Plant Physiol. (1986) 82, 448–453
0032-0889/86/82/0448/06/$01.00/0

Activity of Acyl Carrier Protein Isoforms in Reactions of Plant Fatty Acid Metabolism

Received for publication May 6, 1986

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ABSTRACT

Two forms of spinach acyl carrier protein (ACP-I and ACP-II) have recently been characterized and found to be expressed in a tissue-specific manner (JB Ohlrogge, TM Kuo, 1985 J Biol Chem 260: 8032). To examine possible different functions for these ACP isoforms, we have tested purified preparations of spinach leaf ACP-I and ACP-II and *Escherichia coli* ACP in several *in vitro* reactions of fatty acid metabolism. Total *de novo* fatty acid synthesis and malonyl-CoA:ACP transacylase do not appear to discriminate between acyl carrier protein isoforms. In contrast, the K_m of oleoyl-ACP thioesterase for oleoyl-ACP-II is 10-fold higher than for oleoyl-ACP-I, whereas the K_m of acyl-ACP glycerol-3-phosphate acyl transferase is 5-fold higher for oleoyl-ACP-I than for oleoyl-ACP-II. A characterization of these reactions and a possible role for ACP isoforms in regulation of fatty acid metabolism in plants are described.

Fatty acid synthesis in higher plant plastids requires the presence of acyl carrier protein. The growing acyl chain, during synthesis of C16 and C18 fatty acids, is bound to ACP[1] via a phosphopantetheine prosthetic group. Recent studies of plant ACP have revealed the presence of at least two isoforms in barley (8) and spinach leaves (17). In spinach and castor beans, two major isoforms are expressed in leaves, whereas seeds of these species apparently contain only one major isoform (17). N-terminal amino acid sequence data from barley and spinach, together with amino acid compositions and antibody binding, strongly suggest these isoforms are products of distinct genes (8, 17).

These observations of multiple forms of plant ACP raise questions regarding their physiological significance. A possible function for different ACP structures might involve the different reactions in which ACP must participate. In addition to the six reactions which make up the *de novo* fatty acid synthesis pathway, ACP is also required for desaturation of stearate (13), release of oleate by oleoyl-ACP thioesterase (14, 26), and acyl transfer to glycerol-3-P and monoacylglycerol-3-P (2, 3). In addition, it is known that the elongation of palmitate to stearate requires a different β-ketoacyl-ACP synthetase than that used to assemble the first 16 carbons (24). A recent report from our laboratory demonstrated the existence of two forms of malonyl-CoA:ACP transacylase from soybean (4). The ACP isoforms may vary in reactivity in the reactions presented above and thereby control could be exerted over acyl chain metabolism by manipulation of ACP isoform expression. To investigate this hypothesis we have compared spinach leaf ACP-I and ACP-II in several reactions of plant fatty acid metabolism.

An additional purpose of this study is to further evaluate the activity of *Escherichia coli* ACP with plant fatty acid enzymes. Most *in vitro* studies of plant fatty acid metabolism have used *E. coli* ACP rather than plant ACP as a cofactor. This compromise has been adopted primarily because *E. coli* ACP can readily be purified in good yield, whereas plant ACP is much more difficult to obtain. Although ACP is clearly a highly conserved protein, *E. coli* ACP is only partially cross-reactive with antibodies to spinach ACP (10, 15) and their amino acid sequences are only 40% homologous (11). These data suggest that prokaryotic and eukaryotic ACP may have significant structural differences. The validity of using *E. coli* ACP to study plant enzymes is based primarily on the observations of Simoni *et al.* (27) that bacterial ACP was an effective cofactor for spinach fatty acid synthesis and yielded products similar to those from reactions utilizing plant ACP. However, Simoni *et al.* (27) also observed that plant ACP added to bacterial fatty acid synthetase yielded lower rates of synthesis and an abnormal product distribution. Therefore, ACP structure may, in some instances, strongly influence fatty acid biosynthetic reactions. To address this possibility, we have compared *E. coli* and spinach ACP in several reactions catalyzed by enzymes of plant fatty acid metabolism.

MATERIALS AND METHODS

Reagents and Chromatographic Supports. [2-^{14}C]Malonyl-CoA (45.5 mCi/mmol), [U-^{14}C]palmitic acid (800 μCi/μmol), and [U-^{14}C]oleic acid (900 μCi/μmol) were obtained from New England Nuclear.[2] DEAE cellulose was obtained from Whatman, Inc., octyl sepharose CL4B was from Pharmacia, Accel QMA was from Millipore (Waters and Assoc.), and Affigel 15 was from Bio-Rad. NADPH, NADH, acetyl-CoA, and malonic acid were purchased from Sigma, Inc., and DTT was obtained from Aldrich. Mes and Tricine were from Research Organics, Inc. All other chemicals and reagents were of reagent grade or better.

