Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10639–10643, November 1995
Biochemistry

Modification of the substrate specificity of an acyl-acyl carrier protein thioesterase by protein engineering

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Communicated by Paul K. Stumpf, University of California, Davis, CA, July 24, 1995 (received for review May 23, 1995)

ABSTRACT **The plant acyl-acyl carrier protein (ACP) thioesterases (TEs) are of biochemical interest because of their roles in fatty acid synthesis and their utilities in the bioengineering of plant seed oils. When the FatB1 cDNA encoding a 12:0-ACP TE (Uc FatB1) from California bay, *Umbellularia californica* (Uc) was expressed in *Escherichia coli* and in developing oilseeds of the plants *Arabidopsis thaliana* and *Brassica napus*, large amounts of laurate (12:0) and small amounts of myristate (14:0) were accumulated. We have isolated a TE cDNA from camphor (*Cinnamomum camphorum*) (Cc) seeds that shares 92% amino acid identity with Uc FatB1. This TE, Cc FatB1, mainly hydrolyzes 14:0-ACP as shown by *E. coli* expression. We have investigated the roles of the N- and C-terminal regions in determining substrate specificity by constructing two chimeric enzymes, in which the N-terminal portion of one protein is fused to the C-terminal portion of the other. Our results show that the C-terminal two-thirds of the protein is critical for the specificity. By site-directed mutagenesis, we have replaced several amino acids in Uc FatB1 by using the Cc FatB1 sequence as a guide. A double mutant, which changes Met-197 to an Arg and Arg-199 to a His (M197R/R199H), turns Uc FatB1 into a 12:0/14:0 TE with equal preference for both substrates. Another mutation, T231K, by itself does not effect the specificity. However, when it is combined with the double mutant to generate a triple mutant (M197R/R199H/T231K), Uc FatB1 is converted to a 14:0-ACP TE. Expression of the double-mutant cDNA in *E. coli* K27, a strain deficient in fatty acid degradation, results in accumulation of similar amounts of 12:0 and 14:0. Meanwhile the *E. coli* expressing the triple-mutant cDNA produces predominantly 14:0 with very small amounts of 12:0. Kinetic studies indicate that both wild-type Uc FatB1 and the triple mutant have similar values of $K_{m,app}$ with respect to 14:0-ACP. Inhibitory studies also show that 12:0-ACP is a good competitive inhibitor with respect to 14:0-ACP in both the wild type and the triple mutant. These results imply that both 12:0- and 14:0-ACP can bind to the two proteins equally well, but in the case of the triple mutant, the hydrolysis of 12:0-ACP is severely impaired. The ability to modify TE specificity should allow the production of additional "designer oils" in genetically engineered plants.**

In both animals and plants, an acyl-acyl carrier protein (ACP) thioesterase (TE) terminates fatty acyl group extension by hydrolyzing the acyl moiety from the anabolically active acyl-ACP at the appropriate chain length, releasing free fatty acids (1–3). The plant TEs are of biochemical interest because of the recent progress in the bioengineering of plant oil biosynthesis. A medium-chain acyl-ACP TE (Uc FatB1) from California bay, *Umbellularia californica* (Uc), was isolated (4), and its cDNA was subsequently cloned and expressed in *Escherichia coli* and developing oilseeds of the plants *Arabidopsis thaliana* and *Brassica napus* (5, 6). In all these heterologous systems, large amounts of laurate (12:0) and small amounts of myristate (14:0) were accumulated. These results demonstrated the role of the TE in determining chain length during *de novo* fatty acid biosynthesis and the utility of these enzymes for modifying seed oil contents in higher plants.

To date, a number of cDNAs encoding various plant acyl-ACP TEs have been cloned (refs. 6–10; L.Y., J. Kridl, K. Dehesh, T.A.V., and D.J.H., unpublished data). Sequence analyses of these TEs show high homology, implying similarity in structure and functions (11). Some of these TEs have been expressed in *E. coli*, and their substrate specificities were determined by *in vitro* assays (10). The fact that these enzymes share significant sequence homology yet show different substrate specificities indicates that subtle changes of amino acids may be sufficient to change substrate selectivity.

