Specificities of the Acyl-Acyl Carrier Protein (ACP) Thioesterase and Glycerol-3-Phosphate Acylationtransferase for Octadecenoyl-ACP Isomers

Identification of a Petroselinoyl-ACP Thioesterase in Umbelliferae

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This study was designed to address the question: How specific for double bond position and conformation are plant enzymes that act on oleoyl-acyl carrier protein (ACP)? Octadecenoyl-ACPs with cis double bonds at positions Δ⁶, Δ⁷, Δ⁸, Δ⁹, Δ¹⁰, or Δ¹² and elaidyl (18:1Δ⁹cis)-ACP were synthesized and used to characterize the substrate specificity of the acyl-ACP thioesterase and acyl-ACP:sn-glycerol-3-phosphate acyltransferase. The two enzymes were found to be specific for the Δ⁶ position of the double bond. The thioesterase was highly specific for the Δ⁶ cis conformation, but the transferase was almost equally active with the cis and the trans isomer of 18:1₆⁻ACP. In plants such as the Umbelliferae species coriander (Coriandrum sativum L.) that accumulate petroselinic acid (18:1Δ⁷cis) in their seed triacylglycerols, a high petroselinoyl-ACP thioesterase activity was found in addition to the oleoyl-ACP thioesterase. The two activities could be separated by anion-exchange chromatography, indicating that the petroselinoyl-ACP thioesterase is represented by a distinct polypeptide.

Fatty acid biosynthesis in plants is catalyzed by the fatty acid synthase, a group of at least seven different enzymes (Ohlrogge et al., 1993). Elongation of an acetyl-CoA primer with malonyl-ACP leads to acyl chains with 16 or 18 carbon atoms; throughout this process, the acyl group is attached to the ACP moiety (Cahoon et al., 1992a). In addition, a cDNA of high similarity to the A'-stearoyl-ACP desaturase was discovered in coriander (Cahoon et al., 1992b), and expression of this cDNA in transgenic tobacco led to accumulation of petroselinic acid. As with medium-chain fatty acid synthesis, it is also possible that a specialized acyl-ACP thioesterase is involved in petroselinic acid formation in these plants. In addition to hydrolysis by acyl-ACP thioesterases, fatty acids attached to ACP can also be transferred to glycerol-3-P. Frentzen et al. (1983) reported the occurrence of two different acyltransferases in spinach (Spinacia oleracea L.) that are specific for ACP thioesters and incorporate the acyl chain into plastid membrane lipids. Whereas the GPAT (EC 2.3.1.15) is most active with oleoyl-ACP, the acyl-ACP:1-acyl-sn-glycerol-3-P acyltransferase (EC 2.3.1.51) is specific for palmitoyl-ACP (Frentzen et al., 1983).


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Here we address the question of how the thioesterase and acyltransferase in plants recognize C_{18} monounsaturated acyl-ACPs. Therefore, we measured the OTE and the GPAT activity with octadecenoyl-ACPs with double bonds at different positions and conformations. We also examined whether species of Umbelliferae that produce petroselinic acid have a specific acyl-ACP thioesterase. In addition to providing information on the active site and mechanisms by which enzymes recognize acyl chains, these studies may help to uncover potential limitations on introduction of unusual monoenoic fatty acids into transgenic plants.

MATERIALS AND METHODS

Plant Material and Protein Determination

Plants were grown under greenhouse conditions with natural illumination. For thioesterase assays, extracts from spinach (Spinacia oleracea L.), leaves and seed endosperm of castor bean (Ricinus communis L.), coriander (Coriandrum sativum L.), or dill (Anethum graveolens L.) were obtained by homogenizing 2 g of tissue in 20 mL of 20 mM Tris-HCl (pH 8.5), 2 mM DTT, 5% (v/v) glycerol. For GPAT assays stroma fractions of spinach chloroplasts were prepared as outlined by Lohden and Frentzen (1988), and purified acyltransferase fractions were obtained as described earlier (Frentzen et al., 1983). Protein was measured by the method of Bradford (1976).

