Lipoyl Synthase Requires Two Equivalents of *S*-Adenosyl-L-methionine To Synthesize One Equivalent of Lipoic Acid†

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ABSTRACT: Lipoyl synthase (LipA) catalyzes the formation of the lipoyl cofactor, which is employed by several multienzyme complexes for the oxidative decarboxylation of various α-keto acids, as well as the cleavage of glycine into CO_2 and NH_3, with concomitant transfer of its α-carbon to tetrahydrofolate, generating N^5,N^{10}-methylenetetrahydrofolate. In each case, the lipoyl cofactor is tethered covalently in an amide linkage to a conserved lysine residue located on a designated lipoyl-bearing subunit of the complex. Genetic and biochemical studies suggest that lipoyl synthase is a member of a newly established class of metalloenzymes that use *S*-adenosyl-L-methionine (AdoMet) as a source of a 5′-deoxyadenosyl radical (5′-dA•), which is an obligate intermediate in each reaction. These enzymes contain iron−sulfur clusters, which provide an electron during the cleavage of AdoMet, forming L-methionine in addition to the primary radical. Recently, one substrate for lipoyl synthase has been shown to be the octanoylated derivative of the lipoyl-bearing subunit (E_2) of the pyruvate dehydrogenase complex [Zhao, S., Miller, J. R., Jian, Y., Marletta, M. A., and Cronan, J. E., Jr. (2003) *Chem. Biol. 10*, 1293−1302]. Herein, we show that the octanoylated derivative of the lipoyl-bearing subunit of the glycine cleavage system (H-protein) is also a substrate for LipA, providing further evidence that the cofactor is synthesized on its target protein. Moreover, we show that the 5′-dA• acts directly on the octanoyl substrate, as evidenced by deuterium transfer from [octanoyl-d_{15}]H-protein to 5′-deoxyadenosine. Last, our data indicate that 2 equiv of AdoMet are cleaved irreversibly in forming 1 equiv of [lipoyl]H-protein and are consistent with a model in which two LipA proteins are required to synthesize one lipoyl group.

Lipoyl synthase (LipA)[1] catalyzes the insertion of sulfur atoms into the 6 and 8 positions of protein-bound derivatives of octanoic acid, forming the corresponding lipoyl derivative (Scheme 1) (*1*, *2*). When attached to its appropriate acceptor protein, the lipoyl cofactor plays a central role in the oxidative decarboxylation of glycine and several α-keto acids, which is catalyzed by large multisubunit complexes such as the glycine cleavage system (GCS), the pyruvate dehydrogenase complex (PDC), the α-ketoglutarate dehydrogenase complex (KDC), and the branched chain oxo-acid dehydrogenase complex (BCODC) (*3*, *4*). In each of these complexes, the lipoyl cofactor is bound covalently in an amide linkage to the ϵ-amino group of a target lysine residue on the lipoyl-bearing subunit. This linkage creates a long and flexible appendage that enables the cofactor to deliver intermediates between successive active sites among the subunits of each complex. In contrast to the PDC, KDC, and BCODC, no stable complexes of the GCS have been purified; however, in pea leaf mitochondria, each of the four subunits can be isolated independently of the others. The H-protein is the lipoyl-bearing subunit and sequentially interacts with the other three components of the complex (*5*). Analysis of available sequence information from a number of organisms indicates that lipoyl synthases are found among the bacteria, eukarya, and archaea kingdoms and are present in *Homo sapiens*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, and both Gram-positive and Gram-negative bacteria.

Despite success in elucidating the multiple functions of the lipoyl cofactor in the multienzyme complexes that require it, the unraveling of the pathway by which it is biosynthesized has been challenging. In vivo feeding studies in *Escherichia coli*, initiated as early as 1964, indicated that octanoic acid could serve as a precursor to lipoic acid, requiring removal of only two hydrogens during the transformation, one from C-6 and one from C-8, which are those that are replaced by

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[1] Abbreviations: AdoMet or SAM, *S*-adenosyl-L-methionine; BioB, biotin synthase; BME, 2-mercaptoethanol; bp, base pair; 5′-dA, 5′-deoxyadenosine; 5′-dA•, 5′-deoxyadenosyl radical; ESI^+, electrospray ionization; FeS, iron−sulfur; Flv, flavodoxin; Flx, flavodoxin reductase; GC, gas chromatography or chromatograph; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IS, internal standard; LC, liquid chromatography; LDH, lipoamide dehydrogenase; LHP, [lipoyl]H-protein; LipA, lipoyl synthase; Met, L-methionine; MS, mass spectrometry; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; Ni-NTA, nickel nitrilotriacetic acid; OHP, [octanoyl]H-protein; PCR, polymerase chain reaction; PLP, pyridoxal 5′-phosphate; TCEP, tris(2-carboxyethyl)-phosphine.

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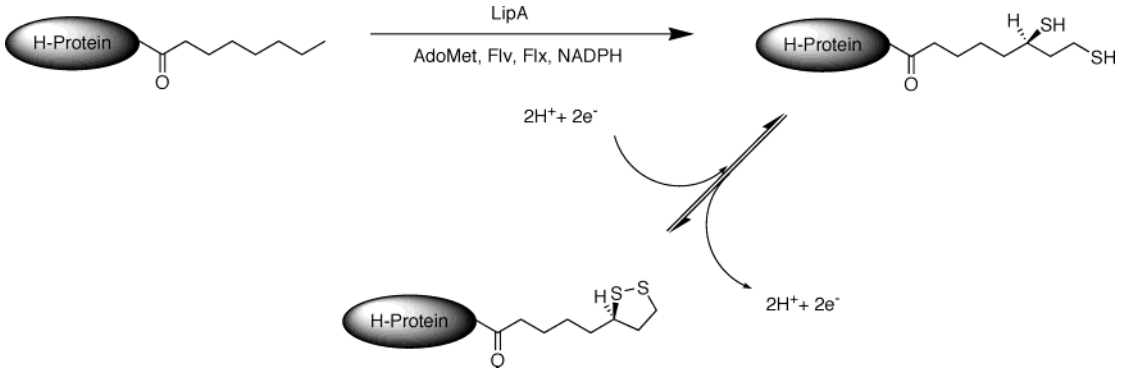
Scheme 1: LipA-Dependent Conversion of [Octanoyl]H-Protein to [Lipoyl]H-Protein and Reversible Oxidation to Its Dithiolane Form



