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Analysis of the Active Site Cysteine Residue of the Sacrificial Sulfur Insertase LarE from *Lactobacillus plantarum*

Matthias Fellner,[†,^] Joel A. Rankin,[†] Benoît Desguin,[⊥] Jian Hu,[†,‡] and Robert P. Hausinger[†,§,]*

[†]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824

[⊥]Institute of Life Sciences, Université catholique de Louvain, B-1348 Louvain-La-Neuve, Belgium

[‡]Department of Chemistry, Michigan State University, East Lansing, MI 48824

[§]Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824

ABSTRACT: LarE from *Lactobacillus plantarum* is an ATP-dependent sulfur transferase that sacrifices its Cys176 sulfur atom to form a dehydroalanine (Dha) side chain during biosynthesis of the covalently-linked nickel-pincer nucleotide (NPN) cofactor (pyridinium-3-thioamide-5-thiocarboxylic acid mononucleotide) of lactate racemase. Coenzyme A (CoA) stabilizes LarE and forms a CoA-Cys176 mixed disulfide with the protein. This study presents the crystal structure of the LarE/CoA complex, revealing protein interactions with CoA that mimic those for binding ATP. CoA weakly inhibits LarE activity, and the persulfide of CoA is capable of partially regenerating functional LarE from the Dha176 form of the protein. The physiological relevance of this cycling reaction is unclear. A new form of LarE was discovered, an NPN-LarE covalent adduct, explaining prior results in which activation of the lactate racemase apoprotein required only the isolated LarE. The crystal structure of the inactive C176A variant revealed an essentially identical fold to that of wild-type LarE. Additional active site variants of LarE were created and their activities characterized, with all LarE variants analyzed in terms of the structure. Finally, the *L. plantarum* LarE structure was compared to a homology model of *Thermoanaerobacterium thermosaccharolyticum* LarE, predicted to contain three cysteine residues at the active site, and to other proteins with a similar fold and multiple active site cysteine residues. These findings suggest that some LarE orthologs may not be sacrificial, but rather they may catalyze sulfur transfer by using a persulfide mechanism or from a labile site on a [4Fe-4S] cluster at this position.

INTRODUCTION

Lactate racemase (Lar) interconverts the D- and L-isomers of lactic acid, a central metabolite of many microorganisms.[1] The enzyme allows for the production of D-lactate for cell wall biosynthesis in bacteria that possesses only L-lactate dehydrogenase and it permits the metabolism of both isomers from racemic mixtures of lactate in microorganisms containing a single form of lactate dehydrogenase.[2-4] The Lar enzyme from *Lactobacillus plantarum* includes the protein LarA (UnitProtKB F9USS9) and a covalently-tethered (via Lys184) cofactor: pyridinium 3-thioamide-5-thiocarboxylic acid mononucleotide with nickel bound to C4 of the pyridinium ring, the two sulfur atoms of the pincer complex, and the His200 side chain (Figure 1).[5, 6] This organometallic complex will subsequently be referred to as the nickel-pincer nucleotide (NPN) cofactor.[7] The NPN cofactor is synthesized from nicotinic acid adenine dinucleotide (NaAD) by the sequential actions of LarB (UniProtKB F9UST0),[8] which carboxylates C5 of the pyridinium ring and hydrolyzes the phosphoanhydride bond, LarE (UniProtKB F9UST4), which converts the two carboxylates to thiocarboxylates,[9] and LarC (UniProtKB F9UST1) which installs the nickel.[8, 10] The process by which the NPN cofactor becomes covalently attached to LarA from *L. plantarum* has not been described, and activation of LarA from *Thermoanaerobacterium thermosaccharolyticum* (UniProtKB D9TQ02) does not require adduct formation.[8]

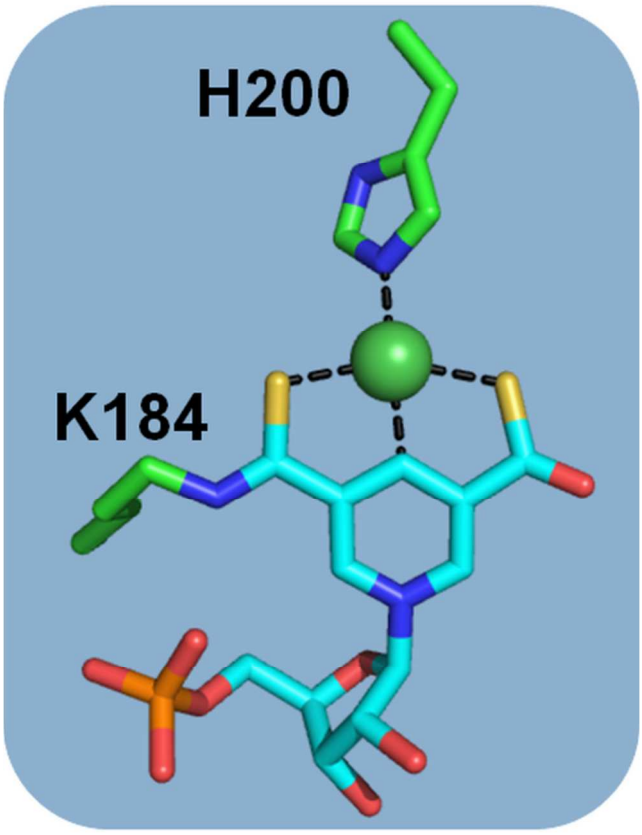


Figure 1. NPN cofactor in lactate racemase. Lys184 of LarA binds pyridinium 3-thioamide-5-thiocarboxylic acid mononucleotide (shown as sticks with cyan carbon atoms; PDB access code 5HUQ). Two sulfur atoms and one carbon atom of the cofactor along with His200 coordinate nickel (green sphere).

This study focuses on LarE, a member of the PP-loop pyrophosphatase family containing a PP-loop SGGxDS motif in its N-terminal region. Transformation of pyridinium-3,5-biscarboxylic acid mononucleotide (P2CMN) to pyridinium-3,5-bisthiocarboxylic acid mononucleotide (P2TMN) by LarE requires two cycles of carboxylate activation (involving ATP-dependent adenylylation) and sulfur insertion, with the sulfur originating from a cysteine residue (Cys176) of the protein, thus making LarE a sacrificial sulfur transferase that acquires a dehydroalanine (Dha) residue.[8, 9]

Here, we examine several unusual and interesting features of LarE focused on its Cys176 residue. First, we investigate the unresolved non-substrate interaction of LarE with coenzyme A (CoA), a cellular component that stabilizes LarE and forms a disulfide with Cys176 but is not believed to be required for LarE activity,[8, 9] by presenting the crystal structure of LarE with bound CoA. Second, we provide evidence that CoA weakly inhibits LarE activity. Third, we demonstrate the ability of CoA-persulfide to regenerate Cys-containing LarE from the Dha176-containing protein ($LarE^{Dha}$). Fourth, we provide evidence for the presence of a novel NPN-adduct of LarE, providing an explanation for earlier confounding observations related to the ability of purified LarE to activate LarA apoprotein. Fifth, we investigate whether the loss of the Cys176 side chain significantly impacts the protein fold by characterizing the structure of the C176A variant. Sixth, we characterize the functions of several other key residues of LarE and summarize all LarE mutagenesis studies to better define the sacrificial sulfur insertion reaction.[9] Lastly, we speculate on whether LarE homologs containing additional active site cysteine residues may operate by a catalytic sulfur transfer process.