Little information is available on the structural and functional divergence among these plant TEs. To the best of our knowledge, the tertiary structure of any plant TE has yet to be determined. Protein engineering has proven to be a powerful tool for understanding the mechanism of substrate recognition and catalysis, leading to the rational design of enzymes with more desirable substrate specificities (12–14). The approach we sought was first to isolate an Uc FatB1 homologue that has different substrate specificity and then identify amino acid residues or regions that could be attributed to substrate recognition. Finally, the information thus generated would be used to guide protein engineering of Uc FatB1. The seeds of camphor (*Cinnamomum camphorum*) (Cc) were particularly interesting for our purpose because this plant belongs to the same family (Lauraceae) as California bay, and its mature seeds accumulate predominantly medium-chain (C10 and C12) fatty acids (15). We report the isolation of a homologue cDNA of Uc FatB1 from camphor and the use of its sequence information† to guide the modification of the substrate specificity of Uc FatB1.

MATERIALS AND METHODS

Materials. The *E. coli* Sure was obtained from Stratagene. Restriction enzymes and DNA ligase were purchased from Boehringer Mannheim. *Taq* DNA polymerase and dNTPs for PCR were from Perkin–Elmer. Acyl-ACP substrates (50–60 μCi/μmol; 1 Ci = 37 GBq) were prepared at Calgene as described (3). Other chemicals were obtained from Sigma in the highest purity available.

Isolation of the TE from Camphor. Developing seeds of *C. camphorum* were collected from local street trees in Davis, CA. For TE analysis, crude extracts of the camphor seeds were prepared and assayed by the procedures of Pollard *et al.* (3). RNA was isolated essentially by a published protocol (16). cDNA was made from 10 μg of total RNA by using oligo(dT) and reverse transcriptase. PCR was used to amplify the regions

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Abbreviations: ACP, acyl carrier protein; TE, thioesterase; Uc, *Umbellularia californica*; Cc, *Cinnamomum camphorum*.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U31813).

that are highly conserved among the bay and safflower TEs (6, 7, 10). To obtain the remaining cDNA sequence, 3′ rapid amplification of cDNA ends was performed as described by Frohman *et al.* (17). An expression construct of Cc FatB1 was made using the pBC SK+ plasmid (Stratagene). The coding region was PCR-amplified and subsequently inserted into *Xba* I and *Sal* I sites of the pBC vector to yield plasmid pCGN5220. The construct was confirmed by DNA sequencing. Plasmid pCGN5220 was designed to closely reassemble the Uc FatB1 expression construct pCGN3823, a plasmid pBS$^-$ (Stratagene) containing a 1.2-kb *Xba* I fragment of Uc FatB1 cDNA as a *lacZ* fusion (5).

Construction of Chimeric Enzymes. Plasmids pCGN3823 and pCGN5220 were used for the construction of the chimeric enzymes. In both cDNAs there is a conserved unique *Kpn* I site at the codon for amino acid residue 177 of the open reading frame (382 total amino acids; see Fig. 1). A second *Kpn* I site is located within the polylinkers of the plasmids at the 3′ of the stop codons in both constructs. Therefore, the interchange of the two *Kpn* I fragments between pCGN3823 and pCGN5220 allows the fusion of the N-terminal region of one TE to the C-terminal region of the other, forming two chimeric enzymes.

Site-Directed Mutagenesis. PCR site-directed mutagenesis (18) was used for amino acid replacements. The sense mutant primers used for the mutagenesis were as follows: **M197R/R199H**, 5′-GGAAATAATGGCCGACGACATGATTTC-CTTGTCC; **T231K**, 5′-GGTTGTCCAAAATCCC; **R327Q**, 5′-GCGTGCTGCAGTCCCTGACC; **R322M/R327Q**, 5′-GAGAGAGTGCACGATGGATAGCGTGCTGCAG-TCCCTGACC, where M, R, H, T, K, and Q (boldface type) are methionine, arginine, histidine, threonine, lysine, and glutamine, respectively (see below), and the mutated nucleotides are underlined.

Enzyme Assays and Kinetics. The transformed *E. coli* cells were grown at 30°C to 0.6 OD_{600} unit, followed by addition of 1 mM isopropyl β-D-thiogalactoside and continuous growth at 30°C for 2 h. The sedimented cells were resuspended and sonicated in the assay buffer, and acyl-ACP hydrolysis was measured as described (5). Sure cells transformed with pCGN3823 and pBC served as positive and negative controls, respectively. One enzyme activity unit is defined as 1000 cpm per min per mg of total protein.

