A MULTIFUNCTIONAL ACYL-ACYL CARRIER PROTEIN DESATURASE FROM HEDERA HELIX L. (ENGLISH IVY) CAN SYNTHESIZE 16- AND 18-CARBON MONOENE AND DIENE PRODUCTS.

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SUMMARY

A desaturase with 83% sequence identity to the coriander $\Delta^4$-16:0-ACP desaturase was isolated from developing seeds of Hedera helix (English ivy). Expression of the ivy desaturase in Arabidopsis resulted in the accumulation of 16:1$\Delta^4$ and its expected elongation product 18:1$\Delta^6$ (petroselinic acid). Expression in E. coli resulted in the accumulation of soluble, active protein that was purified to apparent homogeneity. In vitro assays confirmed $\Delta^4$ desaturation with 16:0-ACP; however, with 18:0-ACP desaturation occurred at the $\Delta^9$ position. The ivy desaturase also converted 16:1$\Delta^9$-ACP and 18:1$\Delta^9$-ACP to the corresponding $\Delta^4,9$ dienes. These data suggest at least two distinct substrate-binding modes: one placing C4 at the diiron active site and the other placing C9 at the active site. In the latter case, 18:0 would likely bind in an extended conformation as described for the castor desaturase with 9-carbons accommodated in the cavity beyond the diiron site. However, $\Delta^3$ desaturation would require the accommodation of 12 carbons for C16 substrates or 14 carbons for C18 substrates. The amino acids lining the substrate-binding cavity of ivy and castor desaturases are conserved except for T117R and P179I (castor/ivy). Paradoxically, both substitutions, when introduced into the castor desaturase, favored the binding of shorter acyl chains. Thus it seems likely that $\Delta^4$ desaturation would require a non-extended, perhaps U-shaped, substrate conformation. A cis-double bond may facilitate the initiation of such a non-extended conformation in the monounsaturated substrates. The multifunctional properties of the ivy desaturase makes it well suited for further dissection of the determinants of regiospecificity.

INTRODUCTION

Acyl acyl carrier protein (ACP) desaturases (EC 1.14.99.6) are responsible for the conversion of saturated fatty acyl-ACPs into monounsaturated-ACPs (1-3). This reaction is the penultimate step in the ACP track of fatty acid biosynthesis in the plastid, and represents the major source of
monounsaturated fatty acids in plant tissues (4). The resulting monounsaturated fatty acids are required to maintain membrane fluidity and in storage tissues are directed into storage triacylglycerols either directly, or after additional desaturation steps. Plant acyl-ACP desaturases belong to a distinct sequence class composed of soluble globular proteins, whereas most fatty acid desaturases, found in fungi and animal and plants, are integral membrane proteins (5). All desaturases identified to date that recognize monoene, diene and polyene substrates are members of the integral membrane class of desaturases. The archetypal $\Delta^9$-18:0-ACP desaturase from Ricinus communis (castor) has been the focus of extensive structure-function studies because it is readily expressed in active form in E. coli and it is the only member of the acyl-ACP desaturase family for which a 3D crystal structure has been determined (6,7). Each monomer of the castor enzyme homodimer contains a boomerang-shaped cavity capable of binding a stearoyl moiety in a gauche conformation adjacent to the active site diiron cluster. While a crystal structure of the desaturase-substrate complex has yet to be published, modeling the substrate into the existing crystal structure suggests that when the methyl group of the stearate is in contact with the bottom of the cavity, C9 and C10 are positioned with their pro-R hydrogens facing the diiron active site (6). This structural model is consistent with the Pro-R Pro-R stereochemistry reported for the desaturase reaction (8) and the location of azide, a peroxo mimic, complexed with the desaturase diiron site (7).

In addition to the archetypal 18:0-$\Delta^9$-ACP desaturase described above, an increasing number of variant desaturases are being isolated from plants that contain unusual fatty acids in their storage lipids. These desaturases exhibit a variety of combinations of chain length and regiospecificity, for instance, for 16:0 desaturases $\Delta^4$, $\Delta^6$ and $\Delta^9$ regiospecificities have been reported (9-11) and several $\Delta^9$ desaturases that recognize 14-, 16- and 18-carbon chain lengths have also been reported (11-15). Together, these enzymes represent a valuable resource for determining the molecular basis for desaturase specificity. For instance, chimeras of the ~360 amino acid sequences of the mature castor $\Delta^9$-18:0- and the Thunbergia $\Delta^9$-16:0-ACP desaturases led to the identification of five residues that when substituted from castor sequence into the corresponding positions in the Thunbergia sequence converted the Thunbergia $\Delta^9$-16:0-ACP desaturase into $\Delta^9$-18:0-ACP desaturase (16). Mutagenesis-selection experiments on the castor enzyme revealed individual determinants of chain length specificity (17), similar attempts to resolve the determinants of regiospecificity have failed. $\Delta^4$ desaturases show the largest difference in desaturation position, 5 carbons distant from that of the archetypal $\Delta^9$ desaturases, making them good candidates for dissecting the determinants of regiospecificity (18). Here we report the isolation of a cDNA and characterization of a $\Delta^4$-16:0-ACP desaturase from English ivy. Unlike the previously described coriander enzyme, the ivy homolog expresses in E. coli, as a soluble and active enzyme suitable for biochemical analysis. In vivo and in vitro evidence confirm its $\Delta^4$-16:0-ACP specificity. In presenting a panel of acyl-ACPs to the ivy desaturase, we discovered that it is multifunctional, desaturating 18:0-ACP to 18:1 $\Delta^9$. In addition, it displays the ability to desaturate monoene-ACP substrates, 16:1 $\Delta^9$-ACP to 16:2 $\Delta^4,9$, and 18:1 $\Delta^9$-ACP to 18:2 $\Delta^4,9$ in a manner similar to that seen for integral membrane front end desaturases.

