Identification and Characterization of *Escherichia coli* Thioesterase III That Functions in Fatty Acid β-Oxidation†

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ABSTRACT: When *Escherichia coli* is grown on oleic acid as the sole carbon source, most of this fatty acid is completely degraded by β-oxidation. However, approximately 10% of the oleic acid is only partially degraded to 3,5-*cis*-tetradecadienoyl-CoA, which is hydrolyzed to 3,5-*cis*-tetradecadienoic acid and released into the growth medium. An investigation of thioesterases involved in this novel pathway of β-oxidation led to the identification of a new thioesterase (thioesterase III) that is induced by growth of *E. coli* on oleic acid. This enzyme was partially purified and identified as the *ybaW* gene product by mass spectrometric analysis of tryptic peptides. The *ybaW* gene, which has a putative consensus sequence for binding the fatty acid degradation repressor, was cloned and expressed in *E. coli.* Thioesterase III was shown to be a long-chain acyl-CoA thioesterase that is most active with 3,5-tetradecadienoyl-CoA, a minor metabolite of oleate β-oxidation. Its substrate specificity and induction by fatty acids agree with its proposed function in the thioesterase-dependent pathway of β-oxidation. Thioesterase III is proposed to hydrolyze metabolites of β-oxidation that are resistant to further degradation and that would inhibit the flux through the pathway if they were allowed to accumulate.

Thioesterases are ubiquitous enzymes that hydrolyze acyl derivatives of CoA, acyl carrier protein (ACP),[1] and other thiol-containing compounds, including proteins and peptides that contain cysteine residues. The best understood function of a thioesterase is in mammalian fatty acid synthesis where the enzyme terminates the process of chain elongation by hydrolysis of fatty acyl-ACP (*1*). The proposed function of this thioesterase is supported by its existence as an integral part of the mammalian fatty acid synthase, which is a multienzyme complex. The search for an enzyme with a similar function in *Escherichia coli* led to the identification and characterization of two long-chain acyl-CoA thioesterases termed thioesterases I and II (*2–4*). However, both thioesterases were shown to hydrolyze long-chain acyl-ACP much more slowly than the corresponding CoA derivatives (*5*) and hence are unlikely to function in fatty acid synthesis. Cloning of *tesA* and *tesB* that encode thioesterases I and II, respectively, facilitated the creation of thioesterase I and II mutants that failed to display unusual growth phenotypes (*6, 7*). Moreover, thioesterase I was identified as a periplasmic enzyme and therefore is unlikely to function in a cytosolic process like fatty acid synthesis (*7*).

An investigation of oleic acid β-oxidation in *E. coli* revealed the involvement of thioesterase(s) in this process (*8*). 2-*trans*,5-*cis*-Tetradecadienoyl-CoA, an intermediate of oleate β-oxidation, is, to a limited extent, converted to 3,5-*cis*-tetradecadienoyl-CoA that cannot be further metabolized because *E. coli* does not contain the necessary enzyme, $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase. The solution to this apparent metabolic block is the hydrolysis of 3,5-*cis*-tetradecadienoyl-CoA to 3,5-*cis*-tetradecadienoic acid that is released into the growth medium. Thioesterase I is most likely not involved in this process because of its periplasmic location. However, thioesterase II might be responsible for hydrolyzing 3,5-*cis*-tetradecadienoyl-CoA because it is highly active with this compound as a substrate (*8*) and it is a cytosolic enzyme and thus has the right intracellular location to participate in β-oxidation.

The aim of this study was to identify the thioesterase that is responsible for the hydrolysis of 3,5-*cis*-tetradecadienoyl-CoA and thereby participates in the thioesterase-dependent β-oxidation of oleic acid. The outcome of this investigation was the characterization of a novel thioesterase, named thioesterase III, which might function in the β-oxidation of oleic acid and perhaps of other unsaturated fatty acids.

EXPERIMENTAL PROCEDURES

Materials. CoASH, acetyl-CoA, butyryl-CoA, octanoyl-CoA, decanoyl-CoA, dodecanoyl-CoA, tetradecanoyl-CoA,

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[1] Abbreviations: ACP, acyl carrier protein; IPTG, isopropyl β-D-thiogalactopyranoside; AP, alkaline phosphatase; NTA−Ni^{2+}−agarose, nitrilotriacetic acid−Ni^{2+}−agarose; HPLC, high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBST, Tris-buffered saline with Tween; kbp, kilo-base pair; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; PCR, polymerase chain reaction; GC−MS, gas chromatography in combination with mass spectrometry; fadR, fatty acid degradation repressor; *fad*, fatty acid degradation; TE, thioesterase.

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palmitoyl-CoA, stearoyl-CoA, and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Life Science Resources (Milwaukee, WI). 5-*cis*-Tetradecenoic acid was synthesized by Cayman Chemical (Ann Arbor, MI). Polyacrylamide ready gels, trans-Blot transfer medium pure nitrocellulose membrane, alkaline phosphatase (AP)-conjugated goat anti-rabbit antibody, AP-conjugated substrate kit, and hydroxylapatite were purchased from Bio-Rad Laboratories. Rabbit anti-*E. coli* thioesterase III antiserum was produced by Pocono Rabbit Farm and Laboratory, Inc. (Canadensis, PA). Amicon Ultrafree Centrifugal Filter Units (NMWL of 10000) were obtained from Millipore, and standard cellulose dialysis tubing was from Spectrum Medical Industries, Inc. DEAE-cellulose, octyl Sepharose CL-4B, oleic acid, and most of the standard biochemicals were obtained from Sigma. The QIAprep spin miniprep kit, the QIAquick PCR purification kit, QIAGEN genomic tips, the pQE-81L expression vector, and NTA−Ni^{2+}−agarose were bought from QIAGEN Inc. (Valencia, CA). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. Restriction endonucleases, T_4 DNA ligase, Taq DNA polymerase, and 2-long DNA ladder were obtained from New England Biolabs Inc. XL10-Gold ultracompetent cells were purchased from Stratagene. *E. coli* strain HC74 (*tesA::kan*^{r} & *tesB::cml*^{r}, *fadE*) and strain LE392 (*hasR, galK, trpR, metB, lacY*) were kindly provided by S. Smith (Children's Hospital Oakland Research Institute, Oakland, CA) and the *E. coli* Genetic Stock Center (Yale University, New Haven, CT), respectively.

