Inhibition of the glucose-6-phosphate transporter in oilseed rape (*Brassica napus* L.) plastids by acyl-CoA thioesters reduces fatty acid synthesis

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Addition of oleoyl-CoA (1 μ M), or other acyl-CoA thioesters with a chain length of C₁₆ or greater, to oilseed rape plastids (*Brassica napus* L.) inhibited the rate of D-glucose 6-phosphate (Glc6P) uptake by 70 % after 2 min. The IC₅₀ value for oleoyl-CoA inhibition of the transporter was approx. 0.2–0.3 μ M. Inhibition was alleviated by the addition of acyl-CoA binding protein (ACBP) or BSA at slightly higher concentrations. Oleic acid (5–25 μ M), Tween 40 (10 μ M), Triton-X 100 (10 μ M) and palmitoylcarnitine (5 μ M) had no effect on Glc6P uptake. The uptake of [1-¹⁴C]Glc6P occurred in exchange for P_i, 3-phosphoglycerate or Glc6P at a typical rate of 30 nmol Glc6P/min per unit of glyceraldehyde-3-phosphate dehydrogenase (NADP⁺). The K_{m(app)} of the Glc6P transporter for Glc6P was 100 μ M. Neither CoA (0.3 mM) nor ATP (3 mM) inhibited Glc6P uptake, but the transporter was inhibited by 72 % when ATP and CoA

were added together. This inhibition was attributable to the synthesis of acyl-CoA thioesters, predominantly oleoyl-CoA and palmitoyl-CoA, by long-chain fatty acid-CoA ligase (EC 6.2.1.3) from endogenous fatty acids in the plastid preparations. Acyl-CoA thioesters did not inhibit the uptake of [2-¹⁴C]pyruvate or D-[1-¹⁴C]glucose into plastids. *In vivo* quantities of oleoyl-CoA and other long-chain acyl-CoA thioesters were lower than those for ACBP in early cotyledonary embryos, 0.7 $\pm$ 0.2 pmol/embryo and 2.2 $\pm$ 0.2 pmol/embryo respectively, but in late cotyledonary embryos quantities of long-chain acyl-CoA thioesters were greater than ACBP, 3 $\pm$ 0.4 pmol/embryo and 1.9 $\pm$ 0.2 pmol/embryo respectively.

Key words: acyl-CoA binding protein, glucose 6-phosphate, oleoyl-CoA, transporters.

INTRODUCTION

Understanding the biochemical basis of the regulation of fatty acid synthesis in plants is important when it comes to designing experiments to manipulate the metabolic pathways underpinning the process. Oilseed rape oil is a major commodity in the global food industry [1] and much effort is being directed towards improving the value of this crop. One objective towards this aim has been to evaluate which carbon precursors are used for the synthesis of fatty acids [2], the acyl component of triacylglycerols. A further aim is to address whether plastidial fatty acid synthesis is metabolically regulated, and if so, how.

In oilseed rape, one of the primary sites for fatty acid synthesis is in the embryos of the seeds, encased within the siliques [3]. Fatty acids are synthesized by the plastids which are, amongst other functions, also responsible for the synthesis of starch, which accumulates transiently during embryo development [4].

In vitro, oilseed rape plastids can utilize a variety of carbon precursors to synthesize long-chain fatty acids [5], the latter being predominantly oleic acid (C_{18:1}) with a little palmitic (C_{16:0}) and stearic acid (C_{18:0}) [6]. These carbon precursors include D-glucose 6-phosphate (Glc6P), malate, pyruvate, dihydroxyacetone phosphate and acetate [5,7]. The majority of the fatty acids synthesized in the plastids are exported [8] and may be modified by enzymes such as the Δ 12 and Δ 15 desaturases on the endoplasmic reticulum [9] before incorporation into storage oil via the enzymes of the Kennedy pathway [10,11]. In the animal kingdom long-chain acyl-CoA thioesters (lcACoAs) regulate fatty acid synthesis by feedback inhibition, acting on acetyl-CoA

carboxylase (EC 6.4.1.2) with a K_i of approx. 5–10 nM [12]. In contrast, little is known about the regulation of fatty acid synthesis in plants, but it is likely to be different because most plants contain a dissociable acetyl-CoA carboxylase located inside the plastid and lcACoAs are generally thought to occur in the cytosol and not to accumulate in plastids [13].