**EXPERIMENTAL PROCEDURES**

**Preparation of cDNA library from developing Hedera helix seeds.** Developing seeds at approximately early to mid-maturity were dissected from fruits of Hedera helix L. (English ivy), frozen in liquid nitrogen, and stored at -80°C until further use. Total RNA was isolated from the developing seeds using Trizol reagent (Invitrogen) according to the manufacturer’s protocol, and polyA+ RNA was
enriched by two passes of the total RNA through oligo dT columns using a QuickPrep mRNA isolation kit (Amersham Biosciences). A unidirectional cDNA library was subsequently prepared from the polyA+-enriched RNA with inserts cloned 5' to 3' in the EcoRI/XhoI sites of pBluescript SK(-), and the library was maintained in plasmid form in E. coli DH10B cells (Invitrogen). The methods used for cDNA library construction were as previously described (19).

**Isolation of a divergent acyl-ACP desaturase cDNA from *Hedera helix* seeds.** Partial acyl-ACP desaturase cDNA sequences were amplified from the library by using fully degenerate oligonucleotides corresponding to the amino acid sequences GDMITEE and EKTIQYL, which are conserved among the majority of known acyl-ACP desaturases. The oligonucleotide sequences and PCR amplification methodology were the same as previously described (10). The PCR reaction products (approximately 215 bp) were subcloned into the pAMP1 vector (Invitrogen) according to the manufacturer’s protocol. Nucleotide sequence was subsequently obtained for ten of the subcloned PCR products. The resulting cDNA class encoding a partial polypeptide of highest identity with the coriander Δ^4-palmitoyl-ACP desaturase (20) was chosen for further characterization. The complete 5' and 3' coding sequences for this cDNA class were amplified from the library by using vector-specific primers in combination with primers specific for the partial *H. helix* cDNA. The primer combinations were: T3 primer and 5'-CTTATTGAGAAGGTCACCATG-3' (for amplification of 5' end) and T7 primer and 5'-ATGGTATTAAGGATGAGACTG-3' (for amplification of 3' end). PCR reactions were conducted using Pfu polymerase (Stratagene), and the longest products were subcloned and sequenced. Based on the sequence information obtained from the PCR products, the following pair of oligonucleotides was designed for amplification of the complete *H. helix* acyl-ACP desaturase cDNA from the developing seed library: 5'-ATTCTAGAAGAAGAAATGGCTTTGAAG and 5'-ATGAGCTCCCTCTGTGCTATCTTC-3' (contains an added XbaI restriction site) and 5'-ATGAGCTCCCTCTGTGCTATCTTC-3' (contains an added SacI restriction site). Three independent PCR reactions were conducted with Pfu polymerase and approximately 350 ng of the *H. helix* developing seed cDNA library as template. Products from each reaction were subcloned and determined to have identical sequence.

**Expression of ivy desaturase in *Arabidopsis***. Ivy desaturase was fused with the castor transit peptide with the use of overlap extension polymerase chain reaction (21). The castor desaturase transit peptide was amplified with the following primers: casmaf AAGGTAAGAAACCCGGATGGCTCTC AAGCTCAATCTC and casivyr GGAGTTAGAGTTGACAGTGAAGCAT GTAGAACTTAAAGATCTGATCTGGA C. The mature portion of the ivy desaturase was amplified with the use of the following primers: casivyf GGCCAGTACCAGATCTCTTATAGGATCTGGTACTGC and ivysacr: CCCTTCCCTCGAGCTCATCTTATTC TCCCGATTGAAAATCCAGC. The two gene fragments were fused by amplification with the use of the casmaf and ivysacr primers described above. The resulting fragment was subjected to restriction digestion with *SmaI* and *SacI* and cloned into the corresponding sites of the *Agrobacterium* binary vector pDATNAP, a derivative of pRD410 (22), to direct seed-specific expression from the napin promoter (23). The vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 pMP90 by electroporation and used to transform *Arabidopsis thaliana* plants by the floral dip method (24).

**Fatty acid analysis**. Seeds were methylated (1 ml of 1 N HCl-methanol, Supelco, 80°C for 1 h), extracted with hexane, and trimethylsilylated (100 µl of BSTFA-TMCS, Supelco, 90°C for 45 min). The BSTFA-TMCS was removed by evaporation and the sample resuspended in hexane. Samples were analyzed on a Hewlett-Packard 6890 gas chromatograph equipped with a 5973
mass selective detector (GC/MS) and a Supelco SP-2340 cyano capillary column (60 m x 250 µm x 0.25 µm). The injector was held at 225°C, the oven temperature was varied (100 to 240°C at 15°C/min followed by 240°C for 5 min), with a helium flow of 1.1 ml/min. Double-bond positions of monounsaturated fatty acid methyl esters were located by GC-MS of dimethyl disulfide (25,26) and pyrrolidine derivatives (27).