Plant Sources and Culture. Spinach (*Spinacia oleracea* L. Hybrid 424) was grown under a 10 to 12 h photoperiod at 20°C in a greenhouse. Plants were watered daily with a 20-20-20 commercial fertilizer solution fortified with 50 μM Fe^{3+}-sequestrene. Achene tissue was obtained from spinach plants subjected to 1 week of continuous illumination using four 400 W metal halide lamps (PPFD 900 μmol m^{-2} s^{-1} at canopy level). Castor

[1] Abbreviations: ACP, acyl carrier protein; FAS, fatty acid synthesis; MCT, malonyl-CoA:ACP transacylase.

[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the United States Department of Agriculture over other firms or similar products not mentioned.

bean plants (*Ricinus communis* L. var. Baker 296) were grown under continuous illumination at 28°C in a greenhouse. Solar radiation was supplemented with one 1000 W metal halide lamp plus four 400 W metal halide lamps (PPFD 1000 μmol m^{-2} s^{-1} at canopy level).

Fatty Acid Synthesis Enzyme Sources. All procedures were conducted at 4°C.

Malonyl-CoA:Acyl Carrier Protein Transacylase. The following procedures are similar to those outlined in Høj and Svendsen (7). One hundred fifty g castor bean leaf tissue (approximately 40 d old) were powered with liquid N_2 and then homogenized in 0.1 M phosphate, 2 mM DTT, 2 mM EDTA, 20% glycerol, and 15% (w/w) insoluble PVP (pH 7.5). The homogenate was passed through two layers of cheesecloth, centrifuged at 5,000*g* for 30 min, and the supernatant was adjusted to 45% $(NH_4)_2SO_4$ saturation.

After centrifugation at 8,000*g* for 45 min, the pellet was discarded and the supernatant adjusted to 80% $(NH_4)_2SO_4$ saturation. Subsequent centrifugation at 8,000*g* for 45 min was followed by resuspending the pellet in 25 mM Tris-HCl, 2 mM DTT, 20% glycerol (pH 7.8), and dialysis against the same buffer overnight (2 buffer changes, 100 volumes each). The dialyzed 45 to 80% $(NH_4)_2SO_4$ fraction was loaded onto a preequilibrated (dialysis buffer) DEAE-cellulose (DE53) column (2.5 cm × 14.0 cm). The flow-through was collected, precipitated with 90% $(NH_4)_2SO_4$, and centrifuged. The pellet was resuspended in 25 mM Tris-HCl, 2mM DTT, 20% glycerol, and dialyzed and stored at −20°C. This preparation was designated MCT_1 as previously described (4). The DE53 column was washed with Tris buffer and subsequently developed with a linear 0.0 to 0.3 M LiCl gradient. Active fractions from the salt gradient were pooled, $(NH_4)_2SO_4$ precipitated, and dialyzed as above. This fraction was designated as MCT_2 (4). The isolation of spinach leaf MCT was similar to that for castor bean, except that activity was not found in the flow-through of the DE53 column. Spinach MCT was pooled from the salt gradient, $(NH_4)_2SO_4$ precipitated, dialyzed and stored at −20°C.

Oleoyl-ACP Thioesterase. One hundred *g* spinach leaves were powdered with liquid N_2 and homogenized in 20 mM phosphate, 2 mM DTT, 1 mM EDTA, and 20% insoluble PVP (w/w) (pH 7.5). Subsequent isolation procedures are similar to those outlined by McKeon and Stumpf (13). Spinach achene tissue (developing fruit) was fractionated in the same manner as leaf tissue except that chromatography was performed on a QMA-Accell ion exchange column and thioesterase was eluted with a linear 0.0 to 0.4 M LiCl gradient.

Fatty Acid Synthetase. Two hundred g spinach leaves were gently homogenized (Tekmar homogenizer, 0.5 speed for 2 min) in 0.1 M Tricine and 0.6 M glycerol (pH 9.0). This and all subsequent steps are similar to those outlined in Høj and Mikkelsen (6). The homogenate was twice squeezed through 1 layer of 31 μm nylon gauze and centrifuged for 5 min at 4,000*g*. The pellet was suspended in 0.1 M Tricine, 0.3 M glycerol, 2.0 mM DTT, 1 mM $MgCl_2$ (pH 9.0), and subjected to a French pressure cell to rupture the plastids. Following centrifugation at 220,000*g* for 45 min, the straw-colored supernatant was brought to 45% $(NH_4)_2SO_4$ saturation and centrifuged at 10,000*g* for 45 min. The resultant supernatant was further fractionated to 70% $(NH_4)_2SO_4$ saturation and the pellet obtained after centrifugation was dissolved and dialyzed in 0.1 M Tricine, 0.3 M glycerol, 2 mM DTT, 1 mM $MgCl_2$ (pH 8.8), and subsequently stored at −20°C.