Synthesis of Substrates and Metabolites. Oleoyl-CoA, 5-*cis*-dodecenoyl-CoA, and 5-*cis*-tetradecenoyl-CoA were synthesized from oleic acid, 5-*cis*-dodecenoic acid, and 5-*cis*-tetradecenoic acid, respectively, by the mixed anhydride method as described by Fong and Schulz (*9*). 3,5-*cis*-Dodecadienoyl-CoA and 3,5-*cis*-tetradecadienoyl-CoA were prepared from the corresponding 5-*cis*-enoyl-CoA thioesters as previously described (*8*). 2-*trans*,5-*cis*-Tetradecadienoyl-CoA (*10*), 2-*trans*-tetradecenoyl-CoA (*8*), L-3-hydroxytetradecanoyl-CoA (*8*), 3-*trans*-tetradecenoyl-CoA (*11*), and 3-ketohexadecanoyl-CoA (*12*) were synthesized by published procedures. All acyl-CoA thioesters were purified by reverse-phase high-performance liquid chromatography (HPLC) on a Waters μBondapak C_{18} column (30 cm × 3.9 mm) that was attached to a Waters gradient HPLC system. The absorbance of the eluate was monitored at 254 nm. The separation of long-chain acyl-CoAs was achieved by linearly increasing the acetonitrile/water (9:1, v/v) content of the 50 mM ammonium phosphate elution buffer (pH 5.5) from 40 to 70% over 20 min at a flow rate of 2 mL/min.

Isolation of fadE Revertants from E. coli Strain HC74. A *fadE* mutation had been introduced into *E. coli* strain LE392 by use of the transposable element Tn*10* (*7*). Subsequently, the Δ*tesA::kan*^{r} mutation followed by the Δ*tesB::cml*^{r} mutation had been introduced into this strain to create strain HC74 (*7*). Strain HC74 is unable to grow on fatty acids as the sole carbon source because β-oxidation is inactive due to the absence of functional electron transferring flavoprotein that is encoded by the *fadE* gene. For the purpose of creating an *E. coli* strain that retained the *tesA* and *tesB* mutations but was able to grow on fatty acids as the sole carbon source, spontaneous revertants of *fadE* were selected by plating strain HC74 on a medium that contained palmitic acid as the sole carbon source in addition to kanamycin and chloramphenicol. Colonies detected after incubation for 7 days were found to grow on long-chain fatty acids as the sole carbon source in the presence of both antibiotics. This strain, named YR1, was used to isolate a novel thioesterase that subsequently was named thioesterase III. The strain was always cultured in the presence of kanamycin (20 μg/mL) and chloramphenicol (20 μg/mL), which did not affect its growth behavior.

Bacterial Growth Conditions and Preparation of Bacterial Extracts. E. coli cells were grown on LB medium from single colonies. The initial culture was diluted 5-fold into M9 minimal medium containing 1% (w/v) tryptone, 2 mM $MgSO_4$, 10 μM $CaCl_2$, 1 μM $FeCl_3$, and additionally oleic acid (0.1, v/v) in the presence of 0.4% Triton X-100. After cultures had grown at 37 °C under shaking at 200 rpm to an absorbance of 1 at 600 nm, they were diluted 20 times into the same growth medium, but without tryptone. The final culture was harvested at an absorbance of 1 at 600 nm by centrifugation at 2300*g* for 30 min at 4 °C. Cell pellets were washed twice with M9 minimal medium and stored at −80 °C. Cell pastes were suspended in 2−3 times their volume of 0.1 M KP_i (pH 7.0) containing 10% glycerol and were sonicated for a total of 2 min (10 s × 12) at 0 °C and centrifuged at 33000*g* for 1 h at 4 °C. The resultant supernatants were collected for enzyme assays and protein purification.

Purification of E. coli Thioesterase III. Thirty-three grams of cell paste of *E. coli* strain YR1 grown on oleate was suspended in 70 mL of 0.1 M KP_i (pH 7.0) containing 10% glycerol, sonicated for a total of 2 min (10 s × 12) at 0 °C, and centrifuged at 33000*g* for 1 h at 4 °C. The resultant supernatant was dialyzed overnight against 0.02 M Tris-HCl (pH 7.8) containing 10% glycerol (buffer A) and applied to a DEAE-cellulose column (41 cm × 2.5 cm) previously equilibrated with buffer A. The column was washed with 300 mL of buffer A containing 50 mM NaCl and then developed with a linear gradient made up of 500 mL each of buffer A containing either 50 or 250 mM NaCl. Fractions eluted from the DEAE-cellulose column were collected and assayed for thioesterase activity with 20 μM myristoyl-CoA as the substrate, and those with high activity were combined, concentrated in an Amicon∗ Ultrafree Centrifugal Filter Unit (NMWL of 10000), and dialyzed against 0.02 M KP_i (pH 6.8) containing 10% glycerol (buffer B) for 2 h. After dialysis, the sample was applied to a hydroxylapatite column (11 cm × 2.5 cm), which had been equilibrated with buffer B. After the column had been washed with 250 mL of buffer B containing 0.1 M KCl, the column was developed with a gradient made up of 250 mL of buffer B containing 0.1 M KCl and 250 mL of 0.07 M KP_i (pH 6.8) containing 10% glycerol and 0.1 M KCl. Active fractions were combined, concentrated, and dialyzed against 1 mM KP_i (pH 7.0) containing 0.5 M $(NH_4)_2SO_4$ (buffer C). The dialyzed sample was applied to an octyl Sepharose CL-4B column (3.0 cm × 1.0 cm) previously equilibrated with buffer C. The column was washed with a linear gradient made up of 15 mL of buffer C and 15 mL of 1 mM KP_i (pH 7.0), washed with an additional 9 mL of 1 mM KP_i (pH 7.0), and then developed with an ethanol gradient made up of 16 mL of 1 mM KP_i (pH 7.0) and 16 mL of 1 mM KP_i (pH 7.0) containing 25% ethanol. Fractions with high thioesterase activity were

combined, concentrated, and, after dialysis against 0.02 M KP_i (pH 7.0) containing 30% glycerol, stored at −80 °C.