Expression of the divergent H. helix acyl-ACP desaturase cDNA in E. coli. The coding sequence for the mature H. helix acyl-ACP desaturase (minus predicted plastid transit peptide) was amplified by using Pfu polymerase and the subcloned full-length cDNA as template. The sense and antisense oligonucleotides used for the PCR reaction were:

5'- TTTCATATGGCTTCCACTGTCAACTC-3' (contains an added NdeI restriction site) and

5'- TTTTAGATCTCTTCCTGTTCATATCTTCAAC-3' (contains an added BglII restriction site). The resulting PCR product was digested with NdeI and BglII and cloned into the NdeI and BamHI restriction sites of vector pET24a (Novagen). The plasmid was transformed into BL21 DE3 Gold (Stratagene) and grown in LB media at 37°C until OD600 ≈ 0.5 at which time IPTG was added to 0.1mM. The temperature was lowered to 30°C and the culture was shaken at 275 RPM for a further 4 hours. Cells were collected by centrifugation and stored at -80°C until further use. Cells were resuspended in five volumes of 7 mM Hepes, 7 mM Mes, 7 mM NaOAc, 4 mM MgCl2 and 6 Kunitz units per ml of DNaseI pH 7.4 per gram of cells and lysed by passage through a French pressure cell with a 104 PSI pressure drop. The lysate was clarified by centrifugation at 48 Kg for 30 minutes. The supernatant was applied to a 1.6 ml Poros 20 CM column equilibrated with 7 mM Hepes, 7 mM Mes, 7 mM NaOAc pH 7.4 (equilibration buffer). After loading, the column was washed with 10 volumes of equilibration buffer before elution with a linear gradient of 0-600 mM NaCl in equilibration buffer. The resulting desaturase was judged to be >90% pure by SDS-PAGE. The resulting enriched desaturase was concentrated and subjected to HPLC size exclusion chromatography with the use of a preparative G-300SW (Toso Haas, Montgomeryville, PA) developed with 20mM Hepes, 70mM NaCl pH 7.0.

Substrate specificity and specific activity. Desaturase preparations were assayed with [1-14C]16:0-, [1-14C]18:0-, [1-14C]16:1∆9- or [1-14C]18:1∆9-ACP substrates with the use of recombinant spinach ACP-I (28). Methyl esters of fatty acids were analyzed by argentation TLC and radioactivity in products quantified as previously described (11,18).

GC/MS desaturase assays. For analysis of double bond positions, assays were performed with the use of unlabeled fatty acid substrates as follows. A typical assay contained approximately 1 nmol purified desaturase, that was incubated with 10 nmol of 14:0-, 16:0-, 16:1∆9-, 18:0-, 18:1∆9- or 18:1∆11-ACP substrates respectively in a 5-fold scale up (total reaction volume 750 µl) of the previously described standard [1-14C] reaction mix (28) except that BSA was omitted to prevent the introduction of additional fatty acids. After incubation the mix was saponified with 0.9 ml of 2.35 M NaOH at 90°C for 1 hour and then neutralized with 2.2 ml of 4 M H2SO4. Free fatty acids were extracted into 3 ml of hexane and dried under nitrogen stream before esterification in 1 ml BCl3-methanol at 90°C for 30 minutes. Following the addition of 1 ml 0.9% NaCl fatty acids were extracted into 2 ml of hexane. The resulting fatty acid methyl esters were concentrated by evaporation under stream of nitrogen before being subjected to GC-MS analysis. Double bond positions were determined as described above (25-27). Kinetic analysis was performed by quantitation of TLC data obtained for assays performed with the use of various substrate concentrations as previously reported for the castor desaturase (17).
Ivy desaturase is a close homolog of a previously described Δ⁴-16:0-ACP desaturase from coriander. A desaturase cDNA was cloned from a library representing mRNA isolated from developing English ivy seeds that accumulate 16:1Δ⁴ and 18:1Δ⁶ fatty acids. A blast search of the amino acid sequence of the ivy desaturase identifies a coriander 16:1Δ⁴-ACP desaturase as its closest match (83% amino acid identity) and somewhat lower identity (74%) to archetypal Δ⁹-18:0-ACP desaturases such as that characterized from castor. A line-up of the sequences of ivy, coriander and castor enzymes can be seen in Figure 1. All three sequences are collinear with ivy and coriander showing a short deletion close to the N-terminus and ivy showing a two-residue insertion near the C-terminus. The high levels of sequence identity together with the observed co-linearity imply that the enzymes share a common three-dimensional fold. We therefore compared the identities of amino acids at positions within the ivy and coriander sequences equivalent to those that had previously been shown to affect substrate specificity in the castor desaturase. We identified sequence changes at positions 117 and 179 (numbered according to the mature castor desaturase sequence (6)) that were previously shown to be important for chain length specificity for the castor desaturase (17,28) (see residues marked ^ in Figure 1). The sequence differences T117R and P179I (castor/ivy) previously shown to increase 16-carbon substrate specificity in the castor desaturase, along with the similarity of the ivy and coriander sequences, suggested that the ivy desaturase is likely a homolog of the coriander Δ⁴-16:0-ACP desaturase and that further investigation of the ivy desaturase was warranted.