Previous studies using isolated plastids have shown that Glc6P is a major substrate for fatty acid synthesis in the early to mid-stages of embryo development, approximately 22–33 days after anthesis [7]. The overall rates of fatty acid synthesis in plastids from carbon derived from Glc6P were 3–4-fold lower in the presence of CoA (0.1–0.3 mM) and ATP (3 mM) [6,8]. The inhibition caused by these concentrations of CoA was alleviated by acyl-CoA binding protein (ACBP). ACBPs bind lcACoAs at high affinity, in a 1:1 ratio, but ACBPs do not bind short-chain acyl-CoA thioesters, non-esterified fatty acids or CoA [14]. ACBPs have been shown to be present in all plant tissues examined including developing oilseed rape seeds [15,16]. The inhibition by CoA and ATP [6,8] provides indirect evidence that the presence of acyl-CoA thioesters can in some way affect the rate of fatty acid synthesis in the plastids. Overall, these previous studies provided strong support for the hypothesis that it was the accumulation of lcACoAs from *de novo* fatty acid synthesis, which was responsible for feedback inhibition of the transporter proteins located on the plastid envelope. In this paper we describe experiments to examine in detail the inhibition of the Glc6P transporter by lcACoAs, and address the question of what the endogenous concentrations of ACBP and lcACoAs are *in vivo* during the maturation of oilseed rape embryos.

Abbreviations used: ACBP, acyl-CoA binding protein; Glc6P, D-glucose 6-phosphate; lcACoA, long-chain acyl-CoA thioester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (NADP⁺); PIM, plastid isolation medium.

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MATERIALS AND METHODS

Preparation of plastids

Oilseed rape plants (*Brassica napus* L. cv Topas) were grown according to the regime described by Kang and Rawsthorne [3]. The embryos (200–300) were harvested from seeds, approximately 22–33 days after anthesis (mid-cotyledonary stage embryos, 1.5–2.5 mg), incubated in 2–3 ml of plastid isolation medium (PIM; 0.5 M sorbitol, 20 mM Hepes/NaOH, pH 7.4, 10 mM KCl, 1 mM MgCl_2 , 1 mM sodium EDTA) [5], without BSA and finely chopped. The solution was passed through two layers of Miracloth (Calbiochem–Novabiochem Ltd, Beeston, Nottinghamshire, U.K.) and the chopping/filtering process repeated five times. The plastids were recovered from the resulting filtrate by centrifugation at 750 *g* for 2 min [3]. Lipid and starch were wiped from the sides of the tube after discarding the supernatant. Repeated additions of PIM were made to slowly resuspend the plastid pellet in a total volume of 10–15 ml and the centrifugation step repeated twice before the plastids were resuspended in 5–6 ml of PIM and maintained on ice prior to carrying out the incubations. When left on ice for approx. 45 min the plastid preparations developed a loose pellet of starch that could be removed with careful pipetting, facilitating an improved plastid preparation. The relative intactness of the plastid incubations was tested by performing latency assays of glyceraldehyde-3-phosphate dehydrogenase (NADP^+) (GAPDH; EC 1.2.1.13), i.e. the enzyme was assayed under conditions in which the osmolarity was maintained at 0.5 M and the activity compared with that obtained in the absence of an osmoticum [5]. Using this procedure values of 80–85% intactness were regularly obtained. It was notable that the current preparative technique resulted in plastid preparations that had a greater intactness and a lower starch content than those prepared according to Kang and Rawsthorne [5] (approx. 60–70% intactness). The method published previously used a Polytron to disrupt the embryos, which tends to result in a high amount of starch in the final pellet and would appear to make uptake assays difficult.

Silicone oil centrifugation and measurement of Glc6P uptake

Sucrose [30 μl , 0.7 M, containing 5% (w/v) trichloroacetic acid as a quenching agent] was transferred into 400 μl microcentrifuge tubes (Life Sciences International Ltd, Basingstoke, Hampshire, U.K.) and centrifuged at 12 500 *g* for 20 s. Silicone oil (AR 200/AP 100; 2:1, v/v; 55 μl ; Wacker-Chemie GmbH., Burghausen, Germany) was layered on to the sucrose and the centrifugation step was repeated. Tubes were stored at 4 °C prior to use; this avoided inversion of layers, which is occasionally encountered. The principal of silicone oil centrifugation, in separating organelles from bathing medium, has been discussed previously by Heldt [17].

Plastids (155 μl in PIM) were pre-incubated with 10 μl of PIM for 90 s at 25 °C. An aliquot of 155 μl was then drawn up into the same pipette tip and mixed 30 s later with 10 μl of [^{14}C]Glc6P (specific radioactivity of 1.48–2.22 GBq/mmol; NEN Life Science Products, Hounslow, U.K.), at a range of concentrations containing on average 5.9 kBq. Upon mixing with the isotope, plastids were immediately removed (155 μl from 165 μl) and maintained in the pipette tip for various time intervals before centrifugation through silicone oil (as described). The plastids which passed through the oil were transferred directly into 10 ml of scintillation fluid (Optiphase HiSafe 3, Fisher Scientific UK, Loughborough, Leicestershire, U.K.). The uptake of Glc6P by plastids was determined as nmol Glc6P/unit of GAPDH [3]. In some experiments, the procedure outlined above was repeated,

except that plastids were incubated at a range of concentrations up to 500 μM , with either [^{14}C]pyruvate containing 4.8 kBq (specific radioactivity 0.37–1.1 GBq/mmol; Amersham Life Science Ltd., Little Chalfont, Bucks., U.K.) or D-[^{14}C]glucose containing 4.9 kBq (specific radioactivity 1.66–2.22 GBq/mmol).