Enzyme Assays. Thioesterase activity was assayed by assessing the release of CoASH from acyl-CoAs with Ellman's reagent (*13*). A standard assay mixture contained 0.175 M KP_i (pH 8.0), 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), and 20 μM acyl-CoA. The progress of the reaction was determined spectrophotometrically at 412 nm, and rates were calculated using an extinction coefficient of 13600 M^{-1} cm^{-1}. Kinetic analyses were performed with purified six-His-tagged thioesterase III with either lauroyl-CoA, 3,5-*cis*-dodecadienoyl-CoA, myristoyl-CoA, 3,5-*cis*-tetradecadienoyl-CoA, 3-hydroxytetradecanoyl-CoA, or palmitoyl-CoA as the substrate. Kinetic constants (K_m and V_{max}) were obtained by nonlinear curve fitting using SigmaPlot 2000. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product in 1 min.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Immunoblotting. Protein solutions were heated for 10 min at 100 °C with equal volumes of SDS sample buffer containing both mercaptoethanol and dithiothreitol (*14*). The samples were subjected to SDS−PAGE on ready gels for 2.0 h at 80 V and then were either stained with Coomassie brilliant blue R or used for immunoblotting. For immunoblotting, the proteins were transferred from ready gels to nitrocellulose membranes by use of a TransBlot SD semidry electrophoretic transfer cell system with a current of 1.0 mA/cm^2 of gel for 1.5 h. To block nonspecific binding sites, we soaked the transferred membrane in 5% nonfat milk in Tris-buffered saline (TBS) for 1 h at 37 °C or overnight at 4 °C. The membrane was washed three times with 0.2% nonfat milk in TBS and then incubated with rabbit antiserum raised against *E. coli* thioesterase III (diluted 500-fold) for 1.5 h under gentle shaking at room temperature. The membrane was washed three times with Tris-buffered saline with Tween 20 (TBST) and then incubated with the second antibody (3000-fold diluted AP-conjugated goat anti-rabbit antibody) for 1 h. After that, the membrane was washed three times with TBST buffer and then incubated with the AP-conjugated substrate until the bands appeared on the membrane.

Peptide Mass Mapping. The partially purified *E. coli* thioesterase III was fractionated by SDS−PAGE into six major and several minor components; three of the major components were subjected to matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) at the Howard Hughes Medical Institute (Columbia University, New York, NY). Proteins were reduced and alkylated and digested with trypsin, and the resulting peptide mixture was analyzed by MALDI-MS without separation. The peptide masses obtained by MS were entered into a search program that scans the database, in most cases NCBI or Genpept, to find a match (*15–18*).

Cloning, Expression, and Purification of Recombinant E. coli Thioesterase III. E. coli thioesterase III was cloned, expressed, and purified to homogeneity. Briefly, purified genomic DNA, which was isolated from *E. coli* strain YR1 with QIAGEN genomic tips, was used for the amplification of the *ybaW* gene by the polymerase chain reaction (PCR). The primers used were 5′-TTGGGATCCGCAAACACAAATCAAAG-3′ and 5′-GGGCTGCAGTAATTATTCCGGGTGTC-3′. The PCR product was purified by use of the QIAquick PCR purification kit, digested with *Bam*HI and *Pst*I, purified by agarose gel electrophoresis, and ligated into plasmid pQE-81L that had been cut with the same restriction enzymes. The resultant expression plasmid, named pQE-81L-TE III, was amplified in XL10-Gold ultracompetent *E. coli* cells and purified by use of a QIAprep spin miniprep kit. Digestion of this plasmid with *Bam*HI and *Pst*I yielded one 500 bp DNA fragment that was of the size predicted for the insert in addition to the 4−4.5 kbp DNA of the original plasmid. Sequencing of the insert of plasmid pQE-81L-TE III confirmed the correct coding sequence for the ybaW protein and the presence of additional termination codons 48 and 91 bp downstream of the *ybaw* termination codon. This plasmid was used to transform *E. coli* BL21 cells according to the method of Chung et al. (*19*). Transformants were grown on LB medium to an absorbance of 0.7 at 600 nm, and the expression of putative thioesterase III was induced by the addition of 1 mM IPTG to the cell suspension for 4 h at 37 °C under shaking at 200 rpm. Cells were harvested by centrifugation, disrupted by sonication, and centrifuged to obtain an extract of soluble proteins, which was expected to contain the ybaW protein. The soluble extract was subjected to chromatography on a NTA−Ni^{2+} column, and in some cases, the protein was further purified by column chromatographies on DEAE-cellulose and octyl Sepharose CL-4B as described for the purification of native thioesterase III from *E. coli*. Fractions were assayed for thioesterase activity with myristoyl-CoA as the substrate, and those with high thioesterase activity were combined, concentrated, and stored at −80 °C after dialysis against 0.05 M KP_i (pH 7.4) containing 30% glycerol.

Isolation and Analysis of Fatty Acids Present in the Growth Medium. E. coli cells were grown to an absorbance of 1 at 600 nm in M9 medium containing oleate (0.1%, v/v) and Triton X-100 (0.4%, v/v). Cells were separated from the growth medium by centrifugation at 2300*g* for 30 min at 4 °C. Fatty acids present in the growth medium were extracted and converted to their methyl esters as described previously (*8*). This fraction was analyzed by gas chromatography in combination with mass spectrometry (GC−MS). Aliquots of 1 μL of the fatty acid methyl esters were injected at 250 °C into a GC−MS instrument (Shimadzu Scientific Instruments) consisting of a gas chromatograph (model GC-17A) interphased with a mass spectrometer (QP-5000) and equipped with a capillary column (30 m; inside diameter, 0.25 mm; film thickness, 0.25 μm; EC-5, Alltech Associates Inc., Deerfield, IL). The oven temperature was increased from 100 to 230 °C at a rate of 5 °C/min and to 300 at a rate of 20 °C/min and then held constant for 6 min. The mass spectrometer served as a detector and was operated at 280 °C.