MATERIALS AND METHODS

Genes, Plasmids, and Cloning. Site-directed mutagenesis of the gene encoding Strep II-tagged wild-type (WT) LarE from *L. plantarum* was performed using a QuikChange mutagenesis kit (Agilent) for creating the K101A, E128A and E223A variants. All of the constructs were verified by DNA sequencing. Details, including instructions for creating the C176A variant, are summarized in our previous work.[9]

LarE Overexpression, Purification, and Characterization. WT, C176A, and the new variant forms of LarE were overexpressed and purified using our previously described pGIR076

Escherichia coli Arctic express – *Strep*-tactin (IBA) system.[9] The final buffer for all samples was 100 mM Tris·HCl, pH 7.5, containing 300 mM NaCl. The activities of WT and variant forms of LarE were assessed by monitoring samples for their abilities to transform P2CMN to P2TMN that was further metabolized by LarC and incorporated into LarA apoprotein from *T. thermosaccharolyticum*; this activated LarA enzyme was then assayed for lactate racemization as described elsewhere in detail.[9]

Crystallization. For both the CoA-bound WT protein and the C176A LarE variant, crystals were obtained after mixing 5 µl of protein samples with 5 µl of reservoir solutions. The hanging drop reservoir contained 100 µl of 30.0% v/v pentaerythritol ethoxylate (15/4 EO/OH), 50 mM Bis-tris, pH 6.5, and 100 mM ammonium sulfate. The CoA-bound sample contained 28 mg/ml of WT LarE mixed with 0.9 mM CoA and was incubated for ~10 min on ice before setting up the drop. The C176A LarE sample contained 8 mg/ml of variant protein. Using the same setup as for C176A, we also set up unsuccessful crystal drops for W97A (~1 mg/ml), S180A (~10, 12, and 15 mg/ml), R212A (~6 mg/ml), and D231R (~3, 11, and 24 mg/ml) variants of LarE using the same procedures.[9]

Diffraction Data Collection, Structure Determination, and Analysis. Datasets were collected at the Advanced Photon Source LS-CAT beamlines (21-ID-F and 21-ID-G). Datasets were processed with xdsapp[11] and iMos,[12] with merging and scaling done using aimless.[13] Phaser molecular replacement[14] was utilized using the WT apoprotein model 5UDQ. Model building and refinement were conducted in Coot[15] and Phenix.[14] Dataset statistics are listed in Table 1. UCSF Chimera[16] was used to create structure figures.

Table 1. Crystallography statistics for the CoA-bound and C176A variant LarE structures

LarE crystals	CoA bound	C176A
Data collection		
Beamline	LS-CAT 21-ID-F	LS-CAT 21-ID-G
Wavelength (Å)	0.979	0.979
Space group	$P4_122$	$P4_122$
Unit cell a, b, c (Å); α, β, γ (°)	107, 107, 320; 90, 90, 90	109, 109, 329; 90, 90, 90
[a]Resolution (Å)	47.95 – 2.09 (2.12 – 2.09)	48.67 – 2.35 (2.39 – 2.35)
Unique reflections	111,492 (5,293)	82,741 (4,345)
[a]Redundancy	6.1 (6.0)	11.3 (11.2)
[a]Completeness (%)	99.7 (97.1)	99.5 (97.3)
[a]$I/\sigma I$	14.7 (1.8)	18.9 (2.1)
[a,b]R_{merge}	0.084 (0.899)	0.102 (1.079)
[a,c]R_{pim}	0.055 (0.599)	0.046 (0.482)
[d]$CC_{1/2}$	0.998 (0.509)	0.999 (0.702)
Refinement		
Protein atoms	11,949	11,420
CoA	288	0
Phosphate	30	30
Sulfate	0	25
H_2O	606	493
[e]R_{work}/R_{free}	0.195/0.234	0.187/0.244
B-factors (Å^2)	43.8	56.3
Protein	43.9	57.0
CoA	59.5	-
Phosphate	38.5	47.7
Sulfate	-	61.5
H_2O	42.5	48.4
R.m.s. deviation in bond lengths (Å)	0.007	0.007
R.m.s. deviation in bond angles (°)	0.975	0.922
Ramachandran plot (%) favored	97.34	97.43
Ramachandran plot (%) outliers	0	0
Rotamer outliers	0	0
PDB ID	6B2M	6B2O

[a]Highest resolution shell is shown in parentheses.

[b]$R_{merge} = \sum_{hkl} \sum_j |I_j(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_j I_j(hkl)$, where I is the intensity of reflection.

[c]$R_{pim} = \sum_{hkl} [1/(N-1)]^{1/2} \sum_j |I_j(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_j I_j(hkl)$, where N is the redundancy of the dataset.

[d]$CC_{1/2}$ is the correlation coefficient of the half datasets.

[e]$R_{work} = \sum_{hkl} | |F_{obs}| - |F_{calc}| | / \sum_{hkl} |F_{obs}|$, where F_{obs} and F_{calc} is the observed and the calculated structure factor, respectively. R_{free} is the cross-validation R factor for the test set of reflections (5% of the total) omitted in model refinement.

Purification of Dha-containing LarE. $LarE^{Dha}$ was purified from *Lactococcus lactis* NZ3900 containing pGIR172 that carries the *larA-larE* genes from *L. plantarum* with *larE* translationally fused to DNA encoding the Strep II tag[5] as previously described.[8] The cells were grown overnight without shaking in 15 mL of M17 medium (Oxoid or Difco) containing 0.5% w/v D-glucose and 10 mg/L chloramphenicol at 30 °C. This inoculum was added to 1.5 L of the same medium, but containing 5 mg/L chloramphenicol, and incubated at 28 °C with shaking (40 RPM) until the OD_{600} reached ~0.3. The culture was supplemented with 1 mM $NiCl_2$, induced with 10 µg/L nisin A (Sigma), and grown for 4 h before harvesting the cells by centrifugation at 5,000 *g* for 10 min. The cell pellet was washed using 100 mM Tris-HCl buffer, pH 7.5, containing 300 mM NaCl and stored until needed at -80 °C. The thawed cells were suspended in 40 mL of 100 mM Tris-HCl buffer, pH 7.5, containing 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF, added as a 100 mM stock in ethanol), and 1 U/mL Benzonase® (Millipore) on ice, then lysed by two passes through a chilled French pressure cell at 16,000 psi. The debris and intact cells were removed by 40 min centrifugation at 27,000 *g* and 4 °C. Purification of $LarE^{Dha}$ was performed by using a 1 mL bed volume of *Strep*-tactin-XT (IBA) resin equilibrated in 20 mM Tris-HCl buffer, pH 7.5, containing 300 mM NaCl at 4 °C. The lysates were loaded at 1 mL/min, the resin was washed by gravity flow with five 1 mL additions of 20 mM Tris-HCl buffer, pH 7.5, containing 300 mM NaCl, and the protein was eluted with six 0.5 mL additions of 20 mM Tris-HCl buffer, pH 7.5, containing 300 mM NaCl and 50 mM biotin (Santa Cruz Biotechnology) that had been neutralized with 50 mM NaOH.

Additional characterization of $LarE^{Dha}$ included analysis of its reactivity with 5,5′-dithiobis(2-nitrobenzoic acid (DTNB) and tris(2-carboxyethyl)phosphine (TCEP) in comparison to that of LarE. To quantify accessible thiol groups, the protein samples were treated with 180 μM DTNB in 100 mM Tris-HCl, pH 7.5, allowed to react for 30 min, and monitored at 412 nm.[17] For testing the reactivity of LarE associated Dha with TCEP,[18] the samples were treated with 5 mM TCEP in buffer and analyzed by mass spectrometry (see below).

LarE Regeneration Reactions. Lysates for use in LarE regeneration experiments were derived from cultures of *L. plantarum* NCIMB8826. Using a 2% inoculum, the cells were grown without shaking at 37 °C in MRS medium (Sigma) supplemented with 0.1% v/v Tween-20. At an OD_{600} ~1, the cultures were induced by adding 200 mM sodium L-lactate as a dry powder or left un-induced and incubated for a further 2 h. The cells were harvested by centrifugation at 5,000 *g*, and the pellets were washed in 100 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and stored at -80 °C. The cells (~500 μL of cell paste) were mixed with glass beads (~500 μL, mean diameter of 100 μm) in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and, for maintaining aerobic conditions,[19] 20 μg/mL glucose oxidase, 2 μg/mL catalase, and 0.3% w/v D-glucose in 1.5 mL screw cap tubes (deaerated by blowing argon over the surface), and lysed by using a Mini-Bead Beater 16 (BioSpec) with two, 60 s pulses separated by 5 min on ice. The debris was removed by 10 min centrifugation at 20,000 *g* and 4 °C.

