"Nickel-pincer cofactor biosynthesis involves LarB-catalyzed pyridinium carboxylation and LarE-dependent sacrificial sulfur insertion."

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ABSTRACT

The lactate racemase enzyme (LarA) of Lactobacillus plantarum harbors a (SCS)Ni(II) pincer complex derived from nicotinic acid. Synthesis of the enzyme-bound cofactor requires LarB, LarC, and LarE, which are widely distributed in microorganisms. The functions of the accessory proteins are unknown, but the LarB C terminus resembles aminimidazole ribonucleotide carboxylase/mutase, LarC binds Ni and could act in Ni delivery or storage, and LarE is a putative ATP-using enzyme of the pyrophosphatase-loop superfamily. Here, we show that LarB carboxylates the pyridinium ring of nicotinic acid adenine dinucleotide (NaAD) and cleaves the phosphoanhydride bond to release AMP. The resulting biscarboxylic acid intermediate is transformed into a bisthiocarboxylic acid species by two single-turnover reactions in which sacrificial desulfurization of LarE converts its conserved Cys176 into dehydroalanine. Our results identify a previously unidentified metabolic pathway from NaAD using unprecedented carboxylase and sulfur transferase reactions to form the organic component of the (SCS)Ni(II) pincer cofactor of LarA. In species where larA is absent, this pathway could be used to generate a pincer complex in other enzymes.

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Nickel-pincer cofactor biosynthesis involves LarB-catalyzed pyridinium carboxylation and LarE-dependent sacrificial sulfur insertion

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Edited by Tadhg P. Begley, Texas A&M University, College Station, TX, and accepted by the Editorial Board March 28, 2016 (received for review January 11, 2016)

The lactate racemase enzyme (LarA) of Lactobacillus plantarum harbors a (SCS)Ni(II) pincer complex derived from nicotinic acid. Synthesis of the enzyme-bound cofactor requires LarB, LarC, and LarE, which are widely distributed in microorganisms. The functions of the accessory proteins are unknown, but the LarB C terminus resembles aminomimidazole ribonucleotide carboxylase/mutase, LarC binds Ni and could act in Ni delivery or storage, and LarE is a putative ATP-using enzyme of the pyrophosphatase-loop superfamily. Here, we show that LarB carboxylates the pyridinium ring of nicotinic acid adenine dinucleotide (NaAD) and cleaves the phosphoanhydride bond to release AMP. The resulting bisscarboxylic acid intermediate is transformed into a bisthiocarboxylic acid species by two single-turnover reactions in which sacrificial desulfurization of LarE converts its conserved Cys176 into dehydroalanine. Our results identify a previously unidentified metabolic pathway from NaAD to NaMN and NaAD. Whereas we detected only very low levels of Lar activity when using NaMN, substantial LarA activation was observed for samples incubated with NaAD and NaHCO3 and the activity increased with added bicarbonate and Ni2+ (Fig. 1B). When the LarB reaction was performed separately from the LarC and LarE reactions, significant Lar activity was only observed when LarB was incubated with NaAD and bicarbonate (Fig. 1C). ATP and Ni2+ were not required for the LarB reaction (Fig. 1C).

**Significance**

Nicotinic acid is a precursor of the ubiquitous cofactors nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Previous studies revealed that nicotinic acid is required for lactate racemization, an enzymatic activity that enables microorganisms to produce or use both isomers of lactate. Lactate racemase (Lar) was shown to harbor a nicotinic acid-derived cofactor that coordinates a nickel ion, forming a pincer complex. The biosynthesis of this cofactor requires three accessory proteins: LarB, LarC, and LarE. Here, we describe this biosynthetic pathway by showing that LarB carboxylates and hydrolyzes the NAD precursor nicotinic acid adenine dinucleotide (NaAD).

Author contributions: B.D., P.S., P.H., and R.P.H. designed research; B.D. performed research; B.D., P.S., P.H., and R.P.H. analyzed data; and B.D., P.S., P.H., and R.P.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. T.P.B. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1600486113/-/DCSupplemental.