RESULTS

Partial Purification of a Novel Thioesterase. In an attempt to determine if thioesterase II is essential for growth of *E. coli* on oleic acid, strain YR1, which is devoid of thioesterases I and II due to deletion mutations in *tesA* and *tesB*, was cultured on oleic acid as the sole carbon source. This thioesterase mutant grew on oleate and, most importantly, produced 3,5-tetradecadienoic acid that was detected in the

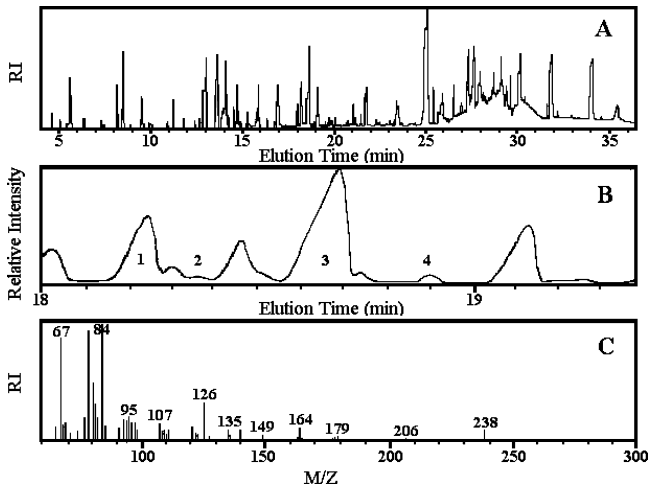


FIGURE 1: Identification of 3,5-tetradecadienoic acid in the medium after growth of *E. coli* strain YR1 on oleic acid as the sole carbon source. (A) Gas chromatogram of the methyl esters of acids extracted from the medium in which *E. coli* strain YR1 was grown to an absorbance of 1 at 600 nm. (B) Region of the gas chromatogram where methyl 3,5-tetradecadienoate would be eluted. Peaks 1−4 have molecular ions with mass-to-charge ratios (*m*/*z*) of 238. (C) Mass spectrum of the material that gave rise to peak 3 of panel B.

growth medium. Acidic compounds were extracted from the growth medium, converted to methyl esters, and analyzed by GC−MS (*8*). As shown in Figure 1A, many acidic compounds were present in the growth medium and the methyl esters of several of them were detected in the 18−20 min region where methyl 3,5-tetradecadienoate would be eluted (Figure 1B). Mass spectra of the materials corresponding to peaks 1−4 were almost identical, and all exhibited a molecular ion with mass-to-charge ratios (*m*/*z*) of 238 as expected of methyl 3,5-tetradecadienoate. Shown in Figure 1C is the mass spectrum of the material corresponding to peak 3, which is identical with the spectrum of synthetic methyl 3,5-tetradecadienoate (*8*). The formation of 3,5-tetradecadienoate by strain YR1, which is devoid of thioesterases I and II, is suggestive of the presence of one or more additional thioesterases in *E. coli*. Direct evidence in support of this idea was obtained by assaying a soluble extract of mutant YR1 cells grown on oleate and detecting a low but significant myristoyl-CoA thioesterase activity of 4.6 milliunits/mg of protein (see Table 1). Hence, this *E. coli* strain must contain one or more additional enzymes with myristoyl-CoA thioesterase activity. The extract was subjected to column chromatography on DEAE-cellulose from which slightly more than half of the myristoyl-CoA thioesterase activity was eluted as a symmetrical peak with a NaCl gradient (data not shown). The myristoyl-CoA thioesterase activity corresponding to this peak was absent when the same *E. coli* strain was grown on glucose or tryptone as the carbon source (data not shown). This myristoyl-CoA thioesterase, henceforth termed novel thioesterase, was further purified by column chromatography on hydroxylapatite followed by chromatography on an octyl Sepharose CL-4B column. The novel thioesterase was eluted from octyl Sepharose CL-4B with a 0 to 25% ethanol gradient (see Figure 2). This purification step increased the specific activity of the novel thioesterase 26-fold and led to the removal of most but not all proteins that were unrelated to this enzyme. As summarized in Table 1, the novel thioesterase was purified almost 900-fold and was obtained in 10% yield on the basis of the total myristoyl-CoA thioesterase activity present in the cell extract. SDS−PAGE analysis of this thioesterase preparation revealed the presence of close to 10 protein bands with six bands considered to be major ones (see Figure 3).

Identification of the Gene Encoding the Novel Thioesterase. The proteins corresponding to bands marked 1−3 (see Figure 3) were considered the more likely ones to have thioesterase activity because they were the most prominent bands. Proteins associated with bands 1−3 were partially digested with trypsin, and the resultant peptides were analyzed by MALDI-MS. Searches of databases for matching sequences revealed the presence of two proteins in each of bands 1 and 3 and of three proteins in band 2. Each of the three bands contained the same protein of unknown function, while the other proteins were well-known housekeeping enzymes like isocitrate dehydrogenase, tryptophanase, aldehyde dehydrogenase, and phenylalanine tRNA synthase that are not known to have thioesterase activity. The protein of unknown function, which was a major component of the materials corresponding to bands 1 and 3, but only a minor component of the proteins associated with band 2, is encoded by the *ybaW* gene. Shown in Figure 4 is the predicted sequence of the ybaW protein that is comprised of 132 amino acid residues and has a molecular mass of close to 15 kDa. Also shown are the peptides that were derived from the partially purified thioesterase and identified by MALDI-MS. All peptides marked in Figure 4 were identified in the tryptic digest of material corresponding to band 1, while the proteins of bands 2 and 3 yielded some of the peptides from the N-terminal and C-terminal regions of the ybaW protein.