Cysteine desulfurase (IscS) was purified from *E. coli* BL21 (DE3) containing pBH402, a plasmid with *E. coli iscS* fused to a sequence encoding a His_6-tag and under the control of the T7 promoter.[20] Overnight cultures of these cells were grown with shaking (300 RPM) at 37 °C in LB containing 50 μg/mL of kanamycin (Gold Biotechnology), then diluted 200-fold into 0.5 L of Lennox LB supplemented with 1 mM pyridoxine (Sigma) plus 50 μg/mL kanamycin and further

grown with shaking at the same temperature. Upon reaching an OD_{600} of ~0.5, the cultures were amended with 0.4 mM isopropyl β-D-1-thiogalactopyranoside and grown another 3 h. The cells were collected by centrifugation, resuspended in 40 mL in buffer A (50 mM Na_2HPO_4, pH 8.0, containing 300 mM NaCl and 20 mM imidazole) supplemented with 2.5 U/mL Benzonase® and 1 mM PMSF. The suspension was chilled on ice and lysed by two passes through a chilled French press cell at 16,000 psi. The cell debris was pelleted by 40 min of centrifugation at 27,000 *g* and 4 °C. The supernatant was applied to a column (10 mL) of Ni-nitrilotriacetic acid-Sepharose that had been equilibrated in buffer A. After washing the column twice with 50 mL buffer A, IscS was eluted at 2 mL/min with a 100 mL linear gradient from buffer A to buffer A containing 500 mM imidazole. The 2.5 mL fractions were collected into tubes containing 2.5 µL of 10 mM EDTA, pH 8.0, to prevent precipitation. The IscS-containing fractions were combined and dialyzed overnight at 4 °C against 2 L of 50 mM potassium phosphate buffer, pH 7.5, containing 5 mM $MgCl_2$, 100 mM KCl, and 0.1 mM EDTA. The protein concentration was quantified using the published $\varepsilon_{280\,nm}$ of 25,400 M^{-1} cm^{-1},[20] and purified IscS was stored at 4 °C.

Organic persulfides were synthesized by incubating 5-20 mM of the disulfides with a 5-fold molar excess of NaHS in 300 mM Tris-HCl (pH 7.5, for making CoA and glutathione persulfides) or 100 mM NaOH (for the Cys persulfide) in an argon atmosphere.[21] The reactions were incubated at 30 °C for 30 min. Persulfide yields were quantified by using a cold cyanolysis procedure,[22] with yields typically of ~30% relative to the starting disulfide concentrations. Persulfide preparations were used immediately without further purification or frozen at -20 ° under argon for later use.

$LarE^{Dha}$ (2.8 µM, 0.087 mg/mL) was incubated in 20 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 20 µg/mL glucose oxidase, 2 µg/mL catalase, and 0.3% w/v D-glucose (to ensure

anoxic conditions), with various additives as specified. Selected mixtures included 10% cell-free lysates from *L. plantarum* cultures that were induced with lactic acid or non-induced. Some mixtures included 2 mM $MgCl_2$ and 1 mM ATP. Individual sulfur sources and a combined mixture of sulfur compounds (1 mM cysteine, 1 mM glutathione, 1 mM 3-mercatopyruvate, 1 mM sodium thiosulfate, 1 mM sodium sulfide, and 200 μM CoA) were tested. In addition, IscS (4.6 μM, 0.21 mg/mL) with 1 mM cysteine was examined. For all samples, the reactions were layered with mineral oil and allowed to incubate at room temperature with 1 mL time points taken at 1 h or overnight. The samples lacking cell-free lysates were directly analyzed by mass spectrometry, but those amended with lysates were loaded by gravity flow at 4 °C onto columns containing 200 μL of *Strep*-tactin-XT resin, the columns were washed using three 1 mL additions of 20 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, and the LarE protein was eluted using six 250 μL additions of the same buffer containing 50 mM biotin neutralized with 50 mM NaOH. These protein samples were concentrated to at least 1 μM LarE using 10 kDa cutoff Amicon centrifuge columns prior to further analysis.

Mass Spectrometry of LarE. The purified LarE protein samples (10 μL in buffer), including those from the LarE regeneration studies, were injected onto a cyano-chemistry HPLC column that was equilibrated in 0.1% formic acid and eluted with an increasing gradient of acetonitrile. The fractions were analyzed by electrospray ionization (ESI)-mass spectrometry (MS) using a XEVO G2-XS instrument in positive ionization mode. The protein masses were derived from the MS data using MaxEnt (Waters Corp.). LarE samples were also purified from *L. lactis* NZ3900 pGIR172 that had been induced for different lengths of time (1, 2, 3, and 4 h) and analyzed by the same ESI-MS approach.

LarE Enzyme Assay. The activity of LarE was assessed indirectly by a four-step process using purified components and assaying for Lar activity. The first step was the production of P2CMN by 3 h incubation of LarB (3 μM) with 1 mM NaAD in 100 mM $NaHCO_3$ with quenching by heat (80 °C for 5 min). The second step involved LarE synthesis of P2TCM; the step 1 mixture (4% of final volume) was incubated with 10 μM LarE, 2 mM reducing agent (Cys, dithiothreitol (DTT), CoA, ß-mercaptoethanol, glutathione, or ascorbic acid), 2 mM ATP, and 20 mM $MgCl_2$ in 100 mM Tris-HCl, pH 7, for 5 min at room temperature, then stopped by heat. Step 3 involved synthesis of the NPN cofactor and generation of active LarA by reacting the step 2 mixture (10% of final volume) with 1.5 μM LarC, 1.5 μM *T. thermosaccharolyticum* LarA apoprotein, 1 mM $MnCl_2$, 0.1 mM CTP, and 40 mM D-lactate in 100 mM Tris-HCl, pH 7, for 5 min at room temperature, with quenching by heat. The final step was to measure L-lactate by an enzymatic kit (Megazyme).

RESULTS

Structural Investigation of Cys176 - Coenzyme A Interaction. Prior studies had demonstrated that the addition of CoA stimulates the activation of LarA apoprotein in mixtures containing P2CMN (the product of LarB), Mg·ATP, LarE, LarC, and LarA apoprotein.[8] Unpublished observations showed a 765 Da adduct present in LarE purified from *L. lactis* cells, which we presumed to be CoA. When LarE (as the Strep II-tagged species lacking the N-terminal Met residue; 31,550 Da) was purified in the presence of CoA, this adduct (32,316 Da) became the majority species and CoA was shown to form a disulfide with the single cysteine residue of LarE (Cys176).[8] Several thiol reductants (sulfide, Cys, DTT, and glutathione) including CoA were able to reduce the disulfide and confer LarE activity in the Lar assay, but

only CoA provided longer-term (20 h) stability to the protein (data not shown). An analogous CoA-protein disulfide (32,274 Da) was formed with the D30A variant of LarE (31,508 Da), but not for the C176A variant (31,520 Da) (Figure 2). CoA is not required for *E. coli*-derived LarE to convert P2CMN to P2TMN.[9] The disulfide-containing form of LarE converts to $LarE^{Dha}$ in the LarA apoprotein activation mixture. While it is clear that CoA interacts with LarE, further studies were required to establish the details of this interaction.

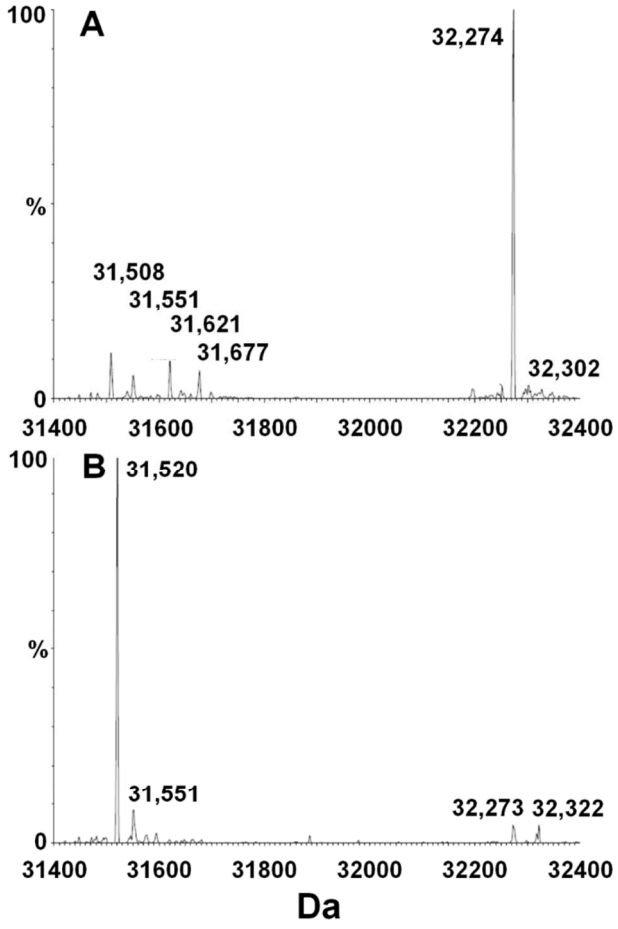


Figure 2. ESI-MS of (A) the CoA-S-S-Cys176 disulfide using D30A LarE and (B) the C176A variant of LarE, both purified in the presence of CoA.