Lactate racemase (Lar) interconverts D- and L-lactic acid, providing microorganisms with the ability to use or produce both isomers even if only one stereospecific lactate dehydrogenase is present. The enzyme (LarA) harbors a covalently bound Ni pyridinium-3-thioamide-5-thioarboxylic acid mononucleotide pincer complex featuring a Ni–C bond (Fig. 1B) (1). A pincer ligand in organometallic chemistry traditionally consists of a central ring disubstituted with two chelating side arms (2). The central atom together with the two donor groups on the side arms bind to the metal ion in a planar fashion, providing both structural and thermal stability (2). Many nickel pincer complexes have been synthesized to stabilize the nickel ion and control its reactivity, some of them exhibiting high catalytic activity (2). Although pincer complexes are ubiquitous in synthetic chemistry, the LarA cofactor is the first example of a pincer compound identified in the natural world. This cofactor, thought to reversibly capture hydride from the substrate, is synthesized from nicotinic acid by a pathway requiring LarB, LarC, and LarE. LarE is able to activate the LarA apoprotein when isolated from cells also producing LarB and LarC (3). This result implies that LarE harbors the Ni-containing cofactor before its transfer to LarA. In this study, we describe the biosynthetic pathway leading to the formation of the lactate racemase (SCS)Ni(II) pincer complex starting from the NAD precursor, nicotinic acid adenine dinucleotide (NaAD).

**Results**

**In Vitro LarA Activation.** We reconstituted LarA cofactor biosynthesis in vitro by incubating extracts of LarB-containing cells with purified LarC and LarE, along with various additives (SI Appendix, Fig. S1A). Lar activity was observed for lysates of cells expressing larB (used directly or after filtration to remove proteins) when incubated with ATP (1 mM), MgCl2 (12 mM), and the other purified proteins, and the activity was stimulated fivefold by inclusion of CoA (10 mM) (SI Appendix, Fig. S1A–D). These results are consistent with the requirement of a small molecule produced by LarB being needed for development of Lar activity. To uncover the substrate of LarB, we replaced the LarB-containing lysates in the LarA activation mixture with purified LarB and potential substrates. Because nicotinic acid is a precursor of the Lar cofactor (1) and LarB features partial sequence identity with PurE, an aminomimidazole ribonucleotide (AIR) mutase/carboxylase (4), we tested the possibility that LarB could carboxylate intermediates of the Press–Handler pathway from nicotinic acid to NAD (5), namely nicotinic acid mononucleotide (NaMN) and NaAD. Whereas we detected only very low levels of Lar activity when using NaMN, substantial LarA activation was observed for samples incubated with NaAD and NaHCO3 and the activity increased with added bicarbonate and Ni2+ (Fig. 1B). When the LarB reaction was performed separately from the LarC and LarE reactions, significant Lar activity was only observed when LarB was incubated with NaAD and bicarbonate (Fig. 1C). ATP and Ni2+ were not required for the LarB reaction (Fig. 1C).
NaAD hydrolysis, NaMN and AMP (Fig. 24). When we added NaHCO₃ to the LarB reaction, however, a species of m/z = 380.04 appeared, with this species shifting to m/z = 381.04 using NaH¹³CO₃ (Fig. 2B). Only the LC fractions containing these species were capable of LarA in vitro activation in the presence of LarC and LarE (SI Appendix, Fig. S2). These species correspond to the mass of NaMN plus a carbonyl group (C₁₂H₁₅NO₁₀P⁺: m/z = 380.038; C₁₁¹³CH₂NO₃P⁺: m/z = 381.045). Fragmentation of these ions yielded products consistent with NaMN being carboxylated on the pyridinium ring (SI Appendix, Fig. S3). In particular, the unlabeled and isotopically labeled suspected pyridinium-3,5-bis(carboxylate mononucleotide (P2CMN) species gave rise to m/z = 168 and 169 positive ion fragments as expected for the corresponding pyridine biscarboxylates (SI Appendix, Fig. S3A). The dicarboxylated pyridinium ring formed with C¹³C can fragment to form NaMN by loss of either the original carbonylate or the ¹³C carbonylate. Equivalence of these two species (SI Appendix, Fig. S3B) is consistent with a symmetric structure, thus indicating the site of carboxylation and confirming that LarB is a NaAD carboxylase-hydrolase-forming P2CMN.