Upstream of the *ybaW* coding region, extending from nucleotide −49 to −33, is a 17 bp sequence that is 53% identical to the consensus sequence for fatty acid degradation repressor (fadR)-binding sites (see Figure 5). The identity of well-characterized fadR-binding sites with the consensus binding sequence is 70−85% (*20*). The existence of a fadR-binding site upstream of the *ybaW* coding region explains why the novel thioesterase is expressed when *E. coli* is grown on oleic acid as the sole carbon source, but not when grown on glucose or tryptone. A review of genes located 50 kbp

Table 1: Purification of a Novel Thioesterase from *E. coli*

step	total protein (mg)	total activity (milliunits)	specific activity (milliunits/mg)	purification (*x*-fold)	yield (%)
cell pellet	33000				
cell extract (after dialysis)	1051	4836	4.6	1.0	100
DEAE-cellulose	95	2148	22.6	4.9	44
hydroxylapatite	6.65	1044	157.0	34.1	22
octyl Sepharose CL-4B	0.12	507	4110	893	10

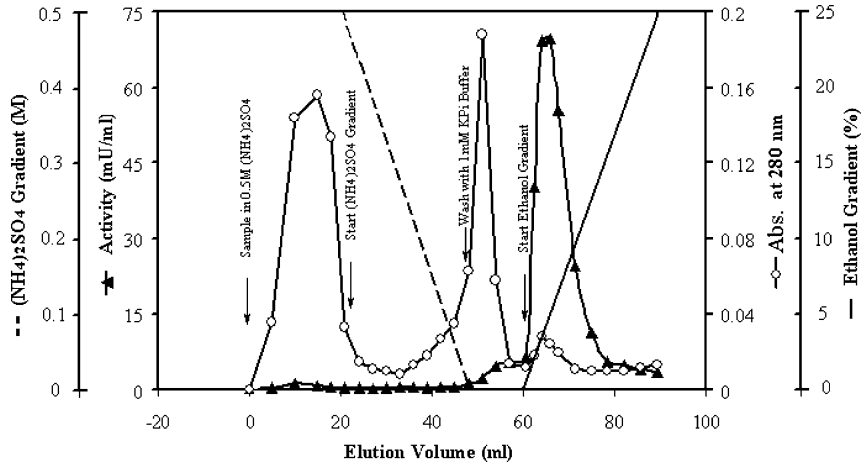


FIGURE 2: Final step in the purification of the novel thioesterase from *E. coli* strain YR1 by column chromatography on octyl Sepharose CL-4B. (▲) Thioesterase activity with myristoyl-CoA as the substrate. (○) Protein concentration. (---) $(NH_4)_2SO_4$ gradient. (−) Ethanol gradient. For details, see Experimental Procedures.

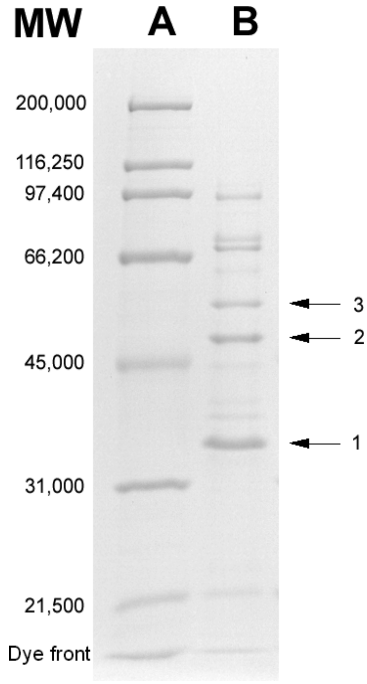


FIGURE 3: SDS−PAGE of the partially purified novel thioesterase from *E. coli* on a 10% gel after staining with Coomassie brilliant blue R: lane A, protein standards to indicate the molecular masses of proteins; and lane B, partially purified novel thioesterase.

MQTQIKVRGYHLDVYQHVNNARYLEFLEEARWDGLENSDSFQWMTAHNIAFVV

VNININYRRPAVLSDLLTITSQLQQLNGKSGILSQVITLEPEGQVVADALITF

VCIDLKTQKALALEGELREKLEQMVK

FIGURE 4: Amino acid sequence of the ybaW protein that was identified as a novel thioesterase from *E. coli.* Underlined are the tryptic peptides of the novel thioesterase that were identified by MALDI-MS and used to establish the identity of the novel thioesterase with the ybaW protein. Arginine and lysine residues are highlighted to indicate potential tryptic cleavage sites.

upstream or downstream from *ybaW*, which is situated at 10 min on the *E. coli* chromosome, did not reveal any functional relationship between them and *ybaW* except for *tesB* that is present 10 kbp downstream from *ybaW*.

Molecular Cloning, Expression, and Partial Characterization of Thioesterase III. It remained to be demonstrated that

Gene	Position																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
fadB	A	T	C	T	G	G	T	A	C	G	A	C	C	A	G	A	T
	+1[a]																+17
fadL (1)	A	G	C	T	G	G	T	C	C	G	A	C	C	T	A	T	A
	-25																-9
fadL (2)	C	A	C	T	G	G	T	C	T	G	A	T	T	T	C	T	A
	-1																+16
fadD (1)	A	G	C	T	G	G	T	A	T	G	A	T	G	A	G	T	T
	-29																-13
fadD (2)	G	G	C	T	G	G	T	C	C	G	C	T	G	T	T	T	C
	-115																-99
fabA	A	A	C	T	G	A	T	C	G	G	A	C	T	T	G	T	T
	-48																-31
Consensus	A	G	C	T	G	G	T	C	C	G	A	Y	N	T	G	T	T
	4/6	3/6	6/6	6/6	6/6	5/6	6/6	4/6	3/6	6/6	5/6	6/6		4/6	3/6	5/6	3/6
YbaW	A	C	C	A	G	T	T	A	T	G	A	C	C	T	C	T	G
	-49																-33

