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The *Escherichia coli lipB* Gene Encodes Lipoyl (Octanoyl)-Acyl Carrier Protein:Protein Transferase

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In an earlier study (S. W. Jordan and J. E. Cronan, Jr., J. Biol. Chem. 272:17903-17906, 1997) we reported a new enzyme, lipoyl-[acyl carrier protein]-protein *N*-lipoyltransferase, in *Escherichia coli* and mitochondria that transfers lipoic acid from lipoyl-acyl carrier protein to the lipoyl domains of pyruvate dehydrogenase. It was also shown that *E. coli lipB* mutants lack this enzyme activity, a finding consistent with *lipB* being the gene that encoded the lipoyltransferase. However, it remained possible that *lipB* encoded a positive regulator required for lipoyltransferase expression or action. We now report genetic and biochemical evidence demonstrating that *lipB* encodes the lipoyltransferase. A *lipB* temperature-sensitive mutant was shown to produce a thermolabile lipoyltransferase and a tagged version of the *lipB*-encoded protein was purified to homogeneity and shown to catalyze the transfer of either lipoic acid or octanoic acid from their acyl carrier protein thioesters to the lipoyl domain of pyruvate dehydrogenase. In the course of these experiments the ATG initiation codon commonly assigned to *lipB* genes in genomic databases was shown to produce a nonfunctional *E. coli* LipB protein, whereas initiation at an upstream TTG codon gave a stable and enzymatically active protein. Prior genetic results (T. W. Morris, K. E. Reed, and J. E. Cronan, Jr., J. Bacteriol. 177:1-10, 1995) suggested that lipoate protein ligase (LplA) could also utilize (albeit poorly) acyl carrier protein substrates in addition to its normal substrates lipoic acid plus ATP. We have detected a very slow LplA-catalyzed transfer of lipoic acid and octanoic acid from their acyl carrier protein thioesters to the lipoyl domain of pyruvate dehydrogenase. A nonhydrolyzable lipoyl-AMP analogue was found to competitively inhibit both ACP-dependent and ATP-dependent reactions of LplA, suggesting that the same active site catalyzes two chemically diverse reactions.

Lipoic acid (1,2-dithiolane-3-pentanoic acid) (Fig. 1) is a cofactor required for the function of key metabolic pathways in most organisms (15). 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 (15). Although the general role of lipoic acid as a coenzyme has been known for decades, 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—pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and glycine cleavage enzyme—that utilize lipoic acid as a cofactor (17, 23). Pyruvate dehydrogenase catalyzes a cycle of three successive reactions in the oxidative decarboxylation of pyruvate to yield acetyl coenzyme A (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 (4). These enzymes also provide biosynthetic intermediates to several essential pathways.

The physiological function of lipoic acid is dependent upon covalent attachment to specific lysine residues of the cognate enzymes. These specific lysine residues are found on protein domains called lipoyl domains that have conserved structures and sequences. These domains are found at the N terminus of a subunit of each of the lipoate-dependent enzymes (15). In *E. coli* there are two independent enzyme systems that modify lipoyl domains (12) (Fig. 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 specific lysine of the lipoyl domain. LplA utilizes ATP to activate lipoic acid to lipoyl-AMP (Fig. 1). LplA then catalyzes the attack of enzyme-bound lipoyl-AMP by the ε-amino group of the lysine of the lipoyl domain to give an amide linkage (Fig. 1). LplA has been shown to be required for *E. coli* to utilize lipoic acid from the environment (11). However, null mutants of *lplA* can be made without creating a deficiency of lipoylated enzymes due to the presence of a second LplA-independent lipoylation pathway (12). This second pathway is dependent on *lipB* (12) and involves a novel enzyme, lipoyl-[acyl carrier protein]-protein *N*-lipoyltransferase (8) that uses the lipoyl thioester of the acyl carrier protein (ACP) of fatty acid synthesis as the acyl donor and also functions with octanoyl-ACP (8). Strains carrying *lipB* mutations lack lipoyltransferase and thus the most straightforward hypothesis was that *lipB* encodes the enzyme. However, the data could also be explained by LipB acting as a positive regulator required for lipoyltransferase expression or activity. Despite a major effort we were unable to either prove or

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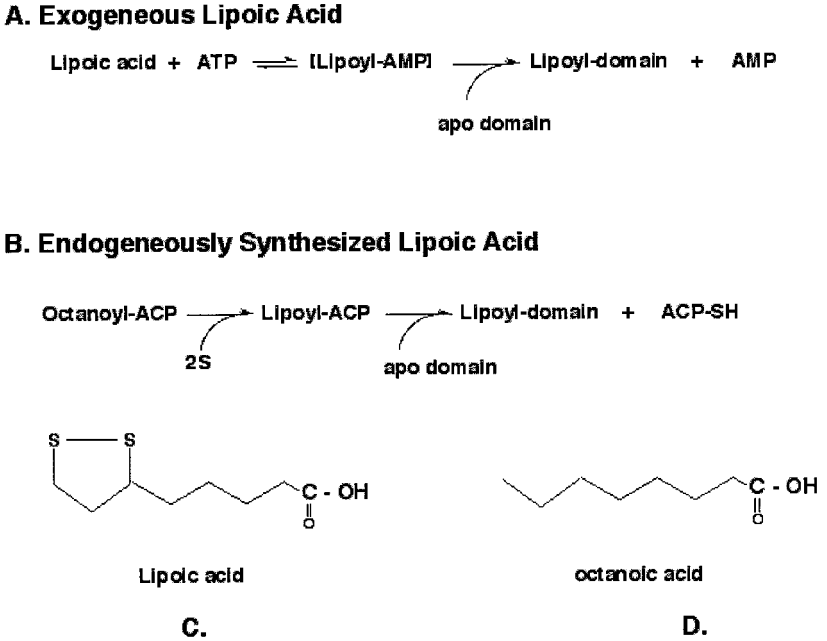