sulfur insertion (*6*−*8*). It is the *pro-R* hydrogen that is removed from C-6; however, sulfur insertion ensues with inversion of configuration at this position. The intrinsic inertness of the octanoyl substrate prompted speculation of the involvement of radical intermediates in the reaction. One hypothesis suggested intermediate hydroxylation followed by activation of the hydroxyl groups and their subsequent displacement with sulfur-derived nucleophiles. The particularly satisfying aspect of the proposal was that it would readily account for the observed inversion of configuration that takes place at C-6, if hydroxylation occurred with retention of configuration. However, in vivo feeding studies with [8-$^2H_2$]-8-hydroxyoctanoic acid, [6(*RS*)-^{2}H]-6-hydroxyoctanoic acid, and [8-$^2H_2$]-(±)-6,8-dihydroxyoctanoic acid indicated that none of the compounds were converted into lipoic acid. By contrast, [8-$^2H_2$]-8-thiooctanoic acid was readily converted into lipoic acid, while [6(*RS*)-^{2}H]-6-thiooctanoic acid was converted 10−20% as efficiently as the former compound (*9*).

A number of parallels can be drawn between LipA and biotin synthase (BioB), which catalyzes a similar reaction. Biotin is formed by insertion of one sulfur atom between C-6 and C-9 of dethiobiotin and also requires removal of two unactivated hydrogen atoms. As described for lipoic acid biosynthesis, the corresponding hydroxylated derivatives of dethiobiotin are not intermediates in the reaction, whereas the thiol-containing derivatives can serve as precursors to biotin under certain conditions of growth (*6*, *10*−*12*). In stark contrast to lipoic acid biosynthesis, sulfur insertion at C-6 of dethiobiotin takes place with retention of configuration (*11*).

Recent genetic, bioinformatic, and biochemical studies indicate that lipoyl synthases belong to a newly defined superfamily of metalloenzymes that use *S*-adenosyl-L-methionine (AdoMet) as a precursor to a 5′-deoxyadenosyl radical (5′-dA•) (*2*, *13*−*15*). In each case, the role of the 5′-dA• is to abstract a hydrogen atom from a protein or small-molecule substrate, either generating a cofactor or initiating a radical-dependent reaction that leads to the formation of the appropriate product (*16*). Enzymes within this "radical SAM" superfamily contain a conserved CXXXCXXC motif, wherein the noted cysteine amino acids coordinate a [4Fe-4S] cluster that is requisite for turnover. In its reduced state ([4Fe-4S]$^{1+}$), it supplies an electron that is necessary for cleavage of AdoMet, generating methionine as a byproduct in addition to the 5′-dA• (*17*−*20*). A second cysteine-containing motif (CXEAXCXNXXEC) is present only in lipoyl synthases and in analogy to biotin synthase, also a radical SAM enzyme, is speculated to coordinate an additional iron−sulfur (FeS) cluster that functions as the sulfur donor in the reaction in some uncharacterized manner (*21*−*25*).

Radical SAM enzymes have been categorized into three classes (*16*). In class I enzymes, AdoMet acts as a true cofactor; its cleavage is readily reversible, and one molecule can support multiple turnovers. In class II enzymes, AdoMet is cleaved irreversibly; however, the 5′-dA• simply abstracts a hydrogen atom from a conserved glycine amino acid on a cognate protein, creating a glycyl-radical cofactor that can support multiple turnovers. Lipoyl and biotin synthases have tentatively been assigned to class III. These enzymes are believed to consume at least 1 equiv of AdoMet for each equivalent of product that is produced. The stoichiometry of AdoMet consumption has been addressed in the BioB reaction; however, conflicting conclusions were reached. Results of one study were consistent with a requirement of 2 equiv of AdoMet/equiv of biotin synthesized (*26*), while the results of a second study indicated that only 1 equiv of AdoMet was required to synthesize biotin (*27*). Herein we describe, for the first time, experiments that address the stoichiometry of AdoMet usage by *E. coli* LipA and conclude that irreversible cleavage of one AdoMet is required for *each* of the two hydrogen atoms that are removed from the substrate.

MATERIALS AND METHODS

Materials. All DNA-modifying enzymes and reagents were purchased from New England Biolabs (Beverly, MA), as was Vent polymerase and its associated 10× reaction buffer. Oligonucleotide primers were obtained from Invitrogen Life Technologies (Carlsbad, CA). *E. coli* BL21(DE3) and expression vector pET-28a were purchased from Novagen (Madison, WI). Adenine, 5′-deoxyadenosine (5′-dA), DL-6,8-thioctic (DL-lipoic) acid, caprylic (*n*-octanoic) acid, porcine heart lipoamide dehydrogenase (LDH), sodium sulfide (nonahydrate), nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide phosphate (NADPH), tris(2-carboxyethyl)phosphine (TCEP), 2-mercaptoethanol, L-(+)-arabinose, pyridoxal 5′-phosphate (PLP), *E. coli* genomic DNA (strain W3110), and ferric chloride were purchased from Sigma Corp. (St. Louis, MO). [d_{15}, 98%]-Octanoic acid was purchased from Cambridge Isotope Laboratories (Andover, MA). Coomassie blue dye-binding

reagent, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), and bovine serum albumin (BSA) standard were purchased from Pierce (Rockford, IL). Nickel nitrilotriacetic acid (Ni-NTA) resin was purchased from Qiagen (Valencia, CA). Sephadex G-25 resin and Nick prepoured gel-filtration columns were purchased from Amersham Biosciences (Piscataway, NJ), while Whatman DE-52 ion-exchange resin was purchased through VWR (West Chester, PA). All other buffers and chemicals were of the highest grade available.

Methods. (*S*,*S*)-*S*-Adenosyl-L-methionine was synthesized using AdoMet synthetase (EC 2.5.1.6) and purified as described elsewhere (*28*). *E. coli* lipoate protein ligase (LplA) (*29*) and the H-protein of the *E. coli* GCS (*4*, *30*) were cloned into the *Nde*I site of expression vector pET-28a, which causes the proteins to be expressed with an N-terminal hexahistidine tag that is separated from the ATG start codon of each protein by a linker of 10 amino acids. *E. coli* genes for flavodoxin (Flv) (*31*) and flavodoxin reductase (Flx) (*32*) were cloned into the intein-based expression vector pTYB1 (New England Biolabs, Beverly, MA) and purified by affinity chromatography. The isolated proteins contained no amino acids that were not part of the natural gene sequence. Details of the cloning and purification of all of these proteins will be provided elsewhere (*33*).