In other experiments acyl-CoA thioesters, oleic acid (as the ammonium salt), palmitoylcarnitine, Tween 40 and Triton X-100 were included in the pre-incubation mixtures. Some incubations also included ACBP, prepared as described in [16] or BSA (fraction V, essentially fatty acid free, Sigma–Aldrich, Poole, Dorset, U.K.). In one series of experiments after 30 s incubation with Glc6P, the plastids, which had been pre-incubated for 2 min with 1 μM oleoyl-CoA, were mixed with 10 μl of PIM ($\pm$ ACBP or BSA) and separated by silicone oil centrifugation immediately (+ 3–4 s), or after 30 or 90 s.

The sorbitol impermeable space of the plastids, which includes the space between the outer and inner plastid envelope membranes, into which sorbitol can pass (sorbitol is impermeable to the inner plastid membrane), was measured as described previously [3].

In order to measure fatty acid synthesis, plastids were incubated with [^{14}C]Glc6P (as described); however, the incubations were for 1 h before extraction of the fatty acids. Fatty acids were saponified in 1 ml of 15% (w/v) KOH in methanol at 80 °C for 40 min, acidified with 6 M HCl and extracted twice into an equal volume of chloroform. The organic phase was concentrated and the fatty acids were purified by TLC and quantified by scintillation counting as described previously [6].

Exchange of labelled phosphate

Plastids prepared as described were incubated with sodium phosphate pre-loading stock in PIM to a final concentration of 5 mM (containing 740 kBq $\text{H}_3^{33}\text{PO}_4$, specific radioactivity 0.87 TBq/mmol) for 10 min; this allowed equilibration of label between the plastids and bathing medium. The plastids (170 μl) were mixed with 30 μl of one of the following unlabelled exchange metabolites: Glc6P, 3-phosphoglycerate and P_i , all at 5 mM; PIM was used as the control. The unlabelled exchange metabolites were made up in sodium phosphate pre-loading stock to avoid changes in P_i concentrations upon mixing. After 20, 60 or 120 s the solutions were centrifuged through silicone oil to stop the reactions. The amount of [^{33}P]P_i remaining in the plastids was determined by scintillation counting.

Measurement of lcACoAs

In some experiments, after incubation of the plastids (as described) lcACoAs were extracted both before the addition of [^{14}C]Glc6P or after a 1 min incubation with the substrate (500 μM); in both cases this was without separation of plastids and supernatant. The extraction procedure and method used for HPLC analysis were both based on that described by Watmough et al. [18].

lcACoAs were also quantified from different stages of embryo development. The embryos (designated early cotyledonary stage, 1–1.5 mg; mid-stage, 1.5–2.5 mg and late stage, 2.5–3.5 mg) were harvested during the day and homogenized in 200 μl of chloroform/methanol (1:2, v/v). The rest of the procedure, following the initial addition of chloroform/methanol, was identical to that used to quantify lcACoAs from plastids, as follows.

Chloroform/methanol (1:2, v/v; 500 μl) was added to the plastid incubations. Heptadecanoyl-CoA (10 pmol in 1 μl of water) was added as the internal standard to assess percentage recovery of the lcACoAs. Ammonium sulphate (50 μl , saturated

solution) was added and the protein allowed to precipitate for 30 min before centrifugation for 10 min at 12 500 g and 4 °C. The precipitate was re-extracted with 200 µl of chloroform/methanol (1:2, v/v). The combined organic phases containing the lcACoAs were transferred to 20 ml glass tubes and concentrated under N₂ before being resuspended in 400 µl of water. This aqueous fraction was extracted three times with 800 µl of diethyl ether and concentrated to, or very near to, dryness under N₂ before being resuspended in 200 µl of water. DEAE-Sephacel (0.5 ml; Amersham Pharmacia Biotech, St. Albans, Herts., U.K.), originally in the chloride ion form, was repeatedly washed with 2 M acetic acid before use. After equilibration with 4 ml of water, the lcACoAs were run through the columns, which were then washed with 4 ml of 80 % (v/v) aqueous methanol. lcACoAs were eluted with 600 µl of 80 % (v/v) methanol containing 0.6 M ammonium acetate and 10 mM acetic acid; this solution was concentrated to near dryness under N₂ prior to resuspension in 600 µl of water. The lcACoAs were separated by HPLC on an Ultra Techsphere ODS 250 mm × 4.5 mm column (5 µm particle size; HPLC Technology Company Ltd., Macclesfield, Cheshire, U.K.). HPLC conditions comprised a flow rate of 1.5 ml/min with an initial solvent composition of 95 % solvent A (50 mM KH₂PO₄, pH 5.6) and 5 % solvent B (acetonitrile). After injection, these conditions were changed to 1 % solvent A and 99 % solvent B after 22 min. After a further 8 min, conditions were returned to the initial solvent composition. UV absorbance was measured at 260 nm. Recoveries of the lcACoAs, calculated from that of the internal standard, were approx. 85 %. The peak area of the internal standard, as a percentage of the total peak area of lcACoAs, was at lowest 3–5 % in those extracts containing the highest concentrations of lcACoAs.

