Regulation of Plant Fatty Acid Biosynthesis[1]

Analysis of Acyl-Coenzyme A and Acyl-Acyl Carrier Protein Substrate Pools in Spinach and Pea Chloroplasts

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ABSTRACT

In previous work (D. Post-Beittenmiller, J.G. Jaworski, J.B. Ohlrogge [1991] J Biol Chem 266: 1858–1865), the in vivo acyl-acyl carrier protein (ACP) pools were measured in spinach (Spinacia oleracea) leaves and changes in their levels were compared to changes in the rates of fatty acid biosynthesis. To further examine the pools of substrates and cofactors for fatty acid biosynthesis and to evaluate metabolic regulation of this pathway, we have now examined the coenzyme A (CoA) and short chain acyl-CoA pools, including acetyl- and malonyl-CoA, in isolated spinach and pea (Pisum sativum) chloroplasts. In addition, the relationships of the acetyl- and malonyl-CoA pools to the acetyl- and malonyl-ACP pools have been evaluated. These studies have led to the following conclusions: (a) Essentially all of the CoA (31–54 μM) in chloroplasts freshly isolated from light-grown spinach leaves or pea seedling was in the form of acetyl-CoA. (b) Chloroplasts contain at least 77% of the total leaf acetyl-CoA, based on comparison of acetyl-CoA levels in chloroplasts and total leaf. (c) CoA-SH was not detected either in freshly isolated chloroplasts or in incubated chloroplasts and is, therefore, less than 2 μM in the stroma. (d) The malonyl-CoA:ACP transacylase reaction is near equilibrium in both light- and dark-incubated chloroplasts, whereas the acetyl-CoA:ACP transacylase reaction is far from equilibrium in light-incubated chloroplasts. However, the acetyl-CoA:ACP transacylase reaction comes nearer to equilibrium when chloroplasts are incubated in the dark. (e) Malonyl-CoA and -ACP could be detected in isolated chloroplasts only during light incubations, and increased with increased rates of fatty acid biosynthesis. In contrast, both acetyl-CoA and acetyl-ACP were detectable in the absence of fatty acid biosynthesis, and acetyl-ACP decreased with increased rates of fatty acid biosynthesis. Together these data have provided direct in situ evidence that acetyl-CoA carboxylase plays a regulatory role in chloroplast fatty acid biosynthesis.

De novo fatty acid biosynthesis in higher plant cells occurs predominantly, if not exclusively, in the plastids, and it is catalyzed by a series of enzymes and ACP[4]. The initial substrates for fatty acid biosynthesis are acetyl-CoA and malonyl-CoA, which are also the immediate precursors for the corresponding acetyl- and malonyl-ACPs. After the initial condensation of acetyl-CoA and malonyl-ACP (13, J.G. Jaworski, D. Post-Beittenmiller, J.B. Ohlrogge, submitted), the intermediates for each step of the fatty acid biosynthetic pathway are acyl-ACPs. Analysis of pools of substrates and intermediates in a pathway can provide direct in vivo evidence needed to identify points of metabolic regulation. Recently, methods have been developed for the analysis of the in vivo levels of ACP-SH and acyl-ACPs, and these methods were used to measure changes in the pool sizes of FAS intermediates during changes in the rates of fatty acid biosynthesis (21). After plants are transferred from the light to the dark, the rate of leaf fatty acid synthesis decreases (2) and is accompanied by a major shift in the pool sizes of acetyl-ACP and ACP-SH (21). The level of acetyl-ACP changes inversely with changes in the rate of fatty acid biosynthesis, increasing 4- to 5-fold in the dark when the rate of fatty acid synthesis is low, and decreasing in the light when the rate is high.

The transfer of the acetyl- or malonyl- moieties from CoA to ACP is catalyzed by acetyl-CoA:ACP transacylase and malonyl-CoA:ACP transacylase, respectively. These reactions are fully reversible, having a thermodynamic equilibrium constant of approximately 2 (33). Although the reversible nature of these reactions suggested that changes in the acetyl- and malonyl-ACP pools would be reflected by similar changes in the acetyl- and malonyl-CoA pools, there have been no in situ measurements available to support this supposition. The rates of these transacylase reactions may determine the availability of substrates for fatty acid biosynthesis. The malonyl transacylase reaction is very rapid in vitro (33), and may, therefore, be near equilibrium in vivo. In contrast, the acetyl transacylase reaction is slow when assayed in vitro (29), and, therefore, is potentially regulatory. However, recent assessments of acetyl-ACP as a primer for in situ chloroplast fatty acid synthesis indicate it to be a very minor substrate

[4] Abbreviations: ACP, acyl carrier protein; GSH-CoA, CoA conjugated to glutathione; FAS, fatty acid synthase; CoA-SH, reduced CoA.

for this pathway (J.G. Jaworski, D. Post-Beittenmiller, J.B. Ohlrogge, submitted). To further examine the metabolic regulation of fatty acid biosynthesis and the in situ activities of the malonyl and acetyl transacylase, we have measured levels of acetyl-CoA, malonyl-CoA, and CoA-SH in isolated chloroplasts and compared these CoA pools to the corresponding ACP pools under conditions with differing rates of fatty acid synthesis.

