The Reaction of LipB, the Octanoyl-[Acyl Carrier Protein]:Protein *N*-Octanoyltransferase of Lipoic Acid Synthesis, Proceeds through an Acyl-Enzyme Intermediate†

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ABSTRACT: The *lipB* gene of *Escherichia coli* encodes an enzyme (LipB) that transfers the octanoyl moiety of octanoyl-acyl carrier protein (octanoyl-ACP) to the lipoyl domains of the 2-oxo acid dehydrogenases and the H subunit of glycine cleavage enzyme. We report that the LipB reaction proceeds through an acyl-enzyme intermediate in which the octanoyl moiety forms a thioester bond with the thiol of residue C169. The intermediate was catalytically competent in that the octanoyl group of the purified octanoylated LipB was transferred either to an 87-residue lipoyl domain derived from *E. coli* pyruvate dehydrogenase or to ACP (in the reversal of the physiological reaction). The octanoylated LipB linkage was cleaved by thiol reagents and by neutral hydroxylamine, strongly suggesting a thioester bond. Separation and mass spectral analyses of the peptides of the unmodified and octanoylated proteins showed that each of the assigned peptides of the two proteins had identical masses, indicating that none of these peptides were octanoylated. However, the one major peptide that we failed to recover was that predicted to contain all three LipB cysteine residues. These three cysteine residues were therefore targeted for site-directed mutagenesis and only C169 was found to be essential for LipB function in vivo. The C169S protein had no detectable activity whereas the C169A protein retained trace activity. Surprisingly, both proteins lacking C169 formed an octanoyl-LipB species, although neither was catalytically competent. The octanoyl-LipB species formed by the C169S protein was resistant to neutral hydroxylamine treatment, consistent with formation of an ester linkage to the serine hydroxyl group. The octanoyl-C169A LipB species was probably acylated at C147. LipB species that lacked all three cysteine residues also formed a catalytically incompetent octanoyl adduct, indicating the presence of a reactive side chain other than a cysteine thiol that lies adjacent to the active site.

Lipoic acid (1,2-dithiolane-3-pentanoic acid) is a cofactor required for the function of key metabolic pathways in most organisms (*1*). The reactive sulfur moieties of lipoic acid occur at the distal ends of long flexible structures, and thus lipoic acid is able to channel reaction intermediates across remarkably long distances between the active sites of large multienzyme complexes. While the general role of lipoic acid as a coenzyme has been known for decades (*2*, *3*), the mechanisms by which lipoic acid is synthesized and becomes attached to its cognate proteins continue to be elucidated. *Escherichia coli* contains three enzyme complexes that require lipoic acid as a covalently bound cofactor, pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and glycine cleavage enzyme (*4*). Pyruvate dehydrogenase catalyzes a cycle of three successive reactions in the oxidative decarboxylation of pyruvate to yield acetyl-CoA plus two reducing equivalents (as NADH). The pyruvate and 2-oxoglutarate dehydrogenases generate energy-rich and reducing equivalents both directly and indirectly (by allowing function of the citric acid cycle), and the activity of these enzyme complexes is vital to aerobically growing *E. coli* cultures that rely upon respiration to provide metabolic energy. These enzymes and glycine cleavage enzyme also provide biosynthetic intermediates to several essential pathways.

The physiological function of lipoic acid is dependent upon its covalent attachment to specific lysine residues of the cognate enzymes (*2*, *3*). These specific lysine residues are found on protein domains called lipoyl domains that have widely conserved structures and sequences (*1*). These domains are found at the N-terminus of a unique subunit of each of the lipoate-dependent enzymes. In *E. coli* there are two independent enzyme systems that modify lipoyl domains (*1*, *5*) (Figure 1). Both enzymes can attach either lipoic acid or octanoic acid (the direct precursor of lipoic acid) to lipoyl domains. The best characterized lipoylating enzyme is *E. coli* lipoate−protein ligase (LplA). LplA utilizes exogenously supplied free lipoic acid to modify the lipoyl domain lysine

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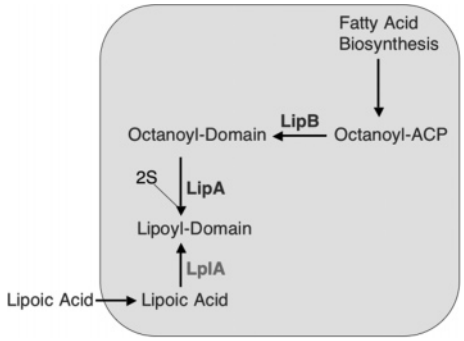

FIGURE 1: Current model of lipoic acid synthesis and attachment in *E. coli* (*5*). The rounded square represents a bacterial cell. The domain is the lipoyl domain of a lipoic acid-dependent enzyme. See text for details.

residue in a two-step reaction in which the enzyme first utilizes ATP to activate lipoic acid to lipoyl-AMP and then catalyzes attack of enzyme-bound lipoyl-AMP by the ϵ-amino group of the lysine of the lipoyl domain to give the amide linkage. LplA has been shown to be required for *E. coli* to utilize lipoic acid from the environment (*6*). However, *lplA* null mutants are not deficient in lipoylated enzyme activities due to the presence of a second LplA-independent lipoylation pathway. This second pathway is dependent on the *lipB* gene and involves a novel enzyme, octanoyl-[acyl carrier protein]:protein *N*-octanoyltransferase that uses the octanoyl thioester of the acyl carrier protein (ACP) of fatty acid synthesis as the acyl donor to acylate lipoyl domains and also functions with lipoyl-ACP (*7*). Strains carrying *lipB* null mutations lack octanoyltransferase activity (*7*), and *lipB* has been demonstrated to encode the octanoyltransferase (*8*, *9*). The octanoyl domain products of LipB have been shown to be the substrates for a novel coenzyme synthetic pathway in which sulfur insertion occurs "on site" (*5*, *10*) (Figure 1). That is, protein-bound lipoic acid has been shown both in vivo and in vitro to be assembled by insertion of two sulfur atoms into the octanoyl moiety of octanoylated domains by the *S*-adenosylmethionine radical enzyme, lipoic acid synthase (LipA) (*11*, *12*). LipB shows no obvious sequence relatedness to proteins other than its homologues which are widely distributed among prokaryotic and eukaryotic organisms. Indeed, yeast mutants that lack their LipB homologues are deficient in lipoate-dependent enzyme activities (*13*, *14*), and a plant mitochondrial LipB has been shown to complement growth of LipB mutants (*15*). Due to its recent discovery and the lack of sequence relatedness to other proteins, little is known of the mechanism of this key enzyme of lipoic acid biosynthesis. We now report that the *E. coli* LipB reaction proceeds through an acyl-enzyme intermediate in which octanoate is first transferred from the thiol of ACP to a specific LipB thiol. The acyl-enzyme thioester bond is then attacked by the ϵ-amino group of the lipoyl domain lysine residue, resulting in the amide-linked octanoylated domains that are the substrates for sulfur insertion (*11*, *12*, *16*).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Media. The bacterial strains used in this study were derivatives of *E. coli* K-12. Strains JK1 and KER184 were described previously (*4*, *17*) whereas the cloning strains TOP10 (Invitrogen), DH5α (Invitrogen), and XL1-Blue (Stratagene) were obtained commercially. Minimal medium E, rich broth, and L broth were the growth media routinely used for growth of bacterial strains and were described previously (*4*, *17*). Solid media contained 1.5% agar. Supplements were added as necessary at the following concentrations: sodium acetate, 5 mM; sodium succinate, 5 mM; thiamin, 1 μg/mL; octanoic acid, 50 μM; and DL-lipoic acid, 5 ng/mL. Glucose (0.4%), acetate (50 mM), or succinate (50 mM) was used as carbon and energy sources in minimal medium. Antibiotics were added at the following concentrations (in mg/L): sodium ampicillin, 100; chloramphenicol, 20; kanamycin sulfate, 50; spectinomycin sulfate, 100; streptomycin sulfate, 50; and tetracycline hydrochloride, 15.