We characterized several properties of LarB-catalyzed P2CMN formation by monitoring the in vitro activation of LarA (SI Appendix, Fig. S4). We showed that the Kₘ for NaAD was 13 ± 3 μM (SI Appendix, Fig. S4A), the Kₘ for MgCl₂ was 0.55 ± 0.18 mM (SI Appendix, Fig. S4B), and the apparent Kₘ for NaHCO₃ was 33 ± 18 mM (SI Appendix, Fig. S4C), which are comparable to the values for AIR carboxylation by PurE (6). We found quite different parameters when examining LarB-catalyzed NaAD turnover by assessing AMP release (SI Appendix, Fig. S5). The rate of product release appeared to exhibit hyperbolic behavior (with a Kₘ of 26 ± 15 μM) superimposed on a reaction rate that was linearly dependent on NaAD concentrations (SI Appendix, Fig. S5A). The Kₘ for MgCl₂ was 3.9 ± 2.2 mM (SI Appendix, Fig. S5B), whereas AMP release was independent of NaHCO₃ concentration (SI Appendix, Fig. S5C). These results suggest that LarB can hydrolyze NaAD directly or it first forms an adduct with NaAD that releases AMP and reacts with CO₂ to generate P2CMN (SI Appendix, Fig. S5D).

**Sulfur Insertion by LarE.** Because the LarA cofactor contains two sulfur atoms (1) (Fig. L4), the role of LarE could be to modify the two carboxylic acids of P2CMN by forming two thiocarboxylic acids. To test this hypothesis, we incubated LarE with P2CMN or ¹³C-labeled P2CMN (P2CMN*) obtained through the in vitro reaction of LarB with NaAD and NaHCO₃ or NaH¹³CO₃, respectively. After incubation, LarE was digested with trypsin, and the solution was analyzed by LC-ESI-MS. We detected species of m/z = 396.02 and m/z = 411.99 when using P2CMN and of m/z = 397.02 and m/z = 413.00 when using P2CMN* (Fig. 2C). The molecular masses of these species are consistent with pyridinium-3-carboxy-5-thiocarboxylic acid mononucleotide (P2TMN) and pyridinium-3,5-bis(thiocarboxylic acid mononucleotide (P2TMN*) (C₁₂H₁₅NO₁₀S₂P⁺: m/z = 396.015 and C₁₂¹³H₁₅NO₁₀S₂P⁺: m/z = 411.993). The two putative P2TMN species (unlabeled and labeled, respectively) similarly gave rise to MS/MS species (m/z = 200 and 201) that were expected for the corresponding pyridine bithiocarboxylate fragments (SI Appendix, Fig. S7). The presence of m/z = 183 and 184 species in the positive-ization mode and of m/z = 333, 334, 393, and 394 species in the negative ionization mode indicates the presence of pyridinium-3-carboxamide-5-thiocarboxylic acid mononucleotide (SI Appendix, Fig. S6), which probably arose as a product of the nucleophilic attack of P2TMN by the ammonia present in solution.

Furthermore, the putative P2TMN and P2TMN* species led to m/z = 334 and 350 negative ion fragments, respectively, arising from loss of COS, which is consistent with sulfur insertion into the carboxylic acid groups of P2CMN (SI Appendix, Figs. S6 and S7), confirming the identity of these species and showing that LarE is responsible for sulfurization of P2CMN into P2TMN.