FIG. 1. Current models of lipoate attachment and synthesis. (A) Lipoate ligase reaction that utilizes exogenous sources of lipoic acid and octanoic acid. (B) Current model of lipoic acid synthesis and attachment. The sulfur insertion step is catalyzed by LipA. LipB then transfers the lipoate moiety from lipoyl-ACP to the lipoyl domains. However, an additional pathway is not precluded in which LipB transfers octanoate from ACP to the lipoyl domain to form the octanoyl-domain, which is recognized as a substrate for LipA-catalyzed sulfur insertion.

disprove that *lipB* encoded lipoyltransferase by purifying the enzyme to homogeneity since *E. coli* extracts contain extremely low levels of the protein (see below). Other workers (16, 22) have reported that *lipB* plays a role in the regulation of *dam*-encoded deoxyadenosine methylase (which in turn regulates a large variety of key cell processes), and thus it was necessary to resolve the function of LipB. We report genetic and biochemical evidence demonstrating that *lipB* encodes the lipoyl-[ACP]:protein *N*-lipoyltransferase (which is also an octanoyl-[ACP]:protein *N*-octanoyltransferase). We also show consistent with prior genetic data that the transfer of lipoic acid and octanoic acid from their ACP thioesters to lipoyl domains can also be catalyzed, albeit very poorly, by LplA.

MATERIALS AND METHODS

Bacterial strains and media. The media used were described previously (23), as were strains TM136 (12) and KER184 (12) and plasmid pKR111 (17).

Isolation of temperature-sensitive *lipB* mutants. Plasmid pKR111, which contains the complete *lipB* gene plus 127 bp of upstream sequence in a high-copy-number plasmid, was mutagenized with hydroxylamine for various time periods according to the protocol of Miller (10). A hydroxylamine treatment time yielding a 50% reduction in the number of ampicillin-resistant transformants was chosen. The hydroxylamine-treated plasmid preparations were then transformed into the *lipB*::Tn*1000*dKan *lplA*::Tn*10* strain TM136, and the transformants were plated on glucose minimal E medium containing sodium ampicillin (100 μg/ml), tetracycline hydrochloride (15 μg/ml), and kanamycin sulfate (50 μg/ml), followed by incubation of the plates at 30°C for 2 days. Eight hundred of the resulting colonies were then tested on glucose-minimal plates containing ampicillin incubated at 30, 37, or 42°C. A parallel set of plates containing 5 ng of lipoate/ml was also prepared and was incubated at 37 or 42°C for 2 days. Three isolates were found that grew at 30°C without lipoate but which failed to grow at 37 or 42°C unless supplemented with lipoate. The plasmids from these strains were designated pSJ65, pSJ67, and pSJ68, and their *lipB* genes, together with that of the parental plasmid pKR111, were sequenced by the Keck Genomics Center of the University of Illinois.

Construction of a plasmid encoding a hexahistidine (His)-tagged LipB. Two plasmids were constructed, each of which contained an *Nco*I site overlapping the *lipB* initiation codon to be tested. Plasmid pSJ118, which encoded a gene beginning with the previously assigned ATG initiation codon, was produced by PCR amplification from plasmid pCTV616 with the primer LIPBATG (5′-CCCCCA TGGATGAATTCACCGATACCCGCCATG-3′) and primer LIPBSTOP (5′-C CCAAGCTTAAGGGGTAATATATTCGAAGTC-3′). The PCR product was cloned into plasmid pCR2.1 by using the TA cloning kit (Invitrogen) to produce plasmid pSJ118. Plasmid pSJ112 resulted from the same manipulations except that primer LIPBTTG (5′-CCCCCATGGATCAGGATAAAATTCTTGTC-3′) was used as the upstream primer and the encoded gene started at the TTG codon (which was changed to ATG in making the *Nco*I site). The *Nco*I-*Hin*dIII *lipB* fragments of plasmids pSJ112 and pSJ118 were ligated to vector pKK233-2 (Pharmacia) cut with the same enzymes to yield plasmids pSJ115 and pSJ119, respectively. The same manipulations, except that vector pET30(a) (Novagen) was used in place of vector pKK233-2, yielded plasmids pSJ120 and pSJ122 derived from plasmids pSJ112 and pSJ118, respectively.

Purification and assay of LipB and LplA. His-tagged LipB was purified by using Qiagen immobilized nickel-agarose matrix according to the protocol supplied by Qiagen. His-tagged LipB was then further purified by chromatography on a Mono-Q anion-exchange column (Pharmacia). The sample was adsorbed to the Mono-Q column by using 50 mM Tris-HCl (pH 7.4) containing 5% glycerol, and His-tagged LipB was eluted by using a gradient of 0 to 250 mM ammonium sulfate in the same buffer. LplA was purified exactly as described previously (11). Lipoylation activity was measured by the pyruvate dehydrogenase activation and gel mobility shift assays described previously (7, 8). In the former assay, lipoylation is measured by conversion of the inactive apo form of pyruvate dehydrogenase to the active lipoylated form (7), whereas in the latter assay lipoylation or octanoylation is measured by shift of the electrophoretic mobility of a purified lipoyl domain derived from *E. coli* pyruvate dehydrogenase (6, 8). Upon acylation of the target lysine, the electrophoretic mobility of the lipoyl domain increases due to loss of the positively charged side chain.