Acyl-ACP Glycerol-3-P Acyltransferase. The procedures outlined in (3) were used to isolate the transferase from spinach leaf tissue.

Acyl Carrier Proteins. The procedures described in Refs. (11) and (17) were used to purify spinach ACP-I and ACP-II to electrophoretic homogeneity except that the heat step was omitted. ACP concentrations were assayed with *E. coli* acyl-ACP synthetase as described previously (12, 16). Isolation of *E. coli* ACP was as described in Ref. (19) and the preparation appeared to be 80% homogeneous by SDS-PAGE.

[^{14}C]Oleoyl-ACP Synthesis and Purification. The procedures outlined in Ref (12) and (18) were used to enzymically synthesize and subsequently purify [^{14}C]oleoyl-ACP. The reaction mixture contained 100 μM fatty acid (10.0% [U-^{14}C]oleic acid, 100 mCi/mmol; 90% unlabeled oleic acid in 10% Triton X-100), 5 mM ATP, 10 mM $MgCl_2$, 2 mM DTT, 0.4 M LiCl, 0.1 M Tris (pH 8.0), 2.0% Triton X-100, acyl-ACP synthetase purified from *E. coli* (18), and 16 μM ACP (either *E. coli*, Spinach I or Spinach II). The reaction was allowed to proceed for 6 h at 37°C and was terminated either by freezing or dilution (20-fold) with 10 mM Mes in 20% isopropanol. Acyl-ACP products were isolated by chromatography on DEAE-cellulose followed by octyl-Sepharose.

Enzyme Assays. *Malonyl-CoA:ACP Transacylase.* Assays were conducted according to a previously published procedure (4). Briefly, each reaction mixture possessed 100 μM malonic acid, 0.1 M Tricine (pH 8.5), 9.7 μM [2-^{14}C]malonyl-CoA, 6.0 μM ACP, and the appropriate amount of enzyme. Reactions were terminated after 1 min at 20°C with ice cold 20% TCA. Precipitated [^{14}C]malonyl-ACP was isolated on glass fiber disc filters and radioactivity was determined.

Oleoyl-ACP Thioesterase. The standard assay for this enzyme contained 1.4 μM oleoyl-ACP (6600 dpm), 0.1 M Tris (pH 8.0), and an appropriate amount of enzyme (13). The reaction was allowed to proceed for 30 min at 20°C after which it was terminated with an equal volume (40 μl) 1 N glacial acetic acid. The acidified mixture was then extracted twice with diethyl ether plus 1.0% unlabeled oleic acid. The ethereal extracts were combined and their radioactivity determined by scintillation counting. Background radioactivity determined with reaction mixtures lacking enzyme was subtracted from the total.

Fatty Acid Synthesis. The standard reaction mixture contained 5.0 μmol Na_2PO_4 (pH 7.9), 1.0 μmol NADPH, 20.0 nmol acetyl-CoA, 2.0 μmol ATP, 1.0 μmol $MgCl_2$, 64 nmol [2-^{14}C]malonyl-CoA (132,000 dpm), and 10 μM ACP as outlined in Ref. (6). The reaction proceeded for 15–60 min at 27°C and was then terminated with 40% KOH plus unlabeled oleic acid (1%) as carrier. After a 40 min saponification at 80°C the reaction mixture was acidified with 40% H_2SO_4 and subsequently extracted three times with hexane. The radioactivity of the combined hexane extracts was determined by scintillation counting and background radioactivity was determined from reaction mixtures lacking FAS enzyme preparation.

Radio HPLC Analysis of FAS Products. Hexane extracts from the FAS reactions were evaporated under N_2 and taken up in methanol. Aliquots were injected onto a 4.6 × 250 mm Zorbax ODS (DuPont) C_{1a} reverse phase column and isocratically eluted in 80% acetonitrile: 20% 30 mM H_3PO_4 at 1 ml per min. Saturated [^{14}C]-free fatty acids were resolved by the use of on-line scintillation counter (FLO-ONE radioactive flow detector). Standard ^{14}C-fatty acids were used to calibrate retention times.

Glycerol-3-P Acyl Transferase. Reaction mixtures (40 μl) contained 0.25 M Mops-NaOH (pH 7.4), 0.2% BSA, 0.6 mM glycerol-3-P, acyltransferase (0.14 μg protein), 13.5 mM Mes-NaOH (pH 6.0), and 0.1 to 1.0 μM C18:1-ACP. After incubation for 1 min at 24°C the reaction products were extracted and separated by TLC (3).