Measurement of ACBP

Quantitative Western blotting, as described by Brown et al. [16], was used to measure the ACBP content of ten embryos from early through to late cotyledonary stage. Briefly, 50 µg of each embryo protein extract (three replicates) was resolved by SDS/PAGE on a 16 % (w/v) polyacrylamide Schagger gel [19] and blotted on to nitrocellulose (100 mA for 1 h). The blots were blocked with 3 % (w/v) BSA in Tris-buffered saline (25 mM Tris/HCl, pH 7.4, 0.14 M NaCl, 2.7 mM KCl, 42 µM Phenol Red) and incubated with a 1:4000 dilution of ACBP antibody. Alkaline phosphatase-conjugated secondary antibody was used at a 1:3000 dilution (Sigma). Following staining using Western Blue (Promega UK, Southampton, U.K.) the staining intensity was determined using a scanning densitometer (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts., U.K.). A standard curve was made by including a range of ACBP mass standards from 12 to 100 ng and the staining response was linear over this range.

RESULTS

Kinetics of Glc6P uptake

In previous studies of the metabolism of Glc6P by oilseed rape embryo plastids it had been assumed that the Glc6P transporter was of the type found in other plastids, i.e. a Glc6P/P_i exchanger. In order to test this, Glc6P was added to plastids which had been pre-loaded with [³³P]P_i and then spun through silicone oil. It was found there was a significant decrease in the amount of label remaining in the plastids (Table 1). This demonstrated that the uptake of Glc6P by plastids occurred in exchange for [³³P]P_i. [³³P]P_i was also exchanged for P_i and the triose-phosphate, 3-phosphoglycerate. When PIM was substituted for either P_i, 3-phosphoglycerate or Glc6P, no significant increase in the export

Table 1 Metabolite dependence on [³³P]P_i export from oilseed rape plastids

After equilibration of plastids with [³³P]P_i an exchange metabolite (at 5 mM) was added to the incubations as described in the Materials and methods section. Plastids were spun through silicone oil to stop the reactions and the amount of [³³P]P_i remaining in the plastids was determined by scintillation counting. The values are means ± S.E.M. for experiments using two separate plastid preparations and three replicate incubations.

Exchange metabolite	Time (s)	[³³ P]P _i export (nmol/unit of GAPDH)
None (control)	20	82 ± 6
	60	76 ± 2
	120	74 ± 7
Glc6P	20	46 ± 9
	60	42 ± 10
	120	31 ± 2
P _i	20	27 ± 2
	60	27 ± 4
	120	20 ± 1
3-Phosphoglycerate	20	36 ± 5
	60	36 ± 5
	120	21 ± 1

Figure 1 Uptake of [¹⁻¹⁴C]Glc6P *in vitro* by oilseed rape plastids and the effect of oleoyl-CoA

After a 2 min pre-incubation ± oleoyl-CoA, the uptake of [¹⁻¹⁴C]Glc6P was measured using the silicone oil centrifugation method over a period of 2 min, as described in the Materials and methods section. Control (▽; PIM, no acyl-CoA), 0.3 µM (▼), 0.6 µM (○) and 1.2 µM (●) oleoyl-CoA. Glc6P uptake (500 µM) was measured as nmol/unit of GAPDH (nmol.U⁻¹.GAPdh). The values are means ± S.E.M. (n = 3).

of [³³P]P_i was observed. These direct measurements of [³³P]P_i exchange, and previous data for the inhibition of Glc6P transport by other metabolites [20], show that the transporter involved is similar to that described for other plant plastids [21].

The uptake of Glc6P *in vitro* reached a plateau after 2 min of incubation. The rate of uptake, determined at 60 s, was equivalent to approx. 1.8 µmol Glc6P/h per unit of GAPDH. This overall uptake would have been sufficient to account for the total utilization of carbon from Glc6P for fatty acid synthesis by these plastids [5,6]. Typically, the rate of fatty acid synthesis from mid-stage embryo plastids ranges from 0.3 to 0.7 µmol Glc6P/h per unit of GAPDH.

Figure 2 Determination of the IC_{50} for oleoyl-CoA inhibition of the Glc6P transporter in plastids

(A and B) The plastids were incubated with differing concentrations of [1-¹⁴C]Glc6P (■, 1000 μM; ▽, 500 μM; ○, 100 μM) and oleoyl-CoA (18:1-CoA; present in the 2 min pre-incubation) for 30 s before being centrifuged through silicone oil to measure the uptake of [1-¹⁴C]Glc6P into the stroma. Inhibition saturated with 0.6 μM oleoyl-CoA and half-maximal inhibition was with approx. 0.2–0.3 μM oleoyl-CoA. Glc6P uptake was measured as nmol/unit of GAPDH (nmol.U⁻¹.GAPDH). The values are means ± S.E.M. ($n = 3$).