**Role of CoA in LarE Activity.** LC-ESI-MS profiles were then performed on LarE to identify the function of CoA in LarA cofactor biosynthesis. These profiles revealed that LarE (31,550 ± 1 Da for sample containing the StreptII-tag residues, ASWHPQFEK, and missing the N-terminal Met) was accompanied by 119 ± 1 Da, 689 ± 1 Da, and 766 ± 1 Da larger species (SI Appendix, Fig. S8, Top). Analogous analysis of LarE purified in the presence of CoA provided a small amount of free protein and a large amount of species larger by 765 ± 1 Da. This species is consistent with a LarE-CoA mixed disulfide, and the 689 ± 1 Da version probably is available in the full-length complex.
the same form after dephosphorylation (the 32,414 ± 1 Da species is attributable to contamination of the sample). LC-ESI-MS and MS/MS analysis of the tryptic digest of LarE confirmed the presence of CoA in disulfide linkage to Cys176 of LarE (SI Appendix, Fig. S9). In particular, a modified peptide of 1,659.51 Da was detected in both negative and positive ion ESI-MS modes that matched for expected for the mass of the peptide corresponding to residues 173–181 (894.41 Da) plus CoA (767.12 Da) less two protons (2.02 Da). Furthermore, several MS/MS products were consistent with this assignment, including some possessing a persulfide, as expected for fragmentation of a mixed disulfide (7) (SI Appendix, Fig. S9). We suggest the LarE-CoA disulfide is an off-pathway species likely to be formed by reaction of the nucleophilic LarE cysteiny1 group with CoA disulfide formed in the aerobic solution (LarE-SH + CoA-S-S-CoA ↔ LarE-S-S-CoA + CoA-SH). Potentially, the P2CMN- and ATP-binding sites of LarE bind CoA to facilitate generation of the LarE-CoA mixed disulfide. This form may stabilize LarE in the absence of P2CMN. We propose that free CoA reduces LarE-CoA to provide the free protein that is needed for converting P2CMN to P2TMN.

Cysteine of LarE Is the Sulfur Donor. We next sought to identify the sulfur donor in the LarE reaction, for which the solution contained only purified LarC and LarE proteins and CoA as potential sulfur sources. Incubation of the LarB reaction mixture with LarE led to the transient development of a distinct LarE species (31,911 ± 1 Da, or 361 ± 1 Da larger than free LarE), followed by all LarE protein being converted to a 31,156 ± 1 species (34 Da less than LarE) by 45 min (Fig. 3A and SI Appendix, Fig. S8). The intermediate species increased by 1 mass unit when using NaH13CO3 in the LarB reaction solution, and the mass was consistent with LarE being linked to P2CMN (SI Appendix, Fig. S8). Neither the intermediate nor the final LarE species was observed in the absence of ATP (SI Appendix, Fig. S8), indicating that ATP is required for these transformations. The final LarE species, with a mass 34 Da smaller than the free protein, was shown to possess a peptide corresponding to residues 173–181 but with Cys replaced by dehydroalanine (Dha) (Fig. 3A). The loss of sulfur from Cys176 accompanies the conversion of P2CMN to P2TMN, consistent with LarE serving as a substrate by offering its cysteiny1 group as the sulfur donor. Even though much of the protein begins as the CoA disulfide, all LarE is converted in this reaction because of the presence of excess CoA in the mixture (i.e., LarE-S-S-CoA + CoA-SH ↔ LarE-SH + CoA-S-S-CoA).

LarE purified from cells expressing the entire larA-E operon was mostly in the desulfurized form (SI Appendix, Fig. S1A), suggesting that the LarE cysteine is not regenerated in vivo. These results lead us to propose a reaction mechanism in which a carboxyl group of P2CMN is first amidylated by ATP [similar to the activation of NaMN by the LarE homolog nicotinamide mononucleotide synthetase (8)], nucleophilic attack by Cys176 of LarE, and Dha formation is coupled to sulfur transfer then creates the final product P2TMN. Thus, two molecules of LarE undergo sacrificial sulfur transfer to create one P2TMN.

![Image](image-url)
trypsin confirmed the presence of K184SVLPGIASYK peptide (38x162)

S12 protecting LarA from oxidation (1), was present (Fig. 4

quiring 90 min to reach maximal activity when sulfite, known to

transferred into the mature cofactor into the LarA apoprotein (3). We observed a
time-dependent increase in Lar activity when the cofactor was

NiCl2 (0.1 mM). (Fig. 4A) Error bars represent the SD (n = 2). (B) LC-ESI-MS of a doubly charged tryptic peptide of LarE before (Upper) and 30 min after (Lower) addition of P2CMN. The sequences of the peptides analyzed by LC-ESI-MS/MS (SI Appendix, Fig. S10) are indicated above the corresponding peaks.

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Fig. 3. Sulfur transfer from Lar to P2CMN. (A) Percentage of each form of LarE as observed by LC-MS-ESI before (Control) and 0, 15, 30, and 45 min after addition of the LarB product (SI Appendix, Fig. S8). Error bars represent the SD (n = 2). (B) LC-ESI-MS of a doubly charged tryptic peptide of LarE before (Upper) and 30 min after (Lower) addition of P2CMN. The sequences of the peptides analyzed by LC-ESI-MS/MS (SI Appendix, Fig. S10) are indicated above the corresponding peaks.