FIGURE 5: Binding sites for the fatty acid degradation repressor (fadR) in the promoters of several fatty acid degradation (*fad*) genes, including *ybaW*. A base was included in the fadR consensus binding site when it was present in at least three of the six compared sequences. The fadR binding sites of the six *fad* genes were experimentally confirmed (*20*), while the *ybaW* sequence is a proposed fadR binding site.

the novel thioesterase isolated from *E. coli* strain YR1 and the ybaW protein are one and the same protein. For this purpose, the *ybaW* gene was cloned and the encoded protein was expressed in *E. coli* as described in Experimental Procedures. After purification on a NTA−Ni^{2+} column, the six-His-tagged ybaW protein had a specific thioesterase activity of 20 units/mg with myristoyl-CoA as the substrate compared to 4.1 units/mg determined with the partially purified novel thioesterase. These two thioesterase preparations, after treatment with SDS, mercaptoethanol, and dithiothreitol for 10 min at 100 °C, were compared by SDS−PAGE and immunoblotting. The purified six-His-tagged ybaW protein gave rise to three bands on SDS-PAGE with the fastest moving material presumed to be due to the 16 kDa subunit of the protein, while the two slower moving bands corresponded to proteins with estimated molecular masses of 43 and 53 kDa (see Figure 6, lane 2). The same pattern of bands was observed when the ybaW protein was subjected to immunoblotting (Figure 6, lane 4). The immunoblot result is not surprising because the six-His-tagged ybaW protein was used to produce the antiserum. The immunoblot of the partially purified novel thioesterase resembled the blot obtained with the ybaW protein, except that the proteins of the former preparation moved slightly faster than the recombinant proteins (compare lanes 4 and 5

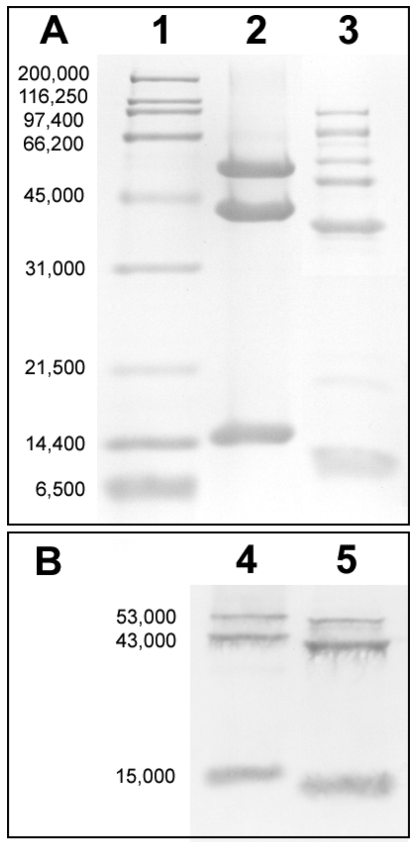


FIGURE 6: SDS−PAGE and immunoblot of the partially purified novel thioesterase and six-His-tagged ybaW protein. (A) A 15% polyacrylamide gel after staining with Coomassie brilliant blue R. (B) Immunoblot after probing with antibodies against *E. coli* six-His-tagged ybaW protein: lane 1, protein standards to indicate the molecular masses of proteins; lanes 2 and 4, purified six-His-tagged ybaW protein; and lanes 3 and 5, partially purified novel thioesterase (native thioesterase III).

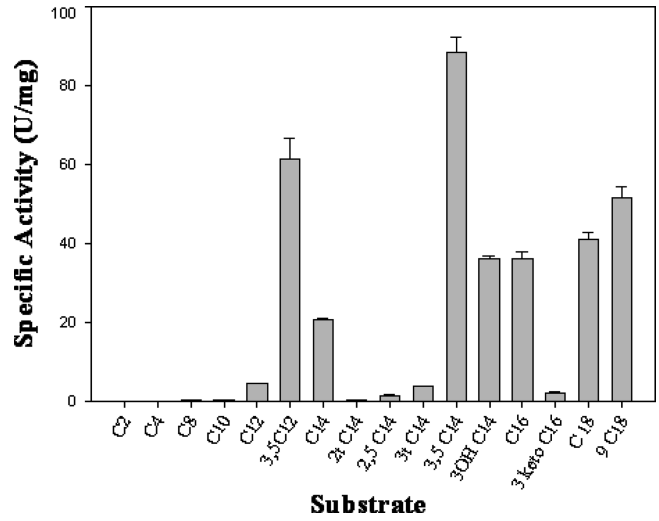


FIGURE 7: Substrate specificity of recombinant thioesterase III. Thioesterase activities were determined with 20 μM: C2, acetyl-CoA; C4, butyryl-CoA; C8, octanoyl-CoA; C10, decanoyl-CoA; C12, dodecanoyl-CoA; 3,5C12, 3,5-*cis*-dodecadienoyl-CoA; C14, tetradecanoyl-CoA (myristoyl-CoA); 2tC14, 2-*trans*-tetradecenoyl-CoA; 2,5C14, 2-*trans*,5-*cis*-tetradecadienoyl-CoA; 3tC14, 3-*trans*-tetradecenoyl-CoA; 3,5C14, 3,5-*cis*-tetradecadienoyl-CoA; 3OHC14, 3-hydroxytetradecanoyl-CoA; C16, hexadecanoyl-CoA (palmitoyl-CoA); 3ketoC16, 3-ketohexadecanoyl-CoA; C18, octadecanoyl-CoA (stearoyl-CoA); and 9C18, 9-*cis*-octadecenoyl-CoA (oleoyl-CoA). Values of enzyme activity are means of three or four determinations ± the standard deviation.

