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Evidence That Isolated Chloroplasts Contain an Integrated Lipid-Synthesizing Assembly That Channels Acetate into Long-Chain Fatty Acids[1]

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High rates of light-dependent fatty acid synthesis from acetate were measured in isolated chloroplasts that were permeabilized to varying extents by resuspension in hypotonic reaction medium. The reactions in hypotonic medium unsupplemented with cofactors were linear with time and were directly proportional to chlorophyll concentration, suggesting that the enzymes and cofactors of fatty acid synthesis remained tightly integrated and thylakoid associated within disrupted chloroplasts. Permeabilized chloroplasts expanded to at least twice the volume of intact chloroplasts, lost about 50% of their stromal proteins in the medium, and metabolized exogenous nucleotides. However, neither acetyl-coenzyme A (CoA) nor malonyl-CoA inhibited fatty acid synthesis from acetate; nor were [1-^{14}C]acetyl-CoA and [^{14}C]malonyl-CoA significantly incorporated into fatty acids. Fatty acid synthesis from acetate was independent of added cofactors but was totally light dependent. Changes in the products of fatty acid synthesis were consistent with the loss of endogenous glycerol-3-phosphate from permeabilized chloroplasts. However, in appropriately supplemented medium, the products of acetate incorporation by spinach (*Spinacia oleracea*) chloroplasts were similar when reactions were carried out in either isotonic or hypotonic medium. Taken together, the results of this study suggest that the enzymes of fatty acid synthesis with chloroplasts are organized into a multienzyme assembly that channels acetate into long-chain fatty acids, glycerides, and CoA esters.

We have previously noted (Roughan et al., 1979a, 1980; Roughan and Slack, 1981) that isolated spinach (*Spinacia oleracea*) chloroplasts synthesize fatty acids from acetate at high rates in hypotonic medium (66 mM sorbitol) usually containing ATP and CoA. This medium, however, causes the organelles to swell and the envelopes to rupture, as indicated by a number of parameters, including the total loss of bicarbonate-dependent oxygen-evolving activity (Roughan et al., 1980). On the other hand, isolation of intact chloroplasts has long been known to be a prerequisite for demonstrating high rates of fatty acid synthesis by chloroplasts in vitro (Stumpf, 1972).

Spinach chloroplasts osmotically shocked by transfer from 330 mM to 40 to 100 mM sorbitol lose the ability to carry out bicarbonate-dependent oxygen evolution (Cockburn et al., 1967) and become permeable to ADP, NADP, and ferricyanide (Reeves and Hall, 1973). Pea chloroplasts become permeable to ADP and ferricyanide when transferred from 400 to 250 mM Suc (West and Wiskich, 1973). Rupture of the chloroplast envelope results in the loss of soluble enzymes, metabolites, and cofactors from the plastids into the surrounding hypotonic medium and effectively prevents biosynthetic processes occurring.

The chloroplast fatty acid synthase is made up of a number of discrete, soluble enzymes (Caughey and Kekwick, 1982; Hoj and Mikkelsen, 1982; Shimakata and Stumpf, 1982) that might be expected to leak from the organelles and be diluted into the hypotonic medium along with ATP and nucleotides. How then, can fatty acid synthesis from acetate proceed apace in osmotically disrupted chloroplasts when enzymes and cofactors are apparently diluted at least 100-fold? The present study shows that spinach and pea (*Pisum sativum*) chloroplasts are capable of high rates of fatty acid synthesis from acetate even when incubated in unsupplemented, hypotonic medium. The characteristics of acetate incorporation into fatty acids in hypotonic medium have prompted us to suggest that acetate may be channeled through acetyl-CoA synthetase and acetyl-CoA carboxylase into a fatty acid synthase assembly that remains tightly integrated and thylakoid associated within disrupted chloroplasts and that is unable to utilize exogenous acetyl-CoA and malonyl-CoA. Such a multienzyme assembly would rival, in complexity at least, that proposed for part of the reductive pentose phosphate cycle in chloroplasts (Sainis and Harris, 1986; Gontero et al., 1988).

MATERIALS AND METHODS

[1-^{14}C]Acetate (60 Ci/mol) and $Na^{14}CO_3$ were from Amersham. [1-^{14}C]Acetyl-CoA and [^{14}C]malonyl-CoA were prepared enzymatically (Roughan, 1994; Roughan and Ohlrogge, 1994) and purified by HPLC. All biochemicals and Percoll were from Sigma. Plants (*Spinacia oleracea, Pisum sativum,* and *Amaranthus lividus*) for chloroplast isola-

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Abbreviation: ACP, acyl carrier protein.

tion were grown as described by Roughan (1987), and chloroplasts were routinely purified by centrifugation on density gradients of Percoll (Roughan, 1994) in low-ionic-strength buffer (Cernovic and Plesnicar, 1984).

Fatty acid synthesis by isolated chloroplasts was measured as the incorporation of [1-^{14}C]acetate into long-chain fatty acids, predominantly oleic and palmitic acids. The basal incubation medium contained 330 mM sorbitol, 25 mM Hepes/NaOH, pH 8.0, 10 mM $KHCO_3$, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM K_2HPO_4, and 0.2 mM [1-^{14}C]acetate (10 Ci/mol). Reactions (0.25 mL) were started by adding chloroplasts equivalent to about 50 μg of Chl, were illuminated with shaking for 10 min at 25°C, and were stopped by adding 0.5 mL of 10% KOH in methanol. Following saponification (80°C for 60 min), fatty acids were recovered and purified by TLC prior to determining radioactivity (Roughan, 1987). Variations to the basal reaction medium, particularly different sorbitol concentrations, were as indicated in the text. In some cases, reactions were stopped by adding 2.5 mL of chloroform-methanol and the different lipid products of [1-^{14}C]acetate incorporation were determined as described by Roughan (1987).