Arabidopsis seed expressing the ivy desaturase accumulate 16:1Δ⁴ and 18:1Δ⁶. Evidence for the involvement of the coriander desaturase in the accumulation of petroselic acid (18:1Δ⁶) was previously provided by its expression in tobacco tissue culture (18). These experiments demonstrated that the accumulation of 18:1Δ⁶ was the result of the coriander enzyme desaturating 16:0 at the Δ⁴ position, followed by elongation to 18:1Δ⁶. To determine the specificity of the ivy desaturase we placed it under the control of a seed specific promoter and used Agrobacterium-mediated transformation to introduce the construct into Arabidopsis. GC analysis of methyl esters from transgenic Arabidopsis seeds revealed two new fatty acids corresponding to the expected elution times of the Δ⁴ and Δ⁶ isomers of 16:1 and 18:1 respectively (Figure 2A and B). The 16:1Δ⁴ and monoene elongation products accumulated a total of ~4% of total fatty acids in transgenic Arabidopsis seed. Positional analysis of dimethyl disulfide adducts of the two monoenes show fragmentation patterns (215 and 147 amu (atomic mass units) for 16:1 and 215 and 175 amu for 18:1) confirming their identities as 16:1Δ⁴ and 18:1Δ⁶ respectively. These data are consistent with a role for the ivy desaturase in petroselic acid biosynthesis.

Expression of ivy desaturase in Escherichia coli resulted in the accumulation of active enzyme. Figure 3A shows the ivy desaturase accumulates to high levels in E. coli. It remains in solution after clarification by high-speed centrifugation and is purified to apparent homogeneity by HPLC cation exchange chromatography followed buy size exclusion chromatography. UV-visible spectroscopy of the purified desaturase reveals the presence of absorption features in the 300-400 nm region, characteristic of ligand-to-metal charge transfer bands arising from the diiron active site (Figure 3B) (29).
enzymatic control of regiospecificity with respect to saturated substrates.

To verify the specificity inferred from \textit{in vivo} expression experiments, \textit{in vitro} desaturase reactions were performed with the use of \textsuperscript{14}C labeled fatty acyl-ACPs and purified ivy desaturase preparations, in which the products separated by argentation TLC (see Figure 4). As expected from the seed expression data, 16:0-ACP was converted to a product with mobility corresponding to that expected for 16:1\(\Delta^4\) (Figure 4, lanes 2,3). GC/MS analysis of the dimethyl disulfide adduct of reaction product methyl ester from assays carried out with (unlabeled) 16:0-ACP (Figure 5A-C) confirmed the expected mass ion, at 362 amu and position of the double bond to be at the \(\Delta^4\) position (ions at 215 and 147 amu) (Figure 5C). We also synthesized the pyrrolidide derivative of the product and subjected it to mass spectral analysis (Figure 5D), and identified mass ion of 307 amu in addition to the diagnostic fragmentation pattern (126/139/152 amu) previously reported for \(\Delta^4\) double bond containing fatty acids (27).

The mobility of the desaturation product of 18:0-ACP was consistent with its location at the \(\Delta^9\) position as confirmed by GC/MS analysis as described above for the 16:1 product (data not shown). In reactions incubated for extended periods of time (Figure 4, lanes 3 and 7), we consistently observed that desaturation of 16:0-ACP gave a single product; whereas desaturation of 18:0-ACP gave two products, one with a mobility indistinguishable from 18:1 formed by the castor desaturase (Figure 4, band marked M, compare lanes 6 and 7 with lane 8) and a second lower mobility product marked D that is clearly visible in lane 7. We note that, as expected, the well-characterized castor 18:0-ACP desaturase converted 18:0 to a single 18:1 product, i.e., without further metabolism even during extended incubations. Below we describe experiments to determine the identity of product D.

Ivy desaturase further metabolizes both 16:1- and 18:1-ACPs. The lower mobility product (Figure 4 lane 7 marked D) formed during extended incubations of ivy desaturase with 18:0-ACP consistently appeared after accumulation of the 18:1-ACP, and its accumulation corresponded to a decrease in 18:1, suggesting a substrate-product relationship between the products marked 18:1 and D in Figure 4. To test this hypothesis, we synthesized 18:1-ACP and incubated it with the ivy and castor desaturases. In the presence of the ivy desaturase, product M decreased and product D accumulated (Figure 6, lanes 4-6). In contrast, in the presence of the castor desaturase, the 18:1-ACP remained unchanged during prolonged incubations (data not shown), consistent with the accumulation of a single product, i.e., 18:1-ACP during prolonged incubations on the castor desaturase with 18:0-ACP (Figure 4, lane 8). To confirm that 18:1-ACP does not bind to the castor desaturase, we performed additional assays with \([\textsuperscript{14}C]\) 18:0-ACP and observed no difference in desaturation rate of 18:0-ACP in the presence of up to 30-fold excess of unlabeled 18:1-ACP (data not shown). Data presented above establish the specificity of the ivy desaturase as \(\Delta^4\) with respect to 16:0 by both heterologous expression in Arabidopsis seed and \textit{in vitro} assay of purified enzyme. The 16:1\(\Delta^4\) product of the ivy desaturase, like the 18:1\(\Delta^9\) product of the castor desaturase, showed no further metabolism during extended incubation periods, suggesting that these are terminal products (Figure 4, lanes 3, 8 respectively). However, because we had observed further metabolism of 18:1\(\Delta^9\)-ACP, we synthesized an equivalent 16-carbon substrate, 16:1\(\Delta^9\)-ACP to investigate whether the ivy desaturase is capable of further metabolizing a shorter chain length monounsaturated substrate. In a surprising result, the ivy desaturase metabolized 16:1\(\Delta^9\)-ACP substrate to a product with equivalent mobility to that metabolized from 18:1\(\Delta^9\)-ACP substrate (Figure 6, lanes 1-3). While the argentation TLC assays are particularly useful for revealing changes in chemical properties of fatty acids, they do not provide diagnostic structural information that can be obtained.
Ivy desaturase converts 16:1Δ⁹- and 18:1Δ⁹-ACPs into equivalent Δ⁴,9-ACP dienes. The identity of products of 16:1Δ⁹- and 18:1Δ⁹-ACPs after incubation with the ivy desaturase was further investigated by incubating unlabeled fatty acyl-ACPs with the ivy desaturase, performing various derivatization reactions and analyzing the product using GC/MS. The scale of these reactions was typically 1-2 orders of magnitude larger than those described above employing ¹⁴C-fatty acid substrates. The reduction of mobility of products observed in argentation TLC the product displayed more polar character in the presence of Ag ions. Based on the reactivity of diiron enzymes, we hypothesized that this could result from the introduction of a second double bond, or epoxide or hydroxyl functionalities. Methyl esters of epoxy and hydroxy fatty acids bind to carbowax GC columns of the type used for these experiments, so the elution of material corresponding to the methyl esters of the new products (without further derivatization) (Figure 7, B and D) discounted the likelihood of epoxy or hydroxyl functionality, leaving a diunsaturated fatty acids as the most likely possibility. Pyrrolidine derivatization and subsequent GC/MS analyses on the products of 16:1- and 18:1-ACP substrates confirmed the presence of a second double bond (Figure 7, E and F). In the presence of ivy desaturase, 16:1Δ⁹-ACP substrate is converted to an abundant product that has longer retention time, (Figure 7B) consistent with our tentative assignment as a diene. Mass spectral analysis of pyrrolidine derivatives of the fatty acid methyl esters revealed that the product has a mass ion of 305, consistent with its assignment as 16:2, and the sequence of mass ions (126, 139 152, 194 and 206) described above for 16:2, showing that it too has bonds at the Δ⁴ and Δ⁹ position (Figure 7F). To test whether diene formation is dependent on the existing double bond being in the Δ⁹ position, we presented the ivy desaturase with 18:1Δ¹¹-ACP, which it converted to 18:2Δ⁴,11 (data not shown).