Progress curves were obtained by scaling up the assay volume and removing 100 μl at 5-min intervals into 0.5 ml of stop solution (10% acetic acid). Kinetic assays were performed at 30°C in 100 mM Tris·HCl, pH 8.0/0.01% Triton X-100/1 mM dithiothreitol/10% (vol/vol) glycerol. After extraction of each reaction mixture with 2.0 ml of dimethyl ether, the radioactivity in 900 μl of the organic fraction was determined by liquid scintillation counting. This procedure allows accurate measurement of the total extractable free fatty acid (^{14}C-labeled) without the interference of interphase between the organic and aqueous fractions. Laurate and myristate production in this assay was linear with respect to time for at least 30 min and with respect to enzyme concentration up to 1 milliunit. All assays were conducted in duplicate. Initial rate data were fitted to the following equations by using kinetics software (K_{cat}) from Biometallics (Princeton): for competitive inhibition, $v = V_{max}S/[K_{m,app}(1 + I/K_{is}) + S]$; for noncompetitive inhibition, $v = V_{max}S/[K_{m,app}(1 + I/K_{is}) + S(1 + I/K_{ii})]$; and for uncompetitive inhibition, $v = V_{max}S/[K_{m,app} + S(1 + I/K_{ii})]$; where v is velocity; V_{max} is maximum velocity; S is substrate concentration; $K_{m,app}$ is apparent Michaelis constant; K_{is} and K_{ii} are slope and intercept inhibition constants, respectively; and I is inhibitor concentration.

Expression of the Mutants in *E. coli fadD* Cells. The *E. coli* fatty acid-degradation mutant strain K27 (*fadD 88*) (19, 20) was obtained from the *E. coli* Genetic Stock Center, Yale University (New Haven, CT) (CGSC 5478) and made competent. The *fadD* cells transformed with either the pBC plasmid or the TE expression constructs were grown overnight at 30°C in LB medium containing chloramphenicol (50 mg/liter) and 1 mM isopropyl β-D-thiogalactoside. The total lipids were analyzed as described (5).

RESULTS

The Camphor TE (Cc FatB1). By combination of homologous PCR and 3′ rapid amplification of cDNA ends, we have isolated a homologue cDNA of Uc FatB1 from *C. camphorum* (Fig. 1). This cDNA encodes a TE that has the same numbers of amino acids as Uc FatB1. Their putative transit peptides (approximately the first 60 amino acids) are almost identical, and the mature proteins share 92% identity (see Fig. 1). When Cc FatB1 cDNA was expressed in *E. coli*, its substrate specificity was found to be predominantly for 14:0-ACP (Fig. 2*B*).

The Chimeric Enzymes. Both cDNAs of the bay and camphor TE contain open reading frames encoding 382 amino acids. Only 31 amino acids are different, and among them more than half are conservative substitutions (Fig. 1). The codon usage is highly conserved between the two genes, suggesting their common origin. By using the unique *Kpn* I sites, which are conserved in both coding regions, and the *Kpn* I sites in cloning linkers of the vectors, two fusion constructs were generated. The resulting chimeric enzymes contain 92 amino acids from the N-terminal of one mature TE and 207 amino acids from the C-terminal portion of the other. The fusion protein containing the C-terminal portion of Cc FatB1 is referred to as chimeric 1 (Ch-1), and the other fusion protein is chimeric 2 (Ch-2) (Fig. 1). TE specific activities of the chimeric enzymes are shown in Fig. 3 *A* and *B*. For Ch-1 the preferred substrate was 14:0-ACP (Fig. 3*A*), whereas for Ch-2 it was 12:0-ACP (Fig. 3*B*). These

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                                                                    50
MATTSLASAFCSMKAVMLARDGRGMKPRSSDLQLRAGNAPTSLKMINGTK
-----------------------------------------Q----------
                                                                   100
FSYTESLKRLPDWSMLFAVITTIFSAAEKQWTNLEWKPKPKLPQLLDDHF
--------K--------------------------------NP--------
                                                                   150
GLHGLVFRRTFAIRSYEVGPDRSTSILAVMNHMQEATLNHAKSVGILGDG
-P------------------------V-----L---A-------------
                                  KpnI                             200
FGTTLEMSKRDLMWVVRRTHVAVERYPTWGDTVEVECWIGASGNNGMRRD
------------I---K----------A----------V-------R-H-
                                                                   250
FLVRDCKTGEILTRCTSLSVLMNTRTRRLSTIPDEVRGEIGPAFIDNVAV
--------------------M---------K--E----------------
                                                                   300
KDDEIKKLQKLNDSTADYIQGGLTPRWNDLDVNQHVNNLKYVAWVFETVP
--E----P-----------------------I------I---D-IL----
                                                                   350
DSIFESHHISSFTLEYRRECTRDSVLRSLTTVSGGSSEAGLVCDHLLQLE
------------I-------M----Q---------------E------