Photosynthetic carbon assimilation was measured in spinach chloroplasts using the basal reaction medium containing different sorbitol concentrations and with $H^{14}CO_3^-$ replacing [1-^{14}C]acetate. Photosynthesis by pea chloroplasts was measured using a medium supplemented with ATP and PPi (Cernovic and Plesnicar, 1984). Reactions (125 μL) contained 130,000 dpm of $H^{14}CO_3^-$ and were started by adding chloroplasts equivalent to 10 μg of Chl. After 10 min of illumination at 25°C the reactions were stopped by adding 75 μL of glacial acetic acid. Acid-stable ^{14}C was determined in aliquots of the acidified mixture. Acetyl-CoA synthetase activity was measured as described by Roughan and Ohlrogge (1994).

Nucleotides were determined by HPLC. Reactions (250 μL) containing about 75 μg of Chl for endogenous nucleotides and 25 to 35 μg of Chl for metabolism of exogenous ADP and NADP were stopped with 50 μL of 1.25 M $HClO_4$, cooled to 0°C, and occasionally shaken for 10 min. After the sample was centrifuged, 250 μL of supernatant were recovered and neutralized to a pH of about 7 with 52 μL of 1 M KOH/0.22 M $KHCO_3$ (Bagnara and Finch, 1972). Twenty to fifty microliters of neutralized extract were injected onto a C_{18}, reverse-phase column (250 × 4.5 mm, Alltima 5μ; Alltech, Deerfield, IL) equilibrated in 0.1 M potassium phosphate, pH 6, containing 0.5% (v/v) acetonitrile and flowing at 1 mL/min. Between 5 and 15 min following injection, the acetonitrile was increased linearly from 0.5 to 20% (v/v). Eluting compounds were detected by A_{254} and were identified by co-elution with standard nucleotides. The main nucleotides of chloroplasts, ATP, ADP, AMP, NADP, and NAD, were normally separated by reverse-phase HPLC in a single run. However, chromatograms of chloroplast extracts were more difficult to interpret than those of standards (Fig. 5). An unidentified compound eluted from the column between ATP and ADP and sometimes unpredictably co-eluted with ADP. AMP and NADP were relatively minor constituents and were sometimes difficult to identify among other compounds with similar mobilities. Where necessary, samples were spiked with standards to ensure that preliminary identifications were correct.

RESULTS

Acetate Incorporation and CO_2 Assimilation by Chloroplasts Incubated in Decreasing Sorbitol Concentrations

Rates of fatty acid synthesis from acetate by intact spinach chloroplasts in unsupplemented reaction medium initially increased as the sorbitol concentration decreased and were greater at 66 than at 330 mM (Fig. 1A). However, at sorbitol concentrations of less than 66 mM, fatty acid synthesis activity declined rapidly and extrapolated to 0 in the absence of osmoticum. *Amaranthus* chloroplasts likewise exhibited higher rates of fatty acid synthesis in 66 than in 330 mM sorbitol (results not shown). By contrast, photosynthetic CO_2 assimilation rapidly declined in spinach chloroplasts as the sorbitol concentration decreased from 230 mM; only 20% of the maximum activity was expressed

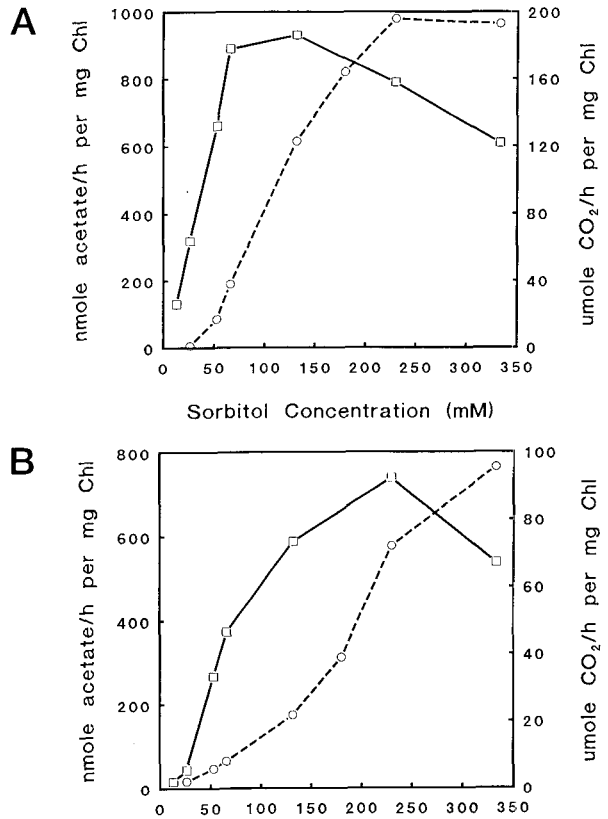

Figure 1. Fatty acid synthesis from acetate and CO_2 fixation by intact chloroplasts isolated from expanding spinach leaves (A) or 8-d-old pea shoots (B) and incubated in increasingly hypotonic medium. Fatty acid synthesis (solid lines) was measured using the basal medium containing [1-^{14}C]acetate and the different sorbitol concentrations shown, and CO_2 fixation (dashed lines) from $H^{14}CO_3^-$ was measured in basal medium (spinach) or in a medium supplemented with ATP and PPi (pea). Reaction times were 10 min.

at 66 mM sorbitol (Fig. 1A). Rates of fatty acid synthesis by pea chloroplasts were higher in 230 than in 330 mM sorbitol but then declined with decreasing sorbitol concentrations and were about 70% of maximum at 66 mM (Fig. 1B). Photosynthetic carbon assimilation by pea chloroplasts was not as active as that in spinach but declined rapidly as the sorbitol concentration decreased from 330 mM (Fig. 1B) and was 10% of maximum at 66 mM.

