Escherichia coli LipA Is a Lipoyl Synthase: In Vitro Biosynthesis of Lipoylated Pyruvate Dehydrogenase Complex from Octanoyl-Acyl Carrier Protein†

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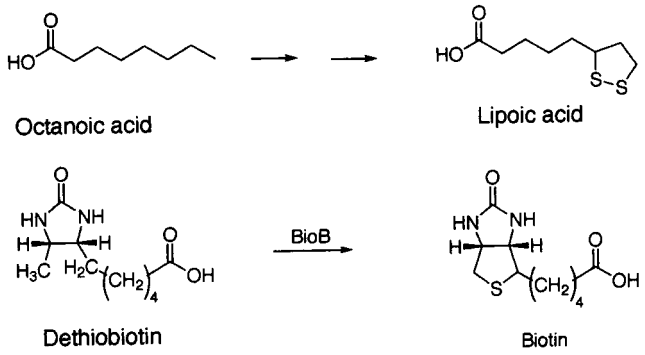
ABSTRACT: The *Escherichia coli lipA* gene product has been genetically linked to carbon−sulfur bond formation in lipoic acid biosynthesis [Vanden Boom, T. J., Reed, K. E., and Cronan, J. E., Jr. (1991) *J. Bacteriol. 173*, 6411−6420], although in vitro lipoate biosynthesis with LipA has never been observed. In this study, the *lipA* gene and a hexahistidine tagged *lipA* construct (LipA-His) were overexpressed in *E. coli* as soluble proteins. The proteins were purified as a mixture of monomeric and dimeric species that contain approximately four iron atoms per LipA polypeptide and a similar amount of acid-labile sulfide. Electron paramagnetic resonance and electronic absorbance spectroscopy indicate that the proteins contain a mixture of [3Fe-4S] and [4Fe-4S] cluster states. Reduction with sodium dithionite results in small quantities of an $S = 1/2$ $[4Fe\text{-}4S]^{1+}$ cluster with the majority of the protein containing a species consistent with an $S = 0$ $[4Fe\text{-}4S]^{2+}$ cluster. LipA was assayed for lipoate or lipoyl-ACP formation using *E. coli* lipoate-protein ligase A (LplA) or lipoyl-[acyl-carrier-protein]-protein-*N*-lipoyltransferase (LipB), respectively, to lipoylate apo-pyruvate dehydrogenase complex (apo-PDC) [Jordan, S. W., and Cronan, J. E. (1997) *Methods Enzymol. 279*, 176−183]. When sodium dithionite-reduced LipA was incubated with octanoyl-ACP, LipB, apo-PDC, and *S*-adenosyl methionine (AdoMet), lipoylated PDC was formed. As shown by this assay, octanoic acid is not a substrate for LipA. Confirmation that LipA catalyzes formation of lipoyl groups from octanoyl-ACP was obtained by MALDI mass spectrometry of a recombinant PDC lipoyl-binding domain that had been lipoylated in a LipA reaction. These results provide information about the mechanism of LipA catalysis and place LipA within the family of iron−sulfur proteins that utilize AdoMet for radical-based chemistry.

Lipoic acid (6,8-thioctic acid) (Scheme 1) is a sulfur-containing cofactor found in many prokaryotic and eukaryotic organisms. This cofactor is utilized by several enzymes involved in oxidative metabolism including: pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain ketoacid dehydrogenases, and the glycine cleavage system (*1*). In each protein, lipoic acid forms an amide linkage via its carboxylic acid moiety to the ϵ-amino of a specific lysyl residue (*2*). During catalysis by these dehydrogenases, protein-bound lipoamide serves as a carrier of activated acyl groups between the active sites of these multienzyme complexes (*1*). Recently, lipoic acid has received attention for its role as a biological antioxidant and has shown some promise in the treatment of diabetes and ischemia reperfusion injury (reviewed in ref *3*).

Scheme 1: Carbon−Sulfur Bond Forming Reactions



Although the existence of lipoic acid has been known for almost 50 years (*4*), the biosynthetic pathway of this important cofactor is poorly understood. In *Escherichia coli* (*5*) and other bacteria (*6*), yeast (*7*, *8*), and plants (*9*), lipoic acid can be synthesized endogenously or scavenged from the environment. Humans and other mammals may synthesize lipoic acid (*10*, *11*), but efficient scavenging from dietary

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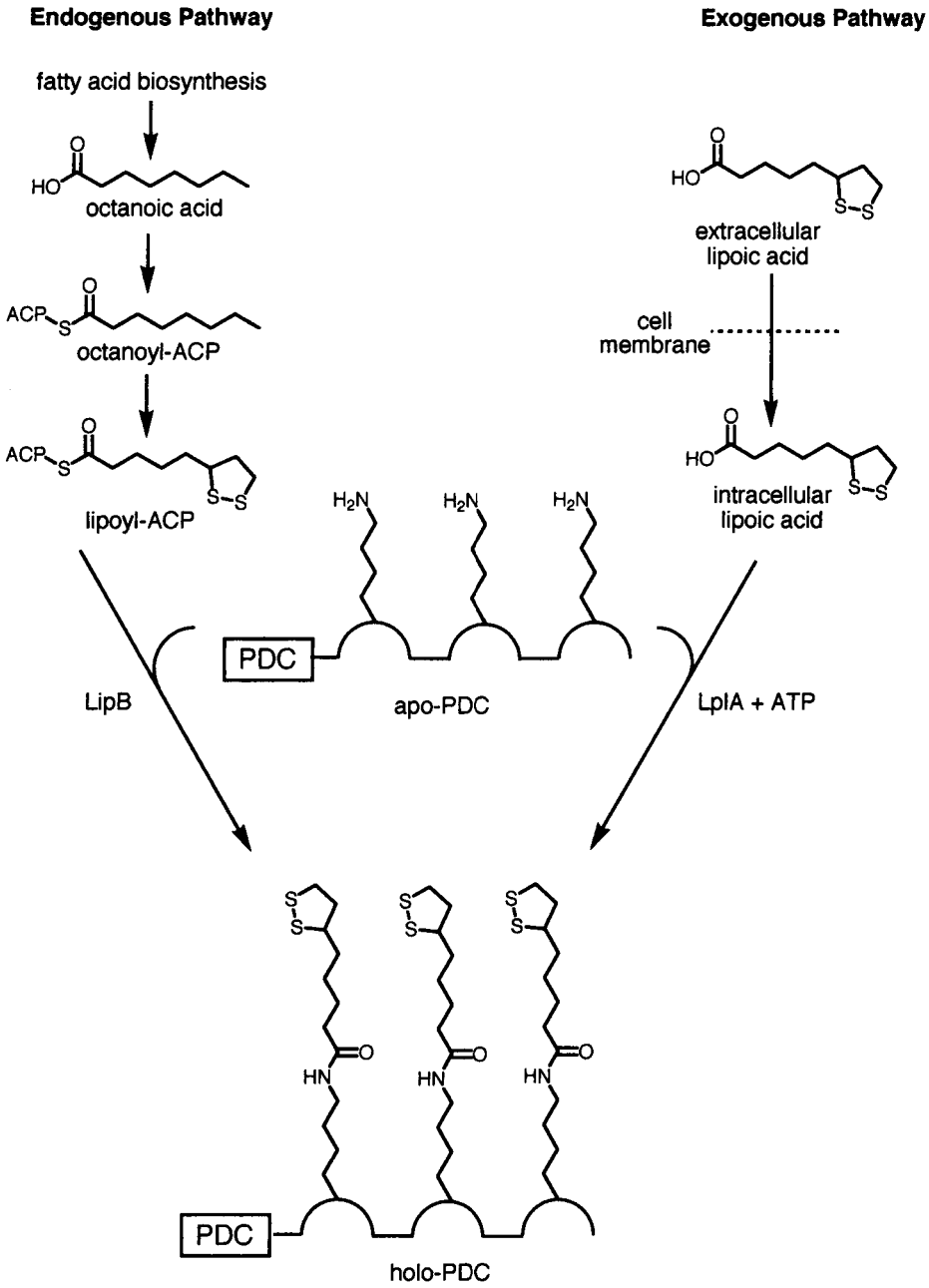
Scheme 2: Complementary Pathways of Protein Lipoylation in *E. coli*[a]



[a] Although PDC is shown as the substrate of LipB and LplA, transfer of lipoyl groups to other lipoic acid-dependent enzymes such as α-ketoglutarate dehydrogenase or the glycine cleavage enzyme is equally likely.

sources by intestinal vitamin transporters (*12*) clouds this conclusion.