RESULTS

Fatty Acid Synthesis. The reactivity of ACP isoforms in the synthesis of fatty acids from malonyl-CoA, was evaluated by using a spinach leaf preparation enriched for stromal FAS pro-

teins (6). Figure 1 displays the effect of ACP concentration on the total amount of hexane extractable radioactivity after a 30 min incubation time. At all ACP concentrations tested (0.11–30.0 μM), we observed only slight differences in reactivity among the two spinach leaf ACP isoforms and *E. coli* ACP.

Simoni *et al.* (27) reported that both *E. coli* and *Arthobacter viscosus* ACP functioned 4 to 10 times more effectively than plant ACP in both FAS and the malonyl-CoA:CO_2 exchange reaction. Høj and Svendsen (7) later observed that at concentrations above 1 μM ACP, *E. coli* yielded approximately 2-fold higher FAS than barley ACP-I. As shown in Figure 1 we have not observed this preference for *E. coli* ACP in the reactions of spinach fatty acid synthesis. One possible explanation for the higher activity of spinach ACP in our study may be the omission of the 80 to 90°C heat treatment step in the initial stages of ACP purification. In an effort to avoid ACP inactivation, we have purified the spinach ACP for this study without a heat treatment, whereas, this step was included in the studies by Simoni *et al.* (27).

Although there appeared to be a consistent difference in the reactivity of ACP-I and ACP-II in FAS, these differences never constituted more than 26%. When ACP was added at 0.19 μM to the reaction mixture, ACP-I yielded 37 pmol/min while ACP-II yielded 28 pmol/min. When ACP concentration was increased, this difference did not appreciably change, suggesting that in the synthesis of fatty acids from [^{14}C]malonyl-CoA ACP-I and ACP-II are equally efficient as cofactors (Fig. 1).

To determine if *E. coli* ACP, spinach ACP-I, or spinach ACP-II influenced the chain length of fatty acids synthesized in the *in vitro* FAS reactions, HPLC analysis of the hexane-extractable products of the reaction was conducted. Table I lists the percent composition of the major products of *de novo* FAS from [^{14}C] malonyl-CoA. Although other minor peaks of radioactivity were detected in our analysis, these constituted less than 10% of the total. With a 15 min reaction time, both ACP-I and ACP-II addition to the *in vitro* FAS resulted in over 90% of the radioactivity incorporated as 16:0 with the remainder in the form of 18:0. *E. coli* ACP after a 15 min reaction yielded 67% 16:0, 30% 18:0, and 3% 14:0. After 1 h, the relative percentages of 18:0 increased above 30% for both ACP-I and ACP-II reaction mixtures (Table I). The bulk of the remaining radioactivity (49–59%) was found in 16:0 with 4 to 5% ^{14}C incorporated into 14:0.

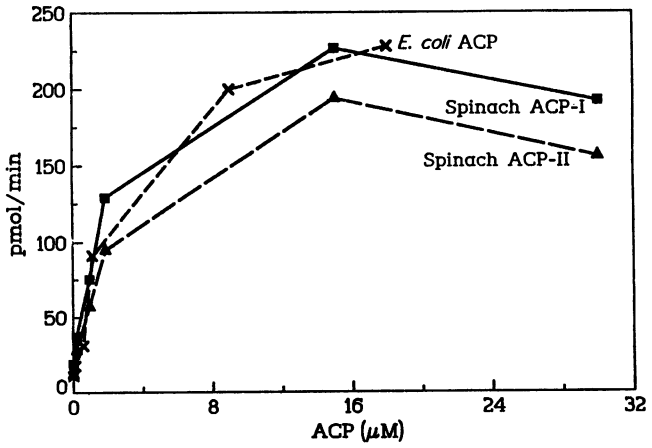

FIG. 1. *De novo* FAS activity *versus* ACP concentration. Incorporation of [^{14}C]malonyl-CoA by a spinach chloroplast stromal FAS preparation was determined with the indicated concentration of *E. coli*, spinach ACP-I, and spinach ACP-II. Values correspond to total hexane-extractable radioactivity and are averages of four determinations. Details of reaction parameters are found in "Materials and Methods." Reaction time = 30 min.

Table I. *Products of de Novo FAS from [^{14}C]Malonyl-CoA*

Fatty acid synthetase was prepared from $(NH_4)_2SO_4$ precipitates of spinach chloroplast extracts. Reaction mixture conditions as outlined in "Materials and Methods."