MATERIALS AND METHODS

Pea (*Pisum sativum*) seed, var Little Marvel, was obtained from Burpee Seeds (Warminster, PA). Spinach (*Spinacia oleracea*) seed, Hybrid No. 7 R Early, was obtained from Sunseeds Genetics, Inc. (Hollister, CA). [1-^{14}C]Acetate, 52.8 mCi/mol, was from Amersham. Percoll, CoA, and its esters were from Sigma. Acetonitrile was of HPLC grade and all other reagents were of analytical grade.

Preparation of Plants and Chloroplasts

Chloroplasts were prepared from spinach grown hydroponically or from pea seedlings grown in soil-less potting media, and in a controlled environment, as previously described (24). Chloroplasts were routinely centrifuged through a cushion of 40% Percoll (18) in low ionic strength buffer (4) to ensure that the preparations contained a high proportion of intact organelles. In some cases, intact chloroplasts were prepared using linear gradients of Percoll (12) in low ionic strength buffer. When chloroplasts recovered by centrifuging through the Percoll cushion were repurified on the Percoll gradient, a single band of intact organelles was resolved. On this basis, we judge our routine chloroplast preparations to be >90% intact. Washed chloroplasts were normally resuspended in the wash buffer to give about 1 mg Chl/mL and were used immediately.

Chloroplast Incubations and FAS Assays

FAS assays were performed according to Roughan (24) with additions as described in the text and with the following modifications. Reactions (0.5 mL) were stopped using an equal volume of 5% TCA rather than chloroform/methanol (1:1), and were placed on ice for 5 to 15 min. Aliquots (50 µL) were removed from the TCA suspension and transferred to 2 mL of chloroform/methanol (1:1), which was then partitioned against 0.9 mL of water. The chloroform phase was recovered, dried, and the lipids saponified in methanolic KOH. Long chain fatty acids were recovered in hexane from the acidified saponification mix and were separated by TLC. [1-^{14}C]Acetate incorporation into long chain fatty acids was determined by liquid scintillation counting. Chl determinations were determined by the method of Arnon (1).

Reverse Phase HPLC Analysis of TCA-Soluble CoAs

CoA-SH and its esters were separated by HPLC using a 4.6 × 250 mm C-18 column (The Nest Group, Southboro, MA) eluted with an interrupted linear gradient of acetonitrile in 0.1 M K-phosphate, pH 5, similar to that described by Corkey (6). Detection was by absorbance at 254 nm. Authentic CoA esters were dissolved at 1 to 2 mg/mL in 10 mM HCl,

and portions of these solutions were diluted 50-fold with 0.1 M K-phosphate, pH 5.0, immediately prior to HPLC analysis. The concentrations of the standards (approximately 25 µM) were determined spectrophotometrically using a millimolar extinction coefficient of 15.4 for absorbance (1 cm light path) at 254 nm. Retention times of the standards were used to identify the peaks on the HPLC tracings. CoA-SH at this concentration and at this pH was significantly oxidized within a few hours at 0°C.

The TCA suspensions from reactions containing [1-^{14}C]-acetate were centrifuged, and 0.85 mL of the supernatants was recovered. Repeated partitioning against diethyl ether (6) removed TCA from the extracts, which were then dried under vacuum. The residues were dissolved in 0.1 M K-phosphate, pH 5, to a final concentration equivalent to 0.5 mg Chl/mL, and aliquots from labeling experiments equivalent to 50 to 100 µg Chl were loaded onto the column. Fractions (1 mL, 1 min) were collected between 24 and 45 min and individually counted to determine radioactivity in the compounds of interest. To confirm that these methods resulted in quantitative recovery of the CoA esters, [1-^{14}C]-acetyl-CoA was added to some of the preparations before TCA precipitation and processing for HPLC analysis. In these preparations, >90% of the radioactivity was recovered in a single peak corresponding to the acetyl-CoA standard.

For the analysis of CoA esters in freshly isolated chloroplasts, washed chloroplasts containing 1 to 2 mg of Chl were resuspended in 1 mL of water and immediately brought to 5% (w/v) with TCA. After centrifuging to separate insoluble material, measured portions of the supernatants were freed of TCA by repeated extractions with diethyl ether and dried under vacuum. The residues were redissolved in K-phosphate, pH 5, so that 1 mL was equivalent to 1 to 2 mg of Chl, and aliquots equivalent to 150 to 250 µg of Chl were injected onto the column. In these cases, the putative CoA esters could be identified by their sensitivity to mild alkali; aliquots (150 µL) of some of the chloroplast extracts were treated with concentrated NH$_4$OH (25 µL) at room temperature for 15 min. The sample was then dried under a stream of N$_2$, the precipitate resuspended in 150 µL of water, and the resulting solution subjected to HPLC analysis. Under these conditions, the acyl-CoAs were hydrolyzed and CoA-SH was oxidized.