Preparation of Acyl-ACPs

Tritiated octadecenoic acids with cis double bonds at positions Δ^6, Δ^7, Δ^8, Δ^9, Δ^11, Δ^12, and trans Δ^9 were kindly provided by Dr. Edward Emken (U.S. Department of Agriculture, Peoria, IL). Fatty acids were methylated with 10% (w/v) boron trichloride in methanol and purified by preparative argentation TLC (Gunstone et al., 1967; Morris et al., 1967). The purity of the fatty acid methyl esters was also confirmed with argentation TLC. Substances on TLC plates were visualized by autoradiography or by spraying with 0.1% (w/v) 2',7'-dichlorofluorescein in methanol (Christie, 1982). Fatty acid methyl esters were quantified by GC on a CPsil88 column (25 mm × 50 m; Chrompack, Middelburg, The Netherlands) with mass determined relative to methyl heptadecanoate. Specific radioactivities (6.3 × 10^{10} to 24.4 × 10^{10} Bq/mol) were calculated after measuring the radioactivity by scintillation counting. The methyl esters were then saponified with 1 N NaOH in 90% (v/v) methanol for 1 h at 80°C (Christie, 1982). Acyl-ACPs were synthesized from these fatty acids and recombinant spinach ACP 1 (Kuo and Ohlrogge, 1984) using Escherichia coli acyl-ACP synthetase (Rock and Garwin, 1979). The acyl-ACPs were purified by chromatography on DE-52 Cellulose (Whatman, Maidstone, UK) and desalted into 10 mM Mes-NaOH (pH 6.1) by gel filtration using a PD-10 column (Pharmacia, Piscataway, NJ).

Acyl-ACP Thioesterase Assay

Approximately 100 μg of plant protein was incubated with 5 μM acyl-ACP in 20 mM Tris-HCl (pH 8.5), 2 mM DTT, 5% (v/v) glycerol at room temperature in a total volume of 100 μL. After 20 min, the reaction was stopped with 50 μL of 1 N acetic acid in isopropanol. The reaction mixture was extracted and the recovered radioactivity was measured by scintillation counting as described by Ohlrogge et al. (1978). The radioactivity in the extract was found to be free fatty acid as judged by TLC on silica plates (data not shown).

Separation of Acyl-ACP Thioesterases from Coriander Seed Endosperm

Approximately 1.7 g of coriander seed endosperm was homogenized as described above. The crude extract was centrifuged (10 min, 15,000g) to separate soluble proteins from insoluble debris and from the fat layer. The protein extract was then centrifuged (30 min, 120,000g) and applied at 0.5 mL/min onto a Mono Q HR 5/5 column (Pharmacia) that had been equilibrated with 20 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol. The column was washed at 1 mL/min with the same buffer and thioesterase activity was eluted at the same flow rate with a linear gradient from 0 to 0.4 M NaCl (in the same buffer) in 80 min. Thioesterase activity in the fractions was measured with petroselinoyl-ACP and oleoyl-ACP.

GPAT Assay

Acyltransferase activity was measured in a manner similar to that described by Frentzen et al. (1983). Briefly, the reaction mixture was composed of 250 mM Mops-NaOH, pH 7.4, 0.3 mM sn-glycerol-3-P, 2 μM acyl-ACP, and the enzyme fraction (100–200 μg of stroma protein and 0.5–2 μg of purified acyltransferase) in a total volume of 800 μL. In some experiments Mops-NaOH, pH 7.4, was replaced by Tricine-NaOH, pH 8.2. After a 10-min incubation at 24°C, the reactions were terminated, the products were extracted, and lysophosphatic acid was separated by TLC (Frentzen et al., 1983).