	Fatty Acid	*E. coli* ACP	Spinach	
			ACP-I	ACP-II
			%[a]	
a) 15 min	14:0	3.0		
	16:0	67.0	93.0	92.0
	18:0	30.0	7.0	8.0
b) 60 min	14:0	2.0	4.0	5.0
	16:0	41.0	49.0	59.0
	18:0	57.0	47.0	36.0

[a] Values represent percent of the major products of FAS as determined by HPLC analysis of hexane-extractable radioactivity.

E. coli ACP reaction mixtures synthesized slightly more 18:0 than 16:0, but the differences were in the range detected for ACP-I and ACP-II. Overall, the incorporation of [^{14}C]malonyl-CoA into hexane-extractable reaction products (14:0, 16:0, 18:0), was not influenced to any major degree by the ACP isoform used in the reaction.

Malonyl-CoA:ACP Transacylase. Recently our laboratory reported the existence of two isozymes of MCT in soybean leaf tissue and one form in developing seed (4). This enzyme catalyzes one of the first reactions of *de novo* FAS in plastids resulting in the commitment of carbon into C_4 to C_{18} fatty acids (25, 28). Because there is a tissue specific expression of both ACP and MCT in higher plants, we evaluated the reactivity of purified ACP-I and ACP-II for MCT_1 and MCT_2 isolated from castor bean leaves and MCT isolated from spinach leaves. MCT isozymes from castor bean were used in this study because of the high specific activity obtained and because there were negligible losses in activity during the course of the experiment. Because spinach leaf extracts do not contain two isozymes of MCT (28), the activity that eluted during the salt gradient was used in this study. The results of this analysis are shown in Figure 2. We observed no significant difference between spinach ACP isoforms or between spinach and *E. coli* ACP in their reactivity in the MCT assay. Therefore, we conclude that this initial reaction of *de novo* FAS synthesis is probably not regulated by ACP isoform expression. Kinetic parameters were not further evaluated for the MCT isozymes because of the apparent lack of difference among ACP isoform reactivity and because of the low availability of spinach ACP.

Oleoyl-ACP Thioesterase. One of the endpoints of *de novo* FAS in plastids involves the hydrolysis of oleoyl-ACP to free oleic acid and ACPSH (29, 30). Figure 3 shows the activity of this enzyme from both leaf and achene spinach tissue. We observed that *E. coli* oleoyl-ACP hydrolysis proceeded at approximately twice the velocity as compared to hydrolysis of oleoyl-ACP prepared from spinach ACP-I. Oleoyl-ACP prepared from spinach ACP-II yielded the lowest reaction rate of the substrates tested (Fig. 3). Of particular interest is the observation that the ACP-II isoform yielded only 10% of the thioesterase activity as compared to ACP-I, when an enzyme preparation from spinach leaves was used. Because ACP-II is the major isoform expressed in spinach seed (17), we further evaluated thioesterase activity from developing achene tissue. Figure 3 shows that ACP-II was only 17% as reactive as ACP-I for the achene oleoyl-ACP thioesterase. These results indicate that oleoyl-ACP-II is a relatively ineffective substrate at the concentration used in the standard assay (1.4 μM) in both leaf and achene tissues.

To further examine the effect of ACP isoform on thioesterase

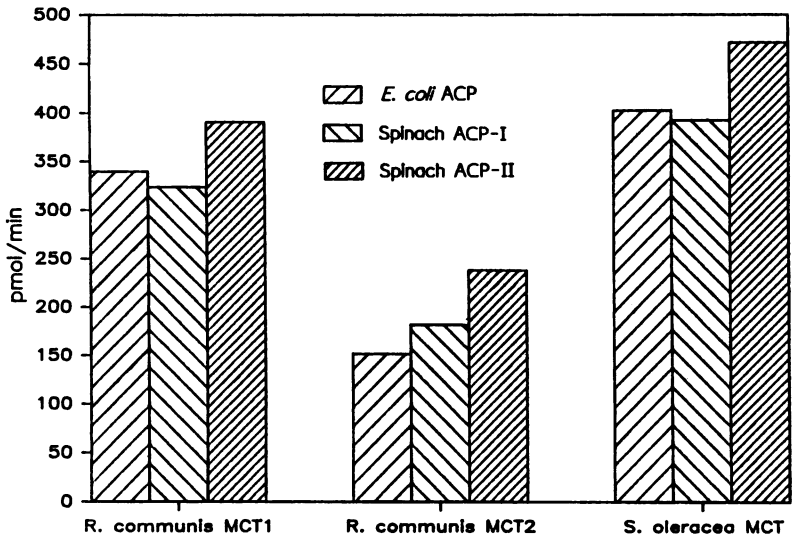

FIG. 2. Malonyl-CoA:ACP transacylase activity *versus* ACP isoform. Two isozymes of MCT were partially purified from leaves of castor bean plants and a single enzyme was obtained from spinach leaves. The reactivity of *E. coli*, spinach ACP-I, and spinach ACP-II was determined by incorporation of radioactivity from [^{14}C]malonyl into acid-precipitable protein. ACP concentration was 6 μM. Values are averages of three determinations.