LarE was isolated from both *L. lactis* and *E. coli* using purification buffers containing CoA, and each protein was crystallographically characterized. All LarE chains of the crystallographic

hexamers had CoA bound. The phosphate group of the 3'-phosphoadenosine portion of the molecule was found at the PP-loop (the ATP-binding site) of LarE (Figure 3A). The adenine moiety forms two hydrogen bonds with the backbone atoms of Ala52 on strand β2. The ribose is stabilized via interactions with the backbone atoms of Ala24 on β1 and Gly123 on β4. The diphosphate group of CoA was well ordered in all structures, but it exhibited slightly different orientations between chain C and all other chains (Figure 3B). The differences were more notable for the remaining pantoic acid-alanine-cystamine portions of CoA; this part of the molecule is less ordered in all chains, does not show direct contacts with the protein, and shows slight variations in chain A, B, D, E, and F (F is shown as a representative example) compared to the very different orientation in chain C (Figure 3B). The distinct orientations of the coenzyme paralleled the differences in side chain orientations of the key residue Trp97.[9] Although MS experiments had previously indicated a disulfide bond between CoA and Cys176 of LarE,[8] no disulfide bond was detected in the electron density maps. This result suggested that photoreduction of the disulfide may have occurred during data collection. Notably, the CoA sulfur atom was in the vicinity of the Cys176 side chain, with the two different orientations having distances of 8.8 and 10.4 Å between the Cys176 and the CoA sulfur atoms. Transitioning between the two orientations of the least ordered portion of CoA would place the CoA sulfur close enough to the Cys176 side chain to form the disulfide.

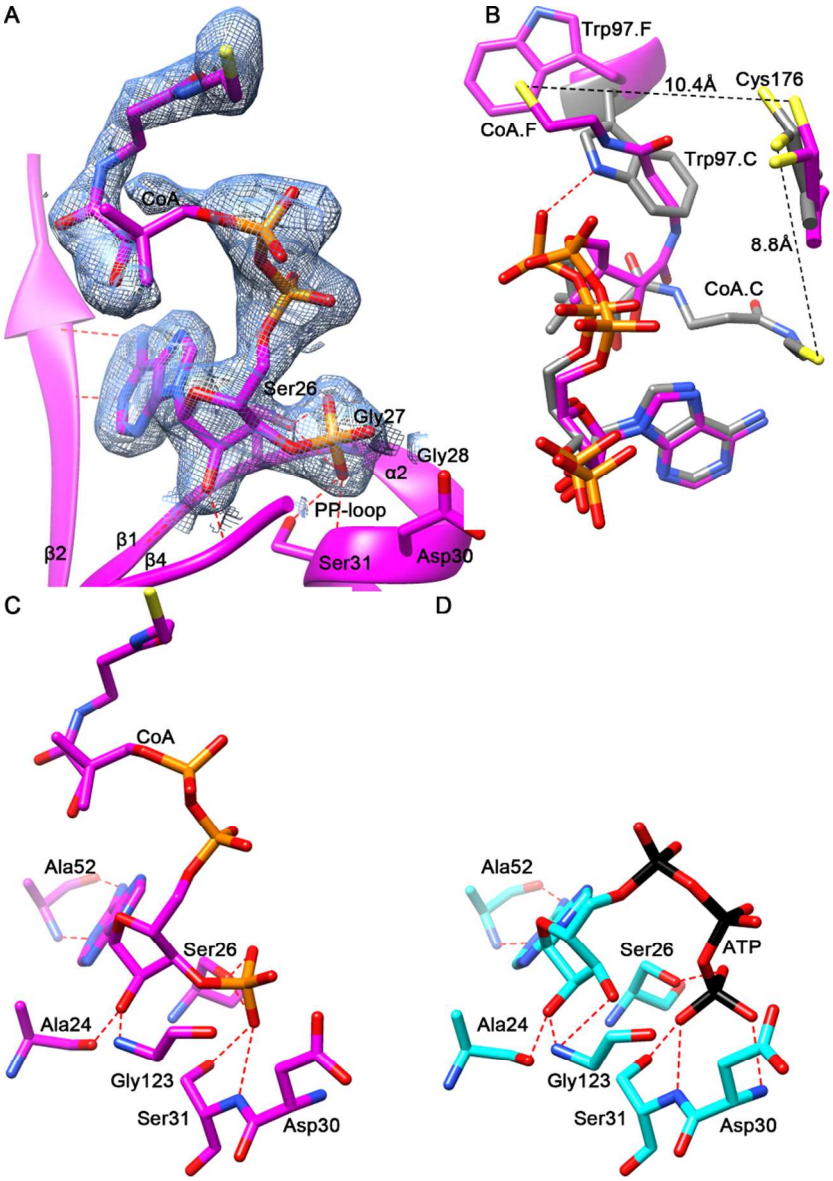


Figure 3. CoA binding to LarE (PDB ID code 6B2M). All comparisons are based on a Cα alignment of the full length protein.[16] (A) The $2F_O$-F_C map of CoA bound to chain F is shown as blue meshes at 1σ. Interacting backbone residues are illustrated as ribbons with secondary structure features labelled, and the PP-loop residue side chains are shown. Hydrogen bonds are indicated as red dashes. (B) Comparison of CoA binding orientations in LarE. Chain F is shown in magenta (representative for chains A, B, D, and E) and compared to chain C shown in grey. The corresponding side chains of Trp97 and Cys176 are illustrated in the same colors with

distances between the CoA and Cys176 sulfur atoms shown as black dashes. (C+D) Comparison of CoA and ATP (PDB ID code 5UDS)[9] binding to LarE. Interacting residues are shown in stick mode with hydrogen bonds indicated by red dashes. The carbon atoms of the CoA- and ATP-bound structures are colored in magenta and cyan respectively. The ligand phosphorous atoms of CoA are shown in orange and those of ATP are black.

The binding mode of the 3'-phosphoadenosine ribose portion of CoA is nearly identical to ATP, with the difference that the triphosphate of ATP is positioned so as to place the γ-phosphate into the PP-loop, whereas it is the ribose-phosphate group of CoA that is tightly associated with the PP-loop residues (Figure 3C and 3D).

CoA Weakly Inhibits LarE Activity. ATP likely has a greater binding affinity for LarE than does CoA, compatible with its greater number of interactions involving the PP-loop, thus overcoming any potent inhibitory effect of the coenzyme. Nevertheless, the apparent LarE activity was lower when using CoA compared to other reductants (Cys, DTT, ß-mercaptoethanol, glutathione, or ascorbic acid) when the protein was subjected to short-term treatment with these reagents and analyzed using the indirect LarA apoprotein activation assay (Figure 4). This result suggests that CoA binding is partly inhibitory to LarE activity.

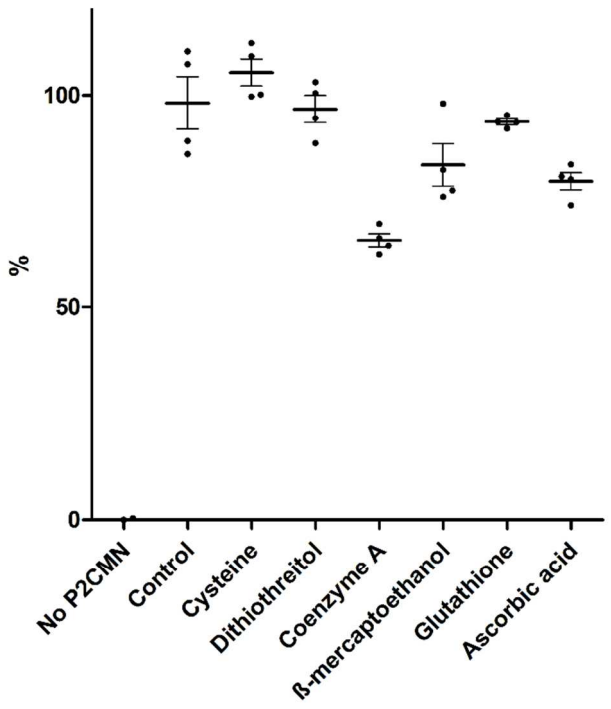


Figure 4. Inhibitory effect of CoA on the apparent LarE activity compared to other reductants. P2CMN was produced enzymatically from NaAD, converted to P2TMN by LarE in the presence of the indicated reductants (2 mM) along with 2 mM ATP and 20 mM $MgCl_2$ in 100 mM Tris-HCl buffer (pH 7), transformed into the NPN cofactor by LarC, incorporated into *T. thermosaccharolyticum* LarA apoprotein, and assayed for Lar activity, shown as percent relative to the no-thiol control.