Posttranslational modification of unlipoylated apoproteins with lipoate occurs by several mechanisms. Higher eukaryotes transfer lipoate to specific apoproteins using two enzymes: lipoate-activating enzyme (*13*), which catalyzes the formation of lipoyl-AMP,[1] and a lipoyl-AMP:N^{ϵ}-lysine lipoyltransferase (*14*), which transfers the lipoyl moiety of lipoyl-AMP to unlipoylated apoproteins. In *E. coli*, two complementary systems for protein lipoylation have been characterized (Scheme 2). Exogenous lipoate is transferred to unlipoylated apoproteins in an ATP-dependent process by lipoate-protein ligase A (LplA) (*15*, *16*). The second pathway in *E. coli* requires the *lipB* gene product (lipoyl-[acyl-carrier-protein]-protein- *N*-lipoyltransferase) to transfer endogenously synthesized lipoate to apoproteins (*16*). LipB utilizes lipoyl-acyl carrier protein (ACP) as a source of lipoyl groups,

[1] Abbreviations: AMP, adenosine monophosphate; ACP, acyl carrier protein; kD, kilodalton; EPR, electron paramagnetic resonance; MALDI, matrix-assisted laser desorption ionization; CoA, coenzyme A; ATP, adenosine triphosphate; PDC, pyruvate dehydrogenase complex; TPP, thiamin pyrophosphate; AdoMet, *S*-adenosyl methionine; SAH, *S*-adenosyl homocysteine; LplA, lipoate-protein ligase A; LipB, lipoyl-[acyl-carrier-protein]-protein-*N*-lipoyltransferase; EDTA, ethylenediaminetetraacetic acid; APAD, 3-acetyl pyridine adenine dinucleotide; BS, biotin synthase; PFL-AE, pyruvate formate lyase activating enzyme; ARNR, anaerobic ribonucleotide reductase; LAM, lysine 2,3-aminomutase; SDS, sodium dodecyl sulfate; kB, kilobase; mTB, modified terrific broth; ACPS, holo-acyl carrier protein synthase; AAS, acyl-acyl carrier protein synthase; IPTG, isopropyl-β-D-thiogalactopyranoside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

although octanoyl groups from octanoyl-ACP can be transferred to apo-lipoylproteins when lipoyl-ACP is limiting (*17*, *18*).

Lipoate biosynthesis studies have focused on *E. coli*, although *Saccharomyces cerevisiae* (*7*), *Rhizobium etli* (*19*), and the plant, *Pisum sativum* (*9*), have also been investigated. Early studies established that octanoic acid is the fatty acid precursor to lipoate synthesized by *E. coli* (*20*−*24*). The results of metabolic feeding studies demonstrated that *E. coli* lipoic acid biosynthesis does not involve desaturation or hydroxylation of octanoate but does result in inversion of stereochemistry at carbon 6 (*24*). Monosulfurated octanoic acids are substrates for in vivo synthesis of lipoic acid in *E. coli*, with greater incorporation of 8-thiooctanoate than 6-thiooctanoate (*23*).

Genetic studies have identified only one *E. coli* gene responsible for the sulfur-insertion steps of lipoate biosynthesis (*5*, *25*−*28*). The *lipA* gene encodes a protein of 36 kD that exhibits approximately 36% identity to the biotin synthase (*bioB*) gene of *E. coli* (*26*, *28*). These proteins both catalyze insertion of sulfur at unactivated carbons (Scheme 1) and are expected to operate by similar mechanisms.

LipA has been overexpressed in *E. coli* and purified from soluble lysates (*29*) and insoluble aggregates that were subsequently refolded and reconstituted with ferrous iron and sulfide (*30*). In both cases, the protein was found to contain two iron and two sulfide atoms per polypeptide. Improved anaerobic techniques and reconstitution procedures resulted in approximately four irons and four acid-labile sulfides per refolded/reconstituted LipA polypeptide (*31*). Electronic absorbance, resonance Raman, and Mössbauer spectroscopic results are consistent with the presence of an iron−sulfur cluster in LipA (*29*, *30*), most likely in the form of a cubane [4Fe-4S] cluster (*31*) with complete cysteinyl ligation (*29*). In this study, overproduction and purification of soluble LipA were improved and a method for purification of a soluble hexahistidine tagged LipA was developed. Results from iron and sulfur stoichiometry, electron paramagnetic resonance (EPR) spectroscopy, and electronic absorbance spectroscopy confirm that the soluble recombinant wild-type and hexahistidine-tagged proteins contain a $[4Fe\text{-}4S]^{2+/1+}$ cluster and that this species is likely essential for lipoate biosynthesis.

The direct involvement of LipA in the sulfur insertion reaction of lipoic acid biosynthesis has been difficult to establish due to the lack of an in vitro assay. Gueguen and colleagues have recently described an in vitro system in which lipoylation of the apo-H protein of the pea mitochondrial glycine cleavage system is monitored by MALDI mass spectrometry (*9*). Recombinant apo-H protein was partially lipoylated and octanoylated in the presence of pea (*Pisum sativum)* mitochondrial extracts, potential sulfur donors, *S*-adenosyl methionine (AdoMet), ACP, coenzyme A (CoA), malonate, and ATP. While this system shows promise as an assay of in vitro lipoate biosynthesis, the presence of endogenous sources of lipoic acid [e.g., lipoyl-ACP (*17*)] in the crude pea mitochondrial extracts makes the demonstration of *de novo* lipoate biosynthesis problematical.

Recent advances in the study of apoprotein lipoylation by LplA and LipB have led to the development of highly sensitive and specific in vitro assays for lipoyl species (*32*). These assays take advantage of the two systems for protein lipoylation in *E. coli* (Scheme 2). Formation of enzymatically active lipoylated-pyruvate dehydrogenase complex (PDC) is monitored spectrophotometrically via reduction of NAD^+ or a suitable analogue in the presence of pyruvate, CoA, thiamin pyrophosphate (TPP), and cysteine. Within a finite range, the rate of reduced pyridine dinucleotide formation is directly dependent upon the amount of lipoylated PDC. The apo-PDC assays are versatile, as both free lipoate and lipoyl-ACP can be detected (*16*, *17*, *32*). In this study, these assays were adapted to the conditions of the LipA reaction and were utilized to detect the first in vitro biosynthesis of a lipoyl species.

LipA is proposed to belong to the growing family of enzymes that utilize a reduced iron−sulfur cluster and AdoMet to generate a deoxyadenosyl radical for further radical-based chemistry. Members of this family include pyruvate formate lyase activating-enzyme (PFL-AE) (*33*), anaerobic ribonucleotide reductase (ARNR) (*34*), lysine 2,3-aminomutase (LAM) (*35*), biotin synthase (BS) (*36*), and possibly the spore photoproduct lyase (*37*) and benzylsuccinate synthase activating enzyme (*38*). This paper presents evidence that LipA is a member of this family of proteins, as LipA is activated by AdoMet and must undergo reduction of the iron−sulfur cluster for lipoyl synthase activity.

MATERIALS AND METHODS

Materials. The following were obtained from Difco Laboratories (Detroit, MI): Bacto-Agar, yeast extract, Tryptone, and Casamino Acids. Ampicillin, 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF/Pefabloc), isopropyl-β-D-thiogalactopyranoside (IPTG), and restriction endonucleases were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Macroprep CHT-II (ceramic hydroxyapatite) and Macroprep CM (cation exchange) resins were purchased from BioRad (Hercules, CA). TSK DEAE 650M anion-exchange resin was purchased from TosoHaas (Montgomeryville, PA). Superdex 200 HiLoad 26/60 and 16/60 gel filtration columns, PD-10 gel filtration columns, and [1-^{14}C]-octanoic acid were purchased from Amersham-Pharmacia Biotech (Piscataway, NJ). Pre-cast 10−20% Tris-glycine SDS polyacrylamide gels and Mark 12 molecular weight standards were purchased from Invitrogen (Carlsbad, CA). Vectors pET23a and pET3a and BL21(DE3) chemically competent *E. coli* were purchased from Novagen, Inc. (Madison, WI). NI-NTA agarose was purchased from Qiagen (Valencia, CA). DE53 resin was purchased from Whatman (Clinton, NJ). Ultrafree 5000 MW and 30 000 MW cutoff spin filters were purchased from Millipore (Bedford, MA). Unless specified, all other reagents were obtained from Sigma Chemical, St. Louis, MO.

Cloning and Overproduction of LipA. The Clarke-Carbon plasmid pLC15−5 (*39*) was digested with *Eco*RV and shotgun cloned into the *Sma*I site of pUC18. From the mixture of pLC15-5 subclones, plasmid pLE71 was isolated for its ability to complement lipoate auxotrophic JRG33-lip9 *E. coli* (*5*). A 3.0 kB *Eco*RV−*Eco*RI fragment containing the *lipA* gene was isolated from pLE71 and subcloned into pUC18 to give pLE61. A *Bam*HI site was inserted 3′ of the *lipA* coding sequence by digestion with *Sna*BI and ligation to a *Bam*HI linker sequence. The LipA coding sequence was excised by digestion with *Bam*HI, and the resulting 2.1 kB LipA expression cassette was ligated into

*Bam*HI-digested pET3a to give pEL21(+). The 2.1-kB *Bam*HI fragment contains 834 bp of untranslated sequence upstream of the start codon and includes a *rho*-independent transcription terminator (*28*). Transformation of the lipoate auxotrophic *E. coli* strain JRG33-lip9 with plasmid pEL21-(+) complements the lipoate auxotrophy of JRG33-lip9 cells.

Construction of a C-Terminal Hexahistidine Tagged LipA Overproduction Strain. The *lipA* coding sequence including 834 bp of 5′-untranslated sequence was amplified from plasmid pEL21(+) by the polymerase chain reaction using the following primers: LipA5′His (CGGATCCCCATCGAAGGAAGCTATG) and LipA3′His (GCGGATCCGACTTAACTTCCATCCCTTTCGC). Primer LipA3′His substitutes a serine codon for the *lipA* stop codon and includes a *Bam*HI restriction site that allows in-frame ligation to the hexahistidine linker sequence of *Bam*HI digested pET23a. The resulting plasmid was designated pLipA-His.