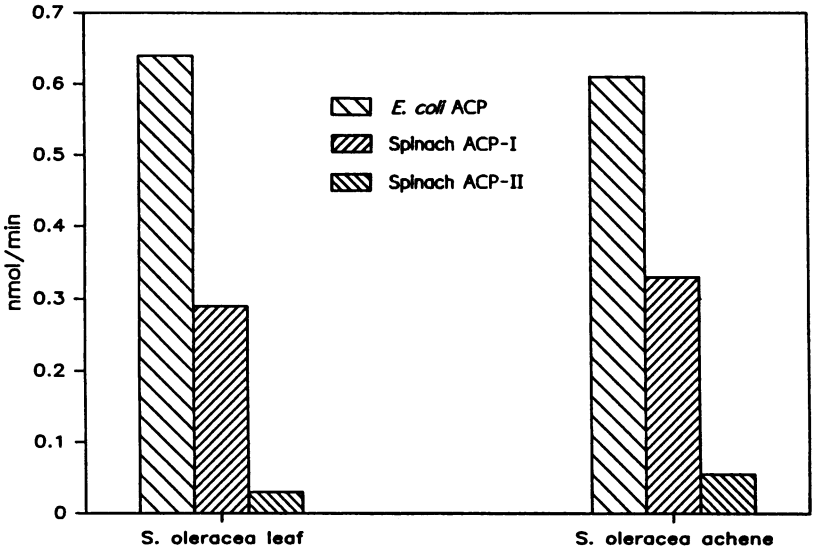

FIG. 3. Oleoyl-ACP thioesterase activity *versus* ACP isoform. Reaction mixture contained 6600 dpm [^{14}C] oleoyl-ACP (1.4 μM), 0.1 M Tris (pH 8.0), and enzyme partially purified from spinach leaf and spinach achene tissue. Details of reaction parameters are found in "Materials and Methods." Values are averages of five determinations.

activity, a series of oleoyl-ACP concentrations were employed in the reaction. These experiments were conducted with achene thioesterase preparations. Figure 4 displays the results of the dependence of thioesterase activity on the concentration of each of the oleoyl-ACP substrates. Consistently, oleoyl-ACP-II was a less effective substrate for the reaction as compared to oleoyl-ACP-I or *E. coli* oleoyl-ACP. At the highest substrate concentration employed (3.24 μM), oleoyl-ACP-II was only 22% as reactive as oleoyl-ACP-I and only 11% as reactive as *E. coli* oleoyl-ACP. Plots of 1/v *versus* 1/s (Fig. 4, insert) revealed that the K_m value for oleoyl-ACP-I was more than 10-fold lower than the K_m for oleoyl-ACP-II (4.8 μM *versus* 50.0 μM). The V_{max} values for thioesterase activity were not significantly different (oleoyl-ACP-I, 2.9 μM min^{-1}; oleoyl-ACP-II, 3.3 μM min^{-1}). For comparison, the K_m and V_{max} of the thioesterase with *E. coli* oleoyl-ACP as substrate were 1.4 μM and 0.7 μM min^{-1}, respectively (Fig. 4, insert).

The results of thioesterase activity *versus* oleoyl-ACP isoform indicate that oleoyl-ACP-II is a relatively poor substrate for the reaction and, furthermore, that the plant enzyme is more reactive with *E. coli* oleoly-ACP than with either spinach isoform. After observing that oleoyl-ACP-II was ineffective as a substrate for both leaf and achene tissue thioesterase, we considered it possible that this substrate was damaged during its synthesis and purification. We therefore synthesized a second preparation of each oleoyl-ACP substrate and again tested for thioesterase activity. Both preparations of oleoyl-ACPs gave essentially identical results.

Glycerol-3-P Acyltransferase. Oleoyl-ACP is at a branch point in plant fatty acid metabolism. In addition to its hydrolysis by oleoyl-ACP thioesterase, the oleoyl acyl chain can also be transferred directly onto the one position of glycerol-3-P. Using *E. coli* ACP, Frentzen *et al.* (3) demonstrated the selectivity of this enzyme for oleate *versus* palmitate and for ACP *versus* coenzyme A thioesters.