Additional Characterization of LarE^{Dha}. Strep-tagged LarE was expressed in *L. lactis* in the context of the entire *lar* operon and purified. As previously described,[8] nearly all of the resulting LarE lacks its Cys176 sulfur atom according to ESI-MS (Figure 5A). This major form of the protein was observed for samples isolated from cultures that had been induced from 1 h to 4 h (data not shown), so LarE^{Dha} is stable in the cells. Along with LarE^{Dha} (31,517 Da) the spectrum reveals likely sodium complexes of the protein (31,540 Da and larger); these forms were greatly

reduced in intensity when using an ammonium bicarbonate buffer. In contrast to the native LarE protein that exhibits DTNB reactivity accounting for 0.93 Cys thiol per protein, no 412 nm peak was detected in the $LarE^{Dha}$ protein. As further support for the presence of Dha, the addition of TCEP to $LarE^{Dha}$ led to partial modification to form a species at 31,767 Da or 250 Da larger than $LarE^{Dhs}$ (Figure S1), consistent with the known reactivity of TCEP towards dehydroalanine,[18] whereas this reagent had no effect on the mass spectrum of LarE.

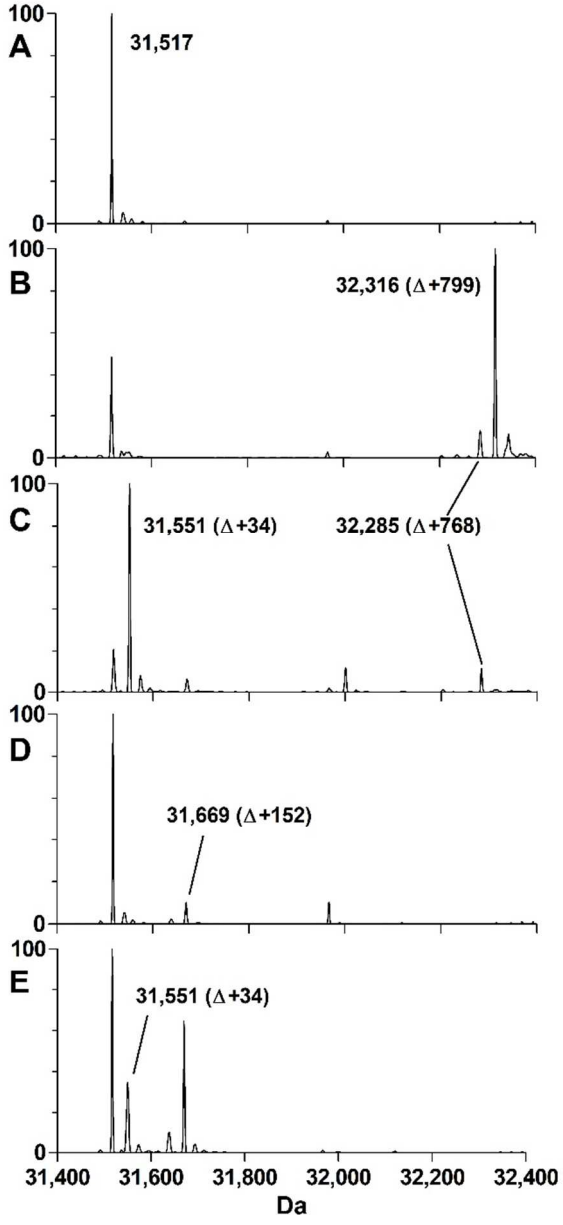
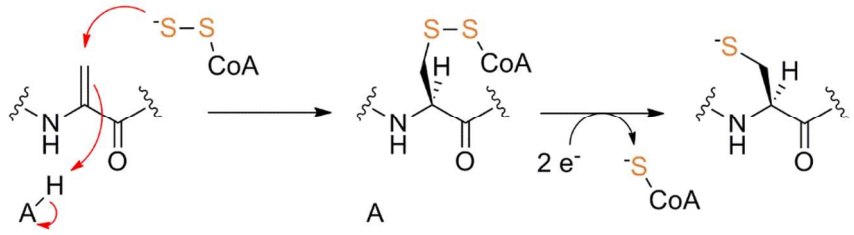


Figure 5. Regeneration of native LarE from LarE^{Dha}. (A) ESI-MS of LarE^{Dha}. (B) LarE^{Dha} treated with CoA persulfide (150 μM, 1 h at room temperature then overnight at 4 °C), revealing conversion to LarE-S-S-CoA. (C) Sample from B reduced with DTT (10 mM, 30 min at room temperature) to generate native LarE. (D) LarE^{Dha} treated with Cys persulfide. (E) Sample from

D treated with DTT. The Δ values are relative to the 31,517 Da of LarE^{Dha}. The y-axes indicate percent relative to the intensities of the maximum peaks.

Regeneration of Cys-Containing LarE from LarE^{Dha} by CoA Persulfide. Regeneration of native LarE from LarE^{Dha} was achieved by using the persulfide of CoA followed by reduction with DTT (Scheme 1). Incubation of LarE^{Dha} with CoA persulfide (Figure 5B) resulted in substantial conversion of the Dha-containing protein (31,517 Da) to a species (32,316 Da) consistent with the LarE-S-S-CoA disulfide. In addition, we noted a minor species (32,285 Da) suggesting the formation of the LarE-S-CoA thioether. Consistent with the thioether interpretation, the same species formed for LarE^{Dha} treated with CoA rather than the CoA persulfide (Figure S2). Subsequent reduction of the CoA persulfide mixture by DTT (Figure 5C) eliminated the putative disulfide species, but not the proposed thioether, and generated a peak (31,551 Da) corresponding to native LarE. In contrast to the robust regeneration observed using the CoA persulfide, the Cys persulfide produced much lower levels of the LarE-S-S-Cys disulfide (31,669 Da, Figure 5D) and corresponding lower amounts of the native LarE after reduction (Figure 5E). DTT reduction of the Cys persulfide-treated sample led to an increase in the 31,669 Da species that we attribute to formation of the thioether between DTT and LarE^{Dha}, which happens to possess the same mass as the LarE-S-S-Cys disulfide, as confirmed by a control reaction of DTT and the Dha-containing protein (Figure S3). No reactivity was detected between the glutathione persulfide and LarE^{Dha} (data not shown). These results suggest the CoA binding site of LarE is used to specifically bind the CoA persulfide during the sulfuration reaction. Although regeneration of native LarE from LarE^{Dha} was demonstrated by these *in vitro* experiments, it remains unclear whether a similar process occurs within *L. plantarum* cells.

Scheme 1



Alternative efforts to reincorporate sulfur into the LarE^{Dha} protein were unsuccessful. These included using a variety of *in vitro* conditions, a range of potential sulfur donors, lysates from native *L. plantarum* cells (induced by addition of L-lactate and non-induced controls), other potential cofactors ($MgCl_2$ and ATP, CoA, and glutathione), , and cysteine plus cysteine desulfurase, a known sulfur source for several other biological sulfur-containing components. In none of these cases did we observe conversion of LarE^{Dha} to the WT species.

Discovery of an NPN Adduct of LarE^{Dha}. [8]Some preparations of LarE^{Dha} possessed a previously undescribed species (31,968 Da) that was 450 Da larger than the Dha-containing protein (31,518 Da) (Figure 6). Of great interest, this species is consistent with the formation of a covalent adduct between NPN and LarE. Such a species, explains a previous confounding observation related to LarA activation. Namely, the incubation of purified LarA (1.4 pmol) with LarE (280 pmol, isolated from cells containing LarB and LarC) was shown to result in activation of ~30% of the lactate racemase apoprotein.[5] We interpret this prior result as arising from a secondary pathway for LarA activation that utilizes this new species with NPN covalently attached to LarE.

