

# The Catalytic Cysteine and Histidine in the Plant Acyl-Acyl Carrier Protein Thioesterases\*

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The plant acyl-acyl carrier protein (acyl-ACP) thioesterases (TEs) play an essential role in chain termination during *de novo* fatty acid synthesis and are of biochemical interest because of their utilities in the genetic engineering of plant seed oils. Biochemical data have shown the possible involvement of an active-site cysteine and a histidine in catalysis, suggesting that these enzymes activate the hydrolysis of the thioester bond using the same basic catalytic machinery as those of proteases and lipases. To identify the cysteine and histidine residues that are critical in catalysis we substituted, in a 12:0 ACP TE (*Uc* FatB1), a conserved cysteine (Cys-320) to an Ala or a Ser, and three conserved histidines (His-140, His-285, and His-345) to an Ala or an Arg. Each Ala mutation caused a substantial loss of enzyme activity. However, only C320A and H285A completely inactivated the enzyme, indicating that these two residues are essential for catalysis. Considerable activity (>60%) still remained when Cys-320 was converted to a Ser, but this mutant (C320S) displayed a reversed sensitivity toward thiol or serine hydroxyl inhibitors compared with the wild-type enzyme. A pH optimal study demonstrates that while the wild-type enzyme has the highest activity between pH 8.5 and 9.5, the mutant H285A shows a shifted optimum to higher pH and a significant increase of activity around pH 12. This result suggests that Arg-285 ( $pK_a$  12) is deprotonated at high pH, thus partially mimicking the role of His-285 for proton abstraction in the wild-type enzyme. We conclude that the Cys-320 of the wild-type enzyme and Ser-320 of the mutant enzyme can attack the thioester bond of the substrate 12:0 ACP, assisted by His-285. Because plant TEs are highly conserved in length and sequence and the residues investigated here are completely conserved in all available TEs, it is reasonable to believe that homologues of Cys-320 and His-285 are present in the active sites of all plant acyl-ACP TEs.

nation during *de novo* fatty acid synthesis and have been proven to be important in plant fatty acid bioengineering (1). In higher plants, these enzymes can be classified into two distinct but related families; FatA represents the commonly found 18:1 ACP TE, and FatB includes TEs preferring acyl-ACPs having saturated acyl groups (2). High amino acid sequence homology is found within each family, and certain regions of the proteins are also highly conserved between the two families (Ref. 2 and see below). Despite the similar enzymatic functions, the plant TEs share no protein sequence homology with the animal and bacterial TEs (3). Also, unlike the animal and bacterial TEs, which have an active-site serine, the plant TEs apparently utilize a catalytic cysteine since the enzymes are sensitive to thiol inhibitors but not to serine hydroxyl-reactive reagents (4, 5). Davies *et al.* (5) also reported that the plant TE lost 97% of its activity when treated with diethylpyrocarbonate, suggesting the existence of an active-site histidine. These data are consistent with a model in which a histidine as a general base enhances the thiol nucleophilicity of a cysteine, thus enabling it to attack on the thioester bond of the acyl-ACP substrate.

By constructing chimeric enzymes and site-directed mutagenesis, our earlier attempt in modifying the substrate specificity of a plant TE has gained some insights into the regions that are critical for substrate recognition (6). However, due to the lack of crystal structure information, very little is known about the active-site residues that are essential for catalysis. To date, more than 30 plant TE sequences are available. This wealth of sequence information allows for the identification of amino acid residues that may be involved in catalysis or substrate binding by virtue of their conservation throughout this divergent set of enzymes. Sequence alignment of 33 plant TEs reveals one conserved cysteine and three conserved histidines. We mutagenized these residues to other amino acids in a 12:0 ACP TE (*Uc* FatB1) and demonstrated their possible roles in catalysis.

## EXPERIMENTAL PROCEDURES

**Site-directed Mutagenesis**—Using polymerase chain reaction-based site-directed mutagenesis as described previously (6, 7), each of the three conserved histidines (Fig. 1) was mutated to an Ala (A) and an Arg (R), and the conserved Cys was converted to an Ala and a Ser (S). The sense mutation primers were as follows (the mutagenized codons are underlined): H140A, 5'-CACTTAATGCTGCGAAGAGTG; H140R, 5'-CACTTAATCGTGCGAAGAGTG; H285A, 5'-CAATCAGGCTGTGAACAACC; H285R, 5'-CAATCAGCGTGTGAACAACC; H345A, 5'-GCGATCGCTTGCTCCAG; H345R, 5'-GCGATCCGTTGCTCCAG; C320A, 5'-GGAGAGAGGCCACGAGGG; C320S, 5'-GGAGAGAGGCACGAGGG.