Overproduction of Wild-Type and Hexahistidine-Tagged LipA in E. coli. Cultures of BL21(DE3) *E. coli* containing the LipA overexpression plasmids pEL21(+) or pLipA-His were grown in a modified Terrific Broth (mTB: 8 mL/L glycerol, 12 g/L casamino acids, 23.9 g/L yeast extract, 5.0 g/L NaCl, 12.3 g/L $K_2HPO_4 \cdot 3\ H_2O$ and 2.2 g/L KH_2PO_4). Single colonies were picked from fresh agar plates and inoculated into 100 mL of mTB containing 300 μg/mL ampicillin. Cultures were incubated at 37 °C with shaking (250 rpm) overnight and were centrifuged at 5000*g* for 10 min and diluted 1:100 in fresh mTB media containing 300 μg/mL ampicillin. Cells were grown at 37 °C with shaking in baffled flasks (250 rpm) or a 12 L New Brunswick Scientific fermenter (300 rpm, 5 L/min aeration). Upon reaching an A_{600} of 0.6, cells were induced by addition of IPTG to a final concentration of 400 μM. The temperature was reduced to 20 °C upon induction and expression was allowed to proceed for 7 h. Cells were harvested by centrifugation at 12000*g* at 4 °C for 15 min and stored at −80 °C for up to 3 months.

Purification of Soluble Wild-Type LipA. All buffers and columns used during purification of LipA were maintained at 4 °C and continuously sparged with purified argon. *E. coli* BL21(DE3) cells (40 g of wet weight) overexpressing wild-type LipA from plasmid pEL21 were thawed at 4 °C and resuspended in 50 mL of buffer A [25 mM HEPES, pH 7.5, 20% (v/v) glycerol, 1 mM benzamidine, 50 μM AEBSF, and 5 mM β-mercaptoethanol]. Cells were disrupted at 16 000 psi using a French pressure cell (SLM Instruments, Rochester, NY) previously chilled to 4 °C. Cell lysate was collected directly into centrifuge tubes under a constant stream of purified argon. The lysate was centrifuged at 100000*g* for 45 min at 4 °C, and the resulting dark brown supernatant was immediately loaded onto a 5 × 21 cm Macro-Prep CHT-II ceramic hydroxyapatite column previously equilibrated with buffer A. The column was washed with 400 mL of buffer A (1.5 mL/min) then eluted with a 750-mL gradient from 0 to 35% buffer B [500 mM potassium phosphate, pH 7.5, 20% (v/v) glycerol, 1 mM benzamidine, 50 μM AEBSF, and 5 mM β-mercaptoethanol] followed by a 275-mL gradient from 35 to 100% buffer B. After 275 mL of the 0−35% buffer B gradient had flowed through the column, the eluate was diverted onto a 5 × 10 cm TSK DEAE 650M anion exchange column previously equilibrated with buffer A. Protein that did not bind to the DEAE column was collected in 12-mL fractions. Dark brown fractions were pooled (∼400 mL) and concentrated to 50 mL under purified argon using an Amicon stirred cell equipped with a YM30 membrane. The concentrated LipA was diluted to 150 mL with buffer A and loaded onto a 2.5 × 25 cm Macro-Prep CM cation exchange column previously equilibrated with buffer A. The column was washed with 200 mL of buffer A at 1.75 mL/min and eluted with a 600-mL gradient from 0 to 30% buffer C [25 mM HEPES, pH 7.5, 20% (v/v) glycerol, 2 M NaCl, and 5 mM β-mercaptoethanol] also at 1.75 mL/min. Fractions (8 mL) were collected starting at the beginning of the sodium chloride gradient. LipA typically eluted between 200 and 300 mM sodium chloride. Dark brown fractions were pooled (∼160 mL) and concentrated to 15 mL or less as previously described. The concentrated LipA was split into two portions and loaded onto two Superdex 200 HiLoad 26/60 gel filtration columns previously equilibrated with buffer D [25 mM HEPES, pH 7.5, 10% (v/v) glycerol, 100 mM NaCl, and 5 mM β-mercaptoethanol]. Fractions (3 mL) were collected at a flow rate of 1.2 mL/min. Monomeric and dimeric LipA constitute the major protein peaks eluting from the gel filtration column and were separately concentrated to 150 μM LipA as described above and stored in 1 mL aliquots at −80 °C. Typical yields were 5−10 mg of dimeric LipA and 30−40 mg of monomeric LipA.

Analytical Gel Filtration. LipA (3 mg) or protein molecular weight standards (750 μg each of β-amylase, alcohol dehydrogenase, bovine serum albumin, egg albumin, carbonic anhydrase, and cytochrome *c* in 1.5 mL of buffer D) were chromatographed on a Superdex 200 HiLoad 16/60 gel filtration column previously equilibrated with buffer D. A standard curve of the logarithm of the molecular weight of the standards vs the elution volume was generated to calculate the apparent molecular weights of monomeric and dimeric LipA.

Purification of Soluble Hexahistidine-Tagged LipA. All buffers and columns used for the purification of LipA-His were sparged with purified argon and maintained on ice in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). *E. coli* BL21(DE3) cells (30 g of wet weight from 3L of culture) expressing hexahistidine-tagged LipA were thawed at 4 °C and resuspended in 40 mL of buffer E [25 mM HEPES, pH 7.5, 500 mM NaCl, 50 mM imidazole, 10% (v/v) glycerol, and 50 μM AEBSF]. Cells were lysed and centrifuged as described for wild-type LipA. Cell-free extract was loaded onto a 2.5 × 7 cm column of Ni-NTA agarose (Qiagen) previously equilibrated with buffer E and subsequently washed with 150 mL of buffer E. LipA-His was eluted in a minimal volume (approximately 10 mL) of buffer F [25 mM HEPES, pH 7.5, 500 mM NaCl, 250 mM imidazole, and 10% (v/v) glycerol]. Imidazole and sodium chloride were removed from the LipA-His samples using a disposable PD-10 gel filtration column previously equilibrated with buffer G [25 mM HEPES, pH 7.5, 100 mM NaCl, and 10% (v/v) glycerol]. Desalted LipA-His was reloaded onto a 1 × 5 cm column of Ni-NTA agarose equilibrated with buffer E. After washing with 50 mL of buffer E, LipA-His was eluted in a minimal volume of buffer F (6 mL or less). LipA-His was desalted into buffer G as described above and stored at −80 °C. Yields were 20−40 mg of pure LipA-His/L of culture. Slightly higher purity

could be obtained by subsequent gel filtration chromatography as described for wild-type LipA; however, this involved some exposure to oxygen and was not routinely performed. Analytical gel filtration analysis of hexahistidine tagged LipA-His was conducted as described above for wild-type LipA with the exception that buffer G was used instead of buffer D.

Analysis of Protein Purity and Concentration. Protein fractions were analyzed for purity by SDS−PAGE using precast 10−20% Tris-Glycine gels and stained with Coomassie Blue R-250. Protein concentrations were determined using the BioRad Bradford protein assay kit standardized with BSA. Quantitative amino acid analysis of purified wild-type LipA (University of Michigan Core Facility) determined that the Bradford assay overestimates the concentration of LipA by 92%. An extinction coefficient of 6000 M^{-1} cm^{-1} at 413 nm was calculated for highly purified dimeric and monomeric LipA based on Bradford assays and visible absorption spectroscopy.

Iron and Sulfide Analysis. Analysis of protein-bound iron and sulfide was conducted using 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (*40*) and *N,N*-dimethyl-*p*-phenylenediamine (*41*), respectively.

Electronic Absorbance Spectroscopy. UV/visible spectra were recorded on a Cary 3E spectrophotometer equipped with a Neslab RTE-100 temperature controller set to 10 °C.

EPR Spectroscopy. Samples were prepared in an anaerobic chamber under an atmosphere of 93% argon/7% hydrogen. Solutions of LipA were degassed using a conventional gas train with 10 vacuum/argon cycles and a 3-min equilibration period between each cycle. Dry reagents were preweighed into 1.7-mL centrifuge tubes that facilitated transfer into the anaerobic chamber. Reagents in solution were placed in Reacti-vials (Pierce, Rockford, IL) fitted with a Teflon cap and made anaerobic by repeated cycles of vacuum/argon prior to transfer to the anaerobic chamber. Stock solutions were prepared in the anaerobic chamber using deoxygenated water. Following preparation, samples were transferred to a Wilmad (Buena, NJ) EPR tube (714-PQ-8), fitted with an airtight septum and immediately frozen and stored in liquid nitrogen for no more than 24 h. All EPR spectra were obtained using a Bruker ESP300E X-band spectrometer equipped with a standard TE102 rectangular cavity and an Oxford instruments ESR-9 helium flow cryostat. Unless otherwise stated, conditions were as follows: temperature, 12K; microwave frequency, 9.49 GHz; microwave power, 1.99 mW; modulation frequency, 100.0 kHz; modulation amplitude, 10.084 G; and time constant 40.96 ms. Spin quantitations were performed by comparison to a Cu-EDTA standard of known concentration as described (*42*).

LipA Assay. All assays were conducted on ice in an anaerobic chamber unless otherwise stated. Any omissions, additions, or changes are described in the text or table legends. LipA (50 μL of a 150 μM solution) was incubated with AdoMet (500 μM final concentration) for 30 s prior to reduction with dithionite (1 mM final concentration). Reduction was allowed to proceed for 1 h after which octanoyl-ACP (500 μM final concentration) or octanoate (500 μM final concentration) was added. After 15 min, any lipoyl product formed was covalently attached to apo-pyruvate dehydrogenase complex (apo-PDC) and quantified as described below.