UV−visible spectra were recorded using Cary 50 or Cary 300 spectrometers (Varian, Walnut Creek, CA) in combination with the associated WinUV software package. HPLC was carried out on an Agilent (Foster City, CA) 1100 HPLC system, which included an autosampler and a variable wavelength detector. Data collection and analysis were performed with the associated ChemStation software package. Sonic disruption of *E. coli* cells was carried out with a 550 sonic dismembrator from Fisher Scientific (Pittsburgh, PA), in combination with a horn containing a $^1/_2$ in. tip. The cable connecting the horn to the power supply was threaded through a port in the anaerobic chamber to allow sonication to be carried out anaerobically. DNA sequencing was performed by The Pennsylvania State University Nucleic Acid Core Facility.

Construction of the LipA Expression Vector. The *lipA* gene was amplified from *E. coli* (strain W3110) genomic DNA using polymerase chain reaction (PCR) technology, which was carried out with a Robocycler thermocycler from Stratagene (La Jolla, CA). The forward amplification primer (5′-GCG GCG TCC ATA TGA GTA AAC CCA TTG TGA TGG AAC GC-3′) included an *Nde*I restriction site (underlined) flanked by an 8-bp GC clamp and the first 27 bp of the *lipA* gene. The reverse primer (5′-GCC GGA ATT CCTT ACT TAA CTT CCA TCC CTT TCG-3′) contained an *Eco*RI restriction site (underlined) flanked by a 4-bp GC clamp and the last 24 bp of the *lipA* gene, including the stop codon. The *lipA* gene was amplified and ligated into expression vector pET-28a by standard procedures (*34*); the correct construct was verified by DNA sequencing and designated pMGS10.

Expression and Purification of LipA. An overnight culture of *E. coli* BL21(DE3) containing plasmids pMGS10 and pDB1282 was used to inoculate 16 L of M9 minimal media (*34*) containing kanamycin (50 μg mL^{-1}) and ampicillin (100 μg mL^{-1}). The culture, which was evenly distributed among four 6 L Erlenmeyer flasks, was allowed to grow at 37 °C. At an optical density (660 nm; OD_{660}) of 0.3, solid arabinose was added to each flask at a final concentration of 0.05% (w/v). At an OD_{660} of 0.6, solid IPTG and ferric chloride were added to each flask at final concentrations of 200 and 50 μM, respectively. The cultures were allowed to incubate further at 37 °C for 4 h, upon which they were cooled in an ice−water bath and harvested by centrifugation at 10000*g* for 10 min at 4 °C. Typical yields were 50−60 g of frozen cell paste, which was stored in a liquid nitrogen dewar until ready for use.

LipA was purified by immobilized metal affinity chromatography (IMAC) using a Ni-NTA solid support. All steps were carried out inside of an anaerobic chamber obtained from Coy Laboratory Products, Inc. (Grass Lake, MI) under an atmosphere of N_2 and H_2 (95%/5%), wherein the O_2 concentration was maintained below 1 ppm via the use of palladium catalysts. Steps involving centrifugation were performed outside of the anaerobic chamber; however, samples were loaded into appropriate centrifuge bottles and tightly sealed before removal. All buffers were prepared using distilled and deionized water that was boiled for at least 1 h and then allowed to cool while being stirred uncapped in the anaerobic chamber for 48 h. All plastic ware was autoclaved, brought inside of the anaerobic chamber while hot, and then allowed to equilibrate overnight inside the anaerobic chamber before use. Buffers were titrated to their final pH using an Accumet (AP61) pH meter (Fisher Scientific, Fairlawn, NJ) that was maintained inside of the chamber.

In a typical purification, 40 g of cell paste was resuspended in 80 mL of buffer A (50 mM HEPES, pH 7.5, 100 μM PLP, 300 mM KCl, 10 mM 2-mercaptoethanol, and 10 mM $MgCl_2$) containing 1 mg mL^{-1} lysozyme and stirred at room temperature for 30 min. After the solution was placed in an ice−water bath and the temperature was allowed to equilibrate to <8 °C, the suspension was subjected to four 1 min bursts of sonic disruption (setting of 7). The supernatant was centrifuged at 50000*g* for 1 h and then loaded onto a Ni-NTA column (2.5 × 7 cm) equilibrated in buffer A. The column was washed with 70 mL of buffer B (50 mM HEPES, pH 7.5, 100 μM PLP, 300 mM KCl, 40 mM imidazole, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 20% glycerol) and subsequently eluted with buffer B containing 250 mM imidazole. Fractions that were brown in color were pooled and concentrated in an Amicon stirred cell (Millipore, Billerica, MA) fitted with a YM-10 membrane (10000 MW cutoff). The protein was exchanged into buffer C (50 mM HEPES, pH 7.5, 100 mM KCl, 20% glycerol, and 10 mM $MgCl_2$) by anaerobic gel filtration, reconcentrated, and stored in aliquots in a liquid N_2 dewar until ready for use.

Reconstitution of LipA. Reconstitution of LipA with iron and sulfide was carried out in a Coy anaerobic chamber using anaerobic buffers and solutions. A typical reconstitution reaction contained, in a final volume of 2 mL, 100 μM LipA and 8-fold molar excesses of $FeCl_3$ and Na_2S. LipA was initially treated with 5 mM DTT for 10 min on ice, followed by addition of $FeCl_3$. Finally, Na_2S was added dropwise over 10 min, and the mixture was allowed to stir gently on ice for an additional 4 h. The solution was placed in airtight centrifuge tubes, removed from the anaerobic chamber, and centrifuged at 14000*g* for 20 min at 4 °C to remove precipitate. The samples were brought back into the anaerobic

chamber, and the supernatants were removed and exchanged into buffer C by gel filtration (Sephadex G-25).