PAGE Analysis of ACPs

For analysis of acyl-ACPs, the TCA-precipitated proteins from chloroplast incubations were pelleted in a microfuge at 4°C. The ACPs were redissolved in 50 mM Mops, pH 7.6, and 10 mM N-ethylmaleimide, and were separated on 1.0 or 5.0 M urea polyacrylamide gels. The ACPs were visualized using either immunoblotting techniques and antibody directed against spinach ACP, or direct autoradiography, as previously described (21).

RESULTS AND DISCUSSION

Essentially all of the CoA Present in Freshly Isolated Chloroplasts Is Acetyl-CoA

Reverse phase HPLC provided a convenient and accurate means to simultaneously resolve and quantitate CoA and its

short chain acyl-esters present in extracts of isolated chloroplasts and whole leaf. Figure 1A represents a typical chromatogram of several CoA standards. With increasing concentrations of acetonitrile, malonyl-CoA eluted first, followed by GSH-CoA, CoA-SH, and acetyl-CoA. CoA-S-S-CoA and acetoacetyl-CoA eluted close together immediately after acetyl-CoA, whereas methylmalonyl-, succinyl-, and hydroxymethylglutaryl-CoAs eluted sequentially between CoA-SH and acetyl-CoA (6). CoA and its esters in chloroplast and leaf extracts were identified by coelution with CoA standards and by their sensitivity to mild alkali treatment with ammonium hydroxide. Quantitation was by peak height with reference to standards of known concentration.

In freshly isolated chloroplasts, essentially all of the TCA-soluble CoA detected was found in the form of acetyl-CoA (Fig. 1B). Malonyl-CoA, GSH-CoA, and CoA-SH were not detected. As shown in Figure 1B, several UV-absorbing peaks were observed which did not coelute with the standards shown in Figure 1A or with other standard CoA esters. These compounds are not believed to be CoA esters since they were unaffected by treatment with mild alkali, did not become labeled when chloroplasts were incubated with [1-^{14}C]acetate, and their masses increased dramatically with time when isolated chloroplasts were incubated at 25°C in media containing no CoA. For instance, the unknown peak eluting immediately before authentic CoA-SH (Fig. 1B) increased in
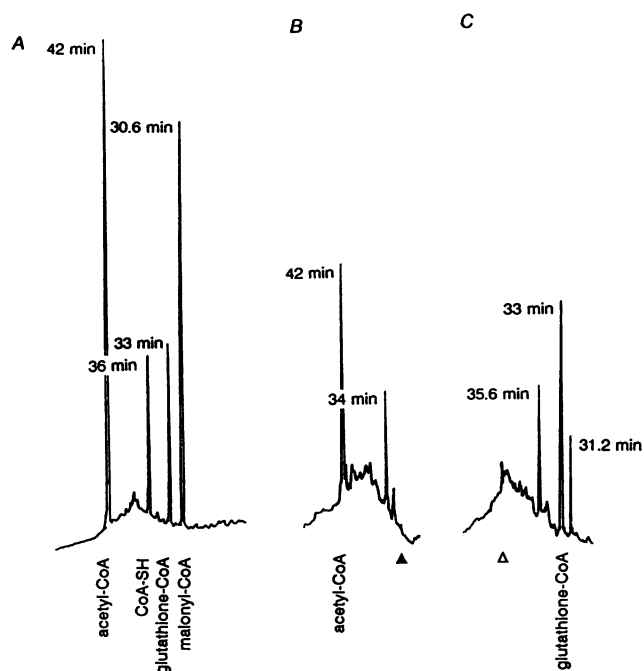

Figure 1. HPLC separations of CoA standards (A) and of TCA-soluble extracts prepared from fresh pea chloroplasts before (B) and after (C) mild NH$_4$OH treatment. Acetyl-CoA, CoA-SH, GSH-CoA, and malonyl-CoA peaks are indicated. Absorbance unit full scale at 254 nm = 0.02. The amounts of chloroplast extracts loaded were equivalent to 79 μg of Chl, and the acetonitrile gradient elution is from right to left. The solid arrowhead indicates the time at which GSH-CoA elutes, and the open arrowhead indicates the time at which acetyl-CoA elutes.

size 4- to 5-fold during the 5-min incubation in the light at 25°C, obscuring detection of endogenous CoA-SH. Acetoacetyl-CoA and hydroxymethylglutaryl-CoA, both precursors of isoprenoids, were not detected either in freshly isolated or in incubated chloroplasts.