To evaluate the reactivity of glycerol-3-P acyltransferase with oleoyl-ACP isoforms, a series of experiments were conducted with oleate derivatives of spinach ACP-I and ACP-II and *E. coli* ACP. Figure 5 shows the effect of substrate concentration on the velocity of the reaction. Oleoyl-ACP-II was consistently a better substrate for the transferase reaction than oleoyl-ACP-I. *E. coli* oleoyl-ACP provided still higher velocities when the concentration of substrate exceeded 0.4 μM. Linearization of the data revealed that the K_m value for oleoyl-ACP-II was approximately 80% lower than the K_m calculated for oleoyl-ACP-I (0.16 *versus* 0.74 μM). V_{max} values differed only slightly among the oleoyl-ACP isoforms (ACP-I = 26.4, ACP-II = 24.0, *E. coli* ACP = 36.0 nmol min^{-1}).

DISCUSSION

In an effort to evaluate possible regulatory consequences of ACP isoform expression in higher plants, we conducted a series

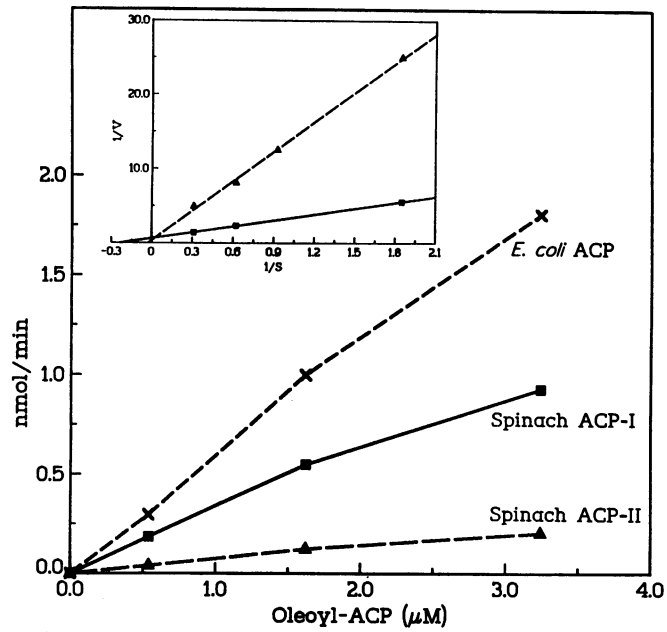

FIG. 4. Oleoyl-ACP thioesterase activity *versus* oleoyl-ACP concentration. Reaction mixture contained oleoyl-ACP (0–3.24 μM) from either *E. coli*, spinach ACP-I, or spinach ACP-II, and an appropriate amount of spinach achene oleoyl-ACP thioesterase in 0.1 M Tris (pH 8.0). Details of reaction parameters are found in "Materials and Methods." Insert: Lineweaver-Burke double reciprocal plot. Values are averages of two determinations.

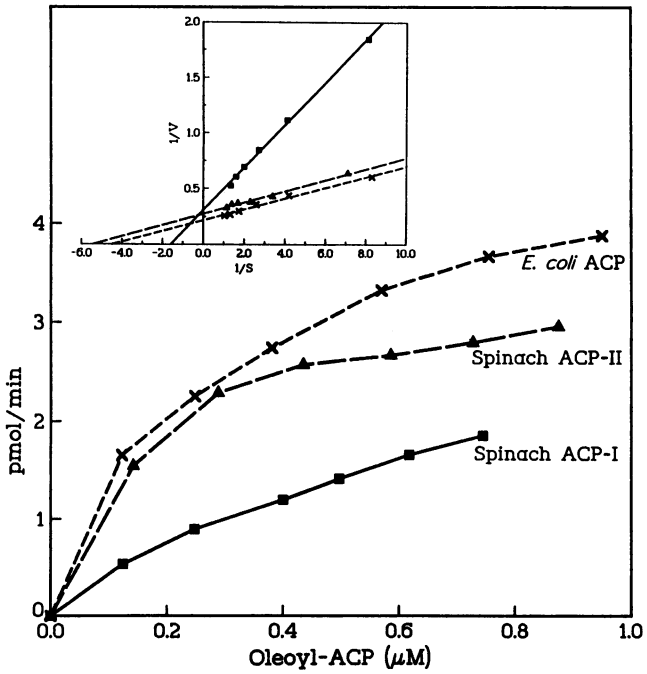

FIG. 5. *sn*-Glycerol-3-P acyltransferase activity *versus* oleoyl-ACP concentration. Reaction mixtures contained oleoyl-ACP (0–1.0 μM) from either *E. coli*, spinach ACP-I, or spinach ACP-II, 0.6 mM glycerol 3-P acyltransferase from spinach leaves, 13.5 mM Mes, and 0.2% BSA in 0.25 M Mops (pH 7.4). Details of reaction parameters are found in "Materials and Methods." Insert: Lineweaver-Burke double reciprocal plot. Values are averages of three determinations.