By contrast, accumulation of acetyl-CoA in the same reaction mixture but in the dark and containing CoA and ATP increased with decreasing sorbitol concentrations (Fig. 2). Therefore, the permeability of both pea and spinach chloroplast envelopes to ATP and CoA, as indicated by the expression of acetyl-CoA synthetase activity, changed little between 330 and 200 mM sorbitol but then increased markedly at lower sorbitol concentrations. Maximum rates of acetyl-CoA synthetase activity were attained when sorbitol concentrations were less than 20 mM, indicating that complete lysis probably occurred when suspensions of intact chloroplasts were diluted 15- to 20-fold with medium containing no osmoticum.

Time Course of Fatty Acid Synthesis in 66 and 330 mM Sorbitol

Rates of fatty acid synthesis from acetate by spinach chloroplasts in basal medium were linear with time for at least 30 min in both 66 and 330 mM sorbitol (Fig. 3). Therefore, even though enzymes, cofactors, and reactants were expected to be leached from the organelles, the reaction rate did not decrease significantly with time. Fatty acid synthesis by pea chloroplasts was also essentially linear for 15 to 20 min in both 330 and 66 mM sorbitol (Fig. 3). In neither case was there a dramatic decline in acetate-incorporating activity as might be expected if chloroplast integrity was progressively deteriorating during the incubation.

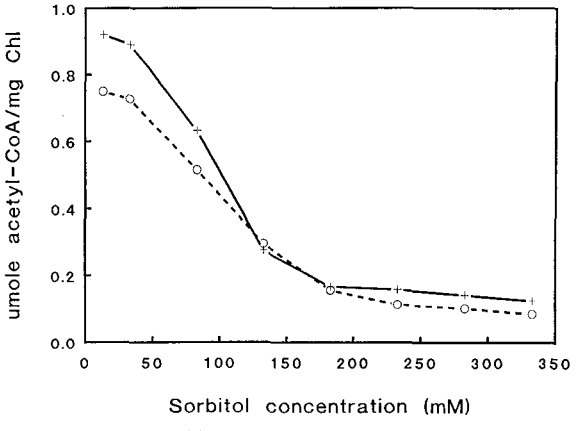

Figure 2. Expression of latent acetyl-CoA synthetase activity by intact chloroplasts isolated from spinach (solid line) and pea (dashed line). Chloroplasts in 330 mM sorbitol were added to the basal reaction medium that contained added 0.2 mM CoA and 2 mM ATP to give the final sorbitol concentrations shown and were incubated for 5 min in subdued light. Acetyl-CoA formed was determined using a filter paper disc assay (Roughan and Ohlrogge, 1994).

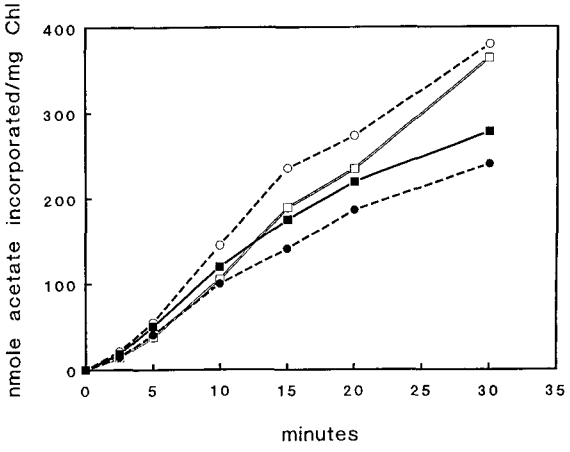

Figure 3. Time course of fatty acid synthesis from acetate by spinach (open symbols) and pea (closed symbols) chloroplasts incubated in 330 mM sorbitol (solid lines) or 66 mM sorbitol (dashed lines). Fifty-microliter samples were removed from 0.5 mL of basal reaction medium containing [1-^{14}C]acetate at the times shown and were saponified with methanolic KOH.

Effect of Chloroplast Concentration on Fatty Acid Synthesis in 66 mM Sorbitol

Rates of fatty acid synthesis from acetate by spinach chloroplasts in the basal medium with 66 mM sorbitol were essentially linear with respect to Chl concentration between 8 and 64 μg of Chl per 0.25-mL reaction (Fig. 4). More significantly, at all Chl concentrations the rate of fatty acid synthesis in 66 mM exceeded the rate in 330 mM sorbitol. Similarly, there was little or no difference in rates of acetate incorporation by pea chloroplasts in 66 mM sorbitol when Chl was 10 to 50 μg/reaction. The volume of spinach chloroplast stroma has been estimated at 66 μL/mg Chl (Winter et al., 1994); therefore, 15 μg of Chl is

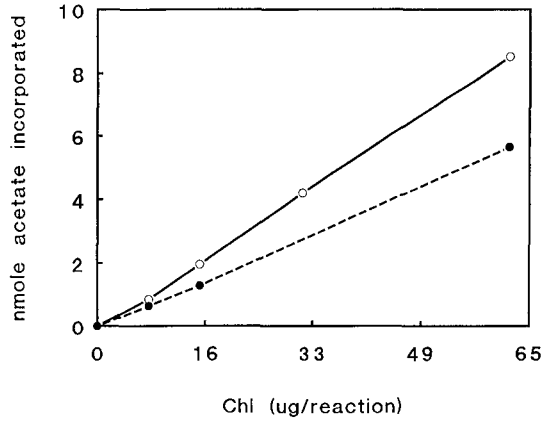

Figure 4. Effect of Chl concentration on fatty acid synthesis by spinach chloroplasts in basal medium containing 66 mM (solid line) and 330 mM (dashed line) sorbitol. Reactions containing chloroplasts equivalent to the amounts of Chl shown were incubated for 10 min and were stopped by adding methanolic KOH. Additional chloroplasts were then added to bring all reactions to the same Chl concentration and to ensure uniform recoveries of fatty acids, which were purified by TLC prior to counting.

equivalent to 1 μL of stroma, and the potential dilution of stromal contents in the standard reaction containing 66 mM sorbitol is 250-fold. Fatty acid synthesis from acetate thus appeared to be independent of the degree of dilution of endogenous cofactors.