The $K_{m(app)}$ for Glc6P uptake by the Glc6P transporter was calculated from a Hanes plot to be 100 μM; a value less than those for the maize endosperm Glc6P transporter (800 μM) and the recombinant pea root Glc6P transporter (700 μM) [21].

Effect of oleoyl-CoA on Glc6P, pyruvate and glucose uptake by plastids

The uptake of Glc6P, over the concentration range measured (100–1000 μM), was progressively inhibited by increasing the concentration of oleoyl-CoA from 0.3 to 1.2 μM (Figure 1). The maximum inhibition of the Glc6P transporter occurred at 1.2 μM oleoyl-CoA. This resulted in a 70% reduction in Glc6P uptake after 2 min in comparison with incubations without oleoyl-CoA, and the IC_{50} value was calculated to be approx. 0.2–0.3 μM (Figure 2A). A Dixon plot of $1/V$ versus oleoyl-CoA concentration (e.g. using data illustrated in Figure 2B) with a range of Glc6P concentrations was complex and suggested that the inhibition was non-linear [22].

In order to determine whether this effect was specific or due to the latter's detergent properties (acyl-CoAs can partition into lipid bilayers through insertion of the acyl chain), we investigated

Table 2 Effect of the chain length of acyl-CoA thioesters on Glc6P uptake by oilseed rape plastids

Plastids were incubated with [1-¹⁴C]Glc6P (500 μM) and acyl-CoA thioesters (all 1 μM). The reactions were terminated after 30 s by silicone oil centrifugation. The values are means ± S.E.M. ($n = 3$).

Acyl-CoA thioesters	C chain length	[1- ¹⁴ C]Glc6P uptake (nmol/unit of GAPDH)
Control		24.4 ± 2.5
Acetyl-CoA	2	21.6 ± 2.4
Malonyl-CoA	3	21.9 ± 1.7
Propionyl-CoA	3	25.3 ± 3.4
Octanoyl-CoA	8	24.9 ± 3.2
Decanoyl-CoA	10	21.1 ± 2.0
Lauryl-CoA	12	16.1 ± 1.1
Myristoyl-CoA	14	9.3 ± 1.8
Palmitoyl-CoA	16	6.5 ± 2.4
Oleoyl-CoA	18	7.9 ± 1.7
Stearoyl-CoA	18	6.9 ± 1.7

the effects of a range of compounds with detergent properties. The ammonium salt of oleic acid had no effect on Glc6P uptake at concentrations of 5–25 μM. At a concentration of 10 μM, the detergents Tween 40 and Triton-X 100, as well as the acyl-CoA derivative palmitoylcarnitine (5 μM), had no effect on Glc6P uptake by these plastids (results not shown).

The inhibition of Glc6P uptake by plastids was dependent on the length of the acyl moiety of the thioester (Table 2). With chain lengths of less than C₁₂, in comparison with controls, no inhibition of Glc6P uptake was observed. However, lauryl-CoA (C_{12:0}) caused 34% inhibition, myristoyl-CoA (C_{14:0}) caused 62% inhibition and longer chain derivatives, e.g. palmitoyl-CoA, caused 73% inhibition of Glc6P uptake at 500 μM into the plastids. CoA and short-chain acyl-CoA thioesters, such as acetyl-CoA or malonyl-CoA, had no detectable effect on the Glc6P transporter.

Addition of oleoyl-CoA at 1, 6 and 10 μM did not inhibit the rate of uptake of either [2-¹⁴C]pyruvate or D-[1-¹⁴C]glucose into plastids over a 2 min period, e.g. after 30 s, with or without oleoyl-CoA in the pre-incubation, pyruvate uptake was 20 ± 4 nmol/unit of GAPDH ($n = 3$, where n is the number of experiments) and glucose was 45 ± 5 nmol/unit of GAPDH ($n = 3$). This concurs with a previous observation, namely that the addition of CoA to plastids utilizing carbon from pyruvate for fatty acid synthesis, in the presence of ATP (3 mM), conditions in which lAcCoAs can be synthesized from *de novo* synthesized fatty acids, does not repress fatty acid synthesis from this substrate [23]. Conversely, synthesis of fatty acids from Glc6P under the same conditions is strongly inhibited.

Effect of cofactors on Glc6P uptake

In order to test whether the Glc6P transporter was inhibited by *de novo* synthesized lAcCoAs, as had been found for fatty acid synthesis from Glc6P [6], plastids were pre-incubated for 2 min with CoA and ATP. Fatty acid-CoA ligase (EC 6.2.1.3), an enzyme present in oilseed rape plastids [24], catalyses the synthesis of lAcCoAs from non-esterified fatty acids; it also requires ATP and CoA. Since uptake measurements of Glc6P were carried out over a short time-course, exogenous oleic acid was added to some of the plastid incubations.