GGSEVLRARTEWRPKLTDSFRGISVIPAEPRV
--------K--------------------SS-
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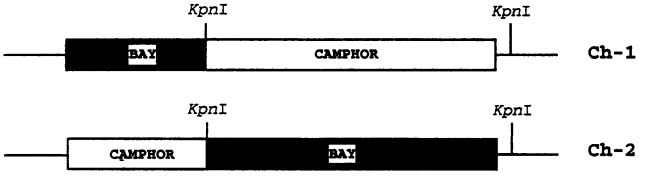

FIG. 1. Amino acid alignment of the TEs and chimeric constructs Ch-1 and Ch-2. The bay TE (Uc FatB1) sequence (GenBank accession no. M94159) is the upper sequence. Residues identical to Uc FatB1 in the camphor TE (Cc FatB1) sequence (lower sequence, GenBank accession no. U31813) are indicated by a dash. An arrow indicates the N-terminal amino acid of the mature protein (6, 10), which was used for the recombinant expression. The *Kpn* I site used for domain switches is shown above the Uc FatB1 sequence. The residues subject to site-directed mutagenesis are shown in boldface type. The chimeric constructs are shown as in-frame fusions of N- and C-terminal portions (from left to right) below the sequences.

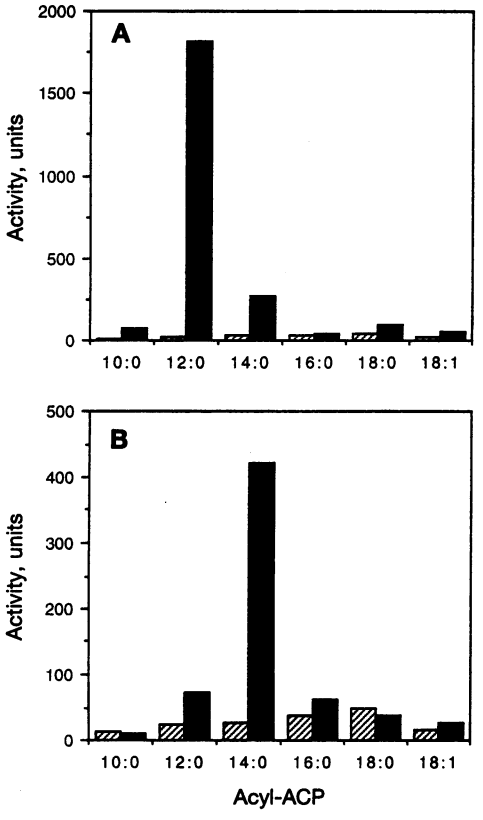

FIG. 2. Substrate specificities (solid bars) of Uc FatB1 (*A*) and Cc FatB1 (*B*). *E. coli* Sure cells transformed with the control pBC SK vector (hatched bars) and TE expression constructs were grown and induced by isopropyl β-D-thiogalactoside, and the cell lysates were assayed. One enzyme activity unit is defined as 1000 cpm per min per mg of total protein.

results indicate that the C-terminal portion of the TE protein determines the substrate specificity.

Internal Substitutions. Site-directed mutagenesis was applied to modify several amino acids of Uc FatB1 by using Cc FatB1 sequence as a guide. The enzyme specificities for two of the mutants are shown in Fig. 3 *C* and *D*. A mutant that changed Met-197 to Arg and Arg-199 to His (M197R/R199H) turned Uc FatB1 into a 12:0/14:0-ACP TE, with equal activity on both substrates (Fig. 3*C*). A second mutant, T231K, and wild type gave identical activity profiles (data not shown). However, the triple mutant M197R/R199H/T231K, which combines the three mutations, switched Uc FatB1 to a 14:0-ACP-preferring TE (Fig. 3*D*).

Two more mutants (R327Q and R322M/R327Q) have also been tested for TE activity. Both mutants showed identical specificities, and their specific activities toward 12:0-ACP and 14:0-ACP decreased about 100- and 30-fold, respectively, compared to the wild-type Uc FatB1. These data indicate that the mutation R327Q is responsible for the decreased activity.