RESULTS

Synthesis of Acyl-ACPs

These studies were conducted to determine the specificity for double bond position and conformation of plant enzymes that act on oleoyl-ACP. Therefore, we have used a series of 3H-labeled octadecenoic acids to prepare 18:1-ACPs with cis double bonds at positions Δ^6, Δ^7, Δ^8, Δ^9, Δ^10, Δ^11, and Δ^12 and trans Δ^9-18:1-ACP. These isomeric octadecenoates were esterified to ACP by the action of the E. coli acyl-ACP synthetase. In all cases the acylation was complete, indicating that the acyl-ACP synthetase did not significantly discriminate between these fatty acid isomers. The purity of the acyl-ACPs was analyzed by PAGE in native gels containing 0, 2.5, or 5.0 M urea using unesterified recombinant spinach ACP 1 as a standard (Post-Beittenmiller et al., 1991). A clear separation of free ACP and acylated ACPs was achieved; however, we were not able to separate the different octadecenoyl-ACP isomers (data not shown).

Acyl-ACP Thioesterase Activity

Acyl-ACP thioesterase activity for different octadecenoyl-ACP substrates was determined with spinach leaf and castor
Thioesterase and Acyltransferase Specificities for 18:1-Acyl Carrier Proteins

Figure 1. Thioesterase specificity for isomeric octadecenoyl-ACPs in protein extracts from castor bean endosperm (A), spinach leaf (B), coriander endosperm (C), and dill endosperm (D).

bean endosperm. A high thioesterase specificity for both the Δ⁹ position and the cis conformation was found (Fig. 1, A and B). Acyl-ACPs with double bonds at positions Δ⁶, Δ⁷, or Δ⁸ were almost completely excluded from hydrolysis, whereas those with Δ¹⁰, Δ¹¹, or Δ¹² double bonds resulted in intermediate activity. These data indicate that plant tissues that produce primarily oleic acid (and its further desaturation products) possess an acyl-ACP thioesterase that is highly specific for oleoyl-ACP. We have also prepared [³H]palmitoleoyl-ACP and measured the in vitro thioesterase activity of castor bean endosperm with this substrate at 5 μM. The thioesterase activity with palmitoleoyl-ACP was about 50% of that with oleoyl-ACP.

Many species of the Umbelliferae, Araliaceae, and Garryaceae families produce high levels of petroselinic acid (18:1Δ⁶cis) instead of oleic acid. Recently, it has been shown that petroselinic acid is produced by an acyl-ACP desaturase (Cahoon et al., 1992b). Therefore, it could be anticipated that petroselinoyl-ACP is a major product of plastid fatty acid synthesis and that these plants may possess an acyl-ACP thioesterase with properties different from those found in spinach or castor bean, etc. To address this question, thioesterase activity with different 18:1-ACPs was also measured in the seed endosperm of the Umbelliferae species coriander and dill (Fig. 1, C and D). In these species a markedly different pattern of thioesterase substrate specificity was observed. Petroselinoyl-ACP, which was the least active substrate with spinach and castor bean, was the most active substrate for coriander and dill. The relative activity with the other octadecenoyl-ACPs, however, was similar to that of spinach and castor bean, and the ratio of the activities for the Δ⁸ cis and trans isomers was not different (data not shown). The additional Δ⁸-specific acyl-ACP thioesterase activity was also found in seed endosperm of other members of the Umbelliferae family including fennel (Foeniculum vulgare Mill.) and marsh parsley (Peucedanum palustre L.) (data not shown).

Characterization of the PTE in Coriander

The high PTE activity found in coriander and dill could be explained by different possibilities: (a) the OTE in these plants may have an altered substrate specificity such that it accepts oleoyl-ACP and petroselinoyl-ACP; (b) the Umbelliferae species may contain an additional thioesterase specific for petroselinoyl-ACP. To distinguish between the two possibilities, the thioesterase activities from coriander were further characterized.

The activities for the OTE and PTE in coriander endosperm were measured at different pH values (Fig. 2). For both activities, a pH optimum between 8.0 and 9.0 was found. An alkaline pH optimum of approximately 9.0 for the OTE has previously been reported (Ohlrogge et al., 1978; Helleyer et al., 1992; Imai et al., 1992).