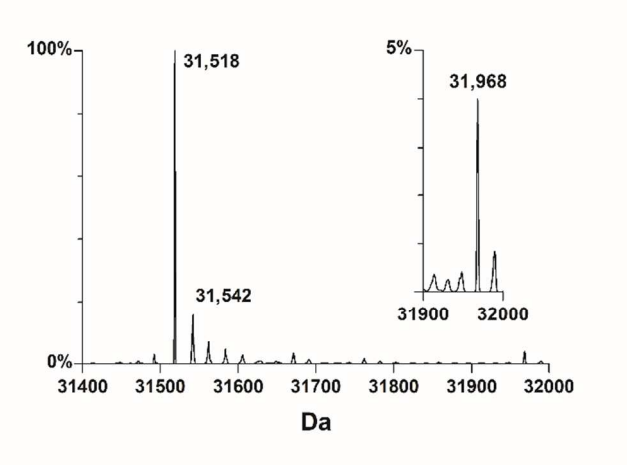


Figure 6. Example ESI-MS spectrum of $LarE^{Dha}$ with an inset depicting an expanded view of the region 31,900 to 32,000 Da.

Structural Characterization of C176A LarE and Functional Characterization of LarE Variants. We attempted to crystallize several LarE variants with largely reduced or abolished activity (W97A, C176A, S180A, and R212A) and the active D231R variant, but had success only with the C176A LarE sample. The W97A variant, thought to be defective in its interaction with the P2CMN substrate,[9] was poorly produced, thus preventing crystallization. The S180A and R212A variants, considered to lack the P2CMN or inorganic phosphate-binding site, were examined using many conditions, but the lack of successful crystallization suggests that a bound phosphate/sulfate molecule at this binding site is required for crystal formation. Although the D231R variant is as active as WT enzyme, we observed no crystal formation suggesting that the LarE trimer/hexamer interface where Asp231 is located may be disturbed, thus preventing crystal growth. The successful case of the C176A variant protein yielded the same crystal morphology and size as the WT enzyme.

The overall fold of the C176A LarE structure (Figure 7A) was identical to that of WT protein. Even the flexible linker between domains on which Cys176 is located matched in both

structures. In addition, the bound phosphate previously noted in WT enzyme[9] was also present in the C176A structure, indicating that hydrogen bonding with the sulfur atom (Figure 7B) is not crucial for binding the phosphate of P2CMN. The loss of activity of the C176A variant must be based solely on the functional role of the thiol group as a sulfur donor as the C176A structure shows no other structural difference to WT enzyme.

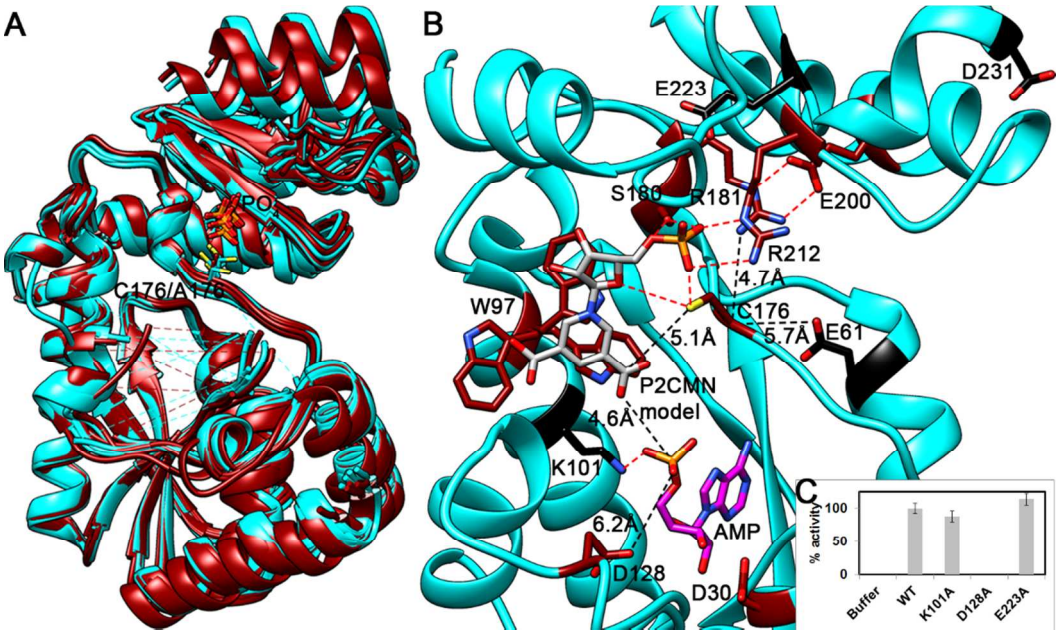


Figure 7. Analysis of LarE variants. (A) Comparison of the six chains of the WT LarE are depicted in cyan (5UDQ[9]) and those of the C176A LarE variant are in dark red (6B2O). The Cys176 side chain and nearby phosphate molecule are shown. (B) The P2CMN/AMP-bound model of LarE based on chain A of the NMN-bound LarE (5UDR) and chain C of AMP-bound LarE (5UDT).[9] Carbon atoms of the P2CMN model are shown in grey, those of AMP in magenta, and the side chains of residues that are altered in inactive variants in dark red (D30A, C176A;[8] W97A, C176A, S180A, R181K, E200Q, R212A;[9] D128A, this publication). In addition, the side chains of residues that were substituted in active variants are shown in black (E61Q, D231R;[9] K101A and E223A, this publication). Hydrogen bonds are shown as red dashes,

and important interactions are illustrated as black dashes with the distances indicated. (C) Activity results of three variants characterized in this publication.

We previously characterized several LarE variants of highly conserved residues to show the importance of the active site cysteine (C176A), the PP-loop (D30A), the P2CMN phosphate-binding site (S180A, R212A), and several key residues in catalysis (W97A, R181K, E200Q), while also ruling out the involvement of other residues (E61Q, D231R) (Figure 7B).[8, 9] Here, we characterized three additional variants to gain an even better understanding of LarE's function. Glu223 is buried in a similar manner as Glu200 in the C-terminal head domain of LarE, which forms trimer/hexamer interactions and binds the phosphate moiety of the substrate, P2CMN. Furthermore, Glu223 forms hydrogen bonds to the nearby Arg214 and Arg221 residues, with Arg214 being directly involved in phosphate binding. The E223A variant exhibited no significant change in activity compared to the WT enzyme. This finding shows that the interaction of Arg214 with phosphate is unlikely to require E223 so that phosphate binding only requires Ser180 and Arg212 (Figure 7B). The Arg214-Glu223 interaction is structurally similar to the nearby Arg181-Glu200 interaction, but in that case the E200Q variant was inactive, highlighting the importance of the Glu200 side chain. Second, we examined Lys101 that is conserved in LarE sequences and forms a salt bridge with AMP (Figure 7B). Surprisingly, the K101A variant was as active as the WT enzyme, suggesting that the lysine is not critical for stabilizing AMP binding and that other residues on a nearby flexible loop might compensate for its absence through other AMP phosphate interaction residues.[9] Asp128 is another highly conserved residue on the flexible loop, but this residue does not directly interact with AMP in the crystal structure (Figure 7B). At a distance of 6.2 Å from the AMP phosphate it is unclear what

role this residue could play; nevertheless, it must be important as the D128A variant was inactive. This result demonstrates a key role of the flexible loop containing Asp128, either in its disordered state found in most structures or as the helical fold in the AMP-bound structure.

DISCUSSION

NPN Biosynthesis, LarE^{Dha} Regeneration, and a New Pathway for LarA Activation. We combine our new results with those from prior studies in a summary depiction of the LarA apoprotein activation process (Figure 8). In the primary pathway, LarB converts NaAD to P2CMN,[8] two molecules of LarE catalyze AMPylation of the pyridinium carboxyl groups followed by sacrificial sulfur transfers from Cys176 to form P2TCM (generating two copies of LarE^{Dha}),[8, 9] LarC inserts nickel by a CTP-dependent process,[8, 10] and the resulting free NPN activates LarA (Figure 8, blue arrow pathway).