Synthesis of lipol-adenylate. The lipoyl-AMP analogue, lipol-adenylate (LipOH-AMP) was made as follows. Lipoic acid was reduced to the alcohol (lipol) with 1.25 eq of BH_3 in tetrahydrofuran (2). This reagent does not reduce disulfides (2). After hydrogen evolution was complete, 15 ml of a 1:1 mixture of water and tetrahydrofuran was added to decompose any remaining reducing agent. The solution was then saturated with potassium carbonate, and lipol was extracted

with ethyl ether. The extracts were dried with sodium sulfate, and the solvent was removed by rotary evaporation to give a quantitative yield of product. The 5′-phosphate of 2′,3′-*O*-isopropylidene adenosine was synthesized from 2′,3′-*O*-isopropylidene adenosine and 2-cyanoethyl phosphate as described by Tenner (21) and purified on a column of DEAE-Sephadex A25 equilibrated in 5 mM ammonium bicarbonate. Any remaining 2′,3′-*O*-isopropylidene adenosine was eluted with 5 mM ammonium bicarbonate, whereas 2′,3′-*O*-isopropylidene adenosine-5′-phosphate was eluted with 0.2 M ammonium bicarbonate. This solution was taken to dryness by rotary evaporation, dissolved in water, and again evaporated to dryness in order to remove ammonium bicarbonate. The 2′,3′-*O*-isopropylidene adenosine-5′-phosphate (2.66 mmol) was then converted to the pyridinium salt (1), which was mixed with a solution of 10 mmol of lipol in dry pyridine. To this mixture 10 mmol of the coupling reagent 1,3-dicyclohexylcarbodiimide was added, and the solution was then stirred for 40 h at room temperature (1). Water was added to decompose any remaining coupling reagent. The solution was filtered and the filtrate was taken to dryness by rotary evaporation. The residue was dissolved in water and again evaporated in order to remove residual pyridine. The residue was then taken up in 150 ml of 20% acetic acid and refluxed for 2 h to remove the isopropylidene protecting group (21). After it cooled, the solution was again filtered and evaporated to dryness. Thin-layer chromatography (silica gel 60) in 2-propanol-concentrated NH_4OH-water (7/3/1, by volume) showed that the major contaminant was AMP. The mixture was then loaded on a DEAE-Sephadex A25 column equilibrated in 20 mM triethylammonium bicarbonate. LipOH-AMP was eluted with 0.2 M triethylammonium bicarbonate (AMP remained on the column) and evaporated to dryness. The product showed a UV absorption maximum at 259 nm for the adenine ring with a shoulder at 302 nm. The shoulder was attributed to the thiolane ring since it disappeared upon treatment with the disulfide reducing agent tris(2-carboxyethyl)phosphine. Electrospray mass spectroscopy gave a mass of 536.2 for the product versus the calculated value of 536.1 (ammonium salt).

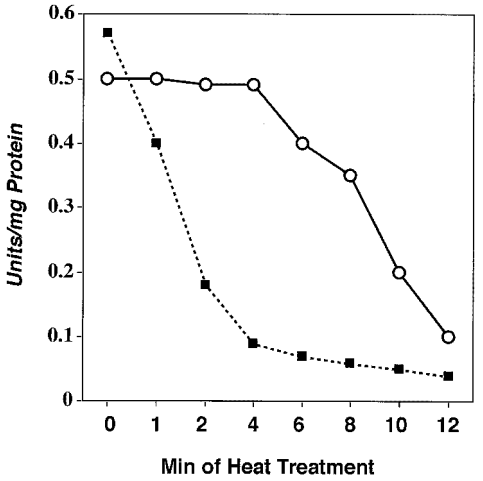

FIG. 2. Thermolability of the lipoyltransferase activity of cell extracts. Cell extracts of the *lipB* strain KER184 carrying either plasmid pKR111, which expresses the wild-type *lipB* allele, or plasmid pSJ67, which expresses the temperature-sensitive *lipB67* allele, were heated at 55°C for the time periods shown before being placed on ice. After all samples had been treated, the samples were assayed for lipoyltransferase activity by activation of apo-pyruvate dehydrogenase. Symbols: ○, activity of the wild-type *lipB* extract; ■, activity of the *lipB67* mutant extract. Note the nonlinear time scale used early in the time course.

RESULTS

Isolation of a temperature-sensitive *lipB* mutant. Given our inability to prove by biochemical means that *lipB* did (or did not) encode the lipoyltransferase, we turned to a classic genetic approach: the isolation of a temperature-sensitive mutant. The argument was that if *lipB* was the lipoyltransferase structural gene, a temperature-sensitive *lipB* mutant strain should encode a lipoyltransferase of increased thermolability. If no change in thermal stability of the lipoyltransferase activity was seen, a regulatory role was indicated for LipB. Plasmid pKR111, which carries a functional *lipB* gene, was mutagenized with hydroxylamine, and the mutagenized plasmids were then transformed into the *lipB* mutant strain KER184 (*lipB*::Tn*1000*dKan). Among these transformants, three strains were found that grew at 30°C without lipoate but which failed to grow at the higher temperatures unless supplemented with lipoate. This phenotype indicated that the temperature-sensitive nature of these strains was due to a mutation in the plasmid-borne *lipB* gene. The plasmids of these three strains were purified and again used to transform strain KER184 to confirm that the temperature-sensitive mutation was carried by the plasmid. The *lipB* genes of the three of these plasmids—pSJ65, pSJ67, and pSJ68—plus the parental plasmid were sequenced. Plasmids pSJ65 and pSJ68 had mutations within the putative promoter region upstream of *lipB*, whereas the pSJ67 *lipB* gene contained a nonsense mutation in codon 74 (CAG to TAG) that we denote as the *lipB67* allele.