Table 1. Accumulation of 12:0 and 14:0 fatty acids in *fadD* cells expressing Uc FatB1, Cc FatB1, and the mutants

TE	Accumulation	
	12:0, nmol/ml	14:0, nmol/ml
Control	0.3	1.6
Uc FatB1	505.5	39.0
Cc FatB1	79.5	318.8
M197R/R199H	123.5	181.1
M197R/R199H/T231K	35.4	352.9

Control is from *fadD* cells transformed with the pBC SK+ vector only. Accumulation is measured as nmol/ml of total culture.

Table 2. Kinetic constants of the wild-type Uc FatB1 and the triple mutant (M197R/R199H/T231K)

Enzymes	$K_{m,app}$, μM*		12:0-ACP K_i, μM†
	14:0-ACP	12:0-ACP	
Uc FatB1	6.4 ± 1.9	1.9 ± 0.5	10.2 ± 1.2 (competitive)‡
Mutant	2.3 ± 0.4	ND	11.6 ± 0.2 (competitive)

ND, not determined.
*Assay conditions: 100 mM Tris·HCl, pH 8.0/0.01% Triton X-100/1 mM dithiothreitol/10% glycerol, at 30°C.
†Slope inhibition constants of 12:0-ACP with 14:0-ACP as varied substrates.
‡Competitive inhibition with respect to 14:0-ACP.

Fatty Acid Accumulation in *E. coli fadD* Cells. When Uc FatB1 was expressed in *fadD* cells, large amounts of laurate (>500-fold above control background) and small amounts of myristate (≈10% of that of laurate) were produced (Table 1). This result is consistent with the previous report (5). When Cc FatB1 was expressed in *fadD* cells, large amounts of 14:0 and much less of 12:0 were produced. When mutant M197R/R199H was expressed in *fadD* cells, the ratio of 12:0/14:0 changed to 1:1.5 (Table 1), reflecting the TE specificity of this mutant (Fig. 3*C*). In contrast to the wild type, the ratio of 12:0/14:0 was completely reversed when mutant M197R/R199H/T231K was expressed in *fadD* cells. This result is also consistent with the enzyme specificity of the mutant (Fig. 3*D*).

Kinetics. Under the same experimental conditions, both Uc FatB1 and the triple mutant have similar values of $K_{m,app}$ with respect to 14:0-ACP (6.4 ± 1.9 μM and 2.3 ± 0.4 μM, respectively, see Table 2). The specific activity of the mutant toward 12:0-ACP was too low to obtain any meaningful kinetic parameters under our assaying system. Inhibition assays using unlabeled 12:0-ACP to compete with the substrate (^{14}C-labeled 14:0-ACP) showed a very similar result for both wild-type and mutant enzyme (Table 3). When equal amounts of inhibitor (12:0-ACP) and substrate (14:0-ACP) were present in the assay, the 14:0-ACP TE activity was reduced about 50%. If the amount of 12:0-ACP was five times that of 14:0-ACP, the 14:0-ACP TE activity was reduced >75%. Inhibition kinetics showed that 12:0-ACP was a competitive inhibitor with respect to 14:0-ACP (K_i values were 10.2 ± 1.2 μM and 11.6 ± 0.2 μM for the wild-type and mutant enzyme, respectively; Table 2).

DISCUSSION

Biochemical data have suggested that the TE activities are responsible for the accumulation of medium-chain fatty acids in maturing seeds of camphor (15). Because the seeds of camphor have a different fatty acid composition compared to those of the bay, we hypothesized the existence of a medium-chain TE that prefers a substrate other than 12:0. By using a homologous PCR approach, we have amplified and subsequently isolated a cDNA encoding a medium-chain TE (Cc FatB1) from camphor. The deduced amino acid sequence of Cc FatB1 reported in this study shows remarkable homology to Uc FatB1 (Fig. 1). However, the two enzymes display different substrate specificities (Fig. 2). The 14:0 specificity of Cc FatB1 was somewhat unexpected since camphor seeds do not accumulate significant amounts of 14:0 (15). We suspect that Cc FatB1 is not involved in the medium-chain fatty acid accumulation but, rather, is a representative of the evolutionary ancient FatB TE probably present in all angiosperms (10). It is possible that C10 and C12 fatty acids in camphor are produced by another TE whose cDNA was not amplified under our relatively stringent PCR conditions. The function of Cc FatB1 could be for "housekeeping," such as myristoylation of proteins, rather than production of storage fatty acids.