*Detection of Lipoate by Lipoylation of apo-PDC***.** Lipoate was transferred to the apo-E2 protein of *E. coli* pyruvate dehydrogenase complex (PDC) with LplA using a modification of the previously described procedure (*32*). The following were added to a 50 μL LipA assay: 1 μL of 1 M potassium phosphate (pH 7.0), 2 μg of purified LplA, 5 μM (final concentration) ATP, and 240 μg of apo-PDC. The reaction mixture was incubated at 37 °C for 8 min and then assayed for holo-PDC activity as described below.

Detection of Lipoyl-ACP by Lipoylation of apo-PDC. Lipoyl groups from lipoyl-ACP were transferred to apo-PDC with hexahistidine tagged LipB (LipB-His) using a modification of the previously described procedure (*17*). The following were added to a 50 μL LipA assay: 1 μL of 1 M potassium phosphate (pH 7.0), 2 μg of LipB-His, and 240 μg of apo-PDC. The reaction mixture was incubated at 37 °C for 8 min and assayed for holo-PDC activity as described below.

Assay of holo-PDC. Lipoylated (holo-) PDC was assayed spectrophotometrically for formation of reduced 3-acetylpyridine adenine dinucleotide (APAD) in the presence of pyruvate, coenzyme A, thiamin pyrophosphate, and cysteine (*17*). APAD was utilized in place of NAD due to the higher extinction coefficient of reduced APAD (*17*). The amount of holo-PDC activity observed was used to calculate the amount of lipoyl species formed by comparison to standard curves determined with lipoate or synthetic lipoyl-ACP.

Lipoylation and Purification of Apo-Lipoyl Domain for MALDI-Mass Spectrometry. LipA-His (75 nmol in 500 μL buffer G) was anaerobically mixed with 2.5 μL of 100 mM AdoMet and 5 μL of 100 mM sodium dithionite or 5 μL of buffer G. After the sample was incubated for 30 min on ice, 1.1 nmol of octanoyl-ACP was added to each tube, and the sample was incubated on ice for 15 min. Potassium phosphate (5 μL of a 1 M, pH 7.5 solution), 50 pmol of LipB-His, and 2 nmol of apo-lipoyl domain (apo-E2 domain) were added to each tube. The mixtures were incubated at 37 °C for 15 min. LipA-His and LipB-His were removed by batch binding to Ni-NTA agarose. Residual modified and unmodified E2 domain was bound to a 1-mL octyl-Sepharose column, washed with 10 mL of water, and eluted with 3 mL of 25% (v/v) 2-propanol in water. Samples were concentrated under vacuum to 20 μL and analyzed by MALDI mass spectrometry.

MALDI Mass Spectrometry. MALDI mass spectra were obtained by cocrystallization of a 1:1 (v/v) solution of matrix [saturated solution of sinapinic acid in 60% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid] and protein sample. Data were collected on a Perseptive Biosystems Voyager DE/STR MALDI instrument operating in linear positive ion mode with delayed extraction.

Preparation of Holo-Acyl Carrier Protein. Acyl carrier protein was overexpressed in *E. coli* strain DK739 (*43*) in 2× YT media containing 50 μg/mL kanamycin and 5 mM pantothenate. Cells were grown at 37 °C to mid-logarithmic phase (A_{600} of 0.6) and induced with IPTG (final concentration 500 μM). ACP was purified from 32 L of fermented culture according to the published procedure (*44*) with the following modifications: cells were lysed by French press at 16 000 psi in 10 mM Tris-Cl, final pH 7.0, and DE53 resin was used for anion exchange chromatography. Each liter of culture yielded approximately 16 mg of pure ACP

with approximately 90% of the ACP in the holoenzyme (phosphopantetheinylated) form. Complete phosphopantetheinylation of ACP was accomplished using coA and partially purified *E. coli* holo-acyl carrier protein synthase (ACPS) as described (*45*). Holo-ACP was precipitated by addition of acetic acid to pH 3.8 and resuspended at a concentration of 10 mg/mL in 10 mM Tris-HCl, pH 7.0. Concentrations of ACP species were determined by the BioRad Bradford protein assay kit using bovine serum albumin and a factor of 2.2 to correct for underestimation of ACP concentration.[2] Holo-ACP was aliquoted and stored at −80 °C until use.

Preparation of Acylated-Acyl Carrier Protein Derivatives. Octanoyl-ACP was synthesized from holo-ACP and octanoic acid using catalytic quantities of partially purified *Vibrio harveyi* acyl-ACP synthetase (AAS) (*46*). Lipoyl-ACP was synthesized in the same manner using 1 mM lipoic acid instead of octanoic acid (*17*). Acylated-ACP derivatives were purified by hydrophobic interaction chromatography on an octyl-Sepharose CL-6B column (2.5 × 10 cm) (*47*) followed by gel filtration chromatography on a Superdex 200 HiLoad 26/60 column equilibrated with 20 mM Tris-Cl, pH 7.4. Purified acyl-ACP derivatives were concentrated using 5000 MW cutoff spin filters, and the pH was adjusted to 7.0 prior to storage at −80 °C. Octanoyl-ACP is stable for at least 3 months. Lipoyl-ACP is readily deacylated and was utilized within two weeks of synthesis.

Urea-PAGE Analysis of Acyl-ACP-Derivatives. Analytical separation of apo-ACP, holo-ACP, and acyl-ACPs was accomplished using 20% polyacrylamide gels as previously described (*44*), except that the pH of the running buffer was 8.8 and 4 M urea (*48*) was included in the separation and sample buffers, respectively.

Preparation of LplA. E. coli LplA was overexpressed and purified from *E. coli* strain TM202 as described (*15*, *49*).

Preparation of LipB-His. Hexahistidine-tagged *E. coli* LipB was overexpressed and purified to >80% purity by immobilized metal affinity chromatography (Ni-NTA agarose) and gel filtration chromatography from BL21(DE3) *E. coli* carrying plasmid pSJ120[2].

Purification of Apoenzyme Complexes. Apo-PDC was purified by differential centrifugation from 22 L of fermented *E. coli* strain TM136 as previously described (*32*). Apo-lipoyl domain (apo-E2 domain) was overexpressed and purified as previously described (*18*).

RESULTS

Expression and Purification of Wild-Type LipA. Plasmid pEL21(+) encodes the *lipA* gene (*28*) under control of an inducible T7 promoter and includes 834 bp of noncoding sequence upstream of the *lipA* start codon. Chemical transformation of lipoate auxotrophic *E. coli* strain KER176 (*lipA)* (*26*) with plasmid pEL21(+) is sufficient to permit growth on lipoate deficient media. KER176 cells similarly transformed with the parent vector pET3a exhibited no growth on lipoate-deficient media. As strain KER176 does not contain the gene for T7 RNA polymerase, expression of *lipA* is likely initiated from the endogenous promoter located within the 834 bp of 5′-noncoding sequence.

[2] Jordan, S. W., and Cronan, J. E., unpublished results.

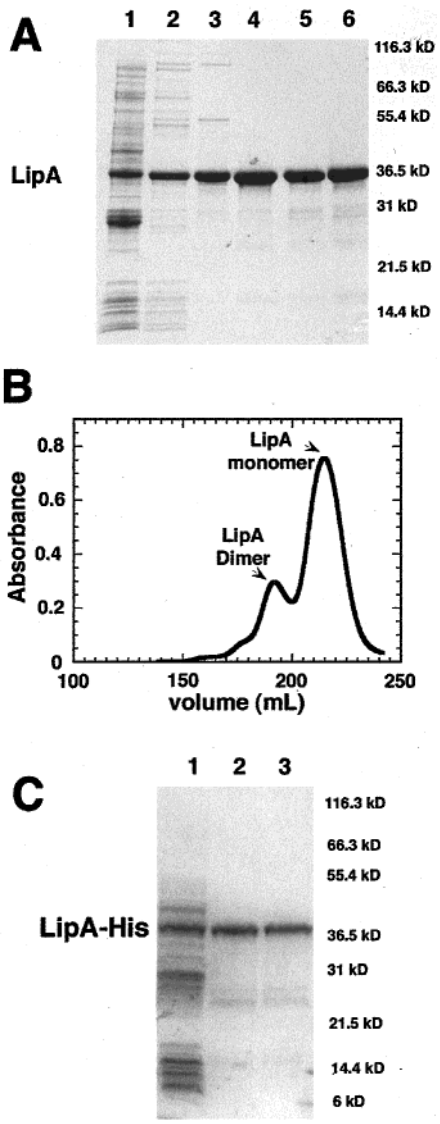


FIGURE 1: Purification of LipA and LipA-His. (A) SDS−PAGE (10−20% Tris-glycine gel) showing LipA during different stages of purification: lane 1, cell free extract; lane 2, hydroxyapatite eluate; lane 3, DEAE unbound proteins; lane 4, post-CM pool; lane 5, LipA dimer after gel filtration chromatography; lane 6, LipA monomer after gel filtration chromatography. (B) S200 gel filtration chromatogram of LipA CM pool. (C) SDS−PAGE (10−20% Tris-glycine gel) showing LipA-His during different stages of purification: lane 1, cell free extract; lane 2, pool from first Ni-NTA column; lane 3, pool from second Ni-NTA column.