The in vitro temperature stabilities of the lipoyltransferases of the mutant strains were tested by treating cell extracts at 55°C for various periods of time before being placed on ice. The extracts were then assayed for lipoyltransferase activity by the pyruvate dehydrogenase activation assay (Fig. 2). The lipoyltransferase activities of strains SWJ175, SWJ178, and SWJ181 (carrying plasmids pSJ65, pSJ68, and pKR111, respectively) had similar temperature stabilities, whereas that of strain SWJ177 (carrying plasmid pSJ67) had a severely decreased temperature stability (Fig. 2). Therefore, the temperature-sensitive *lipB* allele results in the production of a lipoyltransferase of increased thermal lability. These data demonstrate that *lipB* is the gene that encodes the lipoyl (octanoyl) transferase and precludes an indirect regulatory role for the protein in lipoate metabolism.

Overproduction of LipB and lipoyltransferase activity from an altered TTG start codon. Encouraged by the genetic findings we returned to the problematic overproduction of LipB. Previous attempts in this laboratory to overproduce LipB from an open reading frame (ORF) that encoded a 191-residue protein had consistently failed despite testing a large number of transcriptional and translational constructs (17; S. W. Jordan and J. E. Cronan, Jr., unpublished data). However, we had recently tested two constructs containing a putative *Arabidopsis thaliana lipB* homolog for the ability to complement an *E. coli lipB*-null mutant (24). One *A. thaliana* construct contained an ORF with a length similar to that which we and others had assigned for the *E. coli lipB* gene, whereas the second construct included a putative N-terminal leader sequence thought to be a mitochondrial targeting sequence. We expected that only the shorter ORF would complement, but instead the opposite result was obtained; only the longer ORF had complementation activity (24). Inspection of the putative *A. thaliana* leader sequence showed that the deduced amino acid sequence matched the amino acid sequence found upstream of the *E. coli lipB* methionine residue assigned as the initiation codon (Fig. 3). Moreover, the deduced *A. thaliana* sequence contained a methionine codon that was within one residue of alignment with a TTG leucine codon, a known low-efficiency *E. coli* initiation codon (20). Therefore, it seemed probable that the initiation codon had been mistakenly assigned through the

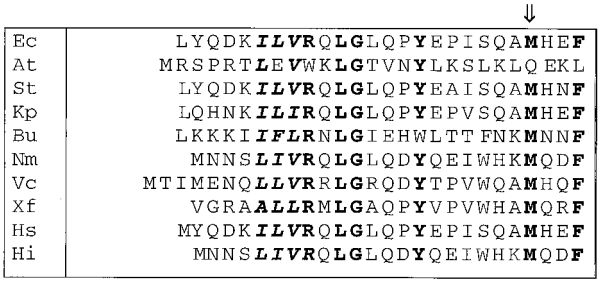

FIG. 3. Alignments of LipB N termini. The amino acid sequences were those deduced from the DNA sequences rather than those deduced during annotation of the gene sequences. The methionine codon that was thought to be the initiation codon is marked with an arrow. The first amino acid residue is given is that normally encoded by that codon to illustrate the use of alternate initiation codons (TTG and GTG, which would be translated as methionine by the translation initiation complex). Residues conserved in at least seven of the eight sequences are given in boldface, and a conserved cluster of hydrophobic residues is given in italic boldface. Sequences: Ec, *E. coli*; At, *Arabidopsis thaliania*; St, *Salmonella enterica* serovar Typhi; Kp, *Klebsiella pneumoniae*; Bu, *Buchnera* sp. strain APS; Nm, *Neisseria meningitidis*; Vc, *Vibrio cholerae*; Xf, *Xylella fastidiosa*; Hs, *Haemophilus somnus*; Hi, *Haemophilus influenzae*.

usual practice of selecting the first methionine codon. Indeed, by aligning the amino acid sequences deduced from the DNA sequences (rather than those of the annotated ORFs) of *lipB* homologues from many diverse bacteria we found excellent conservation of the amino acid sequences upstream of the initiation codons assigned to the genes. Strikingly in organisms closely related to *E. coli* (e.g., *Salmonella enterica* serovar Enteritidis and *Klebsiella pneumoniae*), the leucine codon was the infrequently used TTG codon despite the divergence at the third positions of codons often observed between these bacteria and *E. coli*. Moreover, other bacteria were found to have an ATG methionine codon or another alternative initiation codon, GTG (valine), which aligned within a codon or two of the *E. coli* TTG codon (Fig. 3). Therefore, the TTG codon seemed very likely to be the true *E. coli lipB* initiation codon and would yield a 213-residue protein rather than the 191-residue protein previously tested. We therefore constructed a plasmid in which a *Nco*I site was introduced that overlapped the putative TTG initiation codon of the 213 residue ORF (the TTG codon was converted to ATG by this manipulation) such that conventional protein expression vectors could be used. An analogous construct in which the *Nco*I site overlapped the putative methionine initiation codon of the 191-residue ORF was also made. Upon induction of strains carrying the two plasmids only the 213-residue ORF produced a detectable protein product of the expected size (Jordan and Cronan, unpublished). From these data we concluded that the 213-residue protein was the stable native form, whereas the shorter version was degraded by proteolysis in vivo.