Kinetic analysis of the ivy desaturase. Specific activities of the ivy desaturase with a variety of substrates is shown in Figure 8. The ivy desaturase has the highest specific activity with its natural substrate 16:0-ACP, but also shows activity with 18:0-ACP and 14:0-ACP with lower activities for 16:1-ACP and 18:1-ACP. To investigate the basis for the changes in specificity we determined the kinetic parameters of the ivy desaturase with 16- and 18-carbon, saturated and monounsaturated, substrates in relation to those previously determined for saturated substrates for the castor desaturase (Table 1). For its natural substrate, 16:0-ACP, the ivy enzyme has a lower \( k_{cat} \) and higher \( K_m \) than the castor desaturase for its natural substrate, 18:0-ACP, resulting in a reduced specificity factor (10 versus 92). However, the specificity factor of ivy for 16:1-ACP is 2.5-fold higher than that of the castor desaturase for its second preferred substrate 16:0-ACP.

DISCUSSION

Soluble fatty acid desaturases are remarkable in that they can perform desaturation of fatty acids that lack functional landmarks for the entire length of the saturated acyl chain. The mechanism(s) that specify this exquisite control of regioselectivity remain to be determined. Our strategy for understanding determinants of regioselectivity is to identify closely-related desaturase paralogs that exhibit divergent regiospecificities and to perform comparative structure-function studies on them. The Δ⁴-16:0-ACP desaturases are particularly promising as models for comparative studies because they introduce their double bonds five carbons towards the carboxyl group of the fatty acid substrate.
compared to the $\Delta^9$ double bond introduced by the archetypal desaturases found in all plants. We present evidence that the ivy desaturase has $\Delta^4$-16:0 specificity based on heterologous expression in Arabidopsis, in which it accumulated the two novel fatty acids 16:1$\Delta^4$ and its expected elongation product, 18:1$\Delta^6$, which is consistent with that observed with expression of the coriander homolog in tobacco cell culture (30). The ivy desaturase is more similar in amino acid sequence (83% identity) to the coriander enzyme, which also has $\Delta^4$-16:0 specificity than to the castor $\Delta^9$-18:0 desaturase (78% identity). In contrast to the coriander enzyme the ivy desaturase expresses in functional form in E. coli allowing us to perform a biochemical characterization of its substrate specificity. Consistent with our expectations, the purified ivy desaturase converted 16:0-ACP into 16:1$\Delta^4$-ACP in vitro with a high specificity factor. However, the activity of the ivy enzyme is reduced relative to $\Delta^9$-18:0 desaturases, a result that has been reported for other, presumably newly evolved, desaturases that mediate the accumulation of unusual fatty acids (10,11). The degradation of kinetic parameters (i.e., $k_{cat}$ and $K_m$) of newly-evolved enzymes has been attributed to the stochastic accumulation of mutations that arise in tandem with those that give rise to the new functionality (31). The ability of the ivy desaturase to perform $\Delta^9$-desaturation of 18:0-ACP is consistent the hypothesis that it arose from a $\Delta^9$-18:0-ACP desaturase. Lower $K_m$ values with saturated substrates than those for unsaturated substrates, also supports this view.