When added separately, a 2 min pre-incubation of plastids with either 3 mM ATP or 0.3 mM CoA had no effect on the subsequent uptake of Glc6P by plastids; the rates of uptake after 60 s were 36 ± 2 nmol Glc6P/min per unit of GAPDH (all $n =$

Figure 3 Effect of cofactors on the synthesis of lAcCoAs by oilseed rape plastids

The HPLC chromatograms were obtained from plastids incubated in: (a) the absence of ATP and CoA, and (b) the presence of ATP (3 mM) and CoA (0.3 mM); sample diluted 20-fold to remain on scale, hence no heptadecanoyl-CoA peak; both were without the addition of exogenous fatty acid. The chromatograms were used to calculate the concentrations of lAcCoAs reported in Table 3 ($n = 3$). lAcCoAs were extracted after the 2 min pre-incubation before the addition of Glc6P and analysed by UV absorbance at 260 nm, as described in the Materials and methods section. The large peak at the start of the chromatogram may include various nucleotides. Peak 1, heptadecanoyl-CoA (internal standard); peak 2, linolenoyl-CoA; peak 3, linoleoyl-CoA; peak 4, palmitoyl-CoA; peak 5, oleoyl-CoA; peak 6, stearoyl-CoA.

3) with ATP and 31 ± 4 nmol Glc6P/min per unit of GAPDH. with CoA. Yet, after pre-incubation with both ATP and CoA, unexpectedly, the rate of Glc6P uptake was reduced by 72%

after 60 s in comparison with measurements made in the absence of ATP and CoA (10 ± 1 nmol Glc6P/min per unit of GAPDH). The inference from this observation, since no oleic acid had been added, was that ATP and CoA had caused the synthesis of lAcCoAs from endogenous sources of fatty acids, catalysed by fatty acid-CoA ligase.

In order to test this we developed a method for the extraction and quantification of lAcCoAs from such incubations (see the Materials and methods section). In the absence of ATP and CoA, either before the addition of Glc6P (Figure 3a) or after 60 or 90 s incubation with this substrate (results not shown), no lAcCoAs (> 10 pmol) were synthesized by the plastids. However, in the presence of ATP and CoA, before the addition of Glc6P, lAcCoAs were synthesized by the plastids during the pre-incubation (Figure 3b). The concentrations of lAcCoAs synthesized (Table 3) were sufficient to cause approx. 70% inhibition of the Glc6P transporter after 2 min (Figure 1). For example, the concentration of oleoyl-CoA alone in the incubation containing CoA and ATP was calculated to be $1.7 \mu\text{M}$. The addition of Glc6P followed by the 2 min uptake period did not cause a detectable increase in the concentration of oleoyl-CoA or other lAcCoAs over that found in the absence of the substrate Glc6P (results not shown). The addition of oleic acid as non-esterified fatty acid brought about a small but significant increase in the concentration of oleoyl-CoA synthesized during the pre-incubation (Table 3).

The lAcCoAs synthesized during the 2 min pre-incubation period by the plastids evidently could not have been the result of *de novo* fatty acid synthesis since no substrate was added. Additionally, the rates of synthesis required would have been in excess of the highest rates of fatty acid synthesis from Glc6P that have been recorded for oilseed rape embryo plastids. We conclude that the acyl-CoA thioesters accumulating in incubations with CoA and ATP probably reflected the loss of fatty acids from the plastid membranes, due to hydrolysis of some of the plastid membranes during isolation.

Effect of cofactors on fatty acid synthesis

In conjunction with experiments on Glc6P uptake we routinely measured fatty acid synthesis by plastids, in the presence or absence of cofactors and fatty acids, under very similar conditions to those described. The only difference was that the fatty acids were extracted after a 1 h incubation with $500 \mu\text{M}$ [$1\text{-}^{14}\text{C}$]Glc6P, in order to sufficiently label the fatty acid pool. In the absence of cofactor additions the rate of fatty acid synthesis was $0.39 \pm 0.04 \mu\text{mol/h}$ per unit of GAPDH. (all $n = 3$). With the addition

Table 3 lAcCoAs synthesized by oilseed rape plastids during incubation with cofactors and fatty acids

lAcCoAs were detected by UV absorbance at 260 nm and quantified by HPLC, as described in the Materials and methods section. The concentrations of lAcCoAs listed were those present after the 2 min pre-incubation prior to the addition of Glc6P and are means $\pm$ S.E.M. ($n = 3$). N.D., not detected.

Addition to pre-incubation	lAcCoA (μM)				
	Palmitoyl-CoA	Stearoyl-CoA	Oleoyl-CoA	Linoleoyl-CoA	Linolenoyl-CoA
None	N.D.	N.D.	N.D.	N.D.	N.D.
ATP (3 mM)	N.D.	N.D.	< 0.06	N.D.	N.D.
CoA (0.3 mM)	N.D.	N.D.	< 0.06	N.D.	N.D.
ATP + CoA	0.4 ± 0.1	< 0.1	1.7 ± 0.1	0.2 ± 0.1	0.1 ± 0.05
ATP + CoA + oleic acid*	0.5 ± 0.1	0.1 ± 0.05	2.6 ± 0.2	0.2 ± 0.1	0.1 ± 0.05

* 1 or 10 μM .