Expression and Purification of Recombinant [Apo]- and [Lipoyl]H-Proteins. Plasmid pNMN108, which contained the *E. coli* H-protein cloned into expression vector pET-28a, was transformed into *E. coli* BL21(DE3). A single colony was used to inoculate 100 mL of M9ZB media (*35*) containing 50 μg mL^{-1} kanamycin. A 50 mL portion of this culture was used to inoculate 20 L of the same media, and the culture was allowed to grow at 37 °C until an OD_{600} of 0.6 was attained. Expression was induced by the addition of IPTG (200 μM final concentration) and was allowed to continue for 4 h at 37 °C. The cells were harvested by centrifugation for 10 min at 10000*g* and 4 °C, frozen in liquid N_2, and stored at −80 °C until ready for use. When [lipoyl]H-protein (LHP) was to be isolated, thioctic acid was added to the growth medium at a final concentration of 200 μM.

Recombinant [apo]- or [lipoyl]H-proteins were purified at 4 °C by IMAC. In a typical purification, 20 g of frozen cell paste was resuspended in 60 mL of buffer H (50 mM Na-HEPES, pH 7.5, 300 mM NaCl, and 10 mM imidazole). Lysozyme was added to a final concentration of 1 mg mL^{-1} and allowed to incubate with the suspension at room temperature while being stirred. The suspension was cooled to <8 °C in an ice−water bath and subjected to four 1 min bursts of sonic disruption (setting of 7). The lysate was centrifuged at 50000*g* for 1 h, and the supernatant was loaded onto a Ni-NTA column (2.5 × 7 cm) that was equilibrated in buffer H. The column was washed with 50 mL of buffer H containing 20 mM imidazole and then eluted in buffer H containing 200 mM imidazole. Protein-containing fractions were pooled, concentrated, and exchanged into buffer C (LipA purification) by anaerobic gel-filtration chromatography.

Synthesis of [Octanoyl]H-Protein. The reaction mixture contained, in a final volume of 50 mL, 50 mM Na-HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM ATP, 0.3 mM octanoic acid or octanoic-d_{15} acid, 180 μM H-protein, and 2 mg of LplA. The reaction was allowed to incubate at 37 °C for 40 min, upon which it was diluted to 500 mL with 20 mM potassium phosphate buffer, pH 7.2 (KPB). The diluted protein was loaded onto a DE-52 column (2.5 × 15 cm) that was equilibrated in the same buffer. The column was washed with 200 mL of KPB containing 180 mM NaCl and then eluted with a 500 mL (total) linear gradient of 180−450 mM NaCl in KPB. Protein-containing fractions were pooled and concentrated using an Amicon stirred cell fitted with a YM-3 membrane (3000 MW cutoff). The protein was then brought into the anaerobic chamber and exchanged into buffer C (LipA purification).

Assay for 5′-dA and LHP. The time-dependent formation of 5′-dA and LHP was determined simultaneously using the same assay mixture. The assay contained, in a final volume of 540 μL, 50 mM Na-HEPES, pH 7.5, 20 μM Flv, 5 μM Flx, 1 mM NADPH, 100 μM OHP, 700 μM AdoMet, 50 μM LipA, and 1 mM L-tryptophan. The reaction was initiated by addition of AdoMet after incubation of the other components of the assay mixture at 37 °C for 5 min. For the quantification of 5′-dA, 25 μL aliquots of the assay mixture were removed at designated times and added to 25 μL of 0.2 N H_2SO_4 to quench the reaction. Precipitated proteins were pelleted by centrifugation, and 25 μL aliquots of the supernatant were analyzed by HPLC with UV detection (260 nm), using a Zorbax SB-CN column (4.6 × 250 mm), which was obtained from Agilent. Solvent A consisted of 0.4% trifluoroacetic acid (TFA) titrated to pH 1.76 with triethylamine (TEA), while solvents B and C consisted of 100% acetonitrile and 100% methanol, respectively. The column was equilibrated in 95% solvent A/5% solvent C and eluted for 5 min under the same conditions after sample injection. Over the following 10 min, solvent B was increased linearly to 50%, while solvent C was increased to 30%. Under these conditions and a flow rate of 1 mL min^{-1}, AdoMet eluted at 3.1 min, adenine eluted at 4.5 min, 5′-dA eluted at 6.8 min, methylthioadenosine eluted at 11.1 min, and the tryptophan internal standard (IS) eluted at 12.9 min. The concentration of 5′-dA was then determined from a calibration curve of known concentrations of 5′-dA that was run under identical conditions, using the IS to correct for subtle volume changes between sample injections.

For the quantification of LHP, 100 μL aliquots were removed from the assay mixture, loaded directly onto Nick prepoured gel-filtration columns equilibrated in buffer C, and eluted in a volume of 400 μL according to the manufacturer's specifications. Each assay was carried out at 25 °C and contained, in final volume of 1.1 mL, 50 mM Na-HEPES, pH 7.5, 8 mM TCEP, 2 mM NAD^+, 0.9 μM LDH, and varying amounts of the Nick column eluate. Assays were initiated by addition of LDH after preincubation at 25 °C and were monitored by an increase in absorbance at 340 nm ($\epsilon_{340} = 6220$ M^{-1} cm^{-1}). The concentration of LHP was then determined from a standard curve (0−5 μM) of known concentrations of LHP generated under identical conditions.

GC-MS Analysis of 5′-dA. Assays were carried out as described above, except that [octanoyl-d_{15}]H-protein was substituted for unlabeled substrate, and aliquots of 100 μL were injected onto the HPLC. The 5′-dA produced at each time point was isolated by HPLC as described above, and the solvent was removed in vacuo. Pyridine (50 μL) and MSTFA (100 μL) were added to the remaining pellet, and the mixture was heated at 100 °C for 1 h. The resulting trimethylsilyl (TMS) derivative of 5′-dA was injected onto a Hewlett-Packard 5972 GC-MS without further purification. Separation of the derivatized products was carried out as described previously (*36*) using a J&W DB-5 capillary column (30 m length × 0.25 mm bore × 0.25 μm film thickness).