Construction of the Mutant LipB Proteins. The *lipB* gene of *E. coli* K-12 was amplified by PCR from plasmid pCTV616 (*4*) using primers LipB TTG 1 and LipB TTG 2. The amplified DNA was subcloned into pCR2.1 (original TA cloning kit; Invitrogen, Carlsbad, CA) to give plasmid pYFJ29. A 649 bp *Nde*I−*Hin*dIII fragment of plasmid pYFJ29 that carried the entire *lipB* gene was ligated into the *Nde*I−*Hin*dIII sites of plasmid pQE-2 (Qiagen, Valencia, CA) to give plasmid pYFJ31. The mutant *lipB* genes were constructed by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) with the pYFJ31 as the template. The primers (Table 1) were named for the mutant constructed. The double mutants were made using the single mutant genes as templates and in turn were used as templates for construction of the triply mutant genes. The mutations were verified by DNA sequencing using primers pQE-fwd and pQE-rev done by the Keck Genomics Center, University of Illinois at Urbana−Champaign.

Expression and Purification of Wild-Type and Mutant His-Tagged LipB Proteins. The LipB proteins were expressed as N-terminal His-tag fusions from the T5 promoter of vector pQE-2. The plasmids were introduced into strain KER184, and the resulting strains were cultured in LB broth (20−

Table 1: Oligonucleotide Primers Used

primer name	sequence (5′−3′)
LipB TTG 1	CATATGTATCAGGATAAAATTCTTGTCC
LipB TTG 2	AAGCTTAAGCGGTAATATATTCGAAGTC
C137A	CTATGTTGGGGAAAAGAAAATTGCCTCACTGGGTTTACGTATTCG
C137AR	CGAATACGTAAACCCAGTGAGGCAATTTTCTTTTCCCCAACATAG
C147A	TACGTATTCGACGCGGTGCATCATTCCACGGTCTGGC
C147AR	GCCAGACCGTGGAATGATGCACCGCGTCGAATACGTA
C169A	CCATTTTTACGTATTAATCCTGCTGGGTATGCCGGAATGGA
C169AR	TCCATTCCGGCATACCCAGCAGGATTAATACGTAAAAATGG
C169S	CCATTTTTACGTATTAATCCTAGTGGGTATGCCGGAATGGA
C169SR	TCCATTCCGGCATACCCACTAGGATTAATACGTAAAAATGG
pQE-fwd	CCCGAAAAGTGCCACCTG
pQE-rev	GTTCTGAGGTCATTACTGG

200 mL) to mid-logarithmic growth phase, and LipB overproduction was induced by the addition of 1.0 mM isopropyl β-D-thiogalactopyranoside for 4 h at 30 °C. The cells were harvested by centrifugation at 4000*g* for 10 min, and the pellets were stored at −80 °C. All further purification steps were performed at 4 °C. The His-tagged LipB proteins were purified using Qiagen nickel agarose according to the protocol supplied by Qiagen. Frozen pellets were thawed on ice for 15 min prior to the addition of 1−10 mL of lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole). Lysozyme (1 mg/mL) was added, and after 30 min of incubation on ice the cells were disrupted via sonication (six 10 s pulses). The lysates were centrifuged at 10000*g* for 30 min, and 1−3 mL of Ni-NTA resin (Qiagen, Inc., Valencia, CA) was added to the resultant supernatant. The slurry was rotated slowly at 4 °C for 60 min, and the resin was loaded into a 5 mL column and washed with 8 volumes of wash buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole). The His-tagged protein was eluted with 1 volume of elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 250 mM imidazole). This elution was repeated four times. The His-tagged LipB proteins were then further purified using the Vivapure acidic protein purification kit (Vivascience). The samples were 10-fold diluted in buffer A-Q (25 mM Tris-HCl, pH 8.0) and absorbed to a Vivapure Q Mini H spin column. His-tagged LipB was then eluted by using a step gradient (0.1 M increments) from 0 to 1 M NaCl in the same buffer. The purity of the samples was monitored via SDS−PAGE. Purified His-tagged LipB was then dialyzed against 20 mM sodium phosphate buffer (pH 7.0). If needed, the purified proteins were concentrated using Millipore Amicon Ultra-4 centrifugal filter devices (5000 MWCO). Following dialysis and concentration, 10% glycerol was added prior to storage at −80 °C.

Acyl-ACP Preparation. Acyl-ACPs were synthesized according to the method of Shen et al. (*18*). The 1 mL reaction mixture contained 10 mM ATP, 20 mM $MgSO_4$, 0.1 M Tris-HCl (pH 7.8), 1 mM DTT, 0.66 mg of *E. coli* holo-ACP, 40 μg of His-tagged *Vibrio harveyi* acyl-ACP synthetase purified from *E. coli* (Jiang and Cronan, manuscript in preparation), and either 0.5 mM octanoic acid or 0.19 mM [1-^{14}C]octanoic acid (0.1 mCi/mL, 53 mCi/mmol). The reaction mixtures were incubated at 37 °C for 4 h. Two volumes of acetone was then added to each sample, and the mixtures were allowed to precipitate overnight at −20 °C. The precipitates were pelleted at 20000*g* for 20 min at 4 °C and then washed twice with 3 volumes of acetone. The pellet was air-dried and resuspended in 20 mM Tris-HCl (pH 7.4) at an ACP concentration of 3.3 mg/mL. Completion of the acyl-ACP synthesis reaction was tested either by the gel shift assay (*19*) or by LipB-catalyzed transfer of the octanoyl-group to the apo-E2 domain. Purified samples of *E. coli* holo-ACP and *V. harveyi* acyl-ACP synthetase were the respective gifts of Drs. Jose Solbiati and Yanfang Jiang of this laboratory. Labeled octanoic acid was purchased from American Radiolabeled Chemicals (St. Louis, MO).

In Vitro Assay of LipB Activity. LipB activity was measured by the gel mobility shift assay described previously (*7*, *20*). In this assay, lipoylation or octanoylation is measured by the shift of the electrophoretic mobility of a purified lipoyl domain derived from *E. coli* pyruvate dehydrogenase (*21*). Upon acylation of the target lysine residue, the electrophoretic mobility of the lipoyl domain increases due to the loss of the positive charged side chain. The apo-E2 domain was purified as described previously (*12*), and the octanoyltransferase reaction mixture (20 μL) usually contained 10 mM sodium phosphate buffer (pH 7.0), 0.3 mM DTT, 5.3 μg of octanoyl-ACP, 4 μg of the apo-E2 domain, and various concentrations of His-tagged LipB. The reactions were incubated at 37 °C for 30 min. The reaction products were then analyzed by PAGE using 20% native gels. LipB activity was also measured by a radioactive assay. The reaction was performed essentially the same as the gel shift assay except that [1-^{14}C]octanoyl-ACP was used in place of the nonradioactive donor. The reaction products were then subjected to electrophoresis using 14% sodium dodecyl sulfate (SDS)−polyacrylamide gels, and the ^{14}C-labeled E2 domain was detected by fluorography.