of experiments with spinach ACP-I and ACP-II, and *E. coli* ACP in several reactions of fatty acid metabolism. We have determined that both acyl carrier proteins purified from spinach leaf tissue are effective coenzymes and cosubstrates for *in vitro* FAS starting from [^{14}C]malonyl-CoA and for malonyl-CoA:ACP transacylase (two isozymic forms from castor bean, one from spinach leaf). In addition, we find that in these reactions spinach and *E. coli* ACP have similar kinetics. We have also prepared oleoyl-ACP from the three forms of ACP and tested these as substrates for oleoyl-ACP thioesterase and glycerol-3-P acyltransferase. Surprisingly, for both these reactions *E. coli*-oleoyl-ACP had lower K_m values than the spinach substrates, but V_{max} values were similar. Of particular interest, our comparison of the two spinach ACP isoforms indicates that oleoyl-ACP-I and oleoyl-ACP-II are differentially effective as substrates for two competing endpoint reactions in plastids. From the K_m determinations (Fig. 4), oleoyl-ACP-I was 10-fold more effective as substrate than oleoyl-ACP-II for the oleoyl-ACP thioesterase reaction. In contrast, the glycerol-3-P acyltransferase from spinach leaves was 5-fold more reactive with oleoyl-ACP-II as compared with oleoyl-ACP-I (K_m 0.16 μM *versus* 0.74 μM, respectively).

We have previously estimated the *in vivo* concentration of ACP in spinach chloroplasts to be 8 μM (15). It is difficult to estimate the concentration of acyl-ACP likely to occur *in vivo*, but we believe it to be well below 8 μM. This suggestion is based on our inability to detect acyl-ACP in Western blots of freeze-quenched spinach extracts (17), the absence of substantial acyl-ACP in purified preparations of spinach (11) and barley (7) ACP, and the observation that acyl-ACP constitutes less than 10% of the ACP pool in rapidly growing *E. coli* (20). It is likely that oleoyl-ACP concentrations are sub micromolar. Therefore, the K_m differences we determined *in vitro* (Figs. 4 and 5) are relevant to physiological levels of the acyl-ACPs and may reflect different reactivities of these substrates *in vivo*.

It has frequently been observed that free fatty acids are major products of [^{14}C]acetate incorporation by chloroplasts *in vitro* (21, 29). However, addition of glycerol-3-P to the chloroplasts results in a significant portion of product incorporation into glycerolipids (21). These lipids have a structure characteristic of photosynthetic prokaryotes with oleate esterified primarily at the one position and palmitate esterified exclusively at the two position (5). This acyl chain distribution on glycerol contrasts with the majority of plant glycerolipids in which C_{16}-fatty acids are excluded and oleate is esterified at the two position. These observations together have led to a two pathway model (5, 23) for plant leaf acyl chain mtabolism in which prokaryotic type structures are assembled in the chloroplast through the action of stromal (3) and inner envelope (2) acyltransferases. Eukaryotic type structures require the export of free fatty acids from the plastid, their reesterification to coenzyme A (1, 9), and acyl transfer to phospholipids in the ER. The apportionment of acyl chains between these two pathways may be controlled by a number of factors including glycerol-3-P concentration and the relative activity of thioesterase *versus* acyltransferases. The results from this study suggest that additional control might be exerted by the expression of ACP isoforms. Oleoyl-ACP-II is the preferred substrate for the acylation of the 1-position on glycerol-3-P, while oleoyl-ACP-I is the preferred substrate for the release of free oleic acid from ACP (thioesterase reaction). Together these data suggest that expression of ACP isoforms in plastids may partially determine the metabolic fate of the major end product of *de novo* FAS (oleic acid). Because both the acyltransferase and the thioesterase compete for the same substrate within the plastid, we suggest that fatty acid transport and glycerolipid biosynthesis may be regulated in part by the differential expression or acylation of ACP isoforms.

Seed triacylglycerol biosynthesis has been suggested to occur

through fatty acid biosynthesis in plastids followed by export of acyl chains to the ER for esterification of glycerol (22). Therefore, it might be expected that most acyl chains produced in seeds would be released from ACP as free fatty acids via oleoyl-ACP thioesterase. Because ACP-II appears to be the predominant ACP isoform in spinach seeds, the low reactivity of ACP-II with the oleoyl-ACP thioesterase is not expected. We are currently investigating several possible explanations for these results.

Acknowledgments—The authors thank Dr. T. M. Kuo for assistance in spinach ACP purification, Mrs. D. Elmore and Mr. J. Siedlecki for expert technical assistance, and Mrs. K. Fenster for culturing the plants used in this study.

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