The conservation of amino acids among the enzymes extends throughout the entire mature proteins. This raises the

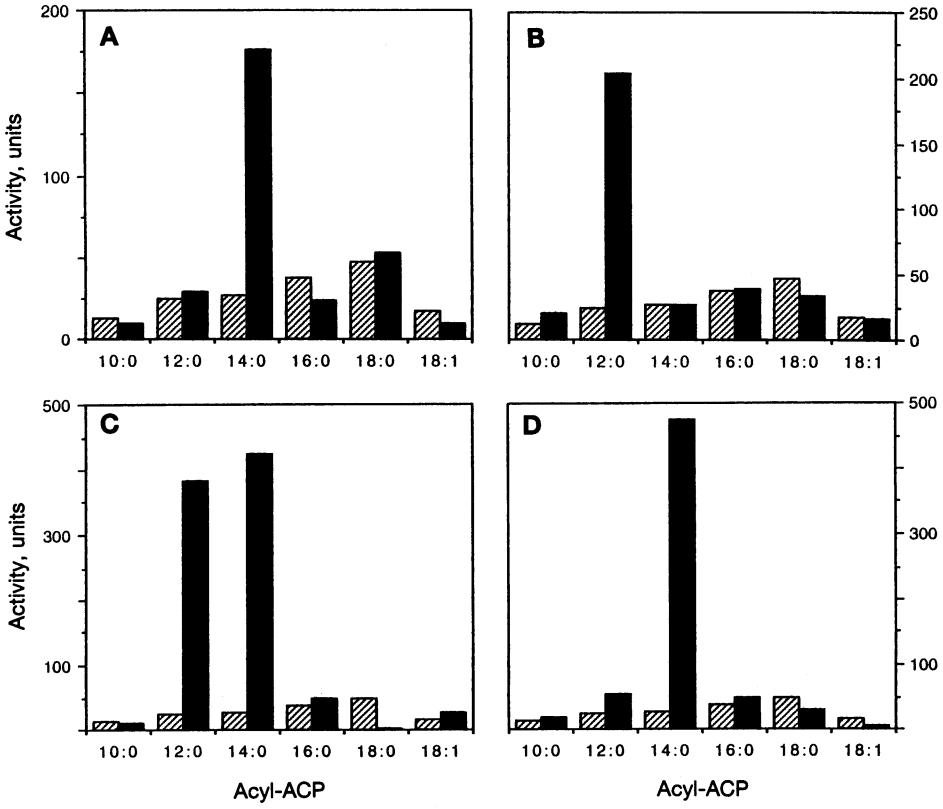

FIG. 3. Substrate specificities (solid bars) of Ch-1 (*A*), Ch-2 (*B*), mutant M197R/R199H (*C*), and mutant M197R/R199H/T231K (*D*). The background activities of the control *E. coli* (transformed with the vector alone) are shown by the hatched bars.

interesting question of which portion of the protein is more critical in determining the substrate specificity. The strategy of using chimeric gene products has been applied to study the structure and function of closely related proteins (21, 22). We have constructed two chimeric enzymes, fusing N- and C-terminal portions of the two enzymes (Fig. 1). Our results show that the C-terminal two-thirds of the protein is critical for substrate specificity; i.e., the chimeric enzyme containing the C-terminal portion of Cc FatB1 (Ch-1) has the same specificity as camphor TE (specific for 14:0), and the other chimeric protein with the Uc FatB1 C terminus (Ch-2) is 12:0-specific (Fig. 3 *A* and *B*).

In the effort to pinpoint the critical amino acid residues determining substrate specificity, the chimeric enzyme data has led us to focus on the C-terminal two-thirds of Uc FatB1. When comparing the sequence alignment, the most striking amino acid substitutions between the two TEs (Uc FatB1 → Cc FatB1) are Met-97 → Arg, Arg-199 → His, Thr-231 → Lys, Ala-293 → Asp, Arg-327 → Gln, Pro-380 → Ser, and Arg-381 → Ser. The mutation of the tripeptide MRR (amino acids 197–199) to RRH is especially interesting. According to secondary structure predictions from methods of Chou and Fasman (23, 24) and Garnier *et al.* (25), this tripeptide follows a β-sheet and a turn anchored by two highly conserved glycines (Gly-193 and Gly-196). When the tripeptide is changed to RRH, mimicking the sequence in Cc FatB1, the activity of the mutant toward 12:0, but not 14:0, is reduced ≈7-fold compared to the wild type. This mutant is a 12:0/14:0 TE that has approximately equal specific activity with respect to the two substrates (Fig. 3*C*). Fully converting Uc FatB1 to a 14:0-ACP TE has been accomplished by making an additional mutation, T231K, in the M197R/R199H mutant (Fig. 3*D*). The resulting mutant enzyme is highly 14:0-ACP-specific. Interestingly, the mutation T231K alone does not affect the specificity of Uc FatB1. The nonadditive combinatorial effect of this mutation on the double mutant suggests that the mutation sites are folded close to each other (26).