We therefore proceeded to construct plasmids encoding N-terminal His-tagged versions of both the 191-residue and the 213-residue ORFs. Upon induction of *lipB* expression, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4A) analysis showed a strong band of ~29 kDa in the strain that expressed the longer protein encoded by plasmid pSJ120. This corresponded well to the expected 28.7-kDa

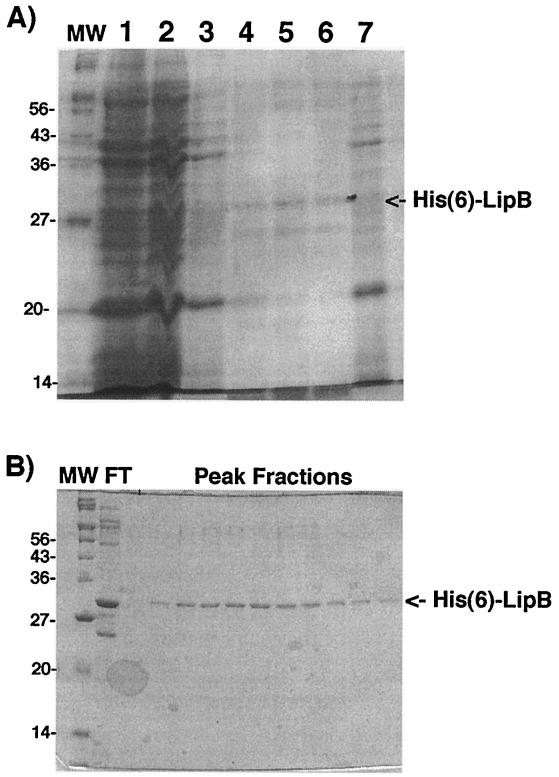

FIG. 4. Purification of His-tagged LipB. (A) SDS-gel electrophoretic analysis of steps in the purification of His-tagged LipB on a column of immobilized nickel cheleate. Lane 1, the cell extract of an uninduced culture; lane 2, flowthrough (unbound) fraction from the column; lanes 3 to 6, fractions from elution of the column with 8 mM imidazole; lane 7, fraction eluted with 80 mM imidazole. (B) Further purification of LipB from the immobilized nickel cheleate column by chromatography on a Mono-Q column. "FT" denotes the flowthrough (unbound) fraction. The fractions containing the peak of lipoyltransferase activity are designated peak fractions. The numbers at the left are the molecular masses of the protein standards in kilodaltons, and the His-tagged LipB bands are indicated by arrows.

mass of the longer His-tagged LipB fusion protein (Fig. 4A). The His-tagged version of the 191-residue protein encoded by pSJ122 gave no obvious band in the expected region of the gel (predicted size of 26.1 kDa) (Jordan and Cronan, unpublished). Therefore, the shorter protein was unstable even when an artificial N-terminal extension (the His tag) was added. However, plasmids (pSJ112 and pSJ118, respectively) that expressed either the 191-residue ORF or the 213-residue ORF both complemented *lipB* null mutants (Jordan and Cronan, unpublished). These results suggest that the additional residues of the 213-residue ORF are not critical for enzyme activity but are necessary for stability of the protein in vivo.

Purified His-tagged LipB has octanoyl-ACP transferase activity. Overexpression of LipB from plasmid pSJ120 increased the specific activity of lipoyltransferase relative to nonoverproduced extracts by ~1,200-fold. The His-tagged LipB was purified directly from cell extracts by immobilized nickel-chelate column chromatography, followed by ion-exchange chromatography to obtain a homogeneous protein (Fig. 4). The purified LipB protein had high levels of lipoyltransferase activity in both assays. The gel mobility shift assay was used to test the

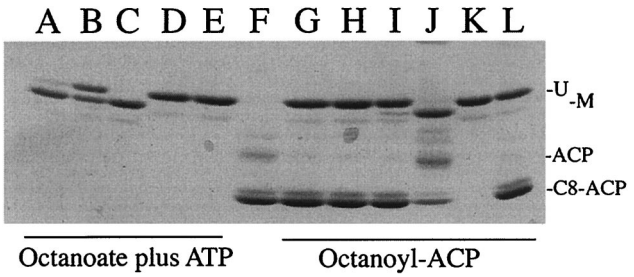

FIG. 5. Gel shift assays of LplA and His-tagged LipB with octanoate plus ATP or octanoyl-ACP. Essentially identical results were obtained with lipoate plus ATP and lipoyl-ACP (data not shown). The assays were performed as described in Materials and Methods. In this assay the modified (M) form of the lipoyl domain protein migrates faster that the unmodified (U) form on these nondenaturing gels due to the increase in net negative charge that results from acylation of the target lysine residue. The substrates in lanes A to E were octanoic acid plus ATP, whereas the substrate in lanes F to L was octanoyl-ACP (C8-ACP), which is the fastest-migrating protein species. The ACP produced upon transfer of octanoate to the lipoyl domain gives a band of intermediate migration rate. Enzyme sources: 1ane A, 10 μg of cell extract protein from the *lipB* strain KER184; lane B, 1 pg of purified LplA; lane C, 10 pg of purified LplA; lane D, 1 ng of purified His-tagged LipB; lane E, 100 ng of purified His-tagged LipB; lane F, reaction lacking lipoyl domain protein; lane G, 10 μg of cell extract protein from the *lipB* strain KER184; lane H, 10 pg of purified LplA; lane I, 1 ng of purified LplA; lane J, 1 pg of purified His-tagged LipB; lane K, 1 pg of purified His-tagged LipB (but the reaction lacked octanoyl-ACP); lane L, reaction lacking enzyme.

ability of LipB to utilize octanoyl-ACP (Fig. 5) or lipoyl-ACP (data not shown) to modify the lipoyl domain. As expected from prior results with cell extracts (9), the purified His-tagged LipB converted the apo form of a lipoyl-domain to the holo domain by using either octanoyl-ACP or lipoyl-ACP. When LipB was tested for the ability to use ATP plus octanoic acid (Fig. 5) or lipoic acid (data not shown), no modification was detected. The ability of the purified LipB preparation to transfer lipoate from lipoyl-ACP to pyruvate dehydrogenase was determined by the activation of apo-pyruvate dehydrogenase. LipB was found to follow Michaelis-Menten kinetics (data not shown) with a K_m for lipoyl-ACP of ~1 μM (Table 1).