Acylation of LipB was detected by utilizing [1-^{14}C]-octanoyl-ACP. The reaction was performed essentially the same as above except that no apo-E2 domain was added to the reaction mixture. The reaction was incubated at 37 °C for 30 min. The reaction products were then subjected to electrophoresis using 14% SDS−polyacrylamide gels, and the ^{14}C-labeled bands were detected by fluorography.

Purification of the Acyl-LipB Intermediate. The [1-^{14}C]-octanoyl-LipB intermediate was purified using the Vivapure acidic protein purification kit (Vivascience) and analyzing the radioactive bands by electrophoresis. The [1-^{14}C]octanoyl-ACP (66 μg) and His-tagged LipB (160−170 μg) reaction mixture (200 μL) was incubated at 37 °C for 1 h, then diluted 10-fold in buffer A-Q (25 mM Tris-HCl, pH 8.0), and absorbed to the Vivapure Q Mini H spin column. The spin column was washed once with 0.4 mL of buffer A-Q, and octanoyl-LipB was eluted with 0.4 mL volumes of increasing NaCl concentrations (0.1, 0.2, and 0.3 M) in the same buffer. After electrophoresis using 14% SDS−polyacrylamide gels the ^{14}C-labeled bands were detected by fluorography.

Assay of Acyl Transfer from LipB to the Apo-E2 Domain and ACP-SH. Transfer of the octanoyl moiety from octanoyl-LipB to apo-E2 domain was tested by mixing the purified [1-^{14}C]octanoyl-LipB intermediate and apo-E2 domain. In a 20 μL reaction system ~2 μg of purified [1-^{14}C]octanoyl-LipB and 4 μg of the apo-E2 domain were mixed in 25 mM Tris-HCl containing 0.2 M NaCl and incubated at 37 °C for 1 h followed by electrophoresis in 14% SDS−polyacrylamide gels and detection by fluorography. Transfer of the octanoyl moiety from octanoyl-LipB to ACP-SH was tested in the same manner except that 30 μg of holo-ACP was substituted for the apo-E2 domain. The incubation was at 37 °C for 1 h or as described in the figure legends. The reaction products were subjected to electrophoresis using 14% SDS−polyacrylamide gels in the absence of reducing agents, and the ^{14}C-labeled bands were detected by fluorography.

Hydroxylamine Treatment. After resolution of proteins by SDS−PAGE the acrylamide gels were fixed and then soaked for 2 h in 1.33 M NH_2OH (pH 7.0, made by mixing 20 mL of 2 M hydroxylamine hydrochloride with 10 mL of 3.5 M NaOH immediately before use). The gels were then fixed again, treated with Enlightning (NEN Life Science Products), dried, and exposed to Kodak BioMax XAR film.

Protease Digestion and Peptide Identification. His-tagged LipB (90 μg) and octanoyl-LipB (40 μg) purified by the Vivapure procedure were dialyzed against 20 mM ammonium acetate buffer, dried, and resuspended in 500 μL of digestion buffer (25 mM Tris-HCl, pH 8.5, plus 1 mM EDTA). Endoproteinase Lys-C (5 μg) (Roche Applied Science) was resuspended in 200 μL of H_2O (25 μg/mL), and 80 μL (2 μg) was added to the LipB preparation and 40 μL (1 μg) to the octanoyl-LipB preparation. Digestion was carried out at 37 °C for 12 h followed by storage at −20 °C. The completion of digestion was tested on precast Novex 16% Tricine gels (Invitrogen). Samples for high-pressure liquid chromatography (HPLC)[1] analyses were thawed in the presence of 0.1% trifluoroacetic acid. The digestion products were separated by reverse-phase HPLC on a C4 column (Brownlee Aquapore, 7 μm particle size, 2.1 × 100 mm) (*22*). The peptides were eluted with a linear gradient of 0−50% acetonitrile (v/v) in 0.1% (v/v) trifluoroacetic acid over 60 min and recovered by vacuum centrifugation for analysis by MALDI-TOF mass spectrometry. Mass spectrometric analyses were done by the Protein Sciences Facility, University of Illinois at Urbana−Champaign.

SDS−PAGE and Fluorography. Tris−glycine gels (14%, Invitrogen) were used for radioactive samples. Dithiothreitol was normally added to the sample buffer to a final concentration of 0.1 M. After electrophoresis, the gels were stained with Coomassie brilliant blue R, destained, treated with Enlightning (NEN Life Science Products), dried, and exposed to Kodak BioMax XAR film.

RESULTS

In the course of preparation of substrates to assay the LipA-dependent lipoic acid synthesis reaction (*12*, *16*), we prepared octanoylated forms of the two *E. coli* 2-oxo acid dehydrogenases, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase, by LipB-catalyzed transfer of octanoyl groups from [^{14}C]octanoyl-ACP radioactively labeled in the octanoyl moiety. Increasing concentrations of a mixture of the two apodehydrogenases were added to reaction mixtures containing constant levels of [^{14}C]octanoyl-ACP and LipB followed by analysis by denaturing gel electrophoresis (Figure 2). As expected, the labeled octanoate was transferred from ACP to the 2-oxo acid dehydrogenase E2 subunits, but an unexpected fourth radioactive band appeared at the molecular weight of LipB. Since the intensity of this band varied inversely with the amount of 2-oxo acid dehydrogenase acceptor protein added and was formed in the absence of any acceptor protein, it seemed likely that the labeled LipB species was an acyl-enzyme intermediate (Scheme 1). Moreover, the fact that the labeled LipB survived denaturing gel electrophoresis indicated that the bond linking the acyl group and LipB was reasonably stable (and/or that the protein was incompletely denatured). Since nothing was known of the LipB reaction mechanism, we decided to determine whether the LipB reaction proceeds through an octanoyl-enzyme intermediate as shown in Scheme 1.

[1] Abbreviations: SDS, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography.

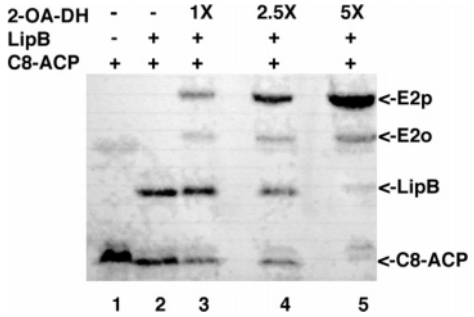

FIGURE 2: Labeling of LipB and the E2 subunits of the pyruvate and 2-oxoglutarate dehydrogenases by transfer of [1-^{14}C]octanoate from [1-^{14}C]octanoyl-ACP. The samples (20 μL reactions) in all lanes contained 5 μg of [1-^{14}C]octanoyl-ACP and lanes 2−5 also contained 4 μg of His-tagged LipB. Lanes 3−5 additionally contained 4, 10, or 20 μg of an apo 2-oxo acid dehydrogenase preparation (a mixture of the pyruvate and 2-oxoglutarate dehydrogenases). After incubation for 30 min at 37 °C the reactions were loaded onto a 10−20% SDS−PAGE gel followed by visualization by phosphorimaging. The symbols E2p and E2o denote the E2 subunits of the pyruvate and 2-oxoglutarate dehydrogenases, respectively. The proteins were prepared as previously described (*16*).