Table 3. Inhibition of 12:0-ACP on 14:0-ACP TE activities

Enzymes	Substrate, μM	Inhibitor, μM	Inhibition, %
Uc FatB1	5	5	53
	5	25	78
M197R/R199H/T231K	5	5	48
	5	25	76

Assays were done in the presence or absence of the inhibitor (unlabeled 12:0-ACP) at the stated concentrations. The substrate (^{14}C-labeled 14:0-ACP) concentration was 5 μM in all assays. Experiments were performed at 30°C in 100 mM Tris·HCl, pH 8.0/0.01% Triton X-100/1 mM dithiothreitol/10% glycerol. After 30 min, the liberated free ^{14}C-labeled fatty acids were extracted and radioactivity was measured.

The changes in specificity of the mutants have been reflected in the total fatty acid profiles of *E. coli fadD* cells expressing the mutant constructs. For Uc FatB1, the *fadD* cells produce large amounts of fatty acid 12:0, with small amounts of 14:0 (5–10% of 12:0) (ref. 5 and Table 1). However, with two amino acid substitutions (M197R/R199H), the mutant enzyme changes the fatty acid accumulation to similar amounts of both 12:0 and 14:0. The triple mutation (M197R/R199H/T231K) has completely reversed the 12:0/14:0 ratio compared to the wild-type Uc FatB1 (Table 1). These data are significant because enzyme activity *in vivo* might have involved sequential interactions or parameters such as lifetime and folding/unfolding rates different from *in vitro* activity assays.

Our results show that 12:0-ACP is a good competitive inhibitor for 14:0-ACP in both the wild-type enzyme and the triple mutant (Tables 2 and 3), indicating that both substrates bind equally well to the two proteins. These results thus suggest that the amino acids Met-197, Arg-199, and Thr-231 are unlikely to directly interact with the substrates. In fact, the Michaelis constants are similar and independent of substrate

chain length for Uc FatB1 and the mutant, suggesting the specificity must be largely determined in the hydrolytic step. Also, the tripeptide MRR could not be the sole determining motif for selectivity toward 12:0-ACP, because it is commonly found in other medium-chain-specific TEs (ref. 10; T.A.V., D.J.H., and L.Y., unpublished data). Therefore, the changes in the mutants may only slightly alter certain secondary structures, similar to what has occurred when the surface loops of *Bacullus stearothermophilus* lactate dehydrogenase were modified (27). This prediction should not be surprising as substrate specificity is rarely the product of a small cassette of structural changes at the point of enzyme–substrate contact (28). Changing the tripeptide from MRR to RRH might have reduced the flexibility of the β-structure immediately following the tripeptide, according to the prediction of chain flexibility in protein devised by Karplus and Schulz (29). This may have led to reduction of the flexibility of the substrate binding pocket and active site.

At the present time it is difficult to speculate on the impacts of the mutations without having the three-dimensional structure of any plant TE. Recently, the structure of a myristoyl-ACP TE from *Vibrio harveyi* has been determined (30). However, this TE, like other bacterial or mammalian TEs, shares no sequence homology with the plant TEs (10). Furthermore, unlike most bacterial and animal TEs, which have an active-site serine, the plant TE apparently utilizes a cysteine for the catalytic role (3, 4, 7). Nevertheless, through protein engineering, we have generated a catalyst with high activity. The results of the site-directed mutagenesis should guide the exploration of certain regions of the protein to understand the enzyme mechanism. High-level expression of Uc FatB1 in Brassica oilseeds has allowed the commercial production of a genetically engineered oil crop "Laurate Canola" (31). Expression of Cc FatB1 in Brassica oilseeds led to accumulation of up to 22% of 14:0 (D.J.H., J. Kridl, and T.A.V., unpublished data). These results lead us to predict that seed-specific expression of the mutant TEs should generate phenotypes corresponding to their specificities. The ability to modify TE specificity is an important step in genetic engineering of plant oils. Ultimately, one might be able to produce a desired fatty acid in oilseeds by introducing an engineered enzyme with tailor-made specificity.