Purified LplA has both lipoyltransferase activity and lipoate ligase activity. Previous studies on LplA indicated that LplA utilized free lipoic acid plus ATP to modify lipoyl domains (11), but at that time lipoyl-ACP had not yet been shown to be a lipoyl donor and thus the ability of LplA to utilize this substrate had not been tested. However, overproduction of LplA was found to allow growth of a *lipB*-null mutant in the absence of exogenous lipoic acid (12), suggesting either that LplA might be able to catalyze the lipoyltransferase reaction at a low rate or that lipoyl-ACP was cleaved to give traces of lipoic acid that could then be salvaged by high levels of LplA. The activity of LplA in the postulated alternate reaction would be low; otherwise, *lipB*-null mutant strains with a normal *lplA* gene would not have a lipoate auxotroph phenotype. Consistent with a low activity, *lipB*-null mutant strains were found to grow very slowly in the absence of lipoic acid (17, 23) and to contain only low levels of lipoylated proteins (12, 17). In strains with null mutations in both *lipB* and *lplA* there is no residual growth in the absence of lipoic acid and no detectable protein lipoylation (12). Although these genetic and physiological data were consistent with LplA possessing a low level of lipoyltransferase activity, direct proof was lacking.

TABLE 1. Kinetic constants of LipB and LplA

Enzyme	Substrate	K_m (μM)	V_{max}[a]
LipB	Lipoyl-ACP	1.0	0.18
LipB	Lipoate + ATP		NA
LplA	Lipoyl-ACP	8.9	0.00010
LplA	Lipoate + ATP	0.9	0.10

[a] The V_{max} values are units of pyruvate dehydrogenase/minute/picogram of enzyme and were measured by the activation of apo-pyruvate dehydrogenase resulting from lipoic acid attachment (7). An activity of 0.18 U in the apo-pyruvate dehydrogenase activation assay is equivalent to conversion of about 30 fmol of apo-lipoyl domain to the holo form in the electrophoretic assay. NA, no activity.

In order to test whether LplA possessed lipoyltransferase activity, we tested the ability of purified LplA to use octanoyl-ACP and lipoyl-ACP as substrates in vitro. We used the gel mobility shift assay to assess the ability of LplA to use octanoyl-ACP as a substrate in place of free octanoic acid plus ATP (Fig. 5). LplA did indeed use octanoyl-ACP as a substrate, although extremely inefficiently. The octanoyl-ACP reaction required ca. 100-fold more enzyme than was required for comparable activity with the ligase substrates, octanoic acid plus ATP. LplA also used lipoyl-ACP as a substrate, as detected by the both the gel mobility shift (data not shown) and pyruvate dehydrogenase activation assays. The latter assay gave a K_m of ~9 μM for the reaction of LplA with lipoyl-ACP (Table 1). Since the activity observed with ACP-bound substrates required high levels of LplA, it seemed possible that activities observed with octanoyl-ACP and lipoyl-ACP as substrates were due to LipB contamination of our LplA preparation. To test this possibility, we purified LplA that was overproduced in a *lipB*-null mutant strain and obtained an enzyme preparation that had the same activity on the ACP-bound substrates as the preparations from a wild-type host strain (data not shown). In another test we synthesized a nonhydrolyzable lipoyl-AMP analogue, lipol-AMP (Fig. 6), that, based on the corresponding aminoacyl-AMP analogues (13, 18), was expected to inhibit the reaction with free lipoic acid (plus ATP) catalyzed by LplA by occupying the active site. Indeed, lipol-AMP was found to be a competitive inhibitor of the reaction, although the analogue was a rather impotent inhibitor (Fig. 7A), suggesting that the lipoate carboxyl group plays an important role in lipoyl-AMP binding. We expected that, if the two reactions catalyzed by LplA used the same active site, then lipol-AMP should also inhibit transfer of lipoate from lipoyl-ACP to apo-pyruvate dehydrogenase. Indeed, the analogue also competitively inhibited the lipoyl-ACP reaction, indicating that both reactions were catalyzed by the same active site (Fig. 7B). Therefore, we conclude that a low level of transferase activity with ACP thioester donors is an intrinsic property of LplA. It should be noted that parallel experiments with purified LipB and lipoyl-ACP showed no inhibition of the LipB-catalyzed lipoate transfer reaction (data not shown).