LC-MS Analysis of the LipA Reaction. Assays were carried out as described above, except that the total volume of each reaction was 500 μL, and each reaction was allowed to incubate for 60 min at 37 °C. The assays were quenched by addition of EDTA to a final concentration of 100 mM and then centrifuged at 14000 rpm for 25 min to pellet precipitated proteins. Under these conditions, the majority of the LipA present in the assay precipitated, while the various forms of the H-protein remained soluble. The supernatant was chromatographed over a NAP column (Amersham Biosciences) in water and subsequently concentrated to a volume of 100 μL using a centricon (Millipore) ultrafiltration device (3000 MW cutoff). The concentrated solution was analyzed directly by electrospray ionization mass spectrometry on a Micromass Quattro II mass spectrometer (Waters, Milford, MA) connected to a Shimadzu (Columbia, MD) LD-10ADvp HPLC system, using a 1 × 10 mm Thermo

Hypersil-Keystone BetaBasic CN guard column (Bellefonte, PA) and elution with a fast gradient of increasing acetonitrile with 0.15% formic acid. Zero-charge-state transformed spectra were calculated from the raw electrospray mass spectra using the MassLynx version 3.5 transform algorithm.

RESULTS

Expression and Purification of LipA. *E. coli* LipA was cloned into a pET-28a expression vector and cotransformed into *E. coli* BL21(DE3) along with plasmid pDB1282. Plasmid pDB1282 confers ampicillin resistance and contains an *E. coli* operon that is involved in the biosynthesis of FeS clusters cloned behind an arabinose-inducible promoter. These genes include *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, and *fdx*. The *iscS* gene encodes a cysteine desulfurase, which activates the sulfur of L-cysteine for use in the formation of FeS clusters. The *iscU* and *iscA* genes are believed to encode proteins that serve as scaffolds for the construction of precursors of mature FeS clusters, while the *hscB* and *hscA* genes encode molecular chaperones that are believed to facilitate incorporation of the FeS clusters into the apoprotein. Finally, the *fdx* gene encodes a ferrodoxin that is believed to be involved in maintaining electron balance during formation of FeS clusters (*37*). The coexpression of *E. coli* LipA under similar conditions has already been reported; the accessory proteins supported a greater yield of soluble LipA, which also contained greater amounts of iron and sulfide (*38*). We obtained very similar results, which are described in detail elsewhere (*21*).

LipA was purified under anaerobic conditions by IMAC. Amino acid analysis indicated that the Bradford reagent, using a commercially available BSA standard, overestimates the protein concentration by a factor of 1.45. This number is in agreement with a previous assessment of the Bradford correction factor for LipA (*39*) but differs a bit from a subsequently reported correction factor, wherein the assay was determined to overestimate the concentration of LipA by almost a factor of 2 (*2*, *39*).

Assay of E. coli LipA. In vitro studies of LipA have been hampered by uncertainty in the identity of the substrates for the reaction. Recent studies indicate that the protein acts on octanoyl derivatives of the target lysine residue of lipoyl-accepting proteins (*40*, *41*). We employed the octanoyl derivative of the H-protein of the *E. coli* GCS as our model substrate because of the ease in which its extent of lipoylation can be quantified. The assay is based on the ability of the lipoylated form of the H-protein to mediate reduction of NAD^+ by dihydrolipoamide dehydrogenase at the expense of TCEP, which allows the extent of lipoylation to be determined spectrophotometrically by measuring the rate of NADH formation and comparing the value to a standard curve of graded concentrations of LHP (*42*). Under the conditions described in Materials and Methods, the assay gives a linear response from 0 to 10 μM LHP.

The cleavage of AdoMet to generate a 5′-dA$^\bullet$ requires the input of one electron, which often can be satisfied by dithionite or 5-deazaflavin plus light. In the *E. coli* cell, the electron is supplied by NADPH via the flavodoxin/flavodoxin reductase reducing system. In the presence of the in vivo reducing system, 50 μM reconstituted LipA catalyzed formation of 18 μM LHP after 20 min at 37 °C, while the as-isolated protein catalyzed formation of 14 μM. In the absence of LipA, AdoMet, or NADPH, 3.40, 5.21, and 6.08 μM LHP was detected, respectively. The product detected in the absence of LipA is attributed to small amounts of contaminating LHP in our OHP stock solution. The slight excess above background produced in the absence of AdoMet is attributed to a fraction of the protein that is isolated with AdoMet bound, while the slight excess produced in the absence of NADPH is attributed to partially reduced forms of flavodoxin and flavodoxin reductase in the assay. Similar behavior with respect to AdoMet and NADPH has been observed previously in studies of LipA (*2*) and BioB (*25*), respectively. In all experiments, detected amounts of LHP that were not dependent on LipA were subtracted from the observed concentrations.

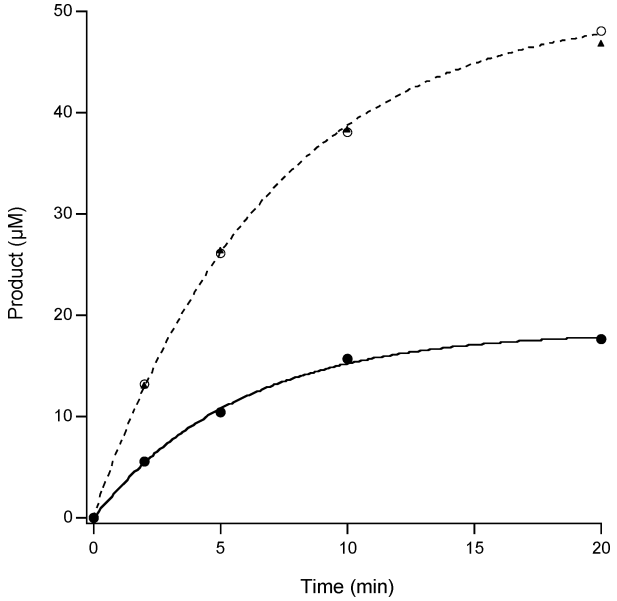


FIGURE 1: Time-dependent production of LHP (closed circles) and 5′-dA (open circles). The reaction was carried out as described in Materials and Methods and contained 50 μM reconstituted LipA, 100 μM OHP, 20 μM Flv, 5 μM Flx, and 700 μM AdoMet. At each time point, the concentration of LHP that was attributed to contamination of the OHP substrate was subtracted from the observed concentration of LHP before the value was plotted. Computed values of 5′-dA (closed triangles) were calculated by assuming two pathways for its formation, one in which $k = 0.175$ min^{-1} and $A = 37$ μM and one in which $k = 0.092$ min^{-1} and $A = 13$ μM.