Scheme 1

$$\text{octanoyl-ACP} + \text{LipB} \rightleftharpoons [\text{octanoyl-LipB}] + \text{ACP-SH}$$

$$[\text{octanoyl-LipB}] + \text{apolipoyl domain} \rightarrow \text{octanoyl-E2 domain}$$

Demonstration of Acyl Transfer from Octanoyl-LipB to a Lipoyl Domain. We modified our prior His-tagged LipB purification (*8*, *12*, *16*) to obtain larger amounts of enzyme of greater purity (Figure 3). MALDI-TOF mass spectrometry of purified LipB gave a mass of 25141.9 (presumably a monosodium adduct of LipB) versus a calculated mass of 25114.8. The purified LipB protein was tested for octanoyl-(lipoyl-) transferase activity using gel mobility shift assay (*7*, *20*). As the terminal acyl acceptor we used an 87-residue lipoyl domain derived from the E2 subunit of *E. coli* pyruvate dehydrogenase in place of the apo 2-oxo acid dehydrogenase acceptor proteins used in Figure 2 (which are difficult to obtain in a highly pure state). This lipoyl domain protein was previously shown to accept the octanoate group from [1-^{14}C]octanoyl-ACP in reactions catalyzed by LipB (*7*, *20*). As expected, addition of the domain to a mixture of LipB and [1-^{14}C]octanoyl-ACP resulted in loss of the labeled LipB band (Figure 4A,B) and appearance of a labeled E2 band (Figure 4B). To directly test the catalytic competence of the putative octanoyl-LipB intermediate, we purified the octanoyl-LipB species away from excess octanoyl-ACP by ion-exchange chromatography (Figure 4C). Upon incubation of the purified labeled octanoyl-LipB with the apolipoyl domain, the label was quantitatively transferred to the apo-E2 domain, thereby demonstrating that the octanoylated LipB species was a true intermediate in the forward reaction (Figure 5). The octanoyl-LipB intermediate also transferred octanoate to ACP (Figure 5), although the extent of transfer was modest, indicating that the equilibrium under these conditions was in favor of the acyl-LipB species. Although we made numerous attempts to detect an octanoylated species by various forms of mass spectrometry, the strong binding of metal ions by this acidic protein spread the spectrum upfield

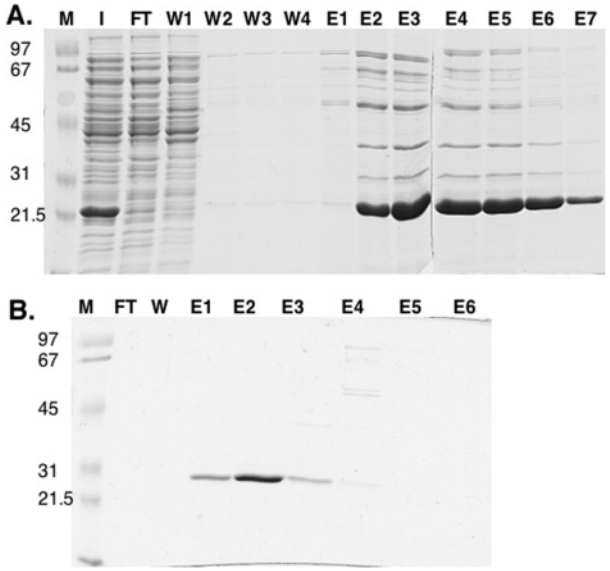

FIGURE 3: Purification of the His-tagged wild-type LipB protein. Panel A: Purification by Ni-NTA column chromatography. Lanes: I, crude extract of the induced sample; FT, flow-through fractions; W1−W4, wash fractions; E1−E7, elution factions. Panel B: Purification by a Vivapure ion-exchange cartridge. Lanes: FT, flow-through fraction; W, wash fractions with buffer A-Q; E1−E6, elution with progressive 0.1 M incremental NaCl concentrations from 0.1 to 0.6 M in buffer A-Q. Lane M is the Bio-Rad prestained protein markers (low range).

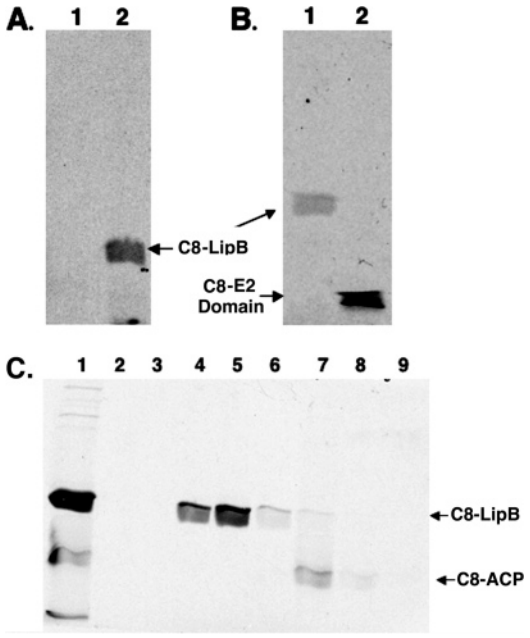

FIGURE 4: Formation and purification of the [^{14}C]octanoyl-LipB intermediate. Reaction details were described in Experimental Procedures. Panel A shows the transfer of the radioactive octanoyl group from octanoyl-ACP to LipB to form an acyl-enzyme intermediate. Lanes: 1, LipB plus [^{14}C]octanoic acid; 2, LipB plus [^{14}C]octanoyl-ACP. Panel B shows transfer of the radioactive octanoyl group from the octanoyl-LipB intermediate to the apo-E2 domain. Lanes: 1, LipB plus [^{14}C]octanoyl-ACP; 2, LipB plus [^{14}C]-octanoyl-ACP plus the apo-E2 domain. Panel C: Purification of the [^{14}C]octanoyl-LipB intermediate. Reaction details are described in Experimental Procedures. Lanes: 1, the LipB plus [^{14}C]octanoyl-ACP reaction mixture; 2, flow-through fraction; 3, wash fraction; 4−9, successive elutions with 0.1−0.6 M NaCl in ascending steps of 0.1 M.

of the peak of the unmodified form that confounded obtaining data of the needed accuracy. Therefore, we returned to radioactive labeling of the octanoyl moiety to study the modification.

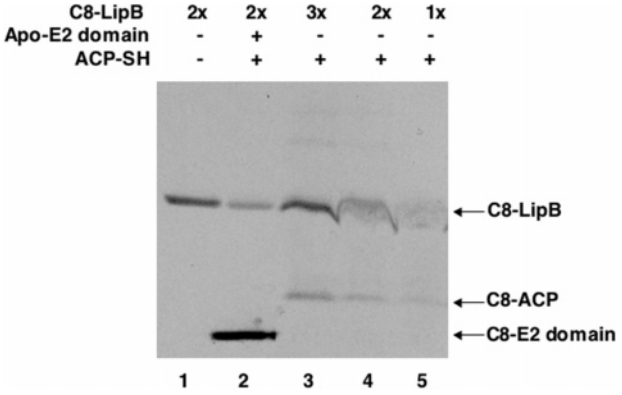

FIGURE 5: Transfer of the [^{14}C]octanoyl group from the [^{14}C]-octanoyl-LipB intermediate to either the apo-E2 domain or ACP. The reaction mixtures contained the following: lane 1, 2 μg of the purified [^{14}C]octanoyl-LipB intermediate; lane 2, 2 μg of the purified [^{14}C]octanoyl-LipB intermediate plus 4 μg of the apo-E2 domain; lane 3, 3 μg of the purified [^{14}C]octanoyl-LipB intermediate plus 30 μg of holo-ACP; lane 4, 2 μg of the purified [^{14}C]octanoyl-LipB intermediate plus 30 μg of holo-ACP; lane 5, 1 μg of the purified [^{14}C]octanoyl-LipB intermediate plus 30 μg of holo-ACP.