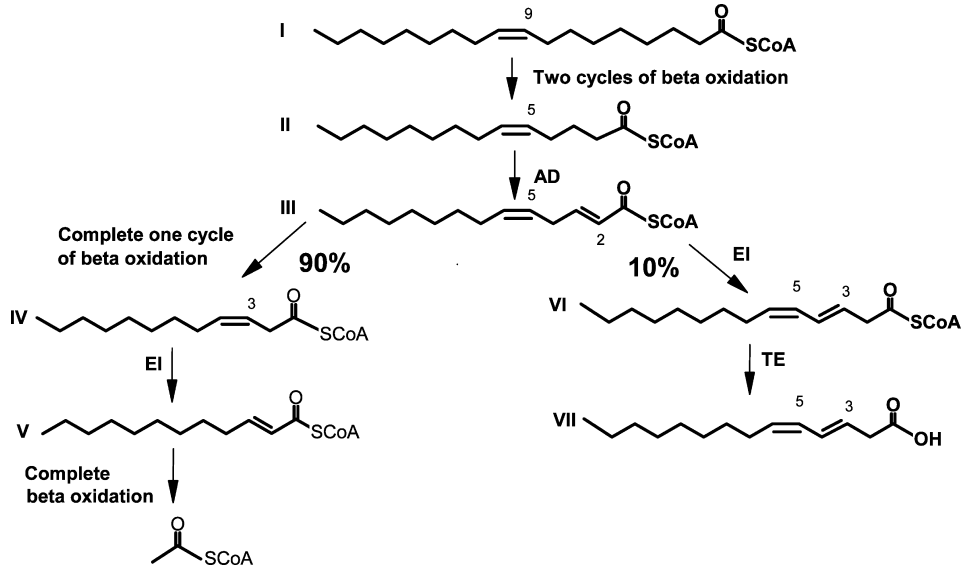
of Figure 6) because they lacked the six-His tags. The partially purified novel thioesterase gave rise to more than six bands on SDS−PAGE (see lane 3 of Figure 6); three of these bands were also detected on the immunoblot (compare lanes 3 and 5 of Figure 6). Because the materials corresponding to three separate bands of the partially purified novel thioesterase had yielded peptides that matched the sequence of the ybaW protein, all seem to be related to the ybaW protein. However, the identity of the materials with molecular masses larger than 15 kDa needed to be established more rigorously. For this purpose, the materials corresponding to the two slower moving bands of the ybaW protein were subjected to limited proteolysis and analyzed by MALDI-MS. Only peptides matching the sequence of the ybaW protein were detected. Hence, the two slower moving materials of the ybaW protein and of the novel thioesterase must be multimeric forms of the protein. Activity measurements together with the SDS−PAGE and immunoblot data strongly support the conclusion that the novel thioesterase and the *ybaW* gene product are the same protein that functions as a long-chain acyl-CoA thioesterase. Because this thioesterase is the third *E. coli* enzyme known to hydrolyze long-chain acyl-CoAs, we propose naming it thioesterase III.

The activity of thioesterase III was measured with different acyl-CoAs, and the kinetic parameters of this enzyme were determined with the best substrates in an attempt to improve our understanding of its metabolic function in β-oxidation. The substrate profile of thioesterase III (see Figure 7) shows that the activity of this enzyme increases with an increase in the length of the substrate's acyl chain. The enzyme is virtually inactive with substrates having acyl chains with fewer than 12 carbon atoms, but it exhibits increasing activity as the acyl chain increases from 12 carbon atoms of lauryl-CoA to 18 carbon atoms of stearoyl-CoA. The 9-*cis* double bond in oleoyl-CoA makes it a better substrate than stearoyl-CoA. Most noteworthy is the fact that the best substrate is 3,5-tetradecadienoyl-CoA, a metabolite of oleic acid that is hydrolyzed during oleate β-oxidation in *E. coli* (*8*). The next best of the substrates is 3,5-dodecadienoyl-CoA, a presumed metabolite of 9-*cis*,11-*trans*-octadecadienoyl-CoA (conjugated linoleic acid). Most other long-chain metabolites of β-oxidation were found to be poor or very poor substrates of thioesterase III except for 3-hydroxytetradecanoyl-CoA that as a substrate is as good as palmitoyl-CoA and much better than myristoyl-CoA (tetradecanoyl-CoA). The kinetic parameters for the hydrolysis of the best substrates were determined and are listed in Table 2. The K_m values for the listed substrates, which are in the low micromolar range, vary only moderately (4-fold). Differences between activities with various substrates are mainly due to different k_{cat} values. For example, the greater catalytic efficiency of the enzyme with 3,5-*cis*-dodecadienoyl-CoA as a substrate compared to its catalytic efficiency with dodecanoyl-CoA (lauroyl-CoA) is due to a 13.5-fold higher k_{cat} value with the former substrate, whereas the K_m values for the two substrates are similar. The substrate specificity of thioesterase III prompts the conclusion that this enzyme is well-suited to hydrolyze of acyl-CoAs with long-chain acyl residues that either are saturated, carry a 3-hydroxy group, or have a 3,5-diene structural element.

Table 2: Kinetic Parameters of the Hydrolysis of Several Acyl-CoAs Catalyzed by Thioesterase III from *E. coli*

substrate	V_{max} (units/mg)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
lauroyl-CoA	6.9 ± 0.2	13.0 ± 1.1	1.6 ± 0.04	0.1
3.5-*cis*-dodecadienoyl-CoA	92.3 ± 1.5	10.8 ± 0.5	21.6 ± 0.4	2.0
myristoyl-CoA	26.4 ± 0.7	6.3 ± 0.6	6.2 ± 0.2	1.0
3,5-*cis*-tetradecadienoyl-CoA	101.3 ± 2.2	3.0 ± 0.3	23.7 ± 0.5	7.9
3-hydroxytetradecanoyl-CoA	45.5 ± 1.6	5.8 ± 0.7	10.7 ± 0.4	1.8
palmitoyl-CoA	43.5 ± 2.2	6.0 ± 0.9	10.2 ± 0.5	1.7

Scheme 1: β-Oxidation of Oleoyl-CoA in *E. coli*[a]



[a] The value of 90% refers to the percentage of oleoyl-CoA that is completely degraded to acetyl-CoA via the main pathway, while 10% indicates the flux through the minor pathway that facilitates the partial degradation of oleoyl-CoA to 3,5-tetradecadienoic acid.