Extent of Chloroplast Disruption in 33 and 66 mM Sorbitol

No intact organelles were recovered when spinach and pea chloroplasts were re-centrifuged on Percoll gradients following resuspension in 66 mM sorbitol; the high density contents had largely been lost. Broken chloroplasts recovered from Percoll gradients were incapable of fatty acid synthesis from acetate in the basal medium. Chloroplasts suspended in 66 mM sorbitol packed (5 min at 2000*g*) to twice (spinach) or 2.5 times (pea) the volume of those suspended in 330 mM sorbitol. Although there was not a linear relationship between A_{546} and sorbitol concentration of chloroplast suspensions (Crofts et al., 1967), the 35% lower absorbance of suspensions in 66 mM sorbitol compared with 330 mM sorbitol coupled with the greater packed volumes suggested that the plastids were swollen to at least twice normal volume in the hypotonic medium. Low rates of CO_2 assimilation (Fig. 1) and expression of latent enzyme activities (Fig. 2) are consistent with loss of chloroplast integrity at lower sorbitol concentrations (Reeves and Hall, 1973). Separation of stromal proteins, enzymes, and endogenous cofactors from plastid membranes may also be used as an indicator of a dysfunctional envelope. Diluting suspensions of intact chloroplasts 25-fold using reaction medium without sorbitol (final sorbitol concentration 13.2 mM) appeared to release all of the stromal protein and acetyl-CoA synthetase activity (Table I; Douce and Joyard, 1982). However, when chloroplasts were transferred to 66 mM sorbitol and then gently centrifuged, only 40 to 60% of total soluble protein and total acetyl-CoA synthetase activity was recovered in the supernatant (Table I). Similarly, of the major endogenous nucleotides, NADP was most readily lost and ATP least readily lost to the 66 mM sorbitol medium, but none was recovered entirely in the medium (result not shown). Therefore, although the results indicate that chloroplasts became leaky in 66 mM sorbitol, some of the soluble constituents remain associated with membranes of gently disrupted plastids. In 33 mM sorbitol, on the other hand, spinach chloroplasts were capable of fatty acid synthesis rates of 50 to 75% of those measured in isotonic medium and both soluble protein and acetyl-CoA synthetase activity were more than 90% separated from the chloroplast membranes (Table I). Therefore, perhaps a better indicator of loss of chloroplast integrity was "expression" of acetyl-CoA synthetase activity by chloroplasts suspended in different sorbitol concentrations (Fig. 2). For example, more than 90% and 60 to 80% of acetyl-CoA synthetase activity was expressed in 33 and 66 mM sorbitol, respectively, indicating the degree of access of exogenous CoA and ATP to the enzyme.

Table I. *Release of stromal protein and acetyl-CoA synthetase activity from intact chloroplasts following incubation in medium with different sorbitol concentrations*

Chloroplasts were incubated for 5 min at room temperature in basal reaction medium containing the sorbitol concentrations indicated and then centrifuged for 5 s in a microfuge ($13{,}000g_{max}$). Suspensions were not illuminated and were mixed by gentle inversion. The results were derived from several experiments using both spinach and pea chloroplasts.

Sorbitol	Soluble Protein	Acetyl-CoA Synthetase
mM	%	%
330	<5	10–15
66	40–60	50–75
33	>90	>90
13.2	100	100

Table II. *Concentrations of major nucleotides endogenous to freshly isolated chloroplasts and to chloroplasts incubated in 330 and 66 mM sorbitol in light*

Chloroplasts equivalent to 70 to 75 μg of Chl were illuminated at 25° for 5 min in 250 μL of the basal medium with unlabeled acetate, and the reactions were stopped by adding 50 μL of 1.25 M $HClO_4$. Nucleotides were analyzed by HPLC as described in "Materials and Methods." The results are means from two separate chloroplast preparations from both spinach and peas.

Treatment	ATP	ADP	AMP	NAPD	NAD
	nmol mg^{-1} Chl				
Fresh					
Spinach	13	17	7	3	1
Pea	15	13	<1	3	6
66 mM Sorbitol					
Spinach	28	12	4	7	3
Pea	28	7	<1	5	7
330 mM Sorbitol					
Spinach	17	14	3	8	2
Pea	19	10	<1	3	5

Fate of Endogenous Nucleotides and Metabolism of Exogenous Nucleotides by Chloroplasts in 66 and 330 mM Sorbitol

Further indications of the extent of metabolic disruption to chloroplasts in hypotonic compared with isotonic medium should be obtained from levels of endogenous nucleotides. Chloroplasts freshly isolated from spinach and peas contained about equal amounts of ATP and ADP (Table II) and lower concentrations of NADP than expected (Heineke et al., 1991). Following 5 min of incubation in fatty acid-synthesizing mode, ATP and ADP concentrations had changed relatively little in photosynthetically competent spinach chloroplasts in 330 mM, but ATP increased dramatically in 66 mM sorbitol (Table II; Fig. 5). The high ATP concentration is probably indicative of reduced ATP utilization in biosynthetic processes by chloroplasts in the hypotonic medium. Although the localization of the ATP was not determined, fatty acid synthesis from acetate in 66 mM sorbitol was probably not ATP deficient. NADP concentrations were significantly higher in spinach chloroplasts following incubation in either 66 or 330 mM sorbitol. Because acid extraction was used to separate nucleotides from proteins, etc., NADPH was not detected in any extract and biosynthetic oxidation of NADPH might