IPTG induction of BL21(DE3) *E. coli* containing plasmid pEL21(+) results in overproduction of a protein that migrates at 36.5 kD on SDS−PAGE (Figure 1A, lane 1). The majority of this protein is found in the soluble fraction of cell lysates. The procedure for purification of LipA described in this paper is a modification of a previous method (*29*) and generates protein of significantly higher purity in much greater yield.

Soluble LipA is purified using hydroxyapatite, anion exchange, cation exchange, and gel filtration chromatography and is approximately 95% pure by SDS−PAGE (Figure 1, panel A, lane 6). LipA is resolved by gel filtration chromatography into two distinct peaks, each dark brown in color (Figure 1, panel B). The apparent molecular masses of these species as determined by analytical gel filtration chromatography are 43.3 ± 1.1 kD and 84.6 ± 6.2 kD, consistent

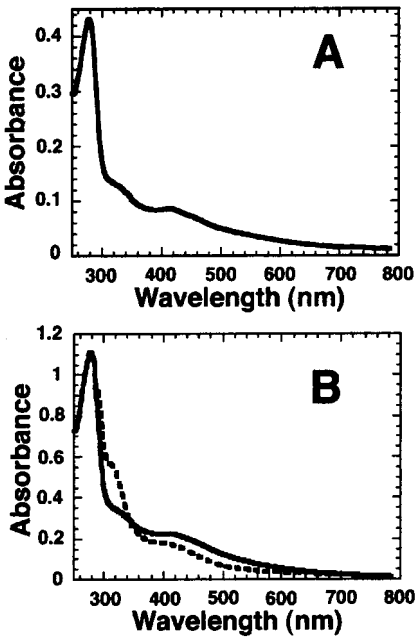


FIGURE 2: Electronic absorbance spectra of purified LipA. (A) Dimeric LipA (15 μM). (B) Monomeric LipA (37 μM) as isolated (solid line) and after reduction with sodium dithionite (300 μM) for 1 h at 0 °C (dotted line).

with monomeric and dimeric forms of LipA (predicted monomeric molecular weight of 36.1 kD). Multiple LipA preparations consistently resulted in 70% monomeric LipA. N-terminal sequence analysis confirmed the identity of the purified protein as LipA (data not shown).

Expression and Purification of LipA Containing a C-terminal Hexahistidine Tag (LipA-His). Purification of wild-type LipA is a relatively lengthy process (>48 h) and involves unavoidable exposure to oxygen. To address these problems, a *lipA* construct containing a C-terminal hexahistidine tag (*lipA*-His) was constructed and expressed in BL21-(DE3) *E. coli*. IPTG induction results in synthesis of large quantities of a protein that migrates at approximately 38 kD on SDS−PAGE (Figure 1, panel C). The protein is efficiently purified (>90% pure by SDS−PAGE) within an anaerobic chamber by two successive rounds of immobilized metal affinity chromatography using Ni-NTA agarose (Figure 1, panel C). Similar to the wild-type protein, LipA-His is isolated as a mixture of 70% monomer and 30% dimer. No differences in spectrophotometric properties or activity are observed between wild-type LipA and LipA-His. *E. coli* lipoate auxotrophic strain KER176 (*lipA*) (*26*), chemically transformed with pLipA-His, can grow in lipoate-deficient media.

Electronic Absorbance Spectroscopy. The electronic absorbance spectra of dimeric and monomeric wild-type LipA are shown in Figure 2, panels A and B, respectively. The spectra are identical and have absorbance maxima at 278 and 413 nm with shoulders present at 343 and 455 nm. The spectra are most similar to that previously reported for soluble monomeric LipA (*29*) and anaerobically purified pyruvate formate lyase activating enzyme (PFL-AE) (*50*) but differ considerably from the featureless spectrum of reconstituted/refolded LipA (*31*). The electronic absorbance spectra are consistent with a mixture of [3Fe-4S] (*51*) and [4Fe-4S] (*52*) clusters in monomeric and dimeric LipA. Addition of sodium dithionite to either form of LipA results in a decrease in the absorbance maxima at 413 nm and a shift to 420 nm (Figure 2, panel B), consistent with reduction of [3Fe-4S] clusters to [4Fe-4S] clusters. The electronic absorbance spectra of LipA reduced with sodium dithionite is similar to that previously reported for soluble dimeric LipA (*29*). In each case, the electronic absorbance spectra of LipA-His are indistinguishable from those of the wild-type protein (data not shown).

Iron and Sulfide Content. Analysis of protein-bound iron and acid-labile sulfide in wild-type LipA shows 3.4 ± 0.4 iron atoms and 4.8 ± 0.8 sulfur atoms/monomer ($n = 3$) and 6.6 ± 0.8 iron atoms and 8.4 ± 1.0 sulfur atoms/dimer ($n = 3$) (Table 1). These results are inconsistent with previous determinations of LipA iron and sulfide content (Table 1) (*29*, *30*). This discrepancy is likely due to oxidative loss of iron−sulfur cluster in the earlier preparations. The iron and sulfide content of soluble LipA are consistent with those recently reported for anaerobic preparations of reconstituted and refolded LipA (Table 1) (*31*). These results corroborate the electronic absorbance spectroscopic results in Figure 2 and support the presence of [3Fe-4S] and/or [4Fe-4S] clusters in both monomeric and dimeric LipA.

Electron Paramagnetic Resonance Spectroscopy of LipA. Monomeric and dimeric LipA were analyzed by electron paramagnetic resonance (EPR) spectroscopy, and both exhibited a nearly isotropic EPR signal centered at $g = 2.006$ (spectrum of monomeric LipA is shown in Figure 3, panel A). The amount of this species varied between successive preparations but never comprised more than 15% of the sample. The characteristics of this signal, fast relaxation (not detectable above 30 K), low anisotropy, and a g value of 2.006, suggest the presence of a $[3Fe\text{-}4S]^{1+}$ cluster in a subpopulation of the LipA molecules. Samples of LipA-His exhibited a nearly identical signal (Figure 3, panel B) and contained only trace amounts (<1% of total iron) of noncluster associated ferric iron (data not shown).

Reduction of wild-type LipA or LipA-His with a 10-fold molar excess of sodium dithionite generates a nearly axial EPR signal with g values of (2.06, 1.95, and 1.92) (spectrum of reduced LipA-His is shown in Figure 3, panel C), similar to that reported previously for refolded/reconstituted LipA reduced with photoreduced 5-deazariboflavin (*31*). This signal is not detected above 30 K, consistent with the relaxation properties of a $S = 1/2$ $[4Fe\text{-}4S]^{1+}$ cluster (data not shown). Addition of a 10-fold excess of AdoMet prior to reduction causes only small changes in the signal features (Figure 3, panel D). The amount of $[4Fe\text{-}4S]^{1+}$ cluster generated by dithionite reduction varies between different preparations of LipA. Spin quantitation using a Cu-EDTA standard shows that the $[4Fe\text{-}4S]^{1+}$ cluster is formed in 1−3% of wild-type LipA polypeptides and up to 5% of LipA-His polypeptides. The addition of AdoMet has no effect on the amount of $[4Fe\text{-}4S]^{1+}$ cluster generated by dithionite reduction.

Development of an in Vitro LipA Assay. Lipoyl-acyl carrier protein and/or lipoate were considered the most likely products of LipA turnover, and a successful in vitro LipA assay system should detect and quantify either product. Previous work has shown that enzymatic lipoylation of *E. coli* apo-pyruvate dehydrogenase complex (PDC) to produce holo-PDC is a sensitive and highly specific means of

Table 1: Iron and Acid Labile Sulfide Content of LipA from Various Preparations

	soluble LipA monomer (current protocol)[a]	soluble LipA dimer (current protocol)[a]	soluble LipA dimer (previous protocol)[b]	refolded reconstituted LipA[c]	refolded reconstituted LipA[d]
iron/polypeptide	3.4 ± 0.4	3.3 ± 0.4	1.8 ± 0.2	1.8−2.3	3.7−4.2
sulfide/polypeptide	4.8 ± 0.8	4.2 ± 0.5	2.2 ± 0.4	1.8−2.3	3.7−4.2

[a] This paper. [b] Ref *29*. [c] Ref *30*. [d] Ref *31*.

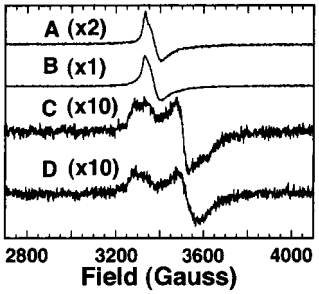


FIGURE 3: Electron paramagnetic resonance spectra of LipA and LipA-His. Conditions and sample preparation were as described in Materials and Methods. (A) LipA as isolated (150 μM). (B) LipA-His as isolated (167 μM). (C) LipA-His (167 μM) after reduction with 1 mM sodium dithionite without exogenous AdoMet. (D) LipA-His (167 μM) and 1 mM AdoMet after reduction with 1 mM sodium dithionite.

detecting lipoate or lipoyl-ACP (*16*, *17*, *32*). This assay is particularly appealing, as apo-PDC exhibits no enzymatic activity and the fact that the lipoyl moieties are used catalytically in holo-PDC gives a large amplification of the signal. Lipoylation of the apo-enzyme generates active holo-PDC that can then be assayed spectrophotometrically for pyruvate-dependent pyridine dinucleotide reduction (*32*).