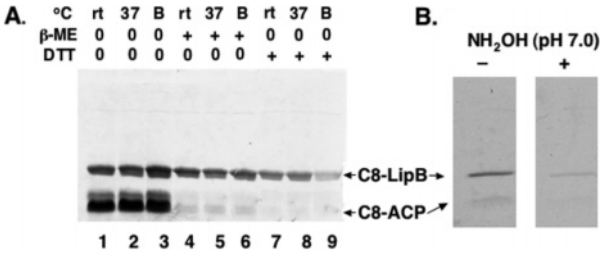

FIGURE 6: Stability of the acyl-enzyme intermediate toward neutral hydroxylamine and thiol reagents. Panel A: Treatment with 0.1 M DTT or 0.125 M 2-mercaptoethanol (β-ME). The thiol reagents were added to samples of completed reactions in which the octanoyl moiety was transferred from [1-^{14}C]octanoyl-ACP to LipB. The samples were then treated at the temperatures given before loading the gels. Symbols: rt, 25 °C for 10 min; 37, 37 °C for 10 min; B, 100 °C for 5 min. Panel B: Treatment with neutral hydroxylamine. Polyacrylamide gels containing samples of an LipB acylation reaction were either exposed (+) to neutral hydroxylamine as described in Experimental Procedures or left unexposed (−).

Stability of the Octanoyl-LipB Linkage. To obtain an indication of the type of linkage involved, LipB was labeled by transfer of the octanoyl moiety from [1-^{14}C]octanoyl-ACP, and the reaction mixtures were incubated under various conditions to assess the ability of various reagents to remove the acyl group from LipB (Figure 6). The residual octanoyl-ACP acted as an internal thioester linkage standard. As expected from the data of Figure 2 the octanoyl-LipB linkage was stable to temperature and quite stable toward 2-mercaptoethanol, although it was cleaved by dithiothreitol (Figure 6A). As expected, the same trend was seen with octanoyl-ACP, although the acyl-ACP thioester was much more labile presumably due to its attachment to the fully solvent accessible 4′-phosphopantotheine prosthetic group. The finding that dithiothreitol gives more efficient cleavage than 2-mercaptoethanol was expected from the work of Stokes and Stumpf (*23*), who showed that attack of dithiothreitol on thioester bonds first results in formation of *S*-acyl-DTT. The acyl group then migrates to the neighboring hydroxyl group to form *O*-acyl-DTT. Acyl migration allows

Table 2: Products of Endoproteinase Lys-C Digestion of His-Tagged LipB Protein Species[a]

position (residue no.)[a]	predicted mass	mass of LipB digest peptide	mass of octanoyl-LipB peptide
15−63	5671.8165	5673.443	5674.164
145−187	4724.3688	N/D[b]	N/D
64−101	4174.1575	4175.098	4174.685
105−143	4157.2604	4157.772	4157.533
193−222	3340.7423	3341.495	3341.073
3−14	1643.7145	1643.77	1643.741
188−192[c]	661.3668	N/D	N/D[b]
102−104	459.3150	N/D	N/D
1−2	278.1533	N/D	N/D
144−144	147.1128	N/D	N/D
188−222	3983.0912[d]	3984.158	3983.485

[a] The residue numbering is that of the His-tagged LipB. [b] N/D, not detected. [c] This peptide is present in the partial digestion products given in the last entry of the table. [d] Partial digest product attributed to the inhibitory K-P sequence of residues 183−184 (*25*).

formation of the cyclic disulfide and thus the cleavage reaction is driven by formation of the sterically favorable 1,2-dithane ring (*23*).

We also examined the sensitivity of the octanoyl-LipB linkage to neutral hydroxylamine, a reagent that cleaves thioesters but not oxygen esters or amides (*24*) (Figure 6B). Hydroxylamine (NH_2OH) also has the advantage that its very small molecular size allows access to sterically restricted environments that thiol reagents may not access. The reagent could not be added to samples before electrophoresis due to the large amounts of salt generated upon neutralization of hydroxylamine hydrochloride. Therefore, we ran two identical denaturing polyacrylamide gels containing [1-^{14}C]octanoyl-LipB in parallel, fixed both gels, and soaked one in neutral hydroxylamine. Both gels were then again fixed, prepared for fluorography, and exposed to film. Hydroxylamine treatment hydrolyzed most of the octanoyl-LipB and the residual octanoyl-ACP consistent with acyl thioester linkages being present in both molecules.

Identification of the Modified Amino Acid Residue. To locate the octanoylated residue, we digested LipB and octanoylated LipB with the lysine-specific protease Lys-C (*25*) and resolved the peptides by high-pressure reverse-phase chromatography. The purified peptides from the two proteins were then analyzed by matrix-assisted laser desorption/ionization mass spectrometry on an Ettan MALDI-TOF instrument using α-cyano-4-hydroxycinnamic acid as the matrix and delayed extraction mode. The mass values obtained were matched to the sequence using the program PeptideMass (www.expasy.ch). All, but one, of the possible large peptides were isolated, and each was matched an expected peptide. The exception was the peptide that corresponded to residues 136−178 of the native protein (residues 145−187 of the His-tagged protein). Numerous attempts to isolate this peptide were fruitless. However, the peptides recovered from the two samples had the same set of mass values within experimental error (Table 2), indicating that none of the recovered peptides carried an octanoyl group (three small peptides of three or less residues were not recovered, but the only modifiable side chains of these peptides that were lysine residues, acylation of which would have blocked digestion and imparted increased mass to another peptide). Thus, by process of elimination, the missing peptide was thought to be that which carried the acyl group. Moreover, consistent with indications of a thioester linkage, the missing peptide contained all three LipB cysteine residues, and thus we targeted these residues for site-directed mutagenesis. Of these cysteine residues C169 (wild-type LipB numbering) seemed the most likely to carry the octanoyl moiety, since it is conserved within a PCG motif found in the plant and all bacterial LipB homologues and as a CG sequence in the putative fungal LipBs (Figure 7). Hence, we mutated C169 to either serine or alanine. As controls, the other two cysteine resides were also mutated to alanine. Plasmids carrying each of the mutated *lipB* genes expressed from the plasmid phage T5 promoter were then tested for the ability to rescue growth of a *lipB* null mutant strain on minimal medium (Figure 8). Although the plasmids encoding the C137A and C147A LipB proteins restored growth, neither of the plasmids encoding the mutated C169 proteins allowed growth, thereby demonstrating that C169 was an essential residue. Each of the mutant proteins was purified by virtue of its His tag and assayed for activity using the gel shift assay (Figure 9 and Table 3). The assay used was to vary the quantity of the mutant LipB proteins to give a level of apo-E2 domain acylation comparable to that of the wild-type protein in the gel shift assay. As expected from the in vivo results, both mutant proteins lacking C169 were defective in catalysis. The C169S protein had no detectable activity whereas the C169A protein had greatly reduced activity (about 1% of the wild-type activity). The proteins having an alanine substitution of C137 or C147 had essentially wild-type activities. Each of the mutant proteins was then tested for the ability to form an octanoyl-enzyme intermediate by transfer of the octanoyl moiety of octanoyl-ACP. Surprisingly, all of the proteins including those lacking C169 formed an octanoylated LipB species (Figure 10).