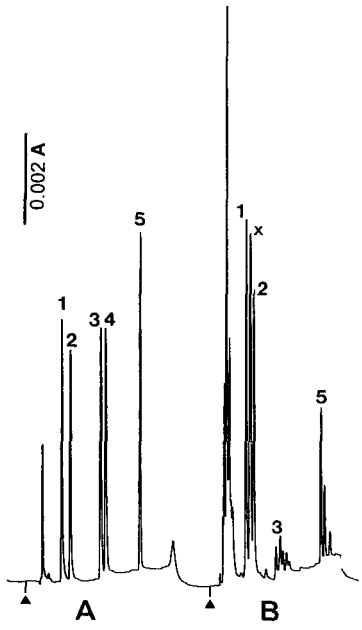

Figure 5. HPLC separation of nucleotides endogenous to fresh pea chloroplasts and typical of the separations from which the data in Table III were calculated. The amount of extract injected here was equivalent to 7.8 μg of Chl. Note the unidentified peak that migrated between ATP and ADP. The standard mixture (B) contained 0.1 nmol each of ATP (1), ADP (2), NADP (3), AMP (4), NADPH (5), and NAD (6). The bars at the left indicate 0.0002 *A* units.

explain the higher concentrations of NADP in incubated chloroplasts.

Utilization of added nucleotides by chloroplasts in hypotonic medium would indicate disruption of the chloroplast envelope. Accordingly, exogenous NADP was not significantly metabolized by illuminated chloroplasts in isotonic medium but was efficiently utilized, reduced, and dephosphorylated to NAD in 66 mM sorbitol (Fig. 6). Exogenous ADP was slowly converted to ATP and AMP in isotonic medium, presumably by adenylate kinase in the envelope (Murakami and Strotmann, 1978), but was rapidly converted to ATP in 66 mM sorbitol. Hence, both ADP and NADP were able to cross the chloroplast envelope in 66 mM sorbitol and react with stromal or thylakoid enzymes.

Effect of Adding Cofactors for Fatty Acid Synthesis and Dependence of Acetate Incorporation on Light

Acetate incorporation into long-chain fatty acids by spinach and pea chloroplasts was insignificant in the dark in both 330 and 66 mM sorbitol (Table III). This light-dependent incorporation of acetate in the basal buffer with 66 mM sorbitol was slightly inhibited by the addition of the full range of cofactors, ACP, ATP, CoA, NAD, and NADPH, required to synthesize fatty acids from acetate. The inhibition may possibly be attributed to a depletion of acetate as acetyl-CoA accumulated when both CoA and ATP were added to reactions in 66 mM sorbitol. The excess acetyl-CoA generated in the reaction may have been unable to interact with the fatty acid synthase, because rates of both acetyl-CoA and fatty acid accumulation remained linear for 20 min (results not shown). However, acetyl-CoA could be expected to be a better substrate than acetate for fatty acid synthesis and rates of fatty acid synthesis would be expected to increase as the concentration of acetyl-CoA in the medium increased to match that of acetate (but see below). Although some acetyl-CoA did accumulate when chloroplasts were incubated in 330 mM sorbitol in the presence of exogenous CoA plus ATP, acetate incorporation into fatty acids was stimulated by up to 50% in the isotonic medium. Acetate incorporation was 50 to 70% inhibited in 33 mM compared with 66 mM sorbitol, even in the presence of the cofactors (Fig. 1; Table III), and remained insignificant in the dark when all cofactors were added to the reaction in 33 mM sorbitol (Table III).

Products of Acetate Incorporation into Isolated Chloroplasts Incubated in 66 and 330 mM Sorbitol

Factors affecting rates and products of acetate incorporation operated similarly in both intact and permeabilized chloroplasts. Added CoA and Triton X-100 stimulated acetate incorporation into spinach chloroplasts both in 66 and 330 mM sorbitol, although the stimulation was greater at the higher sorbitol concentration (Table IV). At both concentrations, fatty acids were converted to acyl-CoA only when both CoA and ATP were provided exogenously. In intact spinach chloroplasts, both the ratio of unesterified

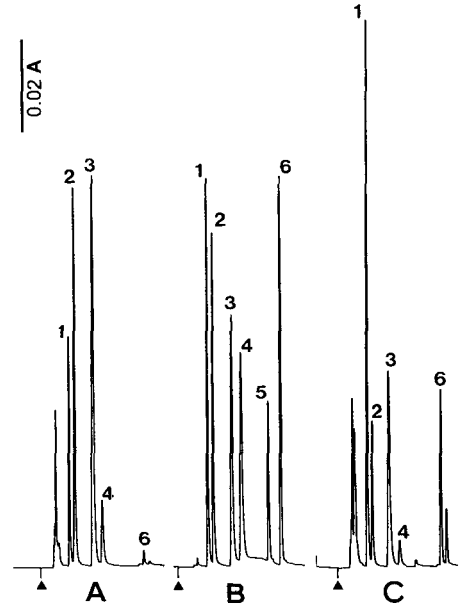

Figure 6. HPLC separations of nucleotides showing different metabolism of exogenous ADP and NADP by spinach chloroplasts incubated in 330 (A) and 66 (C) mM sorbitol. Reactions contained 0.2 mM ADP, 0.4 mM K_2HPO_4, and 0.2 mM NADP in 250 μL of 1.25 $HClO_4$, and the nucleotides were analyzed as described in "Materials and Methods." At Chl concentrations of 50 to 60 μg/250 μL the NADP was completely metabolized. The standard mixture (B) contained 1.25 nmol each of ATP (1), ADP (2), NADP (3), AMP (4), NADPH (5), and NAD (6). The bars at the left indicate 0.02 *A* units.