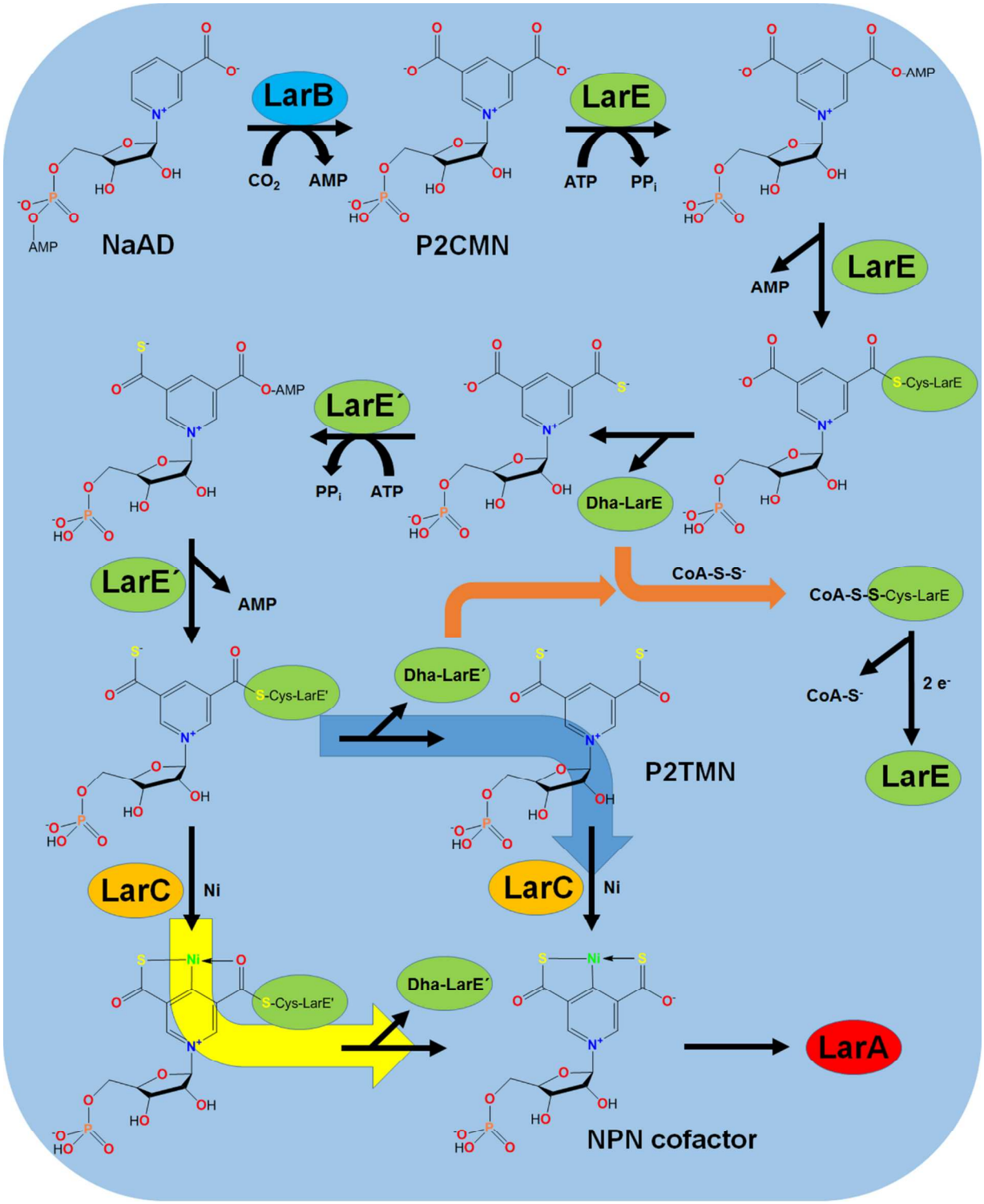


Figure 8. Biosynthesis of the NPN cofactor for LarA apoprotein activation, regeneration of $LarE^{Dha}$, and an alternate pathway to generate lactate racemase activity. In the predominant pathway, LarB carboxylates nicotinic acid adenine dinucleotide (NaAD) and hydrolyzes its

phosphoanhydride linkage, thus forming P2CMN. LarE activates the substrate carboxyl group by adenylylation, forms a thioester linkage with the substrate, sacrifices its Cys176 sulfur atom, and then a second LarE repeats the cycle to produce P2TMN (blue arrow). LarC inserts nickel into this species to generate the NPN cofactor that activates LarA. LarE^{Dha} can be regenerated to native LarE by incubation with CoA persulfide and reductant (orange arrows), but it is unclear whether these reactions are physiologically relevant. A newly identified NPN-LarE intermediate functions in an alternative, minor pathway (yellow arrow) in which LarC installs nickel into the pincer cofactor while it is still bound to LarE. NPN is released from this purified protein for LarA activation.

We have now shown that LarE^{Dha} can be recycled to regenerate native LarE by *in vitro* incubation with CoA persulfide and reductant (Scheme 1 and Figure 8, orange arrows). The CoA persulfide is likely to bind at the CoA binding site of LarE for this reaction because other tested persulfides were inefficient or not effective. Such binding would perfectly position the persulfide to react with Dha. Persulfides are known to have increased nucleophilic character compared to thiols and are of increasing interest in biology.[23, 24] CoA persulfide is found in cells,[25, 26] and has been shown to bind tightly to short-chain acyl-CoA dehydrogenases.[27, 28] Although CoA persulfide is an attractive candidate for recycling LarE^{Dha}, the near exclusive presence of this form of the protein in *L. lactis* cells containing pGIR172 suggests this reaction is not physiologically relevant and LarE is a single turnover enzyme.

Our finding of what appears to be an NPN-LarE adduct suggests that LarC can insert nickel into the incomplete pincer ligand that is still bound in thioester linkage to LarE (Figure 8, yellow arrow). The flexibility of the LarE loop containing Cys176 could reasonably allow the pincer species to be metallated by LarC. The NPN-LarE species is presumed to release NPN,

accounting for the previously reported activation of LarA apoprotein by just LarE,[5] resulting in the generation of the LarE^{Dha} form of the protein. The *in vivo* relevance of the NPN-LarE adduct remains to be clarified; this species may be abundant within the cell, but converts to LarE^{Dha} as the protein is purified.

LarE Homologs: Potential Roles of Additional Cysteine Residues. We had previously noted that many LarE homologs have sequences in which Cys176 appears to be shifted by one residue and points towards two additional cysteine residues, not present in the *L. plantarum* LarE sequence, one of which substitutes for Trp97 (Figure 9A and 9B).[9] We speculate that these alternative LarE versions might catalyze a different mechanism of sulfur transfer that does not require sacrifice of a side chain sulfur atom as observed in the benchmark LarE. Comparison of the location for these cysteine residues in a model of *T. thermosaccharolyticum* LarE (Figure 9B)[9] to the structure of *Thermus thermophilus* TtuA,[29] another PP-loop pyrophosphatase family member that catalyzes sulfur transfer for 2-thiouridine synthesis, reveals the similarity of the LarE ortholog and the [4Fe-4S] cluster-binding site bound by three cysteine residues in TtuA (Figure 9C). In that case, the sulfur transferred to uridine is suggested to derive from the non-[4Fe-4S] cluster sulfide atom (positioned where a fourth cysteine is found for many similar clusters), which must be replaced for each round of synthesis.[29] Structural similarities and sulfur transfer involving a labile extra-cluster sulfide were noted in a study of TtuA from *Pyrococcus horikoshii* that also possesses a [4Fe-4S] cluster bound by three cysteines (Figure 9D).[30] Furthermore, the structure of the more distantly related ortholog adenosine 5'-phosphosulfate reductase from *Pseudomonas aeruginosa* also reveals a [4Fe-4S] cluster at this site;[31] however, in that case four protein cysteine residues are present and the cluster is not thought to donate a sulfur atom (Figure 9E). Rather, this enzyme uses its [4Fe-4S] and flavin to reversibly transfer

electrons from sulfite plus AMP while forming adenylyl sulfate and a reduced electron acceptor. Interestingly, two other structurally-related sulfur transferases, tRNA 2-thiouridylase or MnmA from *E. coli* (Figure 9F)[32] and tRNA 4-thiouridine synthetase or ThiI from *Thermotoga maritima* (Figure 9G),[33] also contain their catalytic cysteine residues in the same region as LarE's Cys176. In these cases, cysteine persulfides are generated, with disulfide exchange involving another cysteine residue allowing for the insertion of a sulfur atom into substrate. Although the structure has not been obtained, ThiI from *Methanococcus maripaludis* was shown to possess a [3Fe-4S] cluster that was needed for sulfur transfer.[34]