The acetyl-CoA peak disappeared when extracts were treated with NH$_4$OH and it was replaced with a peak of comparable height corresponding to GSH-CoA (Fig. 1, B and C). In standard preparations of acetyl-CoA treated with NH$_4$OH, the released CoA is oxidized and forms CoA-S-S-CoA. However, because the chloroplast extracts may contain 100-fold more reduced glutathione (3–5 mM [16]) than the total pool of TCA-soluble CoAs, GSH-CoA, rather than CoA-S-S-CoA, was formed after the NH$_4$OH treatment. Therefore, although CoA-S-S-CoA was detected in aging solutions of reference CoA-SH, it was never detected in extracts of fresh or incubated chloroplasts. That the amount of the newly created GSH-CoA was 88 to 100% of the original acetyl-CoA peak (Fig. 1, B and C) and never larger than the original acetyl-CoA peak provided further evidence that essentially all of the TCA-soluble CoA within chloroplasts was esterified to acetate. CoA-SH and its other esters, if present, would have contributed to the GSH-CoA peak. Levels of acetyl-CoA ranged from 0.8 to 0.9 nmol/mg Chl in freshly isolated spinach chloroplasts and from 1.2 to 1.4 nmol/mg Chl in pea chloroplasts, whether the organelles were prepared using the Percoll cushion or Percoll gradient procedures. Assuming a stromal volume of 26 μL/mg Chl (10), the concentration of acetyl-CoA in the chloroplast was calculated to be 31 to 35 μM (spinach) and 46 to 54 μM (pea).

Turnover of Acetyl-CoA in the Light

When incubated under conditions optimal for fatty acid biosynthesis, isolated chloroplasts can incorporate [1-^{14}C]-acetate into fatty acids at rates of 1 to 2 μmol/h/mg Chl (8, 23, 25, 28), which are equivalent to the in vivo rates of fatty acid synthesis in intact spinach leaves (2, 19). Under these incubation conditions, chloroplasts had levels of acetyl-CoA somewhat lower than the acetyl-CoA levels detected in freshly isolated chloroplasts. The acetyl-CoA concentration was 0.5 to 0.7 nmol/mg Chl (19–27 μM) in light-incubated spinach chloroplasts and 0.6 to 0.9 nmol/mg Chl (23–35 μM) in light-incubated pea chloroplasts. In incubations with [1-^{14}C]acetate, acetyl- and malonyl-CoA concentrations were also determined by collecting peak fractions and measuring their radioactivity by liquid scintillation counting. Calculations of acetyl-CoA concentration either by UV absorbance or by [1-^{14}C]acetate incorporation were comparable at each point on a time course of fatty acid synthesis, indicating that the acetyl-CoA pool was labeled to essentially the same specific radioactivity as that of the supplied [1-^{14}C]acetate. This is the first direct proof that the specific radioactivity of the endogenous acetyl-CoA is the same as that of the supplied [1-^{14}C]acetate. In fact, the supplied [1-^{14}C]acetate equilibrated with the endogenous acetyl-CoA in isolated spinach chloroplasts within 10 s of illumination (Fig. 2). However, the calculated turnover rate is even higher than this. Acetate is incorporated into fatty acids at rates of 1 to 2 μmol/h·mg Chl or 0.3 to 0.6 nmol/s·mg Chl, and the acetyl-CoA con-
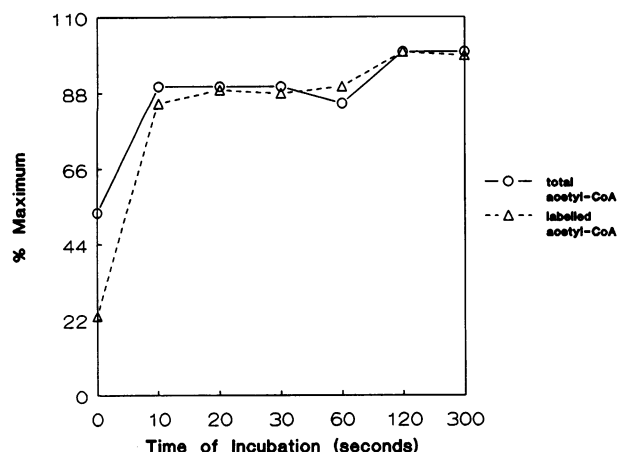
Figure 2. [1-^{14}C]Acetate labeling of acetyl-CoA in light-incubated chloroplasts. Values given are percent of maximum level of either total acetyl-CoA as determined by UV adsorbance or [1-^{14}C]acetyl-CoA as determined by direct liquid scintillation counting of HPLC fractions in this labeling experiment.

centration is 0.5 to 0.9 nmol/mg Chl, so that the total pool of acetyl-CoA (and, therefore, CoA) is equivalent to 1 to 2 s of fatty acid synthesis in the light. (Note that the total pool of ACP in spinach chloroplasts was calculated [30] to be equivalent to 5 s of fatty acid synthesis.) Furthermore, since acetyl-CoA concentrations in incubated chloroplasts were lower than those from freshly isolated chloroplasts and remained constant during fatty acid synthesis, the concentration of acetyl-CoA was not artificially increased in the presence of exogenous acetate. As with freshly isolated chloroplasts, surprisingly, CoA-SH was not detected in incubated chloroplasts. We estimate that the minimum peak of CoA-SH that could be confidently identified in these experiments corresponds to 2 μM.