The polymerase chain reaction conditions are as follows: five cycles were programmed with denaturation for 1 min at 94 °C, renaturation for 30 s at 48 °C, and elongation for 2 min at 72 °C. These first five cycles were followed by 30 cycles using the same program except with renaturation for 30 s at 60 °C.

**Expression of *Uc* FatB1 and the Mutants in *Escherichia coli***—The coding regions of the mature proteins of the wild-type *Uc* FatB1 and its mutants were amplified using polymerase chain reaction (primers: pQE-BTE-f, CAUCAUCAUAAGGATCCATAGCTTGAA; and pQE-

A plant thioesterase (TE)<sup>1</sup> removes the acyl moiety from the acyl-acyl carrier protein (ACP), releasing it as a free fatty acid. The plant acyl-ACP TEs play an essential role in chain termi-

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<sup>1</sup> The abbreviations used are: TE, thioesterase; acyl-ACP, acyl-acyl carrier protein; DTT, 1,4-dithiothreitol; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

			140			285			320			345	
Ct FatA1	-	-	NHA	-	-	NKHVNNV	-	-	YRRECQ	-	-	LHLLRS	
At FatA1	-	-	NHA	-	-	NQHVNNV	-	-	YRRECQ	-	-	LHLLRL	
Bn FatA1	-	-	NHV	-	-	NQHVNNV	-	-	YRRECQ	-	-	LHMLRL	
Ch FatB1	-	-	NHV	-	-	NQHVNNV	-	-	YRRECQ	-	-	QHLLRL	
Cc FatB1	-	-	NHA	-	-	NQHVNNI	-	-	YRRECT	-	-	EHLLQL	
Uc FatB1	-	-	NHA	-	-	NQHVNNL	-	-	YRRECT	-	-	DHLLQL	

FIG. 1. A partial alignment of the conserved histidines and cysteine. Only three representatives of each FatA and FatB type TE were selected for display. These residues are conserved throughout all 33 available plant TEs. *Ct*, *Carthamus tinctorius* (safflower); *At*, *Arabidopsis thaliana*; *Bn*, *Brassica napus*; *Ch*, *Cuphea hookeriana*; *Cc*, *Cinnamomum camphorum* (camphor); and *Uc*, *Umbellularia californica* (bay). The *Uc* FatB1 numbering is used.

BTE-r, CUACUACUACUAAAGTCGACTTACACCCTC). The amplified fragments were subcloned into pQE 32 plasmid (QIAGEN, Inc.) between the *SalI* and *BamHI* restriction sites following the procedures recommended by the manufacturer. The plasmid pQE 32, driven by the *E. coli* phage T5 promoter, allows for the production of the recombinant protein with an N-terminal 6 × His affinity tag. The constructs, confirmed by sequencing, were transformed into *E. coli* strain M15 (pREP4). The transformed cells were grown at 37 °C to an A<sub>600</sub> of 0.7–0.8 before being induced by 2 mM isopropyl-β-D-thiogalactopyranoside for 1–5 h. The cells were then harvested and lysed by sonication. The recombinant enzymes were purified using Ni<sup>2+</sup> resin following procedures recommended by the manufacturer. Affinity-purified recombinant proteins were assayed for enzyme activity and analyzed by SDS gel electrophoresis. Protein concentrations were determined by Bradford assay using the Bio-Rad protein assay reagent with bovine serum albumin as the standard.

**Enzyme Assays and Effects of Inhibitors**—TE activity was assayed essentially as described (4, 6). Approximately 2.5 ng of the purified enzyme was used in the assay at 30 °C for 30 min, with or without inhibitors, in a buffer containing 100 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.01% Triton X-100, and 3 μM <sup>14</sup>C-labeled 12:0 ACP.

## RESULTS AND DISCUSSION

Biochemical data have shown the possible involvement of an active-site cysteine and a histidine in catalysis of the plant TEs (5, 8). An amino acid sequence alignment of all available plant TEs (total of 33) reveals three conserved histidines and one conserved cysteine (Fig. 1). The *Uc* FatB1 is a good model for studying the critical roles of these amino acids because it is highly active and has been subjected to extensive biochemical study (3–6). In the absence of three-dimensional structural information, alanine-scanning mutagenesis can be used to identify structural determinants of catalytic reactivity of an enzyme. By site-directed mutagenesis, we have converted each of the three histidines to an Ala or an Arg and the cysteine to an Ala or a Ser. These mutants were expressed in *E. coli*, purified, and assayed for enzyme activity.