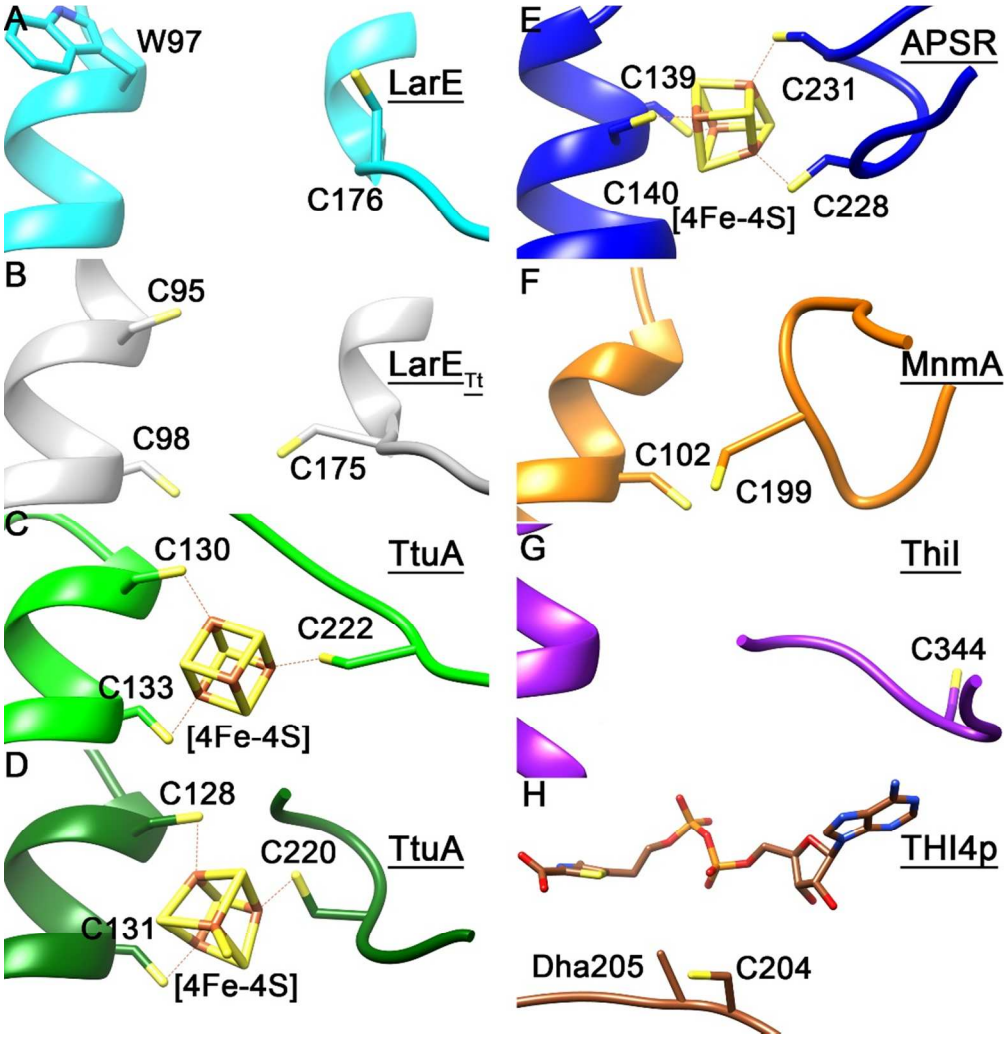


Figure 9. Comparison of active sites of structurally-related sulfur transferases. (A) LarE from *L. plantarum* shown in cyan (5UDR). (B) Homology model of $LarE_{Tt}$ from *T. thermosaccharolyticum* colored in grey. (C) 2-Thiouridine synthetase (TtuA) from *T. thermophilus* shown in light green (5B4E). (D) TtuA from *P. horikoshii* depicted in dark green (5MKP). (E) Adenosine 5′-phosphosulfate reductase from *P. aeruginosa* colored in blue (2GOY). (F) tRNA 2-thiouridylase (MnmA) from *E. coli* shown in orange (2DEU). (G) tRNA 4-thiouridine synthetase (ThiI) from *T. maritima* depicted in purple (4KR6). (H) Functionally-related sacrificial sulfur insertase (THI4p) from *S. cerevisiae* shown in brown with bound adenylylated thiazole (3FPZ).

One precedent exists for a sacrificial sulfur transferase: THI4p that functions in the thiamine biosynthesis pathway of *Saccharomyces cerevisiae*.[35] Although related in function to LarE and similarly thought to catalyze a single turnover, THI4p possesses a distinct fold (Figure 9H). Of further interest, *Methanococcus jannaschii* possesses an ortholog of THI4p that uses exogenous sulfide for thiamin biosynthesis and is catalytic.[36] This example, like that mentioned above for ThiI, illustrates the situation where orthologs of the same protein can exhibit distinct properties. In this case, one example is a sacrificial single-turnover enzyme while the other ortholog exhibits multiple-turnover catalytic activity.

LarE is structurally related to other sulfur transferases that contain [4Fe-4S] clusters or generate persulfides at their active sites, such as TtuA, MnmA, and ThiI. In addition, it is functionally related to sulfur transfer enzymes in thiamin synthesis that utilize two distinct mechanisms, one stoichiometric and one catalytic. Thus, we hypothesize that other LarE orthologs may bind an [4Fe-4S] cluster or generate persulfides and use these species for catalytic

sulfur transfer. LarE homologs that use three active-site cysteine residues to bind a [4Fe-4S] cluster could use the non-ligated sulfur atom for attack of the AMPylated substrate without sacrificing a cysteine residue. Similarly, a persulfide could attack the adenylylated substrate with another cysteine forming a disulfide to allow for sulfur transfer. The active form of the enzyme could then be reconstituted by the normal cellular cluster synthesis machinery or by persulfide formation to allow for additional rounds of sulfur transfer. It will be interesting to assess whether LarE functions catalytically in the multi-Cys proteins.

CONCLUSIONS

We elucidated the structure of the complex between CoA and LarE and unexpectedly observed strong similarities in binding modes for CoA and ATP. The sulfur atom of CoA can adopt multiple positions including formation of the previously identified disulfide with Cys176 of LarE, and the structures provide insights into how CoA provides thermal stability to the protein. The persulfide of CoA was competent for restoring the sulfur atom to $LarE^{Dha}$, whereas other LarE regeneration efforts were poorly effective or unsuccessful. The low extent of LarE recycling *in vitro* and the lack of evidence for *in vivo* recycling are consistent with our prior conclusion that LarE is a sacrificial protein.[9] Only two LarE molecules are required for activation of each cofactor that becomes bound to LarA, so a stoichiometric mechanism may be sufficient for cellular needs. We identified a new species; the NPN adduct of LarE that explains the perplexing prior results in which purified LarE was sufficient to activate LarA apoprotein. Release of NPN from the small proportion of LarE with bound cofactor accounted for LarA activation when using a vast excess of the accessory protein. The observation of this adduct shows that LarC can catalyze the metallation reaction of the organic ligand bound in thioester

linkage to LarE. We determined the structure of the C176A variant and found that the lack of the thiol side chain led to no conformational changes compared to the WT enzyme. We also created three additional active site variants of LarE and discussed their activities in a structural context, along with those of previously described variants. Finally, we carried out a structural comparison of the *L. plantarum* LarE to a homology model for an alternative version of LarE with a predicted cluster of cysteine residues and to other related enzymes that possess [4Fe-4S] clusters or a reactive persulfide in their active sites. From this analysis, we speculate that some orthologs of LarE may bind a [4Fe-4S] cluster providing them with the ability to act catalytically. Our supposition of two forms of an enzyme, one acting stoichiometrically to form $LarE^{Dha}$ while the other acting catalytically, has precedence in thiamin biosynthesis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at …

Figures S1, S2, and S3 provide mass spectrometric results for the interaction of TCEP, CoA, and DTT with $LarE^{Dha}$.

AUTHOR INFORMATION

Corresponding Author

*(R.P.H.) E-mail: hausinge@msu.edu

ORCID

Matthias Fellner 0000-0003-3192-6984

Joel A. Rankin 0000-0002-9775-4508

Jian Hu 0000-0001-6657-9826

Robert P. Hausinger 0000-0002-3643-2054

Present Address

^Department of Biochemistry, University of Otago, PO Box 56, Dunedin, 9054, New Zealand

Author Contributions

M.F. and J.A.R. contributed equally.

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Notes

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