DISCUSSION

In the present study we report genetic and biochemical data demonstrating that *lipB* encodes lipoyl-[ACP]:protein *N*-lipoyl-

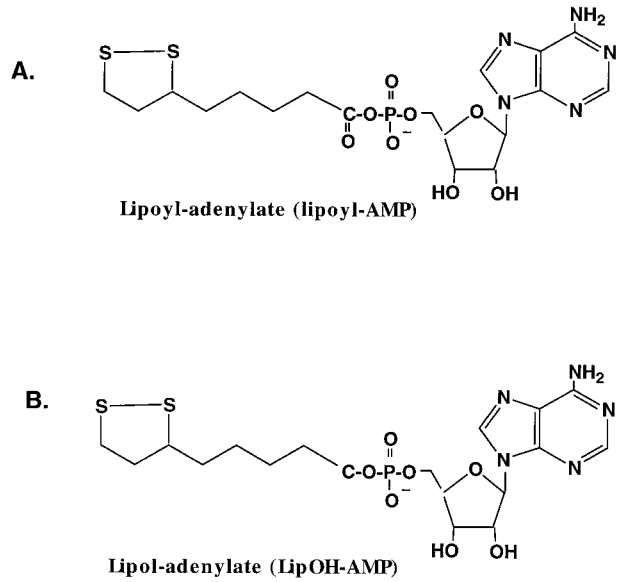

FIG. 6. Structures of lipoyl-AMP and the reduced analogue. (A) Lipoyl-AMP (lipoyl-adenylate). (B) Reduced analog, lipol-AMP (lipol-adenylate). The two compounds differ in the linkage of the lipoyl moiety to the phosphate of AMP. In lipoyl-AMP the linkage is a mixed anhydride, whereas the anhydride linkage is replaced with an ester in lipol-AMP.

transferase. The genetic evidence is based on the temperature-sensitive *lipB67* allele that encodes a lipoyltransferase of greatly decreased thermostability. An unexpected finding was that the mutation was a nonsense (amber) mutation rather than the expected missense mutation. We suspect that the temperature sensitivity is due to insertion of an amino acid residue other than glutamine at residue 74 during read-through of the termination codon. Since the mutant protein has essentially wild-type LipB activity before heating, the read-through of the termination codon is very efficient. Given this efficiency, it seems very likely that our parental strain has acquired a suppressor tRNA mutation during the 30 years since it was derived from strain W3110, a strain lacking known nonsense suppressors. It is also possible that the truncated LipB protein is responsible for the temperature-sensitive behavior. However, this seems very unlikely since the truncated protein would lack many sequences that are highly conserved within the protein family. Moreover, the truncated protein would be very small (73 residues) and hence likely to be degraded in vivo. Although further work is required to understand the basis of the thermolability of the mutant LipB protein, the finding that a temperature-sensitive *lipB* allele encoded a thermolabile lipoyl (octanoyl)-ACP transferase activity indicated that *lipB* was the transferase structural gene.

This genetic evidence caused us to return to the problematical expression of the LipB protein. Sequence alignments with data available from the many genome sequences completed since our original work suggested that we had chosen the wrong initiation codon for *E. coli lipB*. Since a very highly conserved methionine codon is present in the analogous location in most other *lipB* genes, the annotators of the *E. coli* genome sequence (and those of most other genome sequences) had also designated this codon as the initiation codon. However, use of an upstream TTG (leucine) codon gave a stable and functional protein, and thus our prior inability to effectively overproduce LipB was due to degradation of the truncated protein. Vaisvila et al. (22) independently found that only the 213-residue LipB protein was stable upon overproduction (enzyme activity was not measured). The use of alternate initiation codons such as TTG and GTG generally results in lower expression than an ATG codon (20). Indeed, *lipB* is expressed at very low levels in vivo (17), a finding consistent with the use of a poor initiation codon. Consistent with these data, we purified the lipoyltransferase activity by more than 50,00-fold from extracts of wild-type cells by a combination of conventional and affinity chromatographic steps with retention of about half of the original activity but were unable to detect a protein band corresponding to the enzyme (Jordan and Cronan, unpublished). Given the final yield of protein, the molecular weight of LipB, and the mass of the cells extracted, we calculate that LipB is present at a level of <10 molecules per wild-type cell. It is noteworthy that very low levels of protein expression seems characteristic of the *E. coli* enzymes that perform rare protein modifications such as lipoylation (*lipB* and *lplA*), biotinylation (*birA*), and phosphopantetheinylation (*acpS*). Starting with extracts of wild-type cells, each of these enzymes required a purification of several tens of thousands-fold to detect the protein by gel electrophoresis, and the final preparations were often not homogeneous (5, 6, 9). Moreover, each of the genes encoding the enzymes has a low codon adaptation index (19), a property of *E. coli* genes expressed at low to very low levels that indicates frequent use of nonabundant tRNA species in decoding the mRNAs. The values for *E. coli lipB*, *lplA*, *birA*, and *acpS* are 0.269, 0.330, 0.257, and 0.293, respectively, whereas LacI, which is present at 5 to 10 copies/cell (14), has an index of 0.296 and highly expressed proteins have indices of >0.8 (19).

Although the mechanism by which LipB functions in vivo is not yet fully understood, our work demonstrates that *lipB* is indeed the structural gene for the lipoyl (octanoyl) transferase. This enzyme uses the presumed product of lipoic acid biosynthesis, lipoyl-ACP, as a substrate, but it can also use octanoyl-ACP. Probably the most interesting aspect of the enzyme study was the finding that LplA utilizes either lipoyl-ACP or free lipoic acid plus ATP as substrates for lipoylation of apo-pyruvate dehydrogenase (the octanoate species are also active). This is a curious and unexpected result since the linkages attacked by the lipoyl domain lysine ε-amino group differ in the two enzymes. The nucleophilic amino group attacks a mixed anhydride when lipoyl-AMP is the substrate, but it attacks a thioester when lipoyl-ACP is the substrate. How can the LplA active site cope with such different chemistries? One possibility is that LplA protein becomes lipoylated on an active site residue such as a cysteine, serine, or histidine, and it is this linkage that undergoes nucleophilic attack by the lysine amino group. If so, the slow reaction of LplA with lipoyl-ACP could be due to attack of the putative active site residue on the thioester linkage to form the same enzyme-bound intermediate formed from lipoyl-AMP. (Note that the ACP protein moiety need not sterically hinder this interaction since the lipoyl moiety is linked to the protein by the extended phosphopanthetheine prosthetic group of ACP.) Analogous chemical acylations of