Both Malonyl-CoA and Acetyl-CoA Are Present in Light-Incubated Chloroplasts

In contrast to freshly isolated chloroplasts, malonyl-CoA was detectable in chloroplasts incubated in the light. However, because the levels of malonyl-CoA were low, it was difficult to measure accurately the malonyl-CoA concentration by UV absorption. Nonetheless, in light-incubated chloroplasts, the levels of malonyl-CoA could be estimated by [1-^{14}C]acetate incorporation. In spinach chloroplasts, the level of malonyl-CoA ranged from 0.01 to 0.07 nmol/mg Chl (0.4–3 μM). These levels were 7- to 70-fold lower than the corresponding acetyl-CoA levels. Because the thermodynamic equilibrium constant for acetyl-CoA carboxylase favors malonyl-CoA formation (32), the low ratio of malonyl-CoA/acetyl-CoA observed in these experiments indicates that the acetyl-CoA carboxylase reaction is substantially displaced from equilibrium, consistent with its role as a regulatory enzyme (22).

The concentration and distribution of CoA and its esters described above for chloroplasts differs substantially from that reported for *Escherichia coli*, for animal tissues, or for

plant mitochondria. Total CoA concentrations in *E. coli* are reported to be over 300 μM (5). The concentration of total CoA in liver was estimated to be 300 to 500 μM (17) or 80 to 100 μM (9). Budde and coworkers (3) measured CoA pools in pea mitochondria that had been incubated in buffer containing CoA and found that in nonrespiring mitochondria, the total CoA was 150 μM, and in respiring mitochondria, it was 300 μM. In these studies, essentially all of the CoA pool was in the nonesterified form in nonrespiring mitochondria, whereas during respiration, as much as 88% of the total CoA was in the form of acetyl-CoA. Therefore, in incubated mitochondria, there is a major shift in the form of CoA depending on the state of the mitochondria. In contrast, isolated chloroplasts have much lower levels of total CoA, and none of the CoA detected was in the form of CoA-SH. Even when chloroplasts were incubated in the presence of 500 μM exogenous CoA, the acetyl-CoA level increased less than 2-fold (data not shown).

Acetyl-CoA Concentrations Are Maintained in the Dark

Within 2 to 3 min of transferring incubated spinach chloroplasts to the dark, acetyl-CoA concentrations decreased 30 to 50%, but then returned to their original level during the following 6 to 8 min in the dark (Table I). Concomitant with the decrease in concentration was a decrease in the specific radioactivity of acetyl-CoA. Recovery of the specific radioactivity to equal that of the supplied [1-^{14}C]acetate (Table I) indicated that acetyl-CoA was being synthesized from [1-^{14}C]acetate in the dark. Presumably, there was sufficient ATP in dark chloroplasts to drive the acetyl-CoA synthetase reaction. We do not have a plausible explanation at this time for the transient decrease in specific radioactivity.

Table I. *Transient Decreases in Acetyl-CoA Concentrations and Specific Radioactivities When Illuminated Chloroplasts Are Transferred to the Dark*

Chloroplasts were incubated in the basal buffer for 2 min and then transferred to total darkness for the times indicated. For comparison, one pair of tubes was incubated for 7 min in continuous light. The specific radioactivity of the supplied acetate was 116 × 10^3 dpm/nmol.

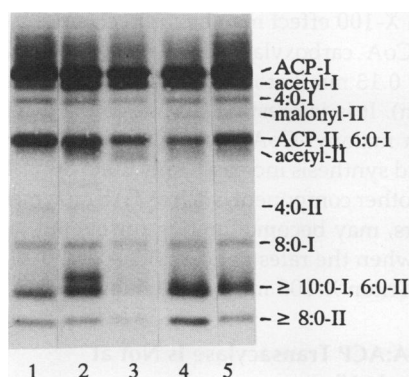| Incubation Time	Acetyl-CoA	Acetyl-CoA
min	pmol/mg Chl[a]	dpm × 10^{-3}/nmol
7 light	522	105
2 light, 1 dark	319	79
2 dark	305	69
3 dark	290	50
4 dark	319	77
5 dark	435	84
6 dark	465	99
7 dark	508	99
8 dark	493	100
9 dark	493	118
10 dark	508	116

[a] Calculated from peak heights.