Probing the specificity of the ivy desaturase with the use of different acyl-ACP substrates revealed its novel multifunctionality and several unexpected results. For instance, when presented with 18:0-ACP it desaturated at the $\Delta^9$ rather than $\Delta^4$ position. This production of different regiospecific products was specific to the substrate chain length in contrast to the Thunbergia $\Delta^6$–16:0-ACP desaturase, which with 16:0 substrate produced 16:1$\Delta^6$, but when presented with 18:0-ACP substrate produced mixture of $\Delta^6$ and $\Delta^9$ products (16). Our interpretation of the ivy result is that the desaturase has distinct binding modes for 16:0 and 18:0 substrates because the hydrogens that are removed from the substrate during desaturation must be located adjacent to the activated oxygen species responsible for desaturation, which is generated at the diiron active site. The location of the diiron site is fixed because its binding ligands are from each of the four helices that constitute the structural core of the enzyme. In the 18:0 binding mode, the substrate-binding cavity must accommodate nine carbons between the ACP and the diiron site and nine carbons between the diiron site and the blind end of the cavity. In contrast, when binding 16:0-ACP, the fatty acid must be inserted deeper into the cavity because it accommodates only four carbons between the carboxyl and the diiron site and 12 carbons between the diiron site and the blind end of the cavity. This means that the ivy 16:0-recognizing enzyme needs a cavity that accommodates three more carbon atoms than that of the 18:0-recognizing castor enzyme. While most of the residues lining the substrate-binding cavity were conserved between the ivy and castor desaturases, we identified two amino acid positions at 117 and 179 containing substitutions T117R and P179I (from castor to ivy). These changes have previously been shown (17,28) to cause increased specific activity for 16:0-ACP relative to 18:0-ACP though both exhibit $\Delta^9$ regiospecificity. Based on the similarity between the castor and ivy desaturases, it seems unlikely that ivy is unable to accommodate 12 carbons in an extended conformation in a cavity shorter than that found in the castor desaturase. We therefore hypothesize that, when performing $\Delta^4$ desaturation, the ivy desaturase binds substrate in a non-extended, perhaps hairpin, conformation similar to that seen for the fatty acid substrate bound to the InhA active site (32).

That 18:1$\Delta^9$-ACP can be further desaturated at the $\Delta^4$ position, while 18:0-ACP is desaturated at the $\Delta^9$ position, means that 18:1$\Delta^9$ can be inserted deeper into the cavity than 18:0 by five carbons. The
accommodation of additional carbons for 18:1 compared to 18:0 substrates is reminiscent of fatty acid chain length specificities of thioesterases described by Voelker, in which he observed that unsaturated substrates appear “shorter” to the enzyme (33). A similar phenomenon might partially explain differences in binding between saturated and unsaturated substrates in the desaturase. The bend in the substrate at the Δ⁹ or Δ¹¹ cis double bond positions could help facilitate the formation of a U-shaped conformation of the substrate as hypothesized above for 16:0. The biochemical data for the ivy desaturase constitute an apparent paradox in that a larger proportion of the substrate has to be accommodated for it to perform Δ⁴ desaturation on 16:0-ACP compared to Δ⁹ desaturation on 18:0-ACP. Crystallographic experiments with the goal of revealing the distinct binding modes that facilitate Δ⁹ and Δ⁴ desaturation will likely provide valuable additional insight.

The existence of two distinct binding modes for the ivy desaturase with 16:0- and 18:0-ACP substrates is in contrast to that seen for the archetypal castor desaturase in which a single binding mode (6) with the fatty acids in an extended conformation can best account for 18:0- and 16:0-ACP desaturation at the Δ⁹ position. Differences in rates of desaturation for various substrates have been correlated to differences in desolvation energy that are a function of their chain lengths (34). The occurrence of distinct substrate binding modes has been implicated for several other fatty acid modification enzymes including the bifunctional oleate 12-hydroxylase/desaturase from Lesquerella (35), and a bifunctional conjugase/desaturase from tung (36).

In vitro desaturation reactions also revealed that 18:1Δ⁹-ACP can be further metabolized to 18:2Δ⁴,9 by the ivy desaturase. Published reports suggest that soluble plastidial desaturases catalyze the introduction of one double bond into saturated acyl-ACPs and therefore do not recognize unsaturated substrates [reviewed in (5,37)]. In addition to recognizing a monounsaturated substrate, the ivy enzyme is capable of performing two sequential desaturations on an 18:0-ACP substrate, the first converting it to 18:1Δ⁹-ACP, the second to 18:2Δ⁴,9-ACP. While a novel finding for the soluble class of desaturases, the introduction of a double bond between an existing double bond and the carboxyl group of a fatty acid has been previously reported for the evolutionary distinct class of integral membrane “front end” desaturases exemplified by the borage desaturase (38). In contrast to the ivy desaturase that performs sequential desaturations with different regiospecificities, the bifunctional Ceratodon purpureus Δ⁶ acetylenase performs two sequential desaturations at the same site, first desaturating at the Δ⁶ position then introducing an acetylenic bond (39).

The observed multifunctionality of the ivy desaturase therefore conveys the capacity for plants to accumulate novel dienes under a specific set of metabolic conditions. Achieving such a metabolic context might involve changes in the activities of other lipid metabolizing enzymes such as endogenous desaturases, thioesterases, condensing enzymes and perhaps acyltransferases (40). As discussed by Dyer, the evolution of multifunctionality can thus be considered as one step in a multi-step process that can result in the evolution of novel metabolic diversity (36).

REFERENCES


FOOTNOTE

Fatty acid nomenclature: \(x:y\), \(x\) is the number of carbon atoms in the fatty acid chain, and \(y\) is the number of double bonds. \(\Delta^x\) indicates the regiospecificity, i.e., the position of the double bond relative to the carboxyl end of the fatty acid. Amu, atomic mass units; ACP, acyl carrier protein. Unless otherwise indicated, amino acid numbering corresponds to the sequence of the mature castor \(\Delta^9\)-18:0-ACP desaturase (see ref. 6). The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number AY998047.