Figure 4 Uptake of [$1\text{-}^{14}\text{C}$]Glc6P by oilseed rape plastids in the presence of oleoyl-CoA and the effect of ACBP and BSA

The proteins ACBP (●) and BSA (Δ) were incubated at a range of concentrations in the pre-incubation step (2 min), which also included $5\text{ }\mu\text{M}$ oleoyl-CoA, before [$1\text{-}^{14}\text{C}$]Glc6P was added to the plastids for 30 s. Reactions were terminated by silicone oil centrifugation. Glc6P uptake was measured as nmol/unit of GAPDH (nmol.U $^{-1}$.GAPdh). The values are means $\pm$ S.E.M. ($n = 3$).

of ATP this value rose, but not significantly, to $0.42 \pm 0.03\text{ }\mu\text{mol/h}$ per unit of GAPDH. CoA added alone caused a slight reduction in fatty acid synthesis to $0.35 \pm 0.03\text{ }\mu\text{mol/h}$ per unit of GAPDH; but with CoA (0.3 mM) and ATP (3 mM) added together the rate dropped to $0.059 \pm 0.02\text{ }\mu\text{mol/h}$ per unit of GAPDH.

Therefore, under conditions in which the highest amounts of lAcCoAs were synthesized, i.e. in the presence of CoA and ATP (Table 3), where there was approx. 70% inhibition of the Glc6P transporter, there was an 85% reduction in overall fatty acid synthesis. The implication is, therefore, that the activity of the Glc6P transporter significantly affects flux of carbon into fatty acids from Glc6P.

Effect of ACBPs on the Glc6P transporter

In another series of experiments it was found that Glc6P uptake in the presence of $5\text{ }\mu\text{M}$ oleoyl-CoA was maintained at control levels by the inclusion of $10\text{ }\mu\text{M}$ ACBP or $6\text{ }\mu\text{M}$ BSA in the pre-incubation (Figure 4). This also occurred when ACBP or BSA was added to plastids incubated with ATP and CoA alone (results not shown). Intermediate levels of inhibition with $5\text{ }\mu\text{M}$ oleoyl-CoA ($500\text{ }\mu\text{M}$ Glc6P) were observed by the inclusion of $1\text{--}6\text{ }\mu\text{M}$ ACBP or BSA. BSA has two specific lAcCoA binding-sites as opposed to one for ACBP, hence full alleviation of the inhibition of the transporter occurred with slightly lower concentrations.

Inhibition of the Glc6P transporter was also found to be reversible following inhibition, since, in another series of experiments after a 2 min pre-incubation with $1\text{ }\mu\text{M}$ oleoyl-CoA, Glc6P uptake after 30 s was approx. $12 \pm 2\text{ nmol/unit}$ of GAPDH (Figure 5). Following the addition of BSA or ACBP (both $5\text{ }\mu\text{M}$), the rate a further 30 s later was $35\text{--}40 \pm 2\text{ nmol/unit}$ of GAPDH, the same as the control value without oleoyl-CoA (result not shown); without addition of ACBP or BSA the rate remained inhibited, approx. 15 nmol/unit of GAPDH after 60 s

Figure 5 Recovery of Glc6P transporter activity by ACBP and BSA after initial inhibition by exogenously added oleoyl-CoA

All plastids were pre-incubated for 2 min with oleoyl-CoA ($1\text{ }\mu\text{M}$). Rates of uptake of Glc6P were determined after 30, 60 and 120 s. In two of the incubations, either ACBP (●) or BSA (Δ) was added to the plastids after 30 s incubation with Glc6P, as described in the Materials and methods section, to give a final concentration of $5\text{ }\mu\text{M}$. In the control experiment (□, broken line), $10\text{ }\mu\text{l}$ of PIM was added instead of the ACBPs. Glc6P uptake was measured as nmol/unit of GAPDH (nmol.U $^{-1}$.GAPdh). The values are means $\pm$ S.E.M. ($n = 3$).

Table 4 Comparison of the quantities of lAcCoAs and ACBP in oilseed rape embryos at three different stages of development

The lAcCoA content of oilseed rape embryos (with testa removed) was quantified by HPLC; the average masses of the embryos are given in brackets. Quantification of lAcCoAs was facilitated by the inclusion of an internal standard (heptadecanoyl-CoA) which formed 20–30% of the total peak area of lAcCoAs. ACBP was determined by quantitative Western blotting. Results are means $\pm$ S.E.M. ($n = 3$). N.D., not detected.

lAcCoA	Cotyledonary stage ... (average mass of embryos) ...	Amount (pmol/embryo)		
		Early (1.5 mg)	Mid (2.4 mg)	Late (3.1 mg)
Palmitoyl-CoA		N.D.	N.D.	0.2 ± 0.05
Stearoyl-CoA		N.D.	N.D.	< 0.1
Oleoyl-CoA		0.7 ± 0.2	1.5 ± 0.5	2.8 ± 0.4
Linoleoyl-CoA		N.D.	N.D.	< 0.1
Linolenoyl-CoA		N.D.	N.D.	< 0.1
ACBP		2.2 ± 0.2	2.5 ± 0.2	1.9 ± 0.2

(Figure 5). This reversibility demonstrated that the inhibition was unlikely to be caused by damage to the Glc6P transporter.