Table III. *Effect of fatty acid synthase cofactors on acetate incorporation into chloroplasts incubated in 66 mM sorbitol*

Cofactors added to the basal medium were MgATP (2 mM), CoA (0.25 mM), NAD (0.01 mM), NADPH (0.2 mM), and ACP (10 μM). The 10-min reactions were stopped by adding methanolic KOH and the long-chain fatty acids, recovered following saponification, were purified by TLC prior to scintillation counting.

Treatment	Acetate Incorporation	
	Spinach	Pea
	nmol h^{-1} mg^{-1} Chl	
No cofactors, 330 mM sorbitol	1010	640
No cofactors, 66 mM sorbitol	1235	529
Cofactors, 66 mM sorbitol	1000	518
Cofactors, 33 mM sorbitol	495	144
Cofactors, 66 mM sorbitol, dark	21	17

fatty acids to glycerides accumulated and the ratio of unsaturated to saturated fatty acids (virtually oleic to palmitic) synthesized were influenced by glycerol-3-P concentrations (Roughan et al., 1979a), whereas only the ratio of unesterified fatty acids to glyceride was influenced by Triton X-100. Similarly in the present study, glycerol-3-P and Triton X-100 reduced acetate incorporation into unesterified fatty acids while increasing incorporation into glycerides in both intact and disrupted chloroplasts (Table IV). In the basal reactions, the shift of acetate incorporation into unesterified fatty acids and away from glycerides in 66 mM sorbitol was consistent with a loss of endogenous glycerol-3-P from disrupted chloroplasts and was reversed by adding glycerol-3-P (Table IV). A marked increase in the ratio of unsaturated to saturated fatty acids accumulated in 33 and 66 mM compared with 330 mM sorbitol (Table V) not only was consistent with a depletion of endogenous glycerol-3-P in permeabilized chloroplasts but also showed that the biosynthetic sequence from acetate to oleate had remained intact. These results suggest that, although small-molecular-weight materials may be lost, acetyl-CoA synthetase, acetyl-CoA carboxylase, fatty acid synthase, palmitoyl-ACP elongase, stearoyl-ACP desaturase, thioesterase, and glycerol-3-P acyltransferase activities, along with their required cofactors, were retained within the disrupted organelles and functioned in a concerted manner.

Utilization of Acetyl-CoA and Malonyl-CoA by Chloroplasts in 66 and 33 mM Sorbitol

Since both ADP and NADP were metabolized by permeabilized chloroplasts, and both CoA and ATP could access endogenous acetyl-CoA synthetase (see above), it could be anticipated that acetyl-CoA and malonyl-CoA should also be metabolized. However, neither acetyl-CoA nor malonyl-CoA (0.05–0.2 mM) significantly inhibited [1-^{14}C]acetate (0.2 mM) incorporation into fatty acids of spinach and pea chloroplasts incubated in 33 or 66 mM sorbitol. Indeed, acetyl-CoA and malonyl-CoA stimulated acetate incorporation, in some instances, in the same way as would CoA-SH. Under the same conditions, neither [1-^{14}C]acetyl-CoA (50 μM) nor [1-^{14}C]malonyl CoA (50 μM) was significantly incorporated into chloroplast fatty acids. These results were consistent with the finding that the acetyl-CoA generated in situ from acetate in hypotonic medium may not have been utilized for fatty acid synthesis (see above) and suggest that acetate is channeled through the initial reactions leading to its incorporation into fatty acids. Roughan et al. (1979a) had earlier reported that 0.16 mM [1-^{14}C]acetyl-CoA was poorly utilized for fatty acid synthesis by spinach chloroplasts in hypotonic buffer. Similarly, avidin might be expected to prevent acetate incorporation in permeabilized chloroplasts by inhibiting acetyl-CoA carboxylase activity, and yet 25 μg of avidin per reaction inhibited acetate incorporation by less than 10% (result not shown). Either avidin could not gain access to the stroma of permeabilized chloroplasts or the acetyl-

Table IV. *Effect of different additions to the reaction medium on the products formed from acetate when spinach chloroplasts were incubated in 66 or 300 mM sorbitol*

Additions to the basal reaction medium were *sn*-glycerol-3-P (0.4 mM), CoA (0.25 mM), ATP (2 mM), and Triton X-100 (0.008%, w/v). The reactions (0.25 mL) were started by adding chloroplasts equivalent to about 50 μg of Chl and were stopped after 10 min by adding 2.5 mL of chloroform-methanol followed by 0.875 mL of 0.25 M HCl in 1% NaCl. Labeled products were analyzed as described by Roughan (1987). DAG, 1,2-Diacylglycerol; FFA, free fatty acids; PL, polar lipids.

Additions to Basal Medium	Sorbitol	Rate of Fatty Acid Synthesis[a]	Percent of Total Incorporation			
			DAG	FFA	PL	Acyl-CoA
	mM					
None	66	897	9	83	5	3
	330	585	15	69	13	3
Glycerol-3-P	66	915	47	31	22	nm[b]
	330	592	42	26	32	nm
CoA	66	1102	10	78	6	6
	330	877	14	69	12	5
Triton X-100	66	1355	19	73	8	nm
	330	1179	29	56	15	nm
CoA + ATP	66	875	14	19	27	40
	330	850	14	30	26	30

[a] nmol acetate incorporated h^{-1} mg^{-1} Chl. [b] nm = Not measured.

Table V. *Effect of sorbitol concentration on fatty acid synthesis and on the ratio of unsaturated fatty acids synthesized by spinach chloroplasts*

Reactions were for 10 min in the light and contained chloroplasts equivalent to 50 μL of basal medium containing [1-^{14}C]acetate but with the sorbitol concentrations shown. Fatty acids were methylated and separated into saturated and monoenoic species on thin layers of silica gel impregnated with silver nitrate.