ACKNOWLEDGMENTS

We thank Dr. J. Setlow for editorial assistance and the Office of Basic Energy Sciences of the U.S. Department of Energy for financial support.
Table I
Kinetic parameters of desaturase with various substrates.

<table>
<thead>
<tr>
<th>Desaturase</th>
<th>Substrate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$k_{cat}$&lt;sup&gt;b&lt;/sup&gt;</th>
<th>$K_m$</th>
<th>Specificity factor&lt;sup&gt;c&lt;/sup&gt; $k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(min&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(µM)</td>
<td>(µM&lt;sup&gt;-1&lt;/sup&gt;.min&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>ivy $\Delta^4/\Delta^9$</td>
<td>16:0</td>
<td>15 (1.3)</td>
<td>1.5 (0.2)</td>
<td>10</td>
</tr>
<tr>
<td>ivy $\Delta^4/\Delta^9$</td>
<td>16:1</td>
<td>2.9 (0.5)</td>
<td>2.1 (0.6)</td>
<td>1.4</td>
</tr>
<tr>
<td>ivy $\Delta^4/\Delta^9$</td>
<td>18:0</td>
<td>6.4 (0.8)</td>
<td>1.5 (0.3)</td>
<td>4.2</td>
</tr>
<tr>
<td>ivy $\Delta^4/\Delta^9$</td>
<td>18:1</td>
<td>0.23 (0.05)</td>
<td>2.0 (0.7)</td>
<td>0.12</td>
</tr>
<tr>
<td>castor $\Delta^9$</td>
<td>18:0</td>
<td>42 (2)</td>
<td>0.46 (0.05)</td>
<td>92</td>
</tr>
<tr>
<td>castor $\Delta^9$</td>
<td>16:0</td>
<td>2.8 (0.1)</td>
<td>5.0 (0.5)</td>
<td>0.56</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fatty acid esterified to ACP.
<sup>b</sup> $k_{cat}$ is reported per diiron site.
<sup>c</sup> Means based on 4 or 5 rates per substrate, numbers in parentheses represent standard errors.
<sup>d</sup> castor data from ref. 17.
FIGURE LEGENDS

Fig. 1. Deduced amino acid sequence comparison of the mature portion of ivy and coriander Δ^4-16:0-ACP desaturases with castor Δ^9-18:0-ACP desaturase. Residues are numbered according to the mature castor amino acid sequence and specific amino acids are indicated as follows: ^ locations in the substrate-binding cavity that differ between ivy and castor desaturases, * diiron binding ligands. Topology is of α-helical and β-sheet sections indicated above the sequences by dashed lines. Alignment was performed by Clustal W, and highlighting by Boxshade.

Fig. 2. Panel A, Fatty acid profiles of methyl esters from fab1 Arabidopsis seeds. Panel B, Fatty acid profile of methyl esters from fab1 Arabidopsis seeds expressing the ivy desaturase. Fatty acid identities are as follows: 1, 16:0; 2, 16:1Δ^4; 3, 16:1Δ^9; 4, 18:0; 5, 18:1Δ^6; 6, 18:1Δ^9; 7, 18:1Δ^11; 8, 18:2Δ^9,12; 9, 20:0. Panel C, MS analysis of the dimethyl disulfide adduct of 16:1Δ^4 and its fragmentation ions: X, CH_3(CH_2)_10CHSCH_3 and Y, CH_3S(CH_2)_6CO_2CH_3; panel D, MS analysis of the dimethyl disulfide adduct of 18:1Δ^6 and its fragmentation ions: X, CH_3(CH_2)_10CHSCH_3 and Y, CH_3S(CH_2)_6CO_2CH_3.

Fig. 3. Purification and characterization of the ivy desaturase. Panel A; SDS-PAGE gel of purification of the ivy desaturase. Lanes, 1, lysate after passage through French pressure cell, 2, lysate after clarification by centrifugation, 3, eluate from ion exchange 20 CM HPLC chromatography, 4, eluate from G300SW size exclusion chromatography. D marks position of desaturase. 10 mg of purified desaturase was loaded in lane 4. Panel B; UV-visible spectrum of purified ivy desaturase. Inset is an expansion of the full profile to show detail in the 320-500 nm region of the ligand to metal charge transfer bands. Arrow marks 375 nm.

Fig. 4. Phosphorimage of TLC separation of [1-^14C] fatty acid methyl esters resulting from desaturation assays. Substrates used, lanes 1-4, 16:0-ACP; 5-8, 18:0-ACP. Enzymes and incubation times: 1,5, control (i.e., no desaturase); 2,6 ivy desaturase for 5 minutes; 3,7 ivy desaturase for 60 min; 4,8 castor Δ^9-18:0-ACP desaturase for 60 min. Letters: S, saturated substrate; M, monoene products; D, putative diene product; O, origin of loaded material.

Fig. 5. In vitro conversion of 16:0 to 16:1Δ^4 by the ivy desaturase. Panel A, GC separation of methyl esters of reactions before and after incubation with purified ivy desaturase. Fatty acid methyl esters 1, 16:0; 2, 16:1Δ^9. Panel C, MS analysis of the dimethyl disulfide adduct of the 16:1Δ^4 product of the ivy desaturase and its fragmentation ions: X, CH_3(CH_2)_10CHSCH_3 and Y, CH_3S(CH_2)_6CO_2CH_3. Panel D, MS analysis of the pyrrolidine adduct of the 16:1Δ^4 product of the ivy desaturase with diagnostic ions labeled.