We thank our colleagues at Calgene, Inc. for technical support and advice and acknowledge the insightful suggestions and critical review of this work by V. Knauf, H. M. Davies, J. Metz, and M. Lassner of Calgene, Inc., and K. Shen of University of California at Davis.

1. Smith, S. (1980) *J. Dairy Sci.* **63,** 337–352.
2. de Renobales, M., Rogers, L. & Kolattukudy, P. E. (1980) *Arch. Biochem. Biophys.* **205,** 464–467.
3. Pollard, M. R., Anderson, L., Fan, C., Hawkins, D. J. & Davies, H. M. (1991) *Arch. Biochem. Biophys.* **284,** 306–312.
4. Davies, H. M., Anderson, L., Fan, C. & Hawkins, D. J. (1991) *Arch. Biochem. Biophys.* **290,** 37–45.
5. Voelker, T. A. & Davies, H. M. (1994) *J. Bacteriol.* **176,** 7320–7327.
6. Voelker, T. A., Worrell, A. C., Anderson, L., Bleibaum, J., Fan, C., Hawkins, D. J., Radke, S. E. & Davies, H. M. (1992) *Science* **257,** 72–74.
7. Knutzon, D. S., Bleibaum, J. L., Nelsen, J., Kridl, J. C. & Thompson, G. A. (1992) *Plant Physiol.* **100,** 1751–1758.
8. Dörmann, P., Voelker, T. A. & Ohlrogge, J. B. (1995) *Arch. Biochem. Biophys. Res. Commun.* **326,** 612–618.
9. Dörmann, P., Kridl, J. & Ohlrogge, J. B. (1994) *Biochim. Biophys. Acta* **1212,** 134–136.
10. Jones, A., Davies, H. M. & Voelker, T. A. (1995) *Plant Cell* **7,** 359–371.
11. Branden, C. & Tooze, J. (1991) *Introduction to Protein Structure* (Garland, New York), p. 249.
12. Blundell, T. L. (1994) *Trends Biotechnol.* **12,** 145–148.
13. Hardy, L. W. & Nalivaika, E. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9725–9729.
14. Ohlrogge, J. B. (1994) *Plant Physiol.* **104,** 821–826.
15. Davies, H. M. (1993) *Phytochemistry* **33,** 1353–1356.
16. Ausubel, F., ed. (1987) *Current Protocols in Molecular Biology* (Wiley, New York), pp. 4.31–4.34.
17. Frohman, M. A., Dush, M. K. & Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8998–9002.
18. Higuchi, R., Krummel, B. & Saiki, R. K. (1988) *Nucleic Acids Res.* **16,** 7351–7367.
19. Klein, K., Steinberg, R., Fiethen, B. & Overath, P. (1971) *Eur. J. Biochem.* **19,** 442–450.
20. Overath, P., Pauli, G. & Schairer, H. U. (1969) *Eur. J. Biochem.* **7,** 559–574.
21. Hjelmstad, R. H., Morash, S. C., McMaster, C. R. & Bell, R. M. (1994) *J. Biol. Chem.* **269,** 20995–21002.
22. Kim, Y.-G. & Chandrasegaran, S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 883–887.
23. Chou, P. Y. & Fasman, G. D. (1974) *Biochemistry* **13,** 222–245.
24. Prevelige, P. & Fasman, G. D. (1989) in *Prediction of Protein Structure and the Principles of Protein Conformation*, ed. Fasman, G. D. (Plenum, New York), pp. 391–416.
25. Garnier, J., Osgutherpe, U. P. & Robson, B. (1978) *J. Mol. Biol.* **120,** 97–120.
26. Sandberg, W. S. & Terwilliger, T. C. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8367–8371.
27. El Hawrani, A. S., Moreton, K. M., Sessions, R. B., Clarke, A. R. & Holbrook, J. J. (1994) *Trends Biotechnol.* **12,** 207–211.
28. Hedstrom, L. (1994) *Curr. Opin. Struct. Biol.* **4,** 608–611.
29. Karplus, P. A. & Schulz, G. E. (1985) *Naturwissenschaften* **77,** 212–213.
30. Lawson, D. M., Derewenda, U., Serre, L., Ferri, S., Szittner, R., Wei, Y., Meighen, E. A. & Derewenda, Z. S. (1994) *Biochemistry* **33,** 9382–9388.
31. Chasan, R. (1995) *Plant Cell* **7,** 235–237.