Mutation to Ala at all four residues caused a substantial decrease of the enzyme activity when compared with the wild-type *Uc* FatB1 (Table I), indicating the functional importance of these conserved amino acids. The specific activity of 9,010 μmol/min/mg for the wild-type enzyme is about 80–900-fold higher than those of most other TEs, e.g. specificity for a 14:0 ACP TE from *Cuphea palustris* is about 120 μmol/min/mg, and for the 8:0/10:0 ACP TE from the same species it is approximately 10 μmol/min/mg.<sup>2</sup> Therefore, even with >90% loss of its activity the bay TE is still as active as many other plant TEs, and the activity can be easily detected in an *in vitro* assay. As shown in Table I, only C320A and H285A were completely inactivated, whereas other Ala mutants displayed detectable activities. These results demonstrate that Cys-320 and His-285

TABLE I  
Specific activities of wild-type *Uc* FatB1 and the mutants toward 12:0 ACP

Enzyme	Specific activity
	μmol/min/mg
<i>Uc</i> FatB1	9010 ± 180
H140A	453 ± 17
H140R	35 ± 3
H285A	0 <sup>a</sup>
H285R	52 ± 3
H345A	236 ± 15
H345R	222 ± 7
C320A	0 <sup>a</sup>
C320S	6100 ± 31

<sup>a</sup> Not detectable when approximately 2.5 ng of the enzyme was used in the assay at 30 °C for 30 min in a buffer containing 100 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.01% Triton X-100, and 3 μM <sup>14</sup>C-labeled 12:0 ACP (6).

are essential for catalysis.

We have also converted Cys-320 to a Ser. This mutant, C320S, retained considerable activity (approximately 60% of that of the wild type, see Table I) but displayed different enzymatic characteristics. The activity of wild-type *Uc* FatB1 was sensitive to thiol reagents such as 5,5'-dithiobis(2-nitrobenzoic acid) and iodoacetamide (Table II), a strong indication of the presence of a catalytic cysteine. Conversely, *Uc* FatB1 was insensitive to phenylmethylsulfonyl fluoride, a serine hydroxyl-reactive compound (Table II and Ref. 4). In contrast, the mutant C320S showed a reversed sensitivity, i.e. its activity was inhibited by phenylmethylsulfonyl fluoride but remained relatively insensitive to the thiol reagents (Table II). In addition, consistent with the presence of an active-site cysteine, 1,4-dithiothreitol is essential for maximum activity of the wild-type *Uc* FatB1 (4). However, DTT is not required for the C320S mutant (data not shown).

Confirming a result previously reported (5), treatment with diethylpyrocarbonate resulted in about 90% loss of activity (Table II), as would be expected for a cysteine enzyme typically dependent on an active-site histidine. Our result of complete inactivation by H285A demonstrates the likelihood of His-285 as a proton acceptor for Cys-320 in catalysis. Supporting evidence for His-285 being the active-site histidine also came from the optimal pH data (Fig. 2). Here we show that the wild-type *Uc* FatB1 has a pH optimum of between 8.5 and 9.5, consistent with what was reported previously (5). On the other hand, mutant H285R shows activity increases as pH increases from 7.0 to 12.3 and, very interestingly, a significant burst of activity at pH >11. This phenomenon can be explained as Arg-285 (pK<sub>a</sub> 12) is deprotonated at high pH, thus partially mimicking the role of His-285 in the wild-type enzyme (9).

Studies on TEs from bacteria, animals, and plants have demonstrated that the enzymatic hydrolysis of the thioester bond proceeds using the same basic catalytic machinery as those of proteases and lipases (5, 8, 10–13). Crystal structure analysis of a myristoyl-ACP TE from the bacterium *Vibrio harveyi* has shown the catalytic triad of Ser, His, and Asp (14). While the active sites of the animal and bacterial TEs resemble that of serine protease such as trypsin, for plant TEs they are likely to be similar to that of papain, a plant cysteine protease in which a thiol acyl enzyme is formed as a covalent intermediate. A histidine residue acts as a general base catalyst activating the cysteine as a nucleophile by partial proton abstraction. Unlike the proteases, in which replacement of naturally occurring active-site serine with a cysteine only produces poor enzymes with low K<sub>cat</sub> values toward natural substrates, the Ser → Cys mutants of animal TEs have been shown to be good catalysts, retaining up to 90% of the activities (11, 12). Here we

<sup>2</sup> K. Dehesh, personal communication.