Stoichiometry of 5′-dA to LHP. In Figure 1, curves depicting the LipA-dependent formation of LHP (closed circles) and 5′-dA (open circles) are displayed as a function of time. Both processes can be fitted to a first-order kinetic equation, allowing extraction of rate constants of 0.175 ± 0.010 min^{-1} for formation of LHP (solid line) and 0.144 ± 0.005 min^{-1} for formation of 5′-dA (dashed line). The amplitudes of each fit reveal the maximum amount of product formed in each first-order process, indicating 50.5 μM 5′-dA, which corresponds to 1.01 equiv with respect to LipA, and 18.4 μM LHP, which corresponds to 0.378 equiv. The concentrations of LHP and 5′-dA formed at each time point are given in Table 1, the ratio of the two varying from 2.38 at early time points to 2.71 at later time points. These ratios can be rationalized by a model in which 2 equiv of both AdoMet and LipA are required to synthesize LHP from OHP if a given fraction of 5′-dA results from nonproductive

Table 1: Time-Dependent Formation of 5′-dA and LHP[a]

time (min)	5′-dA (μM) (observed)	5′-dA (μM) (computed)[b]	LHP (μM)	ratio 5′-dA/LHP
0	0	0	0	
2	13.2	13.1	5.54	2.38
5	26.1	26.4	10.4	2.51
10	38.0	38.4	15.7	2.42
20	48.0	46.8	17.7	2.71

[a] Data are from a single time course. [b] Derived from computing 5′-dA by assuming two independent processes: one with $k = 0.175$ min^{-1} and $A = 37$ μM and one with $k = 0.092$ min^{-1} and $A = 13$ μM.

Table 2: Time-Dependent Deuterium Incorporation into 5′-dA[a]

time (min)	total [5′-dA] (μM)	[5′-dA-d_0] (μM)	[5′-dA-d_1] (μM)	[5′-dA-d_0] (% of total)
0	0			
5	10.4	1.38	9.07	13.2
15	22.4	3.40	19.0	15.2
45	34.5	6.92	27.6	20.0
60	38.6	8.63	30.0	22.3

[a] Data are from a single time course in the presence of [octanoyl-d_{15}]H-protein. Only monodeuterated 5′-dA and unlabeled 5′-dA were detected.

cleavage of AdoMet. From this model, the maximum concentration of LHP that can be synthesized from 50 μM LipA is 25 μM, suggesting that only 74% of our LipA is configured to react productively upon cleavage of AdoMet. Using this constraint, as well as the assumption that the rate constant for productive cleavage of AdoMet must be as large as the rate constant for formation of LHP, the trace for total 5′-dA formation can be reconstructed from two simultaneous and independent first-order processes: one with an amplitude (A) of 37 μM 5′-dA and a rate constant (k) of 0.175 min^{-1} and one with $A = 13$ μM 5′-dA and $k = 0.092$ min^{-1}. Table 1 and Figure 1 (closed triangles) show that the computed values for 5′-dA agree well with the observed values. The slower first-order rate constant that is actually observed for 5′-dA production (0.144 min^{-1}) results from fitting biphasic behavior to a single exponential. Although the data in Figure 1 and Table 1 derive from one time course, repetition of the experiment several times gave almost identical results.

Direct Hydrogen Atom Abstraction by 5′-dA•. To show that the 5′-dA• acts directly on the substrate, [octanoyl-d_{15}]H-protein was substituted for unlabeled substrate, and 5′-dA was isolated, derivatized, and analyzed for its deuterium content by GC-MS. We posited that if a single molecule of AdoMet mediates insertion of both sulfur atoms into the octanoyl group, an increase in 2 atomic mass units (amu) should be observed, since removal of hydrogen atoms from C-6 and C-8 are a prerequisite for sulfur insertion (*8*). By contrast, if insertion of both sulfur atoms is mediated by two different AdoMet molecules, an increase in only 1 amu should be observed. The mass spectral fragmentation behavior of TMS derivatives of 5′-dA has been reported (*36*); the relevant m/z value is 260, which corresponds to the derivatized sugar, after loss of the adenine base, minus a hydrogen. An m/z value of 261 would therefore correspond to the addition of one deuteron to 5′-dA, while an m/z value of 262 would correspond to the addition of two deuterons. However, a small fraction of derivatized, unlabeled 5′-dA contributes to the signal intensity at m/z values of 261 and 262, because of the natural-abundance isotopic distribution of carbon, nitrogen, hydrogen, and oxygen, as well as silicon, which derives from the TMS groups. Chromatograms for four unique m/z values (260, 261, 262, and 263) were collected in single-ion monitoring mode for reactions containing both unlabeled and labeled substrates. The ratios of the intensities at the various m/z values for the unlabeled sample allowed correction for the natural-abundance isotopic distribution at each m/z value for the labeled sample.

In Table 2, the extent of deuterium incorporation into 5′-dA is displayed at several fixed times. Over the course of 60 min, a total of 38.6 μM 5′-dA was formed, which is

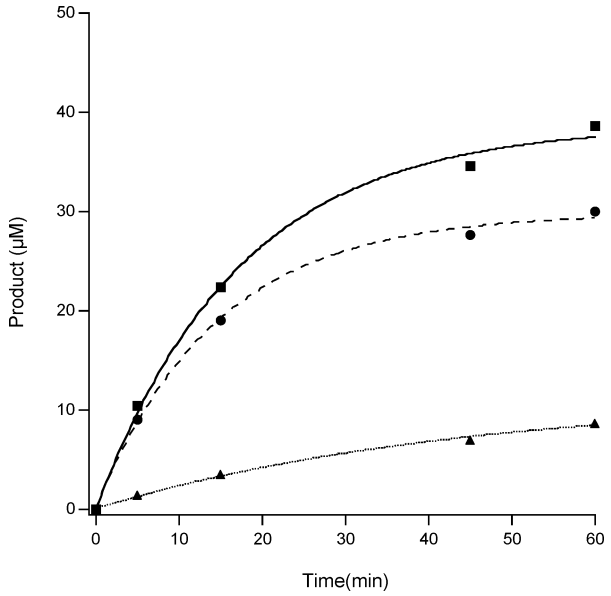


FIGURE 2: Time-dependent production of total 5′-dA (closed squares), monodeuterated 5′-dA (closed circles), and unlabeled 5′-dA (closed triangles). The reaction was carried out as described in Materials and Methods and contained 50 μM reconstituted LipA, 100 μM [octanoyl-d_{15}]H-protein, 20 μM Flv, 5 μM Flx, and 700 μM AdoMet. At each time point, 5′-dA was isolated by HPLC, and its deuterium content was assessed by GC-MS after proper derivatization.