Aberrant Natures of the Acylated Forms of the C169 Mutant LipB Proteins. We assayed each of the acylated mutant LipB proteins for the ability to transfer the octanoyl moiety to the apo-E2 domain (Figure 10). As expected, the wild-type protein and those mutant proteins that retained more than trace levels of activity showed transfer to the acceptor protein whereas the C169A mutant protein only weakly transferred the octanoyl moiety and the C169S protein showed no detectable transfer. Moreover, both C169 mutant LipB proteins also failed to transfer the octanoyl group to ACP whereas the other mutant proteins were active in the reverse acylation reaction (Figure 10). The failure of the C169S mutant protein to transfer the octanoyl moiety indicated that the intermediate formed was a catalytically incompetent (dead-end) adduct rather than a true intermediate whereas the intermediate formed by the C169A protein had only very restricted catalytic competence. Although not apparent in the experiment of Figure 10, upon incubation with [1-^{14}C]octanoyl-ACP the C169S LipB generally formed a more strongly labeled protein band than did the wild-type protein. This finding suggested that the acyl group was bound to the serine hydroxyl through an oxygen ester linkage and that the greater stability of this linkage might account for the more intense labeling. To test the oxygen ester hypothesis, we examined the stability of the C169S acylated species toward neutral hydroxylamine, a reagent that does not cleave aliphatic oxygen esters (unless in especially reactive environments) (*24*). As before (Figure 6), a gel containing the

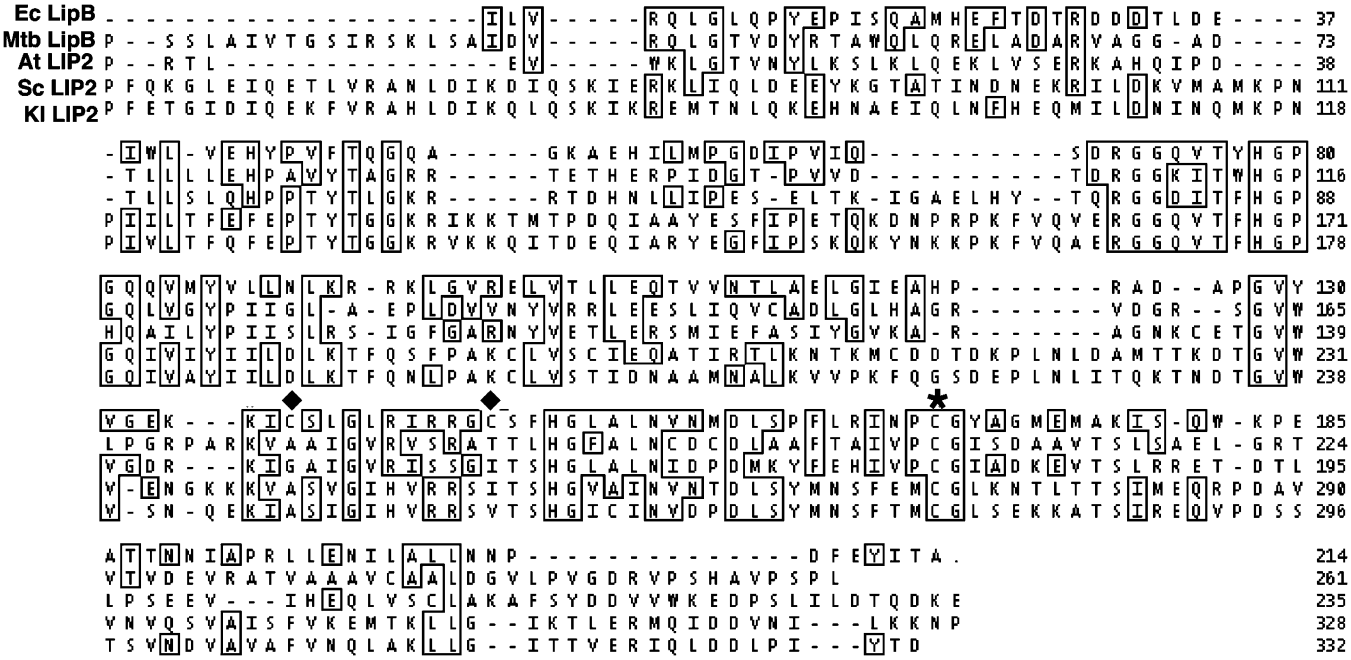

FIGURE 7: Alignment of *E. coli* LipB with LipB homologues of demonstrated biological activity. C169 (*E. coli* numbering) is marked with an asterisk whereas C137 and C147 are marked with a diamond. The *Mycobacterium tuberculosis* (Mtb) *lipB* (Zhao and Cronan, unpublished data) and *Arabidopsis thaliana* (At) *LIP2* genes have been shown to complement growth of *E. coli lipB* mutants (*15*) whereas strains carrying the deletion mutations of the *Saccharomyces cerevisiae* LIP2 (*14*) and *Kluyveromyces lactis* LIPB genes (*13*) are deficient in lipoic acid requiring enzyme activities.

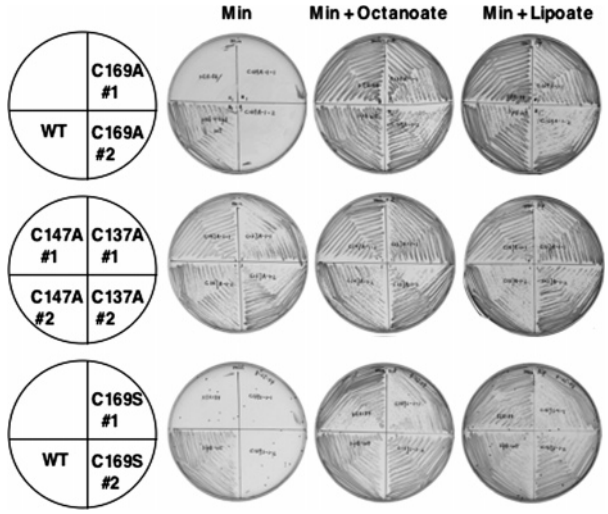

FIGURE 8: Complementation of an *E. coli lipB* null mutant. *E. coli* strain KER184 which carries a null *lipB* mutation (*17*) was transformed with plasmids that expressed the LipB mutant proteins given above from the vector phage T5 promoter. Colonies of the various transformed strains grown on lipoic acid supplemented minimal medium were streaked onto minimal medium glucose plates containing 5 ng/mL lipoic acid (Min + Lip) or 5 μg/mL octanoic acid (Min + Oct) or without supplement (Min). The plates were incubated at 37 °C for 2 days. We used plates divided into quadrants by plastic walls to prevent cross-feeding by excreted metabolites.

denatured proteins was soaked in neutral hydroxylamine solution and compared to an identical gel run in parallel and left untreated. We found (Figure 11) that the bond linking the acyl group to C169S LipB was much more resistant to neutral hydroxylamine than were the intermediates formed by the wild-type and the other mutant proteins. The fact that the acyl group of the C169A mutant protein was released by hydroxylamine treatment suggested that, in the absence of the C169 thiol, another thiol attacked the acyl-ACP thioester to give an acylated protein species.