Figure 3. Comparison of acyl-ACP profiles from spinach leaf and chloroplasts. Acyl-ACP extracts of spinach chloroplasts and leaf were separated using 1 M urea PAGE and analyzed by immuno-blotting techniques. Lane 1, Light-grown spinach leaf; lane 2, fresh chloroplasts; lane 3, dark-grown spinach leaf; lane 4, light-incubated chloroplasts with added acetate; lane 5, light-incubated chloroplasts without added acetate. Roman numerals in the figure refer to the isoform of spinach ACP.

Most of the Acetyl-CoA in Leaves Is Localized in the Chloroplasts

To examine the proportion of total leaf acetyl-CoA associated with chloroplasts, leaf disks (50–80 mg fresh weight) were sampled from spinach leaves during the light portion of a normal growing cycle. The samples were ground in ice-cold 2.5% (w/v) TCA and prepared for HPLC analysis as described for chloroplast preparations. The concentration of acetyl-CoA in total light-grown leaf preparations ranged from 1.08 to 1.11 nmol/mg Chl. Comparison of these values with the acetyl-CoA levels in freshly isolated chloroplasts (0.8–0.9 nmol/mg Chl) indicates that approximately 77% of the total leaf acetyl-CoA can be attributed to the chloroplasts. Thus, in comparison to chloroplasts, the pools of acetyl-CoA in leaf mitochondria, cytoplasm, or other compartments are quantitatively minor. In addition, because most of the leaf acetyl-CoA could be accounted for by that recoverable in chloroplasts, it is unlikely that any substantial proportion of the acetyl-CoA was lost from the chloroplasts during the 5- to 10-min period required for their isolation. In this regard, chloroplasts are similar to mitochondria, which lose their endogenous CoA very slowly (<25% in 48 h) when stored on ice (3, 20).

As with isolated chloroplasts, CoA-SH, GSH-CoA, and malonyl-CoA were not detected in any of the total leaf samples. However, due to the presence of many UV-adsorbing peaks in the HPLC trace, low levels of these CoAs may have been obscured in total leaf samples. CoA ester standards were added to aliquots of some of the leaf samples in an attempt to identify minor peaks, but the results were inconclusive.

Comparisons of Spinach Chloroplast and Leaf Acyl-ACP Profiles

We have recently described the pattern of acyl-ACPs present in spinach leaves during growth in either the light or the dark (21). The acyl-ACP profiles of isolated chloroplasts were determined by urea-PAGE immunoblots and compared to the profiles of intact spinach leaf. In freshly isolated spinach chloroplasts, there were significant levels of acetyl-ACP, but no detectable malonyl-ACP (Fig. 3), a pattern that is similar to leaf during the dark portion of the growth cycle (lane 3). Furthermore, when examined on 5.0 M urea gels (data not shown), there were only very low levels of long chain acyl-ACPs, which is in agreement with work previously reported by Roughan and coworkers (27, 30) demonstrating the light dependency of long chain acyl-ACP concentrations in isolated chloroplasts. In addition, there were some unidentified acyl-ACP bands in freshly isolated chloroplasts that are not found in spinach leaf (Fig. 3, cf. lanes 1 and 2).

The acyl-ACP profiles of chloroplasts incubated in the light with or without added acetate were more similar to profiles from spinach leaf in the light (Fig. 3, compare lanes 1, 4, and 5). Furthermore, incubated chloroplasts (lanes 4 and 5) had a moderately lower level of acetyl-ACP compared to fresh chloroplasts (lane 2), but a somewhat higher level when compared to light-grown leaf (lane 1). Although there are some minor quantitative differences in relative abundance, the patterns of both the long and short chain acyl-ACPs are very similar for both leaves and chloroplasts.

Rates of Fatty Acid Biosynthesis Reflect Changes in the Pools of Acetyl- and Malonyl-CoA and Acetyl- and Malonyl-ACP

Data presented above and previously (21) indicate that regulation of acetyl-CoA carboxylase activity, at least in part, can account for differences in the rates of fatty acid biosynthesis between light and dark spinach leaf. To determine whether changes in acetyl-CoA carboxylase activity can also account for changing rates of fatty acid biosynthesis in isolated chloroplasts, we examined the influence of Triton X-100 treatment and the transition from light to dark on the pools of acetyl- and malonyl-CoA and the corresponding acyl-ACPs.

Low concentrations of Triton X-100 (0.5–0.7 μmol/mg Chl) in the incubation media significantly stimulate rates of long chain fatty acid synthesis in chloroplasts isolated from spinach (25, 28, 31) and pea (8) apparently without affecting the permeability of the plastid envelope. Spinach chloroplasts were incubated with or without added Triton X-100, and the levels of TCA-soluble CoAs and TCA-precipitable acyl-ACPs were measured. In the presence of Triton X-100, the rate of

Table II. Effects of Triton X-100 Addition on Acetyl-CoA and Malonyl-CoA Pools in Light-Incubated Spinach Chloroplasts

Chloroplasts were incubated in the light with or without Triton X-100 (0.13 mM) for 5 min. CoAs were measured as described in "Materials and Methods."