LarC-Mediated Nickel Insertion and Transfer to LarA. Further steps in
the generation of Lar activity include nickel insertion into P2TMN, a process that must use LarC (which purifies with bound Ni) because nickel alone is not inserted (Fig. 4A), and transfer of the mature cofactor into the LarA apoprotein (3). We observed a time-dependent increase in Lar activity when the cofactor was transferred into Lactobacillus plantarum LarA apoprotein, requiring 90 min to reach maximal activity when sulfite, known to protect LarA from oxidation (1), was present (Fig. 4B). Our analysis of the in vitro-activated LarA proteins by LC-ESI-MS revealed the expected mass shift corresponding to the addition of the cofactor to LarA (SI Appendix, Fig. S12A). LC-ESI-MS and LC-ESI-MS/MS analyses of in vitro-activated LarA digested with trypsin confirmed the presence of K184SVLPGIASYK peptide covalently linked to the (SCS)Ni(II) cofactor (SI Appendix, Figs. S12B and S13), as previously described (1). The mass of this peptide was shifted by 1 Da when using LarB-generated P2CMN in the activation mixture (SI Appendix, Figs. S12B and S13), demonstrating that the cofactor in LarA is derived from P2TMN.

Discussion

We propose a scheme for the biosynthesis of active L. plantarum LarA in which LarB converts NaAD into P2CMN, LarE incorporates two sulfur atoms to yield P2TMN, LarC donates Ni, and the mature cofactor becomes covalently attached to LarA apoprotein (Fig. 5). The LarB-dependent carboxylation reaction requires bicarbonate/CO2 (Fig. 1C) and somewhat resembles AIR carboxylation by the LarB homolog PurE (10); however, biological carboxylation of a pyridinium ring was previously unknown. To perform this chemistry, a nucleophilic amino acid of LarB may attack the pyridinium ring of NaAD to neutralize the positive charge and allow for direct attack of the ring C5 on CO2. ATP is not required for the LarB reaction (Fig. 1C), so ATP-dependent activation of bicarbonate does not take place. An alternative hypothesis is that the LarB-catalyzed cleavage of the phosphoanhydride bond of NaAD may activate bicarbonate by formation of carboxy-AMP. This hypothesis would explain why NaMN is not a substrate of LarB (Fig. 1C).

LarE possesses multiple functions: activation of P2CMN by adenylylation, covalent linkage of the protein to P2CMN, sacrificial loss of its Cys176 sulfur atom to form the thioacid of P2CMN, and then repeating the set of reactions with a second LarE protomer to form P2TMN. Although we have not yet detected adenylylated P2CMN or the LarE-PCTMN derivative, we did observe the LarE-P2CMMN adduct and demonstrated Dha in the product LarE. Sulfur-containing cofactors are typically synthesized by using the sulfur atom of free cysteine through the action of a persulfide-forming cysteine desulfurase (11); however, direct sulfur donation from a protein cysteinyl group, also generating a Dha residue, has been reported in the case of thiamine thiazole synthase of Saccharomyces cerevisiae, THI4p (12). Whereas a single molecule of THI4p is used to insert the lone sulfur atom of a

observation of a near 2:1 LarE:LarA ratio for optimal Lar activity (SI Appendix, Fig. S11C).

LarC requirement and Lar activity generation. (A) Lar activity generated upon addition of LarA apoprotein to solutions incubated for 2 h with NaAD (0.5 mM), NaHCO3 (10 mM), LarB (1 μM), LarE (5 μM), MgCl2 (20 mM), ATP (5 mM), and CoA (1 mM) in Tris-HCl buffer (100 mM; pH 8) along with (where indicated) LarC and NiCl2 (0.1 mM). (B) Time course of the in vitro activation of L. plantarum LarA in the absence (−Sulfite) or presence (+Sulfite) of 10 μM sulfite. Error bars represent the SD (n = 3).
Materials and Methods