DISCUSSION

A study of oleate β-oxidation in *E. coli* revealed the existence of a branched pathway (see Scheme 1) that facilitates the complete degradation of 90% of this fatty acid to acetyl-CoA while 10% of it is only partially degraded to 3,5-tetradecadienoyl-CoA (*8*). The latter intermediate (compound **VI** in Scheme 1) is hydrolyzed to 3,5-tetradecadienoic acid (compound **VII** in Scheme 1) that is released into the growth medium (*8*). This result raised the question of which acyl-CoA thioesterase catalyzes the hydrolysis of 3,5-tetradecadienoyl-CoA. Two long-chain acyl-CoA thioesterases, thioesterases I and II, are known to be present in *E. coli*, but only thioesterase II is located in the cytosol where β-oxidation takes place; thioesterase I has a periplasmic location. In an attempt to identify the enzyme(s) that functions in oleate β-oxidation, the growth of *E. coli* strain YR1 with deletion mutations in *tesA* and *tesB* that encode thioesterase I and thioesterase II, respectively, was investigated. Because *E. coli* YR1 cells grew on oleate as the sole carbon source and 3,5-tetradecadienoic acid was detected in the growth medium, a long-chain acyl-CoA thioesterase other than thioesterases I and II must be present and operative in this *E. coli* strain. The presence of such thioesterase was demonstrated by assaying a soluble extract of strain YR1 with myristoyl-CoA as the substrate. This thioesterase, which was expressed when cells were grown on oleate but not when they were grown on glucose or tryptone, was partially purified by a three-step purification procedure. Most effective was the purification by hydrophobic chromatography on octyl Sepharose CL-4B from which the enzyme was eluted with an ethanol gradient. This result is suggestive of an enzyme that is relatively hydrophobic for a soluble protein. Mass spectrometric analysis of peptides derived from several proteins of the partially purified thioesterase preparation provided evidence for this protein being encoded by the *ybaW* gene. This tentative conclusion was verified by cloning and expressing the *ybaW* gene, testing the enzymatic activity of the recombinant protein, and demonstrating its immunological identity with the partially purified thioesterase. As part of a structural genomics project, the ybaW protein had been crystallized and its crystal structure had been determined by X-ray diffraction. Those data were deposited in the Protein Data Bank (Kim et al., entry 1NJK) but have not been published. According to its crystal structure, the ybaW protein is a homotetramer with a hot dog fold. The 15-kDa monomer of this thioesterase, termed thioesterase III, was detected by SDS−PAGE. Two forms of the protein that on SDS−PAGE moved slower than the monomer may reflect the presence of dimeric and tetrameric forms of the protein. The cloning data, the conditions used for SDS−PAGE and immunoblotting, and peptide mapping of the slower moving forms of purified ybaW protein argue against artifacts being responsible for the multiple bands observed with both the partially purified native thioesterase III and its purified recombinant form. It is possible that the hydrophobic property of thioesterase III, inferred from its behavior during hydrophobic chromatography, is related to or even is the cause of its incomplete dissociation in a boiling SDS solution. Such resistance of proteins to dissociation by SDS has

previously been observed and attributed to a very stable structure (*21, 22*).

The search for thioesterase III was prompted by the recognition that one or more additional thioesterases must be present in *E. coli* mutant YR1, which is devoid of thioesterases I and II, the only long-chain acyl-CoA thioesterases known to exist in this organism up to now. The identification of thioesterase III raised the question of whether this enzyme participates in the β-oxidation of oleic acid. The substrate spectrum proves it to be a long-chain acyl-CoA thioesterase, and because it hydrolyzes 3,5-tetradecadienoyl-CoA, which in fact is the best of all tested substrates, thioesterase III most likely functions in oleate β-oxidation. Another clue about the function of this enzyme is its induction when *E. coli* cells are grown on oleic acid as the sole carbon source. The underlying mechanism seems to be the fatty acid degradation repressor (fadR)-dependent regulation of *ybaW* expression. The basis for this hypothesis is the identification of a potential consensus sequence for fadR binding upstream of the *ybaW* coding region. The regulation of gene expression via a 17 bp consensus sequence for fadR binding is well-established (*20*). In the absence of fatty acids, bound fadR blocks the transcription of the target gene. However, when fatty acids are present in the growth medium and especially when they are the only carbon source for generating energy and carbon precursors, long-chain fatty acyl-CoA will bind to fadR and displace it from the promoter, thereby allowing transcription to proceed.

Given that 90% of oleic acid is completely degraded by β-oxidation and only 10% is converted to 3,5-tetradecadienoyl-CoA, it seems justified to ask how important the thioesterase-catalyzed hydrolysis of this metabolite is for β-oxidation to continue unimpeded. Although a definite answer is outstanding, it is reasonable to assume that in the absence of thioesterases 3,5-tetradecadienoyl-CoA would accumulate and thereby tie up free CoA that is required for β-oxidation. If finally insufficient or no free CoA is left, β-oxidation and the energy production supported by this process might come to a halt. It is also possible that 3,5-tetradecadienoyl-CoA inhibits one or more enzymes of β-oxidation and thereby would inhibit β-oxidation before free CoA is depleted. In fact, we previously have observed and reported that 3,5-tetradecadienoyl-CoA, when allowed to accumulate in the presence of free CoA, inhibits the multienzyme complex of fatty acid β-oxidation, most likely by binding to the hydratase/isomerase active site (*8*). Hence, the removal of 3,5-tetradecadienoyl-CoA by hydrolysis seems to be essential for maintaining an optimal rate of β-oxidation and energy production.

In conclusion, a novel long-chain acyl-CoA thioesterase, named thioesterase III, is described that is expressed when *E. coli* is grown on long-chain fatty acids and that seems to function in the thioesterase-dependent pathway of β-oxidation. Thioesterase III effectively hydrolyzes 3,5-tetradecadienoyl-CoA, a minor metabolite of oleic acid in *E. coli*, which would inhibit β-oxidation if allowed to accumulate.

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