Properties of the Multiply Mutated Proteins. We constructed plasmids that encoded proteins having each of the permutations of the three mutated cysteine residues. The plasmids were tested for their abilities to support growth of a *lipB* null mutant strain. The mutant proteins were also purified and assayed for each of the reactions catalyzed by LipB. All proteins that contained the C169S mutation were inactive both in vivo and in all of the in vitro LipB reactions but formed an acylated protein species (Figure 12B). All proteins that contained the C169A mutation failed to complement growth of a *lipB* null mutant strain, but had very weak activities in the overall reaction and in transfer of the acyl group from the acylated protein to the apo-E2 domain, but failed to transfer the LipB-bound acyl group back to ACP (Table 3). Like the singly mutated proteins those that contained both the C137A and C147A mutations behaved similarly to the wild-type protein in all assays. An unexpected finding was that the mutant protein (C137A/C147A/C169A) that lacked all three cysteine residues remained able to form an acylated LipB species (Figure 12B), although the protein was completely inactive in all of the LipB reactions.

DISCUSSION

LipB joins the list of enzymes that form acyl-enzyme intermediates. This part of the lipoic acid synthetic pathway is largely a branch off the fatty acid synthesis pathway, and thus it seems interesting that two enzymes of that pathway, the β-ketoacyl-ACP synthases and malonyl-CoA:ACP transacylases, form acyl-enzyme intermediates (*26*−*29*). In the case of the β-ketoacyl-ACP synthases, an acyl group is transferred from an acyl-CoA or acyl-ACP donor to a cysteine thiol of the protein (*26*, *27*, *30*). The protein-bound thioester is then attacked by a carbanion formed from

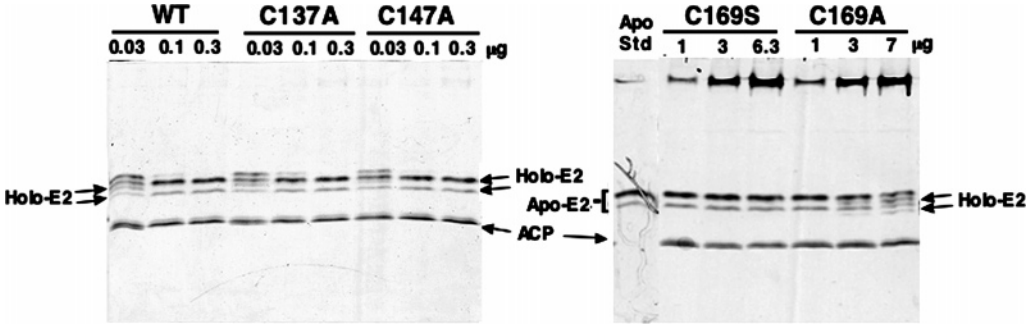

FIGURE 9: In vitro gel shift assay of octanoyltransferase activity. The purified His-tagged mutant LipB proteins were tested for their activities in the overall assay. The reaction details were as described in Experimental Procedures. Different amount of the proteins were added to the reactions as shown in the figure. Apo denotes the apo-E2 domain standard. Note that a more rapidly migrating form of the apo-E2 domain accumulates during storage (presumably a deamidation product) that complicates the patterns since two holo-E2 domain forms are synthesized. The bands at the top of the gels are the LipB proteins.

Table 3: Summary of Site-Directed Mutagenesis Studies

LipB protein	in vivo complementation	in vitro overall transferase activity (%)[a]	formation of acyl-enzyme intermediate	acyl transfer to apo-E2 domain	acyl transfer to ACP
wild type	+[b]	(100)	+	+	+
C137A	+	100	+	+	+
C147A	+	100	+	+	+
C169A	−	1.0	+	±	−
C169S	−	<0.5	+	−	−
C137A/C147A	+	100	+	+	+
C137A/C169A	−	0.4	+	±	−
C137A/C169S	−	<0.5	+	−	−
C147A/C169A	−	<0.3	+	−	−
C147A/C169S	−	<0.4	+	−	−
C137A/C147A/C169A	−	<0.3	+	−	−
C137A/C147A/C169S	−	<0.3	+	−	−

[a] The sensitivities of the assays varied with the concentration of the LipB preparation available. The activity of the wild-type enzyme was set at 100%. The assays were done as in Figures 9 and 11. [b] Symbols: +, growth or activity; −, no detectable growth or activity; ±, very low activity.

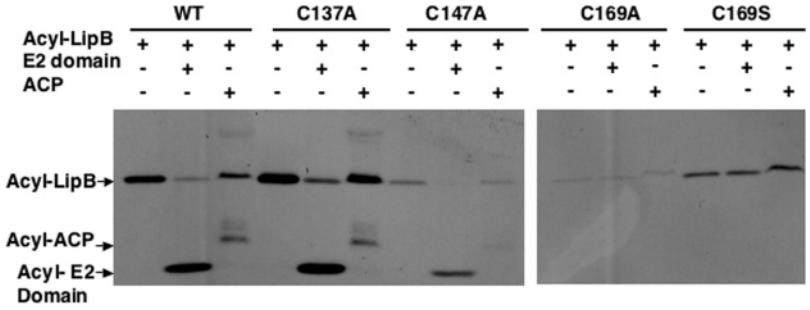

FIGURE 10: Assay of the partial reactions of the mutant LipB proteins. Transfer of the [^{14}C]octanoyl group from the [^{14}C]octanoyl-LipB intermediate to the E2 domain or to ACP was assayed using ~2 μg of the purified acyl-enzyme intermediates of the wild-type and mutant proteins. Either the apo-E2 domain (4 μg) or ACP (30 μg) was added as shown.

malonyl-ACP to give the elongated β-ketoacyl-ACP product. In malonyl-CoA:ACP transacylase the acylated residue is a serine that shuttles malonyl moieties between the thiols of CoA and ACP (*28*, *29*, *31*). A reaction that more closely parallels that of LipB is that of the human protein modification enzyme, *N*-myristoyltransferase (*32*). In this reaction the enzyme cysteine residue is acylated by transfer from a thioester linkage (myristoyl-CoA), and the resulting enzyme acyl-thioester is then attacked by a protein amino group (the N-terminus of the acceptor proteins). However, in contrast to LipB, the cysteine to serine mutant of the *N*-myristoyltransferase was reported to retain appreciable activity in the overall reaction, and the yield of the acyl-enzyme intermediate was greatly diminished by the mutation (*32*). However, it should be noted that the *N*-myristoyltransferase data were obtained by analysis of proteins labeled in the acyl group during expression in bacteria and thus the acyl-enzyme intermediate was not isolated and its catalytic competence was not demonstrated.