TABLE II  
Effects of various inhibitors on the TE activity of wild-type and mutants C320S and H285R

Enzymes (0.1  $\mu$ g) in 100  $\mu$ l of TE assay buffer were preincubated at 25 °C for 5 min with either 5,5'-dithiobis(2-nitrobenzoic acid), iodoacetamide, or diethyl pyrocarbonate and for 30 min with phenylmethylsulfonyl fluoride at the stated concentrations. The remaining TE activities were then assayed as described, and the results were compared with the activities of the positive controls (assayed without the presence of inhibitors).

Inhibitor	Uc FatB1	C320S	H285R
	% inhibition		
5,5'-Dithiobis(2-nitrobenzoic acid) (0.1 mM)	92	15	95
Phenylmethylsulfonyl fluoride (5 mM)	13	90	4
Iodoacetamide (1 mM)	72	20	89
Diethyl pyrocarbonate (1 mM)	90	97	11

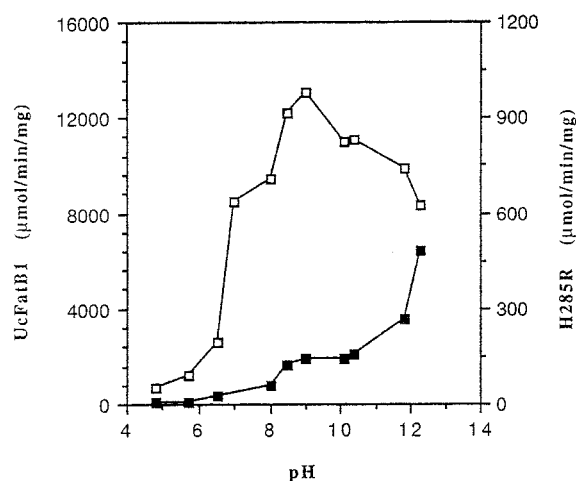


FIG. 2. pH optima of the wild-type *Uc FatB1* and mutant *H285R*. The wild-type (open square) and mutant (solid square) enzymes were assayed for 30 min at 30 °C in buffers with various pH values containing 1 mM DTT, 0.01% Triton X-100, and 3  $\mu$ M  $^{14}$ C-labeled 12:0 ACP using the procedures described (4, 6). Sodium acetate, Tris-HCl, and CAPS buffers (100 mM) were used for the pH ranges 4.8–7.0, 7.0–9.0, and 9.0–12.3, respectively.

report that a Cys  $\rightarrow$  Ser mutant of the plant TE is also highly active (Table I). We also show that His-285 is essential for the nucleophilic attack by the cysteine, because the H285A is the only histidine mutant that inactivates the enzyme. These observations suggest that the Cys-320 of the wild-type enzyme and Ser-320 of the mutant enzyme can attack, assisted by His-285, the thioester bond of the substrate 12:0 ACP. At the present time no data are available for the existence of an Asp or Asn residue forming the classic triad. There are three Asp and four Asn, which are conserved in plant TEs. Although a Ser-His-Asp triad has been found in the myristoyl-ACP TE of *V. harveyi*, the main chain carbonyl of a Gln, instead of an Asp, serves as a hydrogen bond acceptor for the active-site His in a similar enzyme, the *E. coli* malonyl-CoA:ACP Transacylase (15).

The mature proteins of both FatA and FatB classes of plant

acyl-ACP TEs are relatively conserved in length and sequence (approximately 80% within each class and above 50% between classes) (2).<sup>3</sup> The cysteine and three histidines investigated in this report are conserved in all plant TEs found to date. As shown in Fig. 1, the amino acids close to these four residues (especially Cys-320 and His-285) are also highly conserved. In addition, no insertion or deletion in the number of amino acids in the linear polypeptide sequence is found between Cys-320 and His-285 in all available plant TEs. This conserved linear relationship should be reflected in a very similar three-dimensional structure. Therefore, we believe that the results in this study have a general implication. It is reasonable to hypothesize that all these TEs are structurally similar because of their high sequence homology and similar functional characteristics (16). It is thus also reasonable to believe that the active sites of all plant acyl-ACP TEs consist of the homologues of Cys-320 and His-285. We can conclude that these plant enzymes have active-site motifs of NQ(K)HN(S)N and YRR(K)ECG(Q/T), in which the amino acids in parentheses occur relatively infrequently. In fact, these two sequence motifs have been successfully used in polymerase chain reaction amplifications of several TEs from various plant sources (2). The identification of the catalytic cysteine and histidine in plant acyl-ACP TEs is a major step forward in our continuous effort in engineering these important plant enzymes.

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<sup>3</sup> T. A. Voelker and L. Yuan, unpublished data.