Characteristic	Sorbitol (mM)					
	33	66	132	198	264	330
Fatty acid synthesis[a]	606	996	1025	885	694	565
Ratio, unsaturated/saturated	5.0	4.4	3.6	3.3	2.8	2.5

[a] nmol acetate incorporated h^{-1} mg^{-1} Chl.

CoA carboxylase in situ is sequestered in a multienzyme complex in a way that makes it inaccessible to avidin.

Whereas exogenous acetyl-CoA was apparently unable to enter a putative fatty acid synthase complex, acetyl-CoA formed from pyruvate via pyruvate dehydrogenase presumably could, since exogenous pyruvate is utilized for fatty acid synthesis by isolated spinach chloroplasts (Roughan et al., 1979b; Murphy and Walker, 1982). When repeating those earlier experiments we found that 0.5 mM pyruvate was incorporated into fatty acids of spinach and *Amaranthus* chloroplasts at about 33%, and pea chloroplasts at about 40%, of the rate of 0.2 mM acetate in the basal medium; the results were the same whether reactions were carried out in 330 or 66 mM sorbitol. These figures are approximately consistent with our measured values of pyruvate dehydrogenase activity of 0.5 to 1, 1.0, and 2.5 μmol acetyl-CoA h^{-1} mg^{-1} Chl for *Amaranthus*, spinach, and pea chloroplasts, respectively. We currently have no explanation for why acetyl-CoA from pyruvate should behave differently from exogenous acetyl-CoA or excess acetyl-CoA generated in situ by acetyl-CoA synthetase but note that, whereas acetate was converted to acetyl-CoA and malonyl-CoA by broken chloroplast preparations, pyruvate was converted to acetyl-CoA alone (results not shown). It should also be noted that in spinach chloroplasts, fatty acid synthesis from pyruvate was strongly inhibited by low concentrations of acetate, whereas fatty acid synthesis from acetate was only weakly inhibited by higher concentrations of pyruvate (Roughan et al., 1979b; Springer and Heise, 1989). Chloroplasts may not be alone in selectively metabolizing acetyl-CoA, because there is evidence for compartmentation of acetyl-CoA in rat liver mitochondria (von Glutz and Walter, 1975).

DISCUSSION

Perhaps the most striking result in this work is the observation that acetate incorporation into lipids of isolated chloroplasts was maintained under conditions in which other chloroplast functions were seriously impaired. Our results clearly demonstrate that all of the components required to produce high rates of fatty acid synthesis from acetate remain functional under conditions in which (a) chloroplasts have swollen to twice normal volume, (b) CO_2 fixation is reduced by 80%, (c) much of the protein and cofactors have been released into the medium, and (d) ability to sediment through Percoll has been eliminated. A linear rate of fatty acid synthesis in basal hypotonic medium for up to 30 min and an acetate incorporation rate unaffected by extensive dilution of the leaky chloroplasts suggest that the fatty acid-synthesizing system was retained in disrupted chloroplasts in an in situ configuration, possibly as a cluster of sequential metabolic enzymes, also known as multienzyme complexes or metabolons (Sere, 1987; Ovadi and Sere, 1992). That spinach chloroplasts, and to a lesser extent pea chloroplasts, incorporated acetate even more efficiently in hypotonic than isotonic medium suggested the release of some unknown constraint imposed on fatty acid synthesis under isotonic conditions.

The physical state of the chloroplasts in 66 mM sorbitol is problematical. By the standards of Lilley et al. (1975) the plastids are "ruptured," since they are permeable to ADP and NADP. However, they are probably not "envelope free," since the products of the fatty acid synthesis are efficiently metabolized by envelope enzymes. A category of plastids in which the envelopes reseal following rupture and release all or part of the chloroplast's soluble contents might explain some of our results, since such chloroplasts are also impermeable to ferricyanide (Lilley et al., 1975) and presumably to CoA and its esters. However, the efficient metabolism of ADP and NADP by plastids in 66 mM but not 330 mM sorbitol suggests that resealed plastids do not constitute a significant proportion of our preparations. In any case, even if the system under study did consist primarily of resealed plastids, the results here indicate that the enzymes of lipid metabolism are in some way associated because when the bulk of the soluble protein content was released from the membranes (Table I) the bulk of fatty acid biosynthetic capacity was not. It is more likely that chloroplasts transferred from 330 to 66 mM sorbitol swell to at least twice normal volume and that the consequent increase (59%) in surface area destroys the selective permeability of the envelope. In 66 mM sorbitol the chloroplast envelope membrane probably does not detach as vesicles the way it is thought to in very-low-osmolarity medium (Douce and Joyard, 1982).

Although the chloroplast fatty acid synthase is known to consist of a number of discrete enzymes (Caughey and Kekwick, 1982; Hoj and Mikkelsen, 1982; Shimakata and Stumpf, 1982), it has often been assumed (Douce and Joyard, 1982; Shimakata and Stumpf, 1982) that, for catalytic efficiency, these enzymes would be associated into a complex in the chloroplast stroma. For example, at least five of the enzymes of the Benson-Calvin cycle in chloroplast stroma are now believed to be aggregated into a multienzyme complex (Gontero et al., 1988). The present work tends to confirm the expectations for the chloroplast fatty acid synthase but also seems to indicate that an even larger suite of enzymes involved in synthesizing chloroplast lipids from acetate may exist as an integrated assembly that resists dissociation and loss of more mobile constituents from chloroplasts disrupted in hypotonic medium. Although the advantages of such a complex are described by Stafford (1981), the nature of such an integrated assembly for synthesizing chloroplast lipids is far from clear. Other data from transgenic plants indicate that it is possible to introduce foreign enzymes into plastids and these enzymes are able to participate in plastid fatty acid synthesis. For example, when spinach ACP was overexpressed in tobacco, the introduced ACP was acylated in a manner indistinguishable from the endogenous tobacco ACP and, furthermore, a several-fold increase in total acyl-ACP levels in the plants occurred (Post-Beittenmiller et al., 1989). Addition of foreign acyl-ACP thioesterases (Voelker et al., 1992), acyl-ACP desaturases (Cahoon et al., 1992), and acyl-ACP acyltransferases (Wolter et al., 1992) have all resulted in modifications of plastid fatty acid metabolism,