Fig. 6. Phosphorimage of TLC separation of [1-^14C] fatty acid methyl esters from desaturation assays. Substrates used, lanes 1-3, 16:1Δ^9-ACP; 4-6, 18:1Δ^9-ACP. Enzymes and incubation times: 1,4, control (i.e., without desaturase); 2, 5, ivy desaturase for 5 minutes; 3, 6, ivy desaturase for 60 min. Letters as defined in Figure 3.

Fig. 7. In vitro conversion of 16- and 18-carbon monoenes to the corresponding dienes by the ivy desaturase. Panels A-D, GC separation of methyl esters of reactions before (A, C), and after, (B, D), incubation with purified ivy desaturase with monoene substrates, A, B, 16:1Δ^9-ACP, C, D,
18:1Δ⁹-ACP. Fatty acid methyl esters 1, 16:1Δ⁹, 2, 16:2Δ⁴, 3, 18:1Δ⁹, 4, 18:1Δ¹¹, 5, 18:2Δ⁴. Panels E and F, MS analysis of pyrrolidine adducts of the reaction products with diagnostic ions as indicated.

Fig. 8. Specific activity profiles of the ivy desaturase against various substrates. Means of between 5 and 12 assays per substrate, error bars represent standard deviations.
Figure 1.

<table>
<thead>
<tr>
<th></th>
<th>Ivy</th>
<th>Coriander</th>
<th>Castor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ASTVNSNSMVLDNLKSPPN------LQVTHSMPPQKLEIFKSLDDWARNNVLIHLKSVEKSWQPQDYLPDPVSDGFEEQV</td>
<td>ASTLHASPLVFDKLKAG---------R------PEVDELFNSLEGWARDNILVHLKSVENSWQPQDYLPDPTSDAFEDQV</td>
<td>ASTLKSGSKEVENLKKPFMPPREVHVQVTHSMPPQKIEIFKSLDNWAEENILVHLKPVENSWQPQDFLPDPASDGFDEQV</td>
</tr>
<tr>
<td>2</td>
<td>RELRERAREIPDDYFVVLVGDMITEEALPTYMSMLNRCDGIKDETGAEPSAWAMWTRAWTAEENRHGDLLNKYLYLSGRV</td>
<td>KEMRERAKDIPDEYFVVLVGDMITEEALPTYMSMLNRCDGIKDDTGAQPTSWATWTRAWTAEENRHGDLLNKYLYLSGRV</td>
<td>RELRERAREIPDDYFVVLVGDMITEALPTFVMSMLNRCDGIKDNWAEENILVHLKPVENSWQPQDFLPDPASDGFDEQV</td>
</tr>
<tr>
<td>3</td>
<td>DMRKIEKTIQYLIGSGMDIKSENSPYLGFIYTSFQERATFISHANTAKLAQHYGDKNLAHICGSIASDEKRHATAYTKIV</td>
<td>DMRMIEKTIQYLIGSGMDTKTENCPYMGFIYTSFQERATFISHANTAKLAQHYGDKNLAQVCGNIASDEKRHATAYTKIV</td>
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<tr>
<td>4</td>
<td>EKLAEIDPDTTVIAFADMMRKKITMPAHLMYDGSDELLFKHFTAVAQRVGVYSALDYCDILEFLVDKWNVERLTGLSDEG</td>
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<td>5</td>
<td>RKAQEYVCELGPKIRRVEEKVQGKEKKKKAEHPVSFSWIFNRELKI</td>
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<td>KEMRERAKDIPDEYFVVLVGDMITEEALPTYMSMLNRCDGIKDDTGAQPTSWATWTRAWTAEENRHGDLLNKYLYLSGRV</td>
<td>RELRERAREIPDDYFVVLVGDMITEALPTFVMSMLNRCDGIKDNWAEENILVHLKPVENSWQPQDFLPDPASDGFDEQV</td>
</tr>
</tbody>
</table>
Figure 2.
Figure 3.

A

1 2 3 4

-D

B

Absorbance

2

1

320 500

Wavelength (nm)

250 400 550

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Figure 5.

A  
Detector Response

Retention Time

B

C

Relative Abundance
Mass/charge

X
147

Y
215

16:1Δ^4
Pyrrolidide

D  
Relative Abundance
Mass/charge

126
139
152

M^+
307
Figure 6.
Figure 7.

A) 1  
B) 2  
C) 3  4  
D) 3  4  5  

Detector Response  
Retention Time  
Retention Time  
Retention Time  

E)  
F)  

Relative Abundance  
Mass/charge  
Mass/charge  

16:2Δ4,9 Pyrrolidide  
18:2Δ4,9 Pyrrolidide
Figure 8.

Substrate Specific activity (nmol min⁻¹ mg⁻¹)

0 20 40 60 80 100 120 140 160 180

14:0 16:0 18:0 16:1 18:1

Specific activity (nmol min⁻¹ mg⁻¹)

Substrate
A multifunctional acyl-acyl carrier protein desaturase from Hedera helix L. (English ivy) can synthesize 16- and 18-carbon monoene and diene products
Edward Whittle, Edgar B. Cahoon, Satyam Subrahmanyam and John Shanklin

*J. Biol. Chem.* published online June 6, 2005

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