Treatment	FAS Rate[a]	Acetyl-CoA[b]	Malonyl-CoA	Total CoA
Control	978	0.67	0.01	0.68
(+) Triton	1763	0.51	0.12	0.63

[a] FAS rates are nmol acetate incorporated·mg^{-1} Chl·h^{-1}. [b] Values for CoAs are nmol/mg Chl.

fatty acid biosynthesis increased from 30 to 80% over control rates, depending on the chloroplast preparation. The results of a representative experiment are presented in Table II. The levels of malonyl-CoA changed in direct relationship with the rates of fatty acid biosynthesis: i.e. with increasing rates of synthesis, there were significant increases in the levels of malonyl-CoA. In contrast to the changes in malonyl-CoA concentrations, the level of acetyl-CoA changed inversely with the rate of synthesis, decreasing in the presence of Triton X-100. Decreases in acetyl-CoA levels were compensated, in part, by increases in malonyl-CoA levels, maintaining the total TCA-soluble CoA concentration. There were no other CoA esters or CoA-SH detected.

The corresponding acyl-ACP pools in Triton X-100-stimulated chloroplasts or in chloroplasts shifted from light to dark were also examined by autoradiographic analysis (Fig. 4). The increased rate of fatty acid biosynthesis in Triton-treated chloroplasts (about 30%, in this experiment) was accompanied by increases in the levels of malonyl-ACPs (Fig. 4, compare control to Triton-treated), similar to the increased level of malonyl-CoA (Table II). The levels of acetyl-ACP and acetyl-CoA decreased slightly, between 11 and 24%, respectively (Fig. 4; Table II). In chloroplasts that were incubated in the light and then shifted to the dark, there was a marked decrease in the malonyl-ACP levels and a concomitant increase in the levels of acetyl-ACP. These trends continued with increasing time in the dark (cf. 2 and 10 min). Immunoblot and UV/HPLC analyses confirmed that changes in the radioactivity reflect changes in pool sizes (data not shown). Therefore, there was a direct relationship between increases in fatty acid biosynthetic rates and increases in both malonyl-CoA and malonyl-ACP levels, whereas there was an inverse relationship between the rates of fatty acid synthesis and acetyl-CoA and acetyl-ACP levels. These results together suggest that the mechanism of Triton X-100 stimulation of chloroplast fatty acid biosynthesis may be through increased acetyl-CoA carboxylase activity, as suggested for changes in the rates of synthesis in light and dark leaf (21).
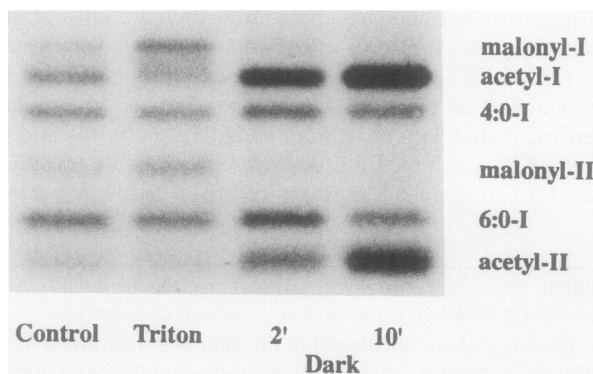

Figure 4. Autoradiograph of spinach [^{14}C]acyl-ACPs separated on a 1 M urea gel. Spinach chloroplasts were incubated under conditions that result in differing rates of fatty acid synthesis: In the light for 2 min without added Triton X-100 (Control); or with Triton X-100; or incubated in the light for 2 min and then shifted to the dark for either 2 or 10 min. Roman numerals in the figure refer to the isoform of spinach ACP.

The Triton X-100 effect may be indirect, since in vitro assays of acetyl-CoA carboxylase do not reveal a stimulation of activity by 0.13 mM Triton X-100 (P. Roessler, personal communication). It is interesting that despite a 5-fold or greater increase in malonyl-CoA and malonyl-ACP levels, the rate of fatty acid synthesis increases only 30 to 80%. This suggests that some other component, such as FAS enzymes, substrates, or cofactors, may become limiting for overall fatty acid biosynthesis when the rates of acetyl-CoA carboxylase are stimulated by Triton X-100 in isolated chloroplasts.