Free lipoate was assayed using purified lipoate-protein ligase A (LplA) and apo-PDC (*16*, *32*). The amount of holo-PDC activity increased with increasing amounts of lipoate and exhibited saturation kinetics, consistent with a limited number of lipoate binding sites on apo-PDC (Figure 4, panel A). PDC showed 50% of maximal activity with 1.9 nmol of lipoate. PDC activity as a function of lipoate concentration is nearly linear in the range of 0−2 nmol of lipoic acid (inset of Figure 4, panel A). Lipoyl-ACP was similarly assayed using LipB-His and apo-PDC (*17*, *32*). Holo-PDC activity was dependent on lipoyl-ACP concentration with 50% of maximal holo-PDC activity observed with 250 pmol of lipoyl-ACP (Figure 4, panel B). The functional linear range of the assay is between 0 and 100 pmol of lipoyl-ACP (inset of Figure 4, panel B). The activity of fully reconstituted PDC differs between Figure 4, panels A and B, due to the use of different apo-PDC preps having different specific activities.

In vitro Lipoyl-Synthase Activity of LipA. LipA or LipA-His were incubated with either octanoic acid or octanoyl-ACP under a variety of conditions (Table 2). Each reaction was supplemented with apo-PDC and either LplA (octanoic acid reaction) or LipB-His (octanoyl-ACP reaction). Active holo-PDC was formed only in assays containing both reduced LipA or LipA-His and octanoyl-ACP. LipA-His exhibits slightly higher activity than the monomeric and dimeric forms of wild-type LipA, which exhibit similar activities. PDC activity increased with increasing amounts of reduced LipA or LipA-His (data not shown). Assays that either contained octanoic acid or that omitted reducing agents produced no active holo-PDC. The former result indicates

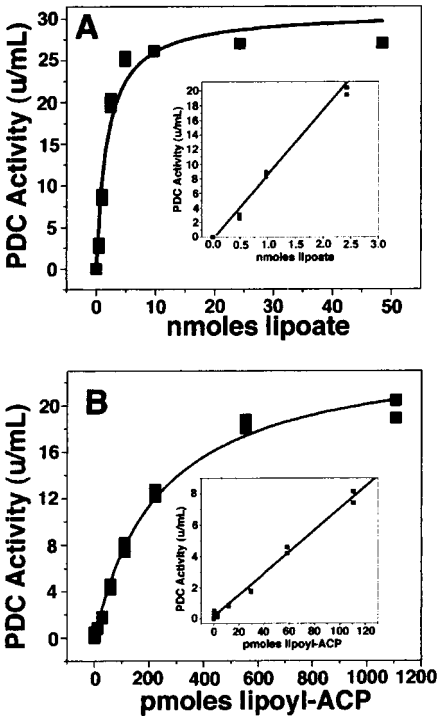


FIGURE 4: Activation of *E. coli* apo-PDC by lipoate and lipoyl-ACP. Assay conditions are described in Materials and Methods. (A) PDC activity resulting from incubation of increasing concentrations of lipoate with apo-PDC and LplA. (Inset) Linear region of assay utilized as a standard curve. (B) PDC activity resulting from incubation of increasing concentrations of lipoyl-ACP with apo-PDC and LipB-His. (Inset) Linear region of assay utilized as a standard curve. The differences in maximum PDC activity between the two assays are due to use of PDC preparations with different specific activities. One unit of PDC activity is defined as 1 μmol of reduced APAD formed/min.

that octanoate is not a substrate for LipA. Addition of AdoMet to LipA results in an approximately 6-fold increase in active PDC (Table 2), whereas a similar quantity of *S*-adenosyl homocysteine (SAH) slightly reduces LipA activity. Inclusion of Fe^{2+}, Fe^{3+}, or S^{2-} resulted in only small increases in activity (data not shown).

Although an excess of octanoyl-ACP substrate is present in each assay, the amount of lipoyl product formed is significantly less than the amount of LipA or LipA-His (Table 2). The errors in these determinations are due to large variations in lipoyl synthase activity among different preparations of LipA. Under the conditions of the assay, nonproductive octanoylation of apo-PDC (*18*) does not appear to reduce the number of available lipoylation sites on apo-PDC, as adding additional octanoyl-ACP after a LipA assay does not alter the amount of lipoyl-PDC produced. Consistent with this observation, incubation of lipoyl-ACP and a 1000-fold excess of octanoyl-ACP with LipB-His and apo-PDC results in less than a 10% decrease in PDC activity relative to that observed without octanoyl-ACP (data not shown). These

Table 2: LipA Assay Results[a]

reaction	LipA species	relative activity[b] (%)	mol of product/ mol of LipA polypeptide
complete	monomer	100	0.017 ± 0.011[c]
complete	dimer	94	0.016 ± 0.010[d]
minus LipA	monomer or dimer	<2	<0.0005
minus C8-ACP	monomer or dimer	<2	<0.0005
minus sodium dithionite	monomer or dimer	<2	<0.0005
minus AdoMet	monomer	15	0.0030[e]
minus AdoMet, plus *S*-Adenosyl homocysteine	monomer	8	0.0017[e]
minus apo-PDC and LipB-His	monomer	<2	<0.0005
minus LipA and LipB-His, plus LplA and octanoic Acid	monomer or dimer	<2	<0.0005
minus LipB-His, plus LplA and octanoic acid	monomer	3	<0.0005
minus LipB-His, plus LplA and octanoic acid	dimer	2	<0.0005
complete	LipA-His monomer and dimer	190	0.032 ± 0.010[f]
minus C8-ACP, plus octanoylated-PDC	LipA-His monomer and dimer	<2	<0.0005

[a] Assays were performed as described in Materials and Methods. The standard reaction mixture contained LipA, sodium dithionite, AdoMet, octanoyl-ACP (C8-ACP), LipB-His, and apo-PDC. Additions or omissions are indicated in the table. [b] Relative activity was calculated as the percentage of holo-PDC activity observed relative to that observed with monomeric LipA and octanoyl-ACP. [c] Average ± standard error from five LipA preparations. [d] Average ± standard error from three LipA preparations. [e] Average of two LipA-His preparations. [f] Average ± standard error from three LipA-His preparations.

Table 3: Equivalence of LipA Lipoyl Product Concentration and $[4Fe\text{-}4S]^{1+}$ Cluster Concentration[a]

sample	LipA conc [μM]	$[4Fe\text{-}4S]^{1+}$ cluster conc [μM]	Lipoyl product [μM]	ratio of product to $[4Fe\text{-}4S]^{1+}$ cluster
wild-type LipA monomer (prep 1)	113	3.8	5.4	1.4
wild-type LipA monomer (prep 2)	141	2.2	2.3	1.0
LipA-His monomer and dimer	167	5.3	6.0	1.1

[a] Samples were prepared from three separate LipA or Lip-His preparations as described. Lipoyl product concentrations were determined by apo-PDC assay as described in Materials and Methods, and $[4Fe\text{-}4S]^{1+}$ cluster concentrations were determined by spin quantitation of EPR spectra using a Cu-EDTA standard.

results suggest the affinity of LipB for octanoyl-ACP is lower than for lipoyl-ACP.

The Amount of Lipoyl Product is Equivalent to the Amount of $[4Fe\text{-}4S]^{1+}$ Cluster Generated by Sodium Dithionite Reduction. Reduction of LipA with sodium dithionite generates substoichiometric quantities of $[4Fe\text{-}4S]^{1+}$ cluster, suggesting that protein reduction is a limiting factor in the formation of the lipoyl product. Spin quantitation of the amount of $[4Fe\text{-}4S]^{1+}$ cluster generated by sodium dithionite reduction and comparison to the amount of lipoyl product formed showed these values were similar in three preparations of wild-type LipA and LipA-His (Table 3).

Identification of a Lipoylated Product of the LipA Reaction. The presumed product of the LipA reaction with octanoyl-ACP is lipoyl-ACP. Lipoyl-ACP is a substrate for LipB, and free lipoyl-ACP is found in cellular extracts from pea (*Pisum sativum*) and the fungus, *Neurospora crassa* (*17*). *E. coli* appears to contain little, if any, free lipoyl-ACP (*17*). Several attempts were made to isolate this species from LipA reactions prior to the addition of LipB and/or apo-PDC. Lipoyl-ACP, octanoyl-ACP, and monothiooctanoyl-ACPs can be separated and detected using polyacrylamide gel electrophoresis (*44*) in the presence of urea (*48*). No lipoyl-ACP or monothiooctanoyl-ACPs were detected by urea-PAGE in LipA reactions that contained lipoyl product as determined by apo-PDC assay (data not shown). This amount of lipoyl product formed by LipA was sufficient for detection by urea-PAGE, as identical quantities of exogenous lipoyl-ACP were readily detected. Additional attempts were made to isolate the lipoyl product by hydrophobic, immobilized metal affinity, and size exclusion chromatography. All were unsuccessful, as the product was unstable to chromatography. Inclusion of LipB-His in the reaction mix did not result in product detection by any of the above techniques. Further, no free lipoate was detected in LipA reactions assayed with LplA and apo-PDC.

Our inability to isolate a free lipoyl-ACP product suggests that the product remains associated with LipA. Consistent with this hypothesis, the lipoyl product and LipA were both retained by membranes that exclude molecules with a mass greater than 30 kDa (data not shown). Exogenous lipoyl-ACP, which has a mass of approximately 9 kD, was not retained by the membrane. The possibility of a covalent lipoyl-product/LipA adduct was tested by reduction of LipA and addition of [1-^{14}C]-octanoyl-ACP and subsequent analysis by SDS−PAGE at neutral pH. No radioactivity was found associated with LipA (data not shown). These results cannot rule out a noncovalent or unstable lipoyl product−LipA complex. This concept will be addressed further below.