To enrich thioesterase activities of coriander, an endosperm extract was applied onto a long-chain alkyl-ACP column as previously described (Dormann et al., 1993). The OTE activity was well bound to this affinity resin, but the petroselinoyl-ACP-specific enzyme was not (data not shown). The PTE thus behaved in a manner similar to the Cuphea medium-
chain acyl-ACP thioesterase, which also did not bind effectively to an alkyl-ACP column (Dormann et al., 1993).

In a further attempt to enrich the PTE activity from coriander, an endosperm extract was applied onto a Mono Q anion-exchange column. Two acyl-ACP thioesterases could be clearly separated with distinct activities for oleoyl-ACP and petroselinoyl-ACP (Fig. 3). The specificity pattern of the 18:1<sup>ad</sup>-ACP-specific enzyme was identical to the OTEs from spinach leaf and castor bean endosperm. In contrast, the second thioesterase (PTE) was active almost exclusively with petroselinoyl-ACP and showed only a low OTE activity (Fig. 4).

We have measured the thioesterase activities with oleoyl-ACP and petroselinoyl-ACP in castor bean endosperm and coriander endosperm extract over a range of substrate concentrations (0.7-15 μM). In castor bean, the activity with oleoyl-ACP was at least 8-fold higher at all concentrations than with petroselinoyl-ACP. In coriander, however, the thioesterase activity with petroselinoyl-ACP was at least 2-fold higher than that with oleoyl-ACP at all concentrations tested. Due to the limited amount of substrate that was available, we could obtain estimates only for the kinetic data. The $V_{\text{max}}$ values for oleoyl-ACP and petroselinoyl-ACP in both castor bean and coriander were about 100 to 400 pmol min<sup>-1</sup> mg<sup>-1</sup>. The $K_m$ for the two substrates in coriander extracts were similar (about 2 μM), whereas the $K_m$ for petroselinoyl-ACP (50 μM) in castor bean was much higher than for oleoyl-ACP (1 μM). These data suggest that in castor bean, in contrast to coriander, the enzyme(s) shows much less affinity for petroselinoyl-ACP than for oleoyl-ACP. The in vivo concentrations of long-chain acyl-ACPs were estimated to be below 2 μM (Post-Beittenmiller et al., 1991), and therefore, the $K_m$ values will be the primary determinant of catalytic efficiency with the various substrates under physiological conditions.

**Specificity of the GPAT**

In addition to the thioesterase, the oleoyl-ACP can also serve as a substrate for the soluble glycerol-3-P acyltransferase. Therefore, we have analyzed this enzyme for its specificity with different 18:1-ACPs. Assays were performed at both pH 8.2, to mimic conditions found in the stroma, and at pH 7.4, which is the optimum for the acyltransferase. At both pH values, the GPAT in spinach showed highest activity for the $\Delta^9$ and lower activity for the $\Delta^{12}$ position of the double bond in octadecenoyl-ACPs (Fig. 5, A and B). 18:1-ACPs with cis double bonds at positions $\Delta^6$, $\Delta^7$, or $\Delta^9$ were almost completely excluded from the transferase reaction. The activities with the cis and trans $\Delta^9$ isomers were very similar, indicating that in contrast to the thioesterase, the acyltransferase did not strongly discriminate based on the conformation of the $\Delta^9$ double bond. The enzyme specificity was found to be more pronounced at pH 8.2 than at pH 7.4, whereas these pH values did not alter its selectivity for oleoyl groups in comparison with both palmitoyl and stearoyl groups (Frentzen et al., 1987). The basis for this pH effect is not known. To assure that the specificity pattern of the purified GPAT was not changed during purification, we also measured the acyltransferase activity in spinach stroma extracts. An identical GPAT specificity was obtained from the crude stroma fraction compared with the purified GPAT (data not shown).
DISCUSSION