noticeably lower than that obtained at 20 min in the presence of unlabeled substrate (48 μM). Also, at each time point, significant amounts of nondeuterated 5′-dA were observed, the fraction thereof increasing as a function of time; however, the dideuterated product was not detected. The intensity of the signal at $m/z = 262$ was approximately 3% of the intensity of the base peak ($m/z = 261$), allowing estimation of a limit of detection of ~2−3% of the intensity of the base peak, or ~1 μM. Unexpectedly, our assay did not detect significant amounts of LHP above the background level, suggesting that there is a significant isotope effect against removing a hydrogen atom from either C-6 or C-8. Curves depicting the time-dependent production of total 5′-dA (solid line), monodeuterated 5′-dA (dashed line), and unlabeled 5′-dA (dotted line) are shown in Figure 2. Fits of each curve to a first-order kinetic equation yield $k = 0.058 \pm 0.005$ and $A = 38.7 \pm 1.2$ for total 5′-dA, $k = 0.068 \pm 0.005$ and $A = 29.7 \pm 0.6$ for monodeuterated 5′-dA, and $k = 0.028 \pm 0.004$ and $A = 11.3 \pm 1.2$ for unlabeled 5′-dA.

LC-MS Analysis of the LipA Reaction. LC-MS analysis of protein products was carried out to confirm that LHP indeed was not synthesized in the presence of OHP-d_{15}. The mass spectrum of unlabeled OHP is shown in Figure 3A

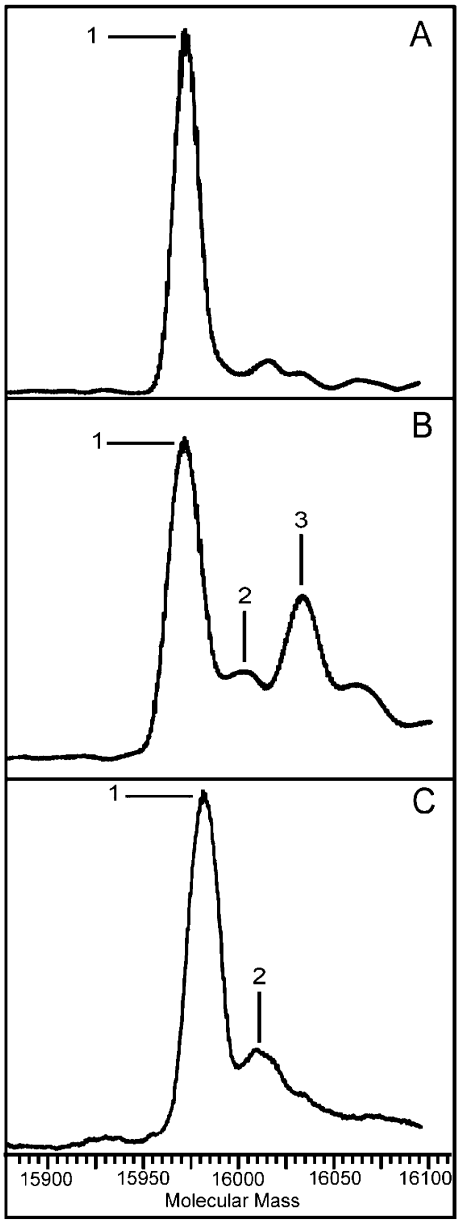


FIGURE 3: Transformed zero-charge-state mass spectra of the LipA reaction. (A) Mass spectrum of starting [octanoyl]H-protein substrate: peak 1 (15971 Da, OHP). (B) Mass spectrum of H-protein after 60 min under turnover conditions using OHP as substrate: peak 1 (15971 Da, OHP); peak 2 (16003 Da); peak 3 (16033 Da, LHP). (C) Mass spectrum of H-protein after 60 min under turnover conditions using OHP-d_{15} as substrate: peak 1 (15986 Da, H-protein containing the octanoyl-d_{15} group); peak 2 (16016 Da). Reactions were carried out at 37 °C as described in Materials and Methods. The H-protein was exchanged into water by gel-filtration chromatography after being quenched in 0.1 M (final concentration) EDTA and then concentrated by ultrafiltration before analysis by LC-MS (ESI^+).

and displays a peak (1) with a molecular mass of 15971 Da, which is the molecular mass predicted by the amino acid composition of the H-protein plus the octanoyl moiety. After incubation of 100 μM OHP with 50 μM LipA at 37 °C for 60 min in the presence of other required components of the assay, a peak at a molecular mass of 16033 Da appears (3), which corresponds to the exact molecular mass of the H-protein plus an oxidized lipoyl group (Figure 3B). A shoulder is also apparent (2) and displays a molecular mass of 16003 Da, which is consistent with a thiooctanoyl group appended to the H-protein. This shoulder is not present in the OHP substrate. In the presence of OHP-d_{15}, the peak at 15971 Da shifts 15 amu (Figure 3C) to 15986 Da, confirming that peak 1 is indeed OHP. However, no peak was observed at a molecular mass of 16046 Da, which would correspond to LHP-d_{13}. The shoulder (2), however, has shifted to a molecular mass of 16016 Da, which is consistent with a monothiolated species. LC-MS was also carried out on V8-protease digests of the H-protein isolated from the reaction containing OHP-d_{15}, which allows a more accurate determination of the mass and relative amounts of all octanoyl species because of the smaller size of the fragment subjected to analysis. The monothiolated species was confirmed and estimated to be ~15−20% of the starting substrate (data not shown).