suggesting that (a) these enzymes have access to an available pool of acyl-ACP intermediates and (b) the stoichiometry of enzymes and ACP in the plastid does not need to be strictly maintained. Taken together, results of these studies imply that the putative assembly of lipid-synthesizing enzymes is flexible enough to accommodate additions of several foreign proteins. This is perhaps not too surprising, because it is well known that a mammalian fatty acid synthase complex will accommodate an acyl-ACP thioesterase from lactating mammary gland, which then directs the production of short- and medium-chain rather than long-chain fatty acids (Libertint and Smith, 1978).

The multienzyme assembly appears to have the ability to channel acetate into lipids and, therefore, must include acetyl-CoA synthetase and acetyl-CoA carboxylase. Acetate channeling through acetyl-CoA synthetase to acetyl-CoA carboxylase and then onto fatty acid synthase is indicated by the inability of exogenous acetyl-CoA and malonyl-CoA either to incorporate into fatty acids or to inhibit acetate incorporation into fatty acids of disrupted chloroplasts. Independent findings also support the concept of acetate channeling; isolated chloroplasts, for instance, incorporate carbon into isoprenoids from CO_2 but not from acetate (Kreuz and Kleinig, 1981; Kleinig, 1989; but see Heintze et al., 1994) even though acetyl-CoA, which is required for isoprenoid synthesis, is readily formed from acetate. This would be explained if acetate was channeled into malonyl-CoA by acetyl-CoA synthetase and acetyl-CoA carboxylase working in concert so that the intermediate acetyl-CoA was retained within a fatty acid synthase multienzyme complex and was not free to enter the isoprenoid pathway. Previous work also indicated that acetyl-CoA was a poor substrate compared with acetate for fatty acid synthesis in disrupted spinach chloroplasts (Roughan et al., 1979a) and that acetate was superior to both acetyl- and malonyl-CoA as a substrate for fatty acid synthesis in sonicated plastids from avocado (Weaire and Kekwick, 1975). The lack of utilization of acetyl-CoA for fatty acid synthesis in plastid preparations that, nevertheless, exhibited high rates of fatty acid synthesis from acetate explained the very low activities of acetyl-CoA carboxylase measured in those same preparations when acetyl-CoA was used as the substrate (Roughan et al., 1979a; Mohan and Kekwick, 1980). The closest parallel we can find to our results is the description of a bicarbonate-dependent, avidin-insensitive synthesis of fatty acids from acetate by a particulate fraction from avocado plastids that did not, however, utilize malonyl CoA (Weaire and Kekwick, 1975; Mohan and Kekwick, 1980).

Since the end products of acetate incorporation were similar in intact and permeabilized chloroplasts, the putative complex includes all of the enzymes required to synthesize oleic acid and to transfer oleate and palmitate from ACP to glycerol-3-P. Fatty acyl-CoA synthetase, 1-acylglycerol-3-P acyltransferase, and phosphatidate phosphatase are all integral proteins on the inner envelope but must also have a close association with the complex.

The dependency on light for acetate incorporation in hypotonic medium coupled with the independence from added cofactors suggests that the hypothetical multienzyme assembly has some close association with thylakoids and that it contains an integral set of cofactors that do not equilibrate with those in the stroma. Isolated chloroplasts contain only a very small pool of total CoA (approximately 1 nmol/mg Chl; 15 μM in the stroma), which occurs almost exclusively as acetyl-CoA (Post-Beittenmiller et al., 1992), and equilibration of the CoA with the hypotonic medium would reduce its concentration in the reaction to about 0.2 μM. Clearly, the working concentration of CoA would be more realistic if it were largely confined within a multienzyme complex. Similarly, if ATP had been able to equilibrate with the medium in 66 mM sorbitol, the final concentration would have been 5 to 6 μM, far less than the concentration required to drive acetyl-CoA synthetase and acetyl-CoA carboxylase at the required rate to accommodate fatty acid synthesis. The first thylakoid association of the complex would presumably be that with the acetyl-CoA carboxylase complex (Mohan and Kekwick, 1980; Saski et al., 1993), the activity of which, in situ, might be driven by a very small pool of ATP recycled by photophosphorylation. Another close association with thylakoids would supply reductant to the fatty acid synthase in a form that did not necessarily equilibrate with the general pool of reductant but that was generated and immediately utilized within the microenvironment of the multienzyme complex. In this regard, the chloroplast stearoyl-ACP desaturase is most active in vitro when Fd is reduced by thylakoid membranes in the light rather than by NADPH/Fd reductase (Jacobson et al. (1974), and perhaps the keto- and enoyl-ACP reductases of fatty acid synthesis respond similarly to thylakoid-generated reductants.

The putative multienzyme system has the potential to link thylakoid and envelope by spanning the stroma. At the beginning of the biosynthetic pathway, thylakoids provide the energy for converting acetate into long-chain fatty acids, and at the terminal end of the pathway, the inner envelope accepts the products for processing into glycerolipids. In this scenario, the products of the complex would be mainly oleic acid and 1-oleoyl-3-phosphoglycerol.

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