Acetyl-CoA:ACP Transacylase Is Not at Equilibrium in Vivo

In spinach leaf, the in vivo levels of acetyl-ACP change 4- to 5-fold during the transition from light to dark (21). In the dark, when the rates of fatty acid biosynthesis are low, the level of acetyl-ACP increases, and in the light, when the rates are high, the level of acetyl-ACP decreases. If the acetyl-CoA:ACP transacylase reaction were near equilibrium in vivo, then the concentration of acetyl-ACP would reflect the concentration of acetyl-CoA. The thermodynamic equilibrium constant for the acetyl-CoA:ACP transacylase reaction has been reported to be approximately 2 (33). However, in light-incubated spinach chloroplasts, the level of acetyl-CoA was found to be 20- to 30-fold higher than the level of acetyl-ACP. We have estimated that the concentrations of substrates and products for this reaction are 19 to 27, 0.5 to 1, 3 to 6, and <2 μM for acetyl-CoA, acetyl-ACP, ACP-SH, and CoA-SH, respectively, in the light (21, and this work). Therefore, the mass action ratio is <0.01, indicating that the reaction is far from equilibrium. In dark-grown leaves or dark-incubated chloroplasts, the level of acetyl-ACP increases and the level of ACP-SH decreases such that the mass action ratio increases to approximately 0.15, but the reaction is still substantially displaced from equilibrium. In contrast, a similar comparison between malonyl-CoA and malonyl-ACP levels indicates that malonyl-CoA:ACP transacylase is much closer to equilibrium in vivo in either the light or the dark.

Our previous interpretation of the 4- to 5-fold increase in acetyl-ACP concentration that occurs in the dark was that it reflected a similar increase in acetyl-CoA levels brought about by a decrease in acetyl-CoA carboxylase activity. However, the data in this study demonstrate that major increases in acetyl-ACP pools can occur without similar changes in the acetyl-CoA pools. For this reason, it now seems likely that the increase in the acetyl-ACP pool that occurs in the dark is the result of the acetyl-CoA:ACP transacylase reaction coming closer to equilibrium. We estimate that there is at least a 10-fold difference in the mass action ratio for the transacylase reaction between light and dark conditions.

Based on in vitro assays (29), the acetyl-CoA:ACP transacylase reaction is the slowest reaction of de novo fatty acid biosynthesis. Thus, it is perhaps not surprising that the reaction catalyzed by acetyl-CoA:ACP transacylase is far from equilibrium in vivo. Further evidence related to this observation and to the involvement of acetyl-ACP in fatty acid metabolism has recently been obtained. Jaworski and coworkers (submitted) have shown that the flux of carbon through the acetyl-ACP pool is at least 10-fold slower than

the rate of fatty acid synthesis. Thus, acetyl-ACP appears to play only a minor role as a primer for fatty acid synthesis. However, acetyl-ACP levels do decrease in the light, and it appears likely that this is the result of the slow utilization of acetyl-ACP by 3-ketoacyl-ACP synthase-I. In the light, when malonyl-ACP is available, acetyl-ACP can serve as a substrate (although slowly) for condensation by 3-ketoacyl-ACP synthase-I. In the dark, acetyl-ACP utilization is further slowed by the absence of malonyl-ACP and, therefore, the pool size of acetyl-ACP increases, bringing the reaction closer to equilibrium. Thus, the major light/dark changes in acetyl-ACP pools reflect a reduction in malonyl-ACP availability brought about by changes in acetyl-CoA carboxylase activity.

CONCLUSIONS

Despite their fundamental role in metabolism, very little information is available on the absolute concentration of CoA and acetyl-CoA in plant tissues. Results from this study indicate that the major form of CoA in both chloroplasts and whole leaves is acetyl-CoA. In addition, the levels of acetyl-CoA do not differ greatly in light and dark. Thus, it is likely that the concentration of this key metabolite of intermediary metabolism is maintained at a relatively constant level, providing a pool of precursors for fatty acid, amino acid, isoprenoid, and other biosynthetic pathways. Furthermore, it seems unlikely that modulation of acetyl-CoA levels in leaves is a major form of metabolic regulation of these pathways in chloroplasts.

The major use for acetyl-CoA in chloroplasts is for fatty acid biosynthesis via acetyl-CoA carboxylase. Although numerous previous studies have suggested a rate-determining role for acetyl-CoA carboxylase in the regulation of rates of fatty acid biosynthesis (7, 15, 26; for review see ref. 11), convincing evidence for this hypothesis has been lacking. Examination of the changes in pool sizes of intermediates in a pathway can provide in situ evidence for points of metabolic regulation. Regulatory steps are characterized by increases in their substrate concentration and decreases in product concentration when the flux through the pathway is reduced. In contrast, pools of substrates of nonregulatory enzymes in a pathway frequently show little change or decrease in concentration when the flux is reduced (22). Results from this study provide in situ evidence that directly implicates the role of acetyl-CoA carboxylase in regulation of plastid fatty acid biosynthesis. Malonyl-CoA levels were undetectable in dark-incubated chloroplasts, but rose manyfold in the light and increased further when fatty acid synthesis was stimulated in the light by Triton X-100. In addition, the level of acetyl-CoA was reduced concomitantly with the increases in malonyl-CoA. Thus, the changes in both the substrate and product pools of the acetyl-CoA carboxylase reaction provide direct evidence for its role in determining the flux through the fatty acid biosynthetic pathway.

ACKNOWLEDGMENTS

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