Concentration of lAcCoAs and ACBP in embryos

The demonstration of the inhibition of the Glc6P transporter and its reversal by ACBP raises the important question of the *in vivo* concentrations of lAcCoAs and ACBP during embryo development. We quantified lAcCoA and ACBP in embryos from early, mid and late cotyledonary stages. At any given stage the amounts of lAcCoAs and ACBP are broadly similar and comparing equivalent stages together are not statistically different from one another at $P > 0.05$ (Student's *t* test). During embryo

development the ACBP level stays fairly constant, whereas the concentration of lcACoAs increases significantly ($P = 0.02$) by approx. 4-fold (Table 4).

Estimation of the concentrations of lcACoAs and ACBP requires knowledge of the subcellular volumes, which are not available for developing seeds of oilseed rape. Estimations of the cytosolic volume have been made for mesophyll cells from barley and spinach and were found to be 3 and 6% of the total volume respectively [25,26]. If we assume that the cytosolic volumes of cells in a developing oilseed rape embryo are similar, and that the ACBP and lcACoA pools are cytosolic, then we estimate that the lcACoAs are at a concentration of 10–15 μM in the early and mid-cotyledonary stages, and at 20–30 μM in the late cotyledonary stage. Conversely, again assuming the former estimations of cytosolic volume, the ACBP concentration was about 30–45 μM in the early to mid-stages, falling to approx. 15–20 μM in the late stage. It is known that lcACoAs can freely partition into membranes [27] and therefore this may potentially affect the cytosolic concentration of lcACoAs *in vivo* where there is an excess of lcACoA over ACBP. However, it has been established that the addition of an equimolar concentration of ACBP completely displaces lcACoAs from membranes as they become bound to the protein [27].

DISCUSSION

The high sensitivity of the plastidial Glc6P transporter to low micromolar concentrations of lcACoAs with a chain length greater than C_{16} (IC_{50} 0.2–0.3 μM) could have physiological significance now that we have demonstrated that both lcACoA and ACBP are present at broadly similar concentrations. However, without an extremely accurate determination it is not possible to be certain of the actual concentration of unbound lcACoAs in the cytosol. The flux of fatty acids through the acyl-CoA pool in a developing embryo is approximately 280 pmol/min [28], hence the pool turns over in about 200 ms. It could be suggested that so long as the concentration of lcACoAs remains just below that of the ACBP, a high flux through the pathway to the acyltransferases will be maintained. A small decrease in the use of acyl-CoAs by the acyltransferases would very rapidly lead to an excess of acyl-CoA above the concentration of ACBP; this would cause inhibition of the supply of Glc6P as carbon skeletons for stromal glycolysis and a source of reductant for fatty acid synthesis [6]. The inhibition would be removed once the supply of fatty acids has been reduced enough to match the rate of use. The presence of ACBP in the embryo cell, amongst other functions, therefore allows for regulation of the Glc6P transporter. The latter is very sensitive to small (submicromolar) changes in acyl-CoA concentration, but ACBP allows for the maintenance of a relatively high concentration (10–20 μM) of acyl-CoAs available for the acyltransferases in the endoplasmic reticulum which can use lcACoAs bound to ACBP [16].

The data suggest that on average the concentration of ACBP in the early and mid-cotyledonary stages may well maintain an extremely low or non-existent pool of free lcACoA, and that the Glc6P transporter is not inhibited by lcACoAs. However, the late-stage embryos would appear to have an excess of lcACoAs and thus the Glc6P transporter may be inhibited at this time. This may partly reflect the observation that *in vitro* there is an increase in the utilization of carbon from pyruvate over Glc6P for fatty acid synthesis in plastids isolated from late cotyledonary stage embryos [7]. Although many other factors are likely to be involved, this could indicate an increase in cytosolic rather than plastidial glycolysis (*viz*, increased pyruvate utilization), one of

the results of which may be the inhibition of the plastidial Glc6P transporter.

Inhibition of the kind described here, though not described in plants before, is not unique, since lcACoAs also inhibit the Glc6P transporter in rat liver microsomes [29]. In the latter, the reduction in Glc6P transporter activity by palmitoyl-CoA led to the inhibition of glucose-6-phosphatase (EC 3.1.3.9), the last enzyme in the gluconeogenic and glycogenolytic pathway. The K_i for the inhibition of Glc6P transport by palmitoyl-CoA was 40–50 μM , which is significantly higher than the IC_{50} value calculated for plastids *in vitro*. In rat liver microsomes palmitoyl-CoA lowered both the V_{max} and K_m of the transporter for Glc6P. The addition of ATP and CoA, without added fatty acid, also inhibited Glc6P uptake and was due to the conversion of endogenous fatty acids into lcACoAs, as found in the current work.

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