Direct evidence for a lipoyl-containing product of the LipA reaction was obtained by transfer of the lipoyl moiety of the product to a stable acceptor protein. LipB-His was utilized to catalyze transfer of the acyl moiety of the LipA reaction product to a recombinant lipoyl-binding domain of the E2 subunit of PDC (apo-E2 domain). The apo-E2 domain is approximately 8.8 kD and contains a single lipoylation site (*18*). The small size of the E2 domain facilitates analysis of the acylation state by mass spectrometry.

LipA-His with a 10-fold molar excess of AdoMet was reduced with sodium dithionite, or an equivalent volume of water was added for 1 h prior to addition of octanoyl-ACP, LipB-His, and an excess of the apo-E2 domain. The resulting acylated and apo-E2 domains were purified and subsequently analyzed by MALDI mass spectrometry to determine the identity of the acyl moieties (Figure 5). A species with a mass identical to lipoylated-E2 domain (8974.1 Da) was observed when sodium dithionite reduced LipA-His was

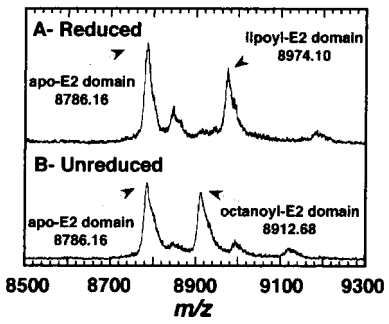


FIGURE 5: Reduced LipA catalyzes synthesis of lipoyl groups from octanoyl-ACP. MALDI mass spectra of purified E2 domains after incubation with octanoyl-ACP, LipB-His, apo-E2 domain, AdoMet, and (A) LipA-His reduced with sodium dithionite or (B) LipA-His.

included in the assay (Figure 5, panel A). Reactions containing unreduced LipA-His do not produce any detectable lipoyl product from octanoyl-ACP (Table 2) and in this assay result in an acylated E2-domain identical in mass to octanoyl-E2 domain (8912.7 Da, Figure 5, panel B). The difference in mass between the acylated E2-domains in Figure 5, panels A and B, is 62 Da, consistent with insertion of two sulfur atoms and loss of two hydrogen atoms in the sample containing reduced LipA (Schemes 1 and 2). Apo-E2 domain is present in excess of octanoyl-ACP and thus is detected in both reactions. No species corresponding to the octanoyl or monosulfurated products (i.e., 6-thiooctanoyl-E2 domain or 8-thiooctanoyl-E2 domain) were observed with reduced LipA.

DISCUSSION

The family of proteins that utilizes iron−sulfur clusters and AdoMet to perform radical chemistry is rapidly growing. Although the chemistry catalyzed by each protein in this family [e.g., glycyl radical formation in pyruvate formate lyase by PFL-AE (*53*) or sulfur insertion in BS (*54*, *55*)] is different, certain characteristics are shared by all members of the family: (i) all contain a CxxxCxxC iron−sulfur cluster binding motif which is the only sequence similarity between many members of this family; (ii) all contain an iron−sulfur cluster that cycles between various redox states; and (iii) all require AdoMet for full activity. Lipoyl synthase had conformed to only two of these requirements as it contains the requisite cluster binding sequence motif (*26*, *28*) and an iron−sulfur cluster (*29*−*31*). However, the lack of a functional activity assay had prevented the determination of a requirement for AdoMet and precluded classification of LipA as a member of this protein family. The results described in this manuscript demonstrate in vitro lipoyl synthase activity with purified LipA and place LipA in the family of AdoMet-dependent iron−sulfur proteins.

Since our initial report on LipA (*29*), advances in the expression and purification of LipA have been made that greatly increase the yield and purity of this protein. These modifications allow separation of LipA into monomeric and dimeric forms (Figure 1, panel B). It is unclear whether one or both oligomeric forms of LipA are physiologically relevant, as both show identical lipoyl synthase activities and spectroscopic properties. We previously reported differences in the electronic absorbance spectra and cluster content between these two species (*29*); however, these differences now appear to be the result of oxidative damage to the protein during purification. LipA exhibits extreme oxygen sensitivity, as observed by ourselves[3] and others (*30*, *31*). Although the purification procedure for wild-type LipA produces large quantities of protein, a faster method of purification adaptable to an anaerobic chamber was needed. The closely related *E. coli bioB* (biotin synthase) gene product has been expressed and purified with a C-terminal hexahistidine tag with no loss in activity or change in properties (*56*). On the basis of the success of these results, a hexahistidine tag was incorporated into the C-terminus of LipA. The hexahistidine-tagged protein shows lipoyl synthase activity and is indistinguishable from wild-type soluble LipA in oligomeric state and spectroscopic properties.

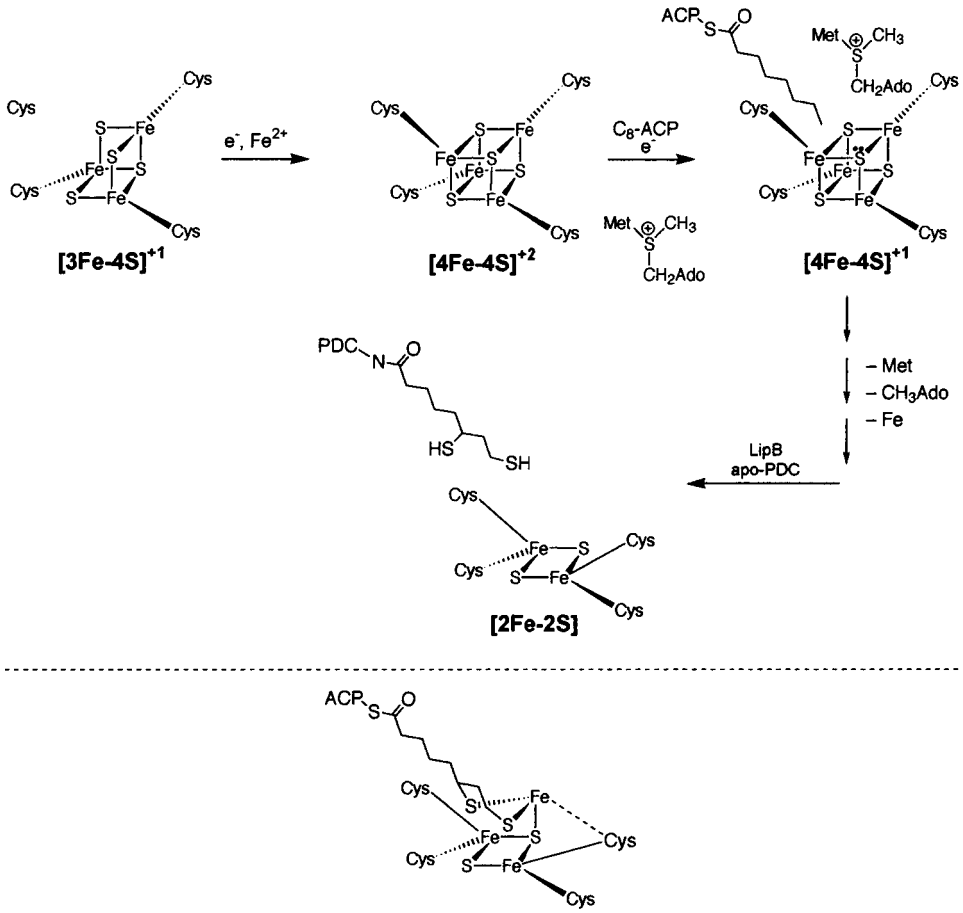
The members of the AdoMet-dependent iron−sulfur protein family exhibit multiple forms of redox active iron−sulfur clusters including [2Fe-2S], [3Fe-4S], and [4Fe-4S] (*55*, *57*−*59*). Soluble LipA, as isolated, contains a mixture of $[3Fe\text{-}4S]^{1+}$ clusters and EPR-silent clusters consistent with the electronic absorbance spectroscopic properties of $[4Fe\text{-}4S]^{2+}$ clusters. Reduction of LipA with sodium dithionite results in a mixture of EPR active $[4Fe\text{-}4S]^{1+}$ and EPR-silent clusters with an electronic absorbance spectrum consistent with a $[4Fe\text{-}4S]^{2+}$ cluster state. This conversion occurs in the absence of exogenous ferrous iron and does not require addition of exogenous AdoMet. Reduction of LipA to the $S = 1/2$ $[4Fe\text{-}4S]^{1+}$ state has been reported for refolded/reconstituted LipA (*31*), and we provide confirmation that the native protein behaves similarly. The small amount of $[4Fe\text{-}4S]^{1+}$ cluster generated by sodium dithionite reduction of LipA is not an indication of inactive or damaged protein, as we have recently determined that photoreduced 5-deazariboflavin can generate greater quantities of the $[4Fe\text{-}4S]^{1+}$ cluster than sodium dithionite.[4] Additionally, protein components present in *E. coli* lysates are observed to increase LipA activity severalfold.[5] These results suggest that the in vitro LipA assay described within this paper is a starting point for development of a more robust system.