DISCUSSION

Lipoyl and biotin synthases belong to a unique subclass of radical SAM enzymes in which the proteins, themselves, serve both as catalyst and as substrate (*43*, *44*). Consequently, they are unable to catalyze multiple turnovers in the absence of a suitable system for regenerating the active sulfur donor. Indeed, BioB catalyzes no more than one turnover, even after 4 h at 37 °C (*25*, *27*, *44*), while LipA has been reported to catalyze formation of no more than 0.05 equiv of product after 15 min at 37 °C (*2*). Herein, we report that reconstituted LipA can catalyze formation of 0.38 equiv of LHP with an apparent first-order rate constant of 0.175 min^{-1} in the presence of the physiological reducing system. This rate constant, though slow, compares favorably with the rate constant (0.07 min^{-1}) for BioB-catalyzed sulfur insertion into dethiobiotin under similar conditions (*25*). Moreover, we posit that the 0.38 equiv of product that we obtain actually corresponds to 74% of the maximum theoretical yield, suggesting that each LipA polypeptide donates only one sulfur atom to the product, thereby requiring 2 equiv of polypeptide to synthesize one lipoyl group. Our basis for this stoichiometry stems from the large number of time-dependent assays conducted (>10), spanning several different preparations of LipA, each of which gave 1 ± 0.05 equiv of 5′-dA after 20 min for the reconstituted protein, rather than the expected 2 equiv required for production of one LHP. In one case, 1.2 equiv was obtained under slightly different conditions. Since the low product yield per monomer could be attributable to incomplete FeS-cluster assembly, we assessed whether this stoichiometry is independent of whether LipA is expressed in the presence of plasmid pDB1282. We found that inclusion of the plasmid only resulted in higher levels of LHP produced by as-isolated (nonreconstituted) LipA. The ratio of 5′-dA to LHP was always 2.3−2.7, regardless of whether LipA was reconstituted and/or expressed in the presence of plasmid pDB1282. Careful iron and sulfide analysis in combination with several forms of spectroscopy is consistent with the Bradford correction factor used in determining the concentration of LipA (*21*), which is also similar to one reported previously (*39*) and well within a factor of 2 of another (*2*).

Stoichiometry of the Lipoyl Synthase Reaction. Clearly, the stoichiometry of 5′-dA to LHP at each time point is consistent with a model in which 2 equiv of AdoMet is required to synthesize 1 equiv of LHP from OHP. Additionally, the absence of a burst phase of 5′-dA production, the

absence of a lag phase in LHP production, and the relatively constant ratio of 5′-dA to LHP throughout the kinetic trace suggest that the apparent first-order rate constant for cleavage of AdoMet to install the first sulfur is significantly slower than the second. We had hoped to address the stoichiometry of AdoMet usage by simply counting the number of deuterium atoms in isolated 5′-dA when perdeuterated OHP was substituted for unlabeled OHP. Although no dideuterated 5′-dA was detected, which would support usage of one AdoMet per LHP synthesized from OHP, unexpectedly, LHP also was not detected. If we define an abortive process as one that is unrelated to chemistry involving the substrate, which corresponds to the fraction of 5′-dA-d_0 at each time point, our deuterium transfer studies would indicate that less than 10% of the 5′-dA produced in the presence of unlabeled OHP would stem from such a process, which takes place with a rate constant of $\sim$0.028 min^{-1}. If only one AdoMet were used to synthesize LHP from OHP, it would be expected that a much larger fraction of the 5′-dA at each time point would be produced with this significantly slower rate constant, since >50% would derive from abortive processes. Our kinetic studies are not consistent with a model wherein >50% of the 5′-dA is produced with a rate constant of 0.028 min^{-1}. In biotin synthase, abortive cleavage of AdoMet was observed to occur in the absence of substrate when 5-deazaflavin plus light, a low-potential source of reducing equivalents, was used in the reaction (*19*). Additionally, DTT was observed to greatly stimulate cleavage of AdoMet by the activating enzyme of the *E. coli* anaerobic ribonucleotide reductase (*20*, *45*). To minimize nonproductive cleavage of AdoMet, our studies were carried out in the absence of DTT and with the physiological reducing system. The observed stoichiometry is surprisingly similar to that obtained in one of the two studies that addressed the stoichiometry of AdoMet usage in the BioB reaction (*26*) and suggests that the abortive cleavage of AdoMet in each system might derive from some innate reactivity associated with this subclass of radical SAM enzymes.

Intermediates in the Lipoyl Synthase Reaction. Our results indicate that 24% of the 5′-dA produced after 20 min does not lead to LHP formation during the time span of the assay. Of that 24%, 10%, obtained by extrapolation of the data in Table 2 to a 20 min time point, results from hydrogen atom abstraction by the 5′-dA$^{\bullet}$ from a nonsubstrate molecule. Therefore, the nature of the reaction that accounts for the other 14% is in question. In vivo feeding studies in *E. coli* have shown that there is a significant intramolecular isotope effect against removing tritium from [8-^{3}H]octanoic acid during the synthesis of lipoic acid (*8*), which might explain our failure to observe LHP in our in vitro assay when using OHP-d_{15} as substrate. The effect of isotopic substitution at C-6 has not yet been addressed in a quantitative manner. Nevertheless, monodeuterated 5′-dA is indeed formed from OHP-d_{15} with a rate constant that is $\geq$0.058 min^{-1}, which is not an insignificant number when compared to the rate constant for LHP formation in the presence of unlabeled substrate (0.175 min^{-1}). Abstraction of this deuterium from substrate is either unrelated to LHP formation (i.e., it does not derive from C-6 or C-8 of the octanoyl group) or represents a slower pathway to LHP formation. We prefer the latter hypothesis since it has been shown that octanoic-d_{15} acid can be converted into lipoic acid in vivo (*7*, *41*). If this rate constant corresponds to sulfur insertion at C-6, then its magnitude would be expected to be $\geq$0.058 min^{-1} in the presence of unlabeled substrate. If 6-mercaptooctanoic acid is a much worse substrate than 8-mercaptooctanoic acid, as in vivo feeding studies have indicated, then this slower pathway would have an inhibitory effect on the total amount of LHP formed during the time span of our in vitro assays. Therefore, our observation that only 74% of our LHP is configured to react productively might simply be a function of the magnitude of rate constants that govern abstraction at C-8 versus C-6 of the OHP substrate. When sulfur insertion at C-8 is slowed, insertion at C-6 becomes the dominant step; however, 6-mercaptooctanoic acid is a relatively poor substrate for LipA, thus supporting little or no formation of LHP during the time span of our assays. Our mass spectral data are very much consistent with this premise. Monothiolated species are seen in reactions containing labeled or unlabeled OHP. In the presence of unlabeled OHP, the monothiolated species is significantly less in intensity than the lipoylated species. In the presence of labeled OHP, the monothiolated species is still present; however, the lipoylated species cannot be detected. Almost all facets of our model are experimentally testable and are currently being evaluated. In particular, synthesis of the proposed monothiolated intermediates will allow kinetic studies to be undertaken, which may facilitate simulation of the kinetics of the entire reaction.

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