The finding that the C169A LipB mutant protein formed a hydroxylamine-sensitive acyl-enzyme species that transferred its octanoyl moiety to the apo-E2 domain (although not to ACP) suggests the presence of a second cysteine thiol located close to the active site. In the absence of a nucleophilic side chain on residue 169 this thiol attacks the octanoyl-ACP thioester bond. The LipB thioester thus formed can be attacked (albeit poorly) by the ϵ-amino group of the apo-E2 lysine but is not attacked by the less nucleophilic ACP thiol. C147 seems the more likely of the two remaining cysteine residues to form this adduct because introduction of the C147A mutation abolished the trace activity of the C169A protein whereas the doubly mutated C137A/C169A

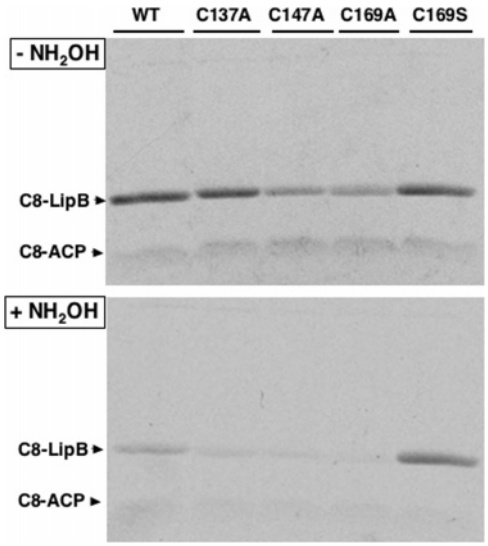

FIGURE 11: Relative sensitivities of acyl-enzyme intermediates of the mutant LipBs to hydroxylamine. Polyacrylamide gels containing octanoylated wild-type and mutant LipBs were exposed (+NH_2-OH) to neutral hydroxylamine as described in Experimental Procedures or left untreated (−NH_2OH). About 5 μg of each of the LipB proteins given above the figure was loaded.

protein retained the trace activity (Figure 12A, Table 3). Moreover, the C147A/C169A double mutant protein formed considerably less acylated enzyme than did the protein containing the C137 and C169A mutations (Figure 12B). The residual labeling of the C147A/C169A protein cannot be attributed to acylation of C137 because the triple mutant (C137A/ C147A/C169A) that lacked all three cysteine residues somehow is acylated (Figure 12B). Hence, there must also be a non-thiol side chain located close to the LipB active site that can attack octanoyl-ACP. This adduct was unable to transfer the acyl group in either direction, suggesting the formation of a bond insensitive to nucleophilic attack. Therefore, the LipB active site seems to contain a pecking order of nucleophilic site chains that can attack the reactive octanoyl-ACP thioester. C169 is the active site LipB nucleophile and the only residue that upon acylation is competent to transfer the acyl group both to the apo-E2 domain and to ACP. The specificity of acylation seems preserved when C169 is converted to serine, although the acylated protein is catalytically frozen. When residue 169 cannot be acylated (the C169A mutant protein), another cysteine, probably C147, can attack the octanoyl-ACP thioester, but this acylated protein is also catalytically compromised and inactive in vivo. Finally, when the mutant protein lacks cysteine residues (the C137A/C147A/C169A LipB), an unknown residue becomes acylated to form yet another dead-end adduct.

There is a precedent to our finding that another side chain can weakly replace the role of C169. Dreier et al. (*31*) reported that conversion of S97 of the *Streptomyces coelicolor* malonyl-CoA:ACP transacylase to alanine does not totally abolish transacylase activity. Complete loss of activity was expected since S97 is the residue that is transiently

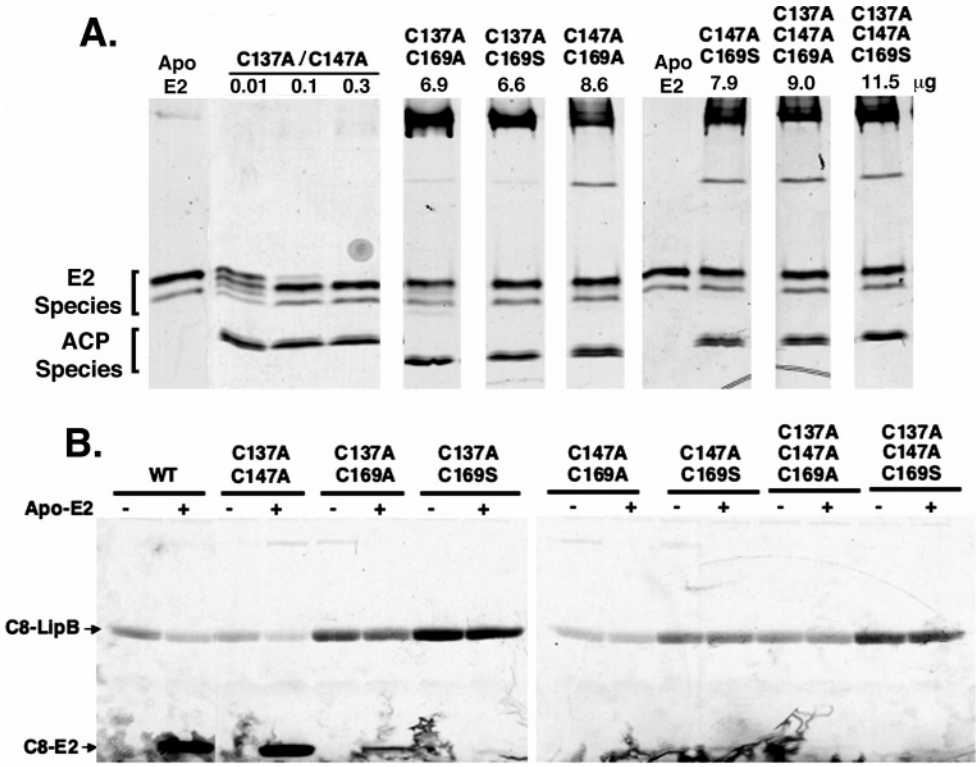

FIGURE 12: Properties of the doubly and triply mutated proteins. Panel A: In vitro gel shift assay of octanoyltransferase activities of the mutant proteins. The purified His-tagged mutant LipB proteins were tested for their activities in the overall assay. The reaction details were as described in Experimental Procedures. Different amounts of the proteins (in μg) were added to the reactions as shown in the figure. Apo denotes the apo-E2 domain standard. Note that a more rapidly migrating form of the apo-E2 domain accumulates during storage (presumably a deamidation product) that complicates the patterns since two holo-E2 domain forms are synthesized. The bands at the top of the gels are the LipB proteins. Panel B: Accumulation of [^{14}C]octanoyl-LipB species and assay of transfer of the octanoyl moiety to the apo-E2 domain. Note that although cracks in the gel that formed during drying for fluorography obscure the octanoyl-E2 band regions of some lanes, transfer can also be evaluated by the relative intensities of the octanoyl-LipB bands in the lanes plus or minus apo-E2. The reaction details were as described in Experimental Procedures.

malonylated during the transacylation reaction. However, the S97A enzyme forms a catalytically competent acyl-enzyme intermediate by acylating an alternative nucleophile, H96 (*31*).

Lipoic acid synthesis has been proposed to be an appropriate pathway for intervention by therapeutic agents, especially against apicomplexan protozoa (e.g., the organisms that cause malaria) (*33*, *34*). If so, then the LipB reaction would seem an appropriate target. Cerulenin specifically inhibits the long-chain β-ketoacyl-ACP synthases of fatty acid synthesis by an irreversible covalent modification of the active site thiol (*35*). Hence, a search for LipB inhibitors that have analogous modes of action might be fruitful.

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