The substrate specificities of the OTE and the GPAT, two enzymes that are involved in acyl-ACP metabolism, were analyzed with octadecenoyl-ACPs with double bonds at different positions and conformations. The results presented here suggest that the OTE and the GPAT recognize the acyl moiety in a different manner. Both enzymes are specific for the Δ9 position, but the GPAT also shows considerable activity with 18:1Δ10ts-ACP, 18:1Δ11ts-ACP, and particularly 18:1Δ12sn-ACP. In addition, the GPAT accepts both the cis and trans isomer of 18:1Δ9ts-ACP almost equally well, whereas the OTE discriminates against the trans double bond. Both enzymes display a very low activity with 18:1Δ9cn-ACP, 18:1Δ10sn-ACP, and 18:1Δ12sn-ACP. Isomeric monounsaturated fatty acids have previously been used to analyze the specificity of enzymes involved in lipid metabolism. Ohlrogge et al. (1976) could show that in E. coli 18:1Δ9ts and 18:1Δ11ts were the most effective substrates for cyclopropane acid formation from cis octadecenoic acids. In another study, safflower acyl-CoA:1-acyl-glycero-3-P acyltransferase was found to be specific for unsaturated cis Δ9 acyl-CoA (Ichihara et al., 1987).

Previous studies have indicated a specificity of the GPAT and OTE for oleoyl-ACP over stearoyl- and palmitoyl-ACP (Ohlrogge et al., 1978; McKeon and Stumpf, 1982; Löthen and Frentzen, 1988; Alban et al., 1989; Helleyer et al., 1992; Imai et al., 1992; Knutzon et al., 1992). In addition, the OTE was found to be much more specific for oleoyl-ACP versus saturated long-chain acyl-ACPs than the acyltransferase (Löthen and Frentzen, 1988). The question of which amino acid residues are involved in recognizing the acyl moiety still remains unsolved. Presumably, the two enzymes contain a hydrophobic binding domain for the acyl chain like many enzymes that are involved in lipid metabolism (e.g. lipoxigenases; Slone et al., 1991). The structure of this domain is most likely responsible for the specificity for the acyl chain length and the position and conformation of the double bond.

The cis/trans conformation of the double bond has a major impact on the structure of the acyl chain. Whereas a cis double bond within a fatty acid leads to a bend at this position, a trans double bond does not. The OTE clearly prefers acyl chains that have a Δ9cis double bond. Therefore, we speculate that the binding domain itself may be sensitive to the cis conformation. Furthermore, the observation that castor bean thioesterase activity with palmitoleoyl-ACP was approximately 50% of that with oleoyl-ACP suggests that both the chain length and the double bond position are important for the recognition of the acyl group by the thioesterase. The specificity of the GPAT for octadecenoyl-ACPs, however, seems to be more complex. The Δ9cis and Δ12trans conformations do not alter the activity significantly, and acyl-ACPs with double bonds at positions Δ10, Δ11, or Δ12 are also good substrates. In this case, the acyltransferase active site may be more sensitive to the presence of a double bond in the Δ9 to Δ12 positions than to the conformation of the acyl chain. Because high expression of these enzymes in E. coli has recently been obtained, a more complete understanding of the problem of how the two enzymes recognize the length, double bond position, and conformation of the acyl chain may now be obtained by x-ray crystallography.

In species of the Umbelliferae family we found an additional acyl-ACP thioesterase that is highly specific for 18:1Δ9ts-ACP. This thioesterase has a very low activity with octadecenoyl-ACPs with double bonds at positions Δ7, Δ8, Δ10, Δ11, or Δ12. The fact that the PTE can easily be separated from the OTE by anion-exchange chromatography may indicate that these two enzymes are not closely related. Along with a specific desaturase (Cahoon et al., 1992b), this thioesterase is the second enzyme found to date that is specifically involved in the biochemical pathway that leads to the production of petroselinic acid in Umbelliferae species.

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LITERATURE CITED

Dörmann P, Spener F, Ohlrogge JB (1993) Characterization of two...
acyl-acyl carrier protein thioesterases from developing Cuphea seeds specific for medium-chain- and oleoyl-acyl carrier protein.

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