Although progress on the biophysical characterization of LipA reported here and by others (*31*) has been made, further research on LipA requires both an in vitro assay that can detect a lipoyl product(s) and the demonstration of lipoyl synthase activity by the recombinant protein. We took advantage of the endogenous protein lipoylation pathways of *E. coli* (Scheme 2) as a source of assay components (*32*). These assays are sensitive and quantitative (Figure 4) and offer the benefit of detecting free lipoate and lipoyl-ACP. The principal disadvantage is that the assays are indirect and detect lipoylation of apo-PDC rather than the lipoyl species itself. Thus, the exact identity of the lipoyl product of the LipA reaction cannot be determined by the assay.

Previous biochemical (*17*) and genetic (*16*) studies of *E. coli* lipoate biosynthesis have indicated that octanoyl-ACP is the immediate precursor to lipoate. Indeed, free octanoate is not a substrate for LipA, whereas octanoyl-ACP is (Table 2). Observation of lipoyl synthase activity requires only a strong reductant, octanoyl-ACP, LipB, and a lipoyl-accepting

[3] Busby, R. W., and Marletta, M. A., unpublished results.
[4] Miller, J. R., Henshaw, T. F., Cheek, J., Broderick, J. B., and Marletta, M. A., unpublished results.
[5] Miller, J. R., and Marletta, M. A., unpublished results.

Scheme 3: Proposed Mechanism of the LipA-catalyzed Reaction Showing a Hypothetical Intermediate of the LipA Reaction[a]



[a] The first three species shown in the upper portion of the scheme have all been observed. Although we have evidence consistent with the formation of an ACP-lipoate-[FeS] complex similar to the hypothetical structure shown in the lower portion of the scheme, we have not directly observed this species. The formation of the final product, lipoylated-PDC, was detected by PDC activity and by mass spectrometry of an isolated lipoyl domain from the E2 subunit of PDC (Figure 5). Note that although the final step of the mechanism is shown as transfer to apo-PDC, transfer to other lipoic acid-dependent enzymes such as α-ketoglutarate dehydrogenase or the glycine cleavage enzyme can also occur.

apoprotein (e.g., pyruvate dehydrogenase complex). It has not been determined if a lipoyl product is formed by LipA in the absence of LipB and apo-PDC. LipA activity increases from a basal level upon addition of AdoMet and is decreased by the AdoMet analogue *S*-adenosyl homocysteine. These results suggest that LipA copurifies with less than a full complement of AdoMet and that the AdoMet that is bound to LipA can be displaced by *S*-adenosyl homocysteine. This is consistent with results obtained with other members of the AdoMet-dependent family of iron−sulfur proteins (*34*, *54*, *58*, *60*). Analogous to biotin synthase (*61*−*63*), no exogenous sulfur source is required for lipoyl synthase activity, suggesting that the source of the sulfurs in the dithiolane ring of lipoate is the LipA protein itself. It should be noted that octanoyl-PDC is not a substrate for LipA in vitro, consistent with previous in vivo experiments (*26*).

As the apo-PDC assay for lipoyl synthase activity is indirect, it was desirable to confirm lipoyl synthesis via a separate analytical technique. Additionally, knowledge of the identity of the LipA reaction product could provide information about the reaction mechanism. The logical product of the LipA reaction is lipoyl-ACP, as octanoyl-ACP is the only known LipA substrate (Table 2), and LipB transfers only ACP-esterified acyl groups to unlipoylated apoproteins (*17*). Multiple attempts to isolate free lipoyl-ACP, thiooctanoyl-ACP, or free lipoate from the LipA reaction mixtures were unsuccessful. Further, no evidence for a stable covalent association of the lipoyl product with LipA was observed. In every case, the product behaves as if it is tightly associated with LipA but is unstable to chromatographic analysis. We hypothesized that enzymatic transfer of the lipoyl product to an unlipoylated apoprotein would facilitate detection. When LipB−His and recombinant apo-E2 domain (*18*) were added to the LipA assay, lipoylation of the E2 domain was observed by MALDI mass spectrometry (Figure 5). The mass of the lipoylated E2 domain formed in the LipA reaction is consistent with an oxidized lipoyl moiety. This does not imply that an oxidized lipoyl species is the immediate product of the LipA reaction, as the acylated E2 domains were purified aerobically. Under these conditions, a dihydrolipoyl (reduced) product would be readily oxidized.

No monothiooctanoyl-E2 domains were detected in this reaction or on urea polyacrylamide gel electrophoresis, suggesting that monosulfurated-ACP products are not released from LipA as intermediates. However, we cannot rule out the possibility that the high ratio of LipA to octanoyl-ACP in these experiments precludes detection of small concentrations of intermediates or that free monothiooctanoic acids are released as intermediates not detectable by our assay techniques. These results are in marked contrast to BS, in

which both free biotin and an unidentified intermediate (proposed to be a 9-mercaptodethiobiotin derivative) can be isolated from reaction mixtures (*64*).

Reduction of LipA is required for product formation, as evidenced by the lack of E2-domain lipoylation in the unreduced protein sample shown in Figure 5. The amount of product formed is approximately equal to the amount of LipA in the $[4Fe\text{-}4S]^{1+}$ state (Table 3). These results suggest that the $[4Fe\text{-}4S]^{1+}$ cluster is the active form of the protein and that one LipA monomeric unit is responsible for both sulfur insertion reactions within a single turnover. These hypotheses are currently under investigation.

Ugulava and colleagues have proposed for dimeric BS that each monomer containing a [4Fe-4S] cluster is responsible for formation of one of the two C−S bonds in biotin (*56*). Thus, BS dimers that lack full cluster occupancy could release the 9-mercaptodethiobiotin derivative described above as an intermediate. A second molecule of BS could rebind this intermediate and convert it to biotin. This hypothesis is consistent with the 2:1 stoichiometry of AdoMet consumption to biotin formation (*36*, *64*). Such a scenario may not occur with LipA, as both monomeric and dimeric LipA are competent for lipoyl product formation, and no monosulfurated-ACP products or other reaction intermediates were detected.

A mechanism for LipA lipoyl biosynthesis that is consistent with the results reported above is shown in the top portion of Scheme 3. The mixture of $[3Fe\text{-}4S]^{1+}$ and $[4Fe\text{-}4S]^{2+}$ clusters in the protein as isolated is reduced to the active $[4Fe\text{-}4S]^{1+}$ state by dithionite in the in vitro assay. However, flavodoxin and flavodoxin (ferredoxin) reductase (*65*) are likely the physiological reductants. AdoMet and octanoyl-ACP are proposed to bind the reduced protein, and the $[4Fe\text{-}4S]^{1+}$ cluster is likely used to reductively cleave AdoMet to generate methionine and a 5′-deoxyadenosyl radical. This deoxyadenosyl radical could then abstract a hydrogen atom from the alkyl chain of octanoyl-ACP generating an alkyl radical that would recombine with a cluster sulfide to form a carbon−sulfur bond. Repetition of this process could generate a lipoyl-ACP−iron−sulfur cluster similar to the hypothetical species shown in the bottom portion of Scheme 3. This species remains a part of the cluster until the lipoyl moiety is removed by LipB in an undetermined manner and transferred to an unlipoylated apoprotein (e.g., apo-PDC). This leaves a [2Fe-2S] cluster in LipA that is inactive for additional lipoyl synthesis until reconstituted, thus allowing only a single turnover to occur in our in vitro LipA assay system.

The proposed LipA mechanism is appealing as it accounts for our inability to isolate a free lipoyl product from the LipA reaction. However, the instability of the lipoyl product observed in our in vitro reactions is difficult to reconcile with a lipoyl-ACP−iron−sulfur species such as that shown in the lower portion of Scheme 3. This lipoyl-ACP−iron−sulfur cluster should either remain stably associated with LipA or decompose to release free lipoyl-ACP. Little chemical precedent exists for the mechanism in Scheme 3. However, the use of iron−sulfur cluster sulfides to catalyze nucleophilic chemistry has some biochemical precedent in the reaction of spinach thioredoxin reductase (*66*). Staples and colleagues proposed nucleophilic attack by a μ_3-sulfide of a $[4Fe\text{-}4S]^{1+}$ cluster on an active site disulfide to initiate thiol-disulfide interchange chemistry resulting in reduced thioredoxin (*67*, *68*). Recently, Bui and colleagues have shown that in BS, [2Fe-2S] clusters can be converted to [4Fe-4S] clusters by the NifS (cysteine desulfurase) protein of *Azotobacter vinelandii* (*69*). The BS produced by this reaction is fully functional. As *E. coli* contain proteins of similar function (*70*, *71*), LipA might use a similar mechanism to regenerate an active [4Fe-4S] cluster from the [2Fe-2S] cluster formed in the last step of Scheme 3, thus allowing multiple turnovers. The mechanism in Scheme 3 raises some intriguing questions that can now be addressed experimentally using purified active LipA.

Redox roles for iron−sulfur centers are well-established. The results reported here extend this redox chemistry to include the donation of sulfur atoms to alkyl carbons that may result in disassembly of the iron−sulfur cluster as illustrated in Scheme 3. The chemistry involved in the formation of the proposed deoxyadenosyl radical(s) is likely analogous to that proposed for other members of the AdoMet dependent iron−sulfur protein family. The formation of two C−S bonds with sulfur atoms from the iron−sulfur cluster would be unprecedented chemistry for this cofactor, and experiments are underway to further characterize this reaction.

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