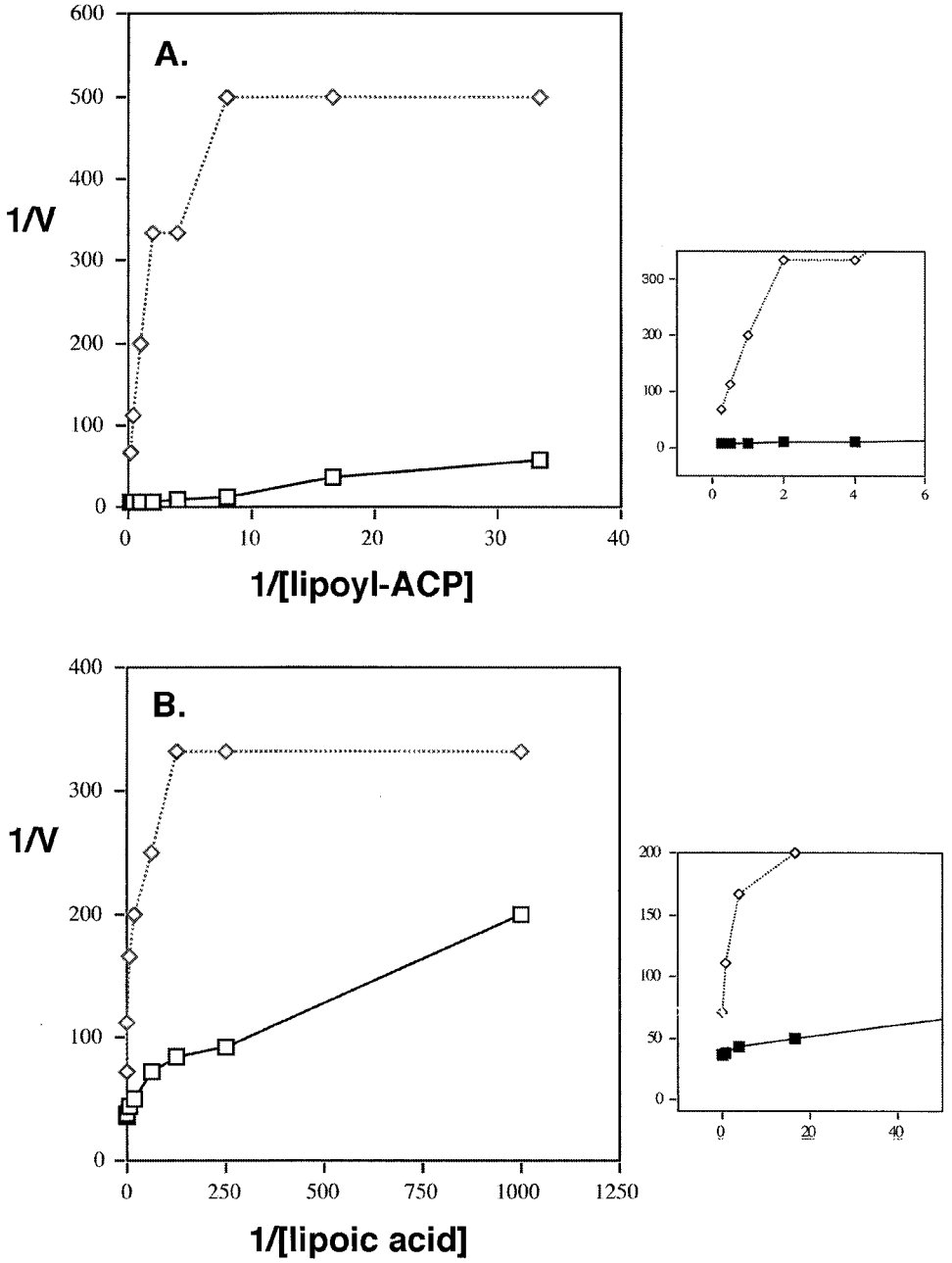

FIG. 7. Inhibition of LplA by lipol-AMP. (A) LplA-catalyzed transfer of lipoic acid from lipoyl-ACP to apo-pyruvate dehydrogenase. (B) LplA-catalyzed ATP-dependent attachment of lipoic acid to apo-pyruvate dehydrogenase. The small graphs to the right of the panels are magnifications of the regions close to the axis so that the competitive inhibition character can be more readily seen. The inhibitor was added at 1 mM. The velocity (V) values are apo-pyruvate dehydrogenase activation units (see Table 1), and the substrate concentrations are given in micromolar amounts. The inhibition constant (K_i) for the lipoic acid plus ATP reaction was 370 μM, whereas the K_i for the lipoyl-ACP reaction was 666 μM. Lipol-AMP (1 mM) had no detectable effect on the rate of LipB-catalyzed transfer of lipoic acid from lipoyl-ACP to apo-pyruvate dehydrogenase over a range of substrate concentrations. Symbols: ◇, reactions in the presence of 1 mM lipol-AMP; □ and ■, reactions lacking inhibitor.

active site residues are known for the 3-ketoacyl-ACP synthases and malonyl-CoA:ACP transacylases involved in the elongation steps of fatty acid synthesis (3). If this scenario applies, then lipoyl-ACP could be acting as a model substrate of LplA presumably due to binding of the lipoyl moiety by the enzyme active site. This model reaction would be very inefficient (as we have observed) and thus would be physiologically useful only upon high levels of LplA overexpression (12). We are currently studying the reaction mechanisms of LipB and LplA.

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