl chains. Similarly, reverse-phase chromatography of total acyl-ACP fatty acid methyl esters revealed discrete bands of radioactivity which corresponded to authentic decanoate, laurate, myristate, palmitate plus oleate and stearate. However, the recovery of components with ≤12 carbons was variable, presumably because of their higher volatility and this would account for the difference in radioactivity between 'total acyl-ACPs' and long-chain acyl-ACPs (fig.1). Oleate accounted for >95% of the radioactivity recovered in the monoenoic fraction from argentation TLC.

Exogenous CoA and Triton X-100 increased rates of fatty acid synthesis in spinach chloroplasts and also elevated the steady state concentrations of long-chain acyl-ACPs (table 1). On the other hand, increasing concentrations of added G3P reduced steady-state concentrations of acyl-ACPs, particularly oleoyl-ACP, by up to 66% (table 2) but decreased the rate of fatty acid accumulation by only 15–33%. Since added G3P stimulated the incorporation of long-chain fatty acids into glycerides (table 2), it may be inferred that the lower concentrations of acyl-ACPs resulted from a direct transfer of acyl groups from ACP to G3P. However, Triton X-100 also stimulated glyceride synthesis (tables 1,2) and in these cases acyl-ACP concentrations were elevated compared to controls. Steady-state concentrations of myristoyl-ACP were relatively unaffected by the treatments which resulted in marked changes in concentrations of longer-chain homologues (table 2).

After 2 min incubation there was no further increase in the labelling of the fatty acid methyl esters derived from the acyl-ACP fraction whereas [1-^{14}C]acetate incorporation into total fatty acids continued linearly (fig.1). Consequently we assume that the specific radioactivity of the acyl carbons within acyl-ACPs was the same as that in the supplied [1-^{14}C]acetate. Maximum steady-state concentrations of the different acyl-ACPs were then calculated from the data in table 2 to be: $C_{14:0}$, 0.33 μM; $C_{16:0}$, 1.60 μM; $C_{18:0}$, 0.99 μM; and $C_{18:1}$, 2.08 μM. This calculation assumes a chloroplast volume of 47 μl . mg chl^{-1} [12] and the sum of the acyl-ACPs at 5 μM compares favourably with 8 μM for total ACP in spinach chloroplasts [5] based on a chloroplast volume of 36 μl . mg chl^{-1}.

4. DISCUSSION

These results suggest that steady-state concentrations of various long-chain acyl-ACPs can be measured relatively easily in isolated spinach chloroplasts. However, more work will be needed to obtain reliable measurements for short-chain thioesters. The steady-state concentration of a particular acyl-ACP represents the balance between its rate of synthesis and its rate of utilization. Therefore, the increased concentrations resulting from the inclusion of CoA and Triton X-100 in incubation media indicate that these substances stimulated the fatty acid synthetase activity rather than those reactions utilizing oleoyl-ACP as suggested [6]. On the other hand, the decreased concentrations of endogenous acyl-ACPs in the presence of added G3P indicates an increase in the rate of utilization, possibly through acyl transfer directly from acyl-ACP to G3P [4]. Since there must have been a constant amount of total ACP in the chloroplasts in any one experiment, treatments which caused an increase in esterified ACP would logically result in a decrease in unesterified ACP. Therefore, unesterified ACP concentrations would have been considerably higher in control compared to CoA- and Triton-stimulated reactions judging from a comparison of our maximum concentrations of acyl-ACP and a previous estimate of total ACP in spinach chloroplasts [5]. The increased rates of chloroplast fatty acid synthesis in response to exogenous CoA and Triton X-100 cannot then, be attributed to increased concentrations of unesterified ACP [6]. As a corollary to this, added G3P probably caused an increase in unesterified ACP concentrations but we have never observed an increase in rates of fatty acid synthesis in highly-active chloroplasts in the presence of exogenous G3P [7,9].

The recent discovery that spinach leaf cells contain 1 mM acetate [13] does not significantly influence these results. We calculate that in a 'worst possible case' the specific radioactivity of the added [1-^{14}C]acetate would have been diluted $< 5\%$ by unlabelled acetate added with the isolated chloroplasts.

We repeatedly observed that lower overall rates of [1-^{14}C]acetate incorporation into chloroplast lipids were accompanied by lower concentrations of long-chain acyl-ACPs and since these concentrations were quite low (~ 0.5–2.5 nmol acetate · mg chl^{-1}) anyway, it was imperative to use highly active chloroplast preparations for these studies.

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