Biological Materials and Growth Conditions. Bacterial strains and plasmids used in the present study are listed in SI Appendix, Table S1. We grew Lactococcus lactis in M17 broth supplemented with 0.5% glucose at 28 °C. NiSO₄ (1 mM) was added to cultures expressing pGIR051, and chloramphenicol (10 mg/L) was added to cultures expressing pGIR072. DH10B was grown with agitation (200 rpm) at 37 °C in LB medium containing 150 mM NaCl (buffer W). Cells were resuspended in 15 mL of W buffer and transferred by 0.5-mL aliquots into 0.5-mL suspensions of glass beads (106 μm; Sigma) in W buffer using 2-mL microtubes. Lysis was accomplished by using a Mini-Beadbeater-16 (BioSpec Products) twice for 1-min periods, with 2 min of cooling on ice between the runs. After lysis, the soluble fractions were collected by centrifugation at 20,000 × g for 15 min at 4 °C. Affinity chromatography was performed with gravity-flow Strep-Tactin Superflow high-capacity columns of 1.5 mL, as described (17), with buffer W. For purification of LarE in the presence or CoA, 0.5 mM CoA was added to all buffers. Protein concentrations were measured by Bradford assay (18).

DNA Techniques. General molecular biology techniques were performed according to standard protocols (13). Transformation of E. coli and L. lactis was performed by electroporation (14, 15). PCR amplifications used Phusion high-fidelity DNA polymerase (New England Biolabs). The primers used in this study were purchased from IDT and are listed in SI Appendix, Table S1. Plasmid pGIR026, derived from pGIZ660 (4), bears a translational fusion of larB and DNA encoding the StrepII tag for expression in E. coli. pGIZ660 was PCR-amplified with the primers BT_A and BT_B, digested with NheI, and self-ligated, generating a 30-bp in-frame insertion of a fragment encoding the StrepII tag at the C terminus of LarB. The ligation mixture was treated with DpnI before transformation into E. coli to digest the original pGIZ660 plasmid template. Plasmids bearing variants of larE for expression in L. lactis were derived from pGIR072 (3) (i.e., pGIR072M1-M2) and provided the two variant forms (D30A, C176A) of LarE. For each construction, pGIR072 was first methylated by Dam methylase using 5-adenosyl methionine (New England Biolabs), and PCR amplification was performed to obtain a fragment comprising the mutated plasmid. The PCR product was digested with DpnI before transformation in L. lactis according to the Quickchange mutagenesis protocol (16). The plasmid sequences were confirmed by sequencing with primer UP_PNZB048.

Cell Lysate Preparation. A total of 1.5 L of L. lactis cultures expressing pNZB048 (empty vector) or pGIR022 (encoding LarB) were collected and the pellets were washed twice in 100 mM Tris-HCl at pH 7.5 (buffer T), resuspended in 15 mL of buffer T with 15 g L⁻¹ of lysozyme, incubated 30 min at 37 °C, and cooled 10 min on ice. This treatment was followed by sonication at maximum amplitude (Branson Sonifier 450) using two series of 10 one-second pulses with an intervening 1-min pause on ice. Clear supernatants were obtained after centrifugation at 12,000 × g for 25 min at 4 °C. For obtaining denatured cell lysates, the supernatants were placed into an Amicon Ultra-15 centrifugal filter unit (10-kDa cutoff; Merck Millipore) and spun, and the flow-through fraction was collected.

Protein Purification. LarE from L. plantarum (LarEₑₕ) or Thermoanaerobacterium thermosaccharolyticum (LarEₕₕ) Lactococcus lactis, LarE, and LarC were purified from 1.5 L of the appropriate L. lactis cultures (containing plasmids that are listed in SI Appendix, Table S1), which were collected by centrifugation (5,000 × g for 10 min) and washed twice with 50 mL of 100 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl (buffer W). Cells were resuspended in 15 mL of W buffer and transferred by 0.5-mL aliquots into 0.5-mL suspensions of glass beads (106 μm; Sigma) in W buffer using 2-mL microtubes. Lysis was accomplished by using a Mini-Beadbeater-16 (BioSpec Products) twice for 1-min periods, with 2 min of cooling on ice between the runs. After lysis, the soluble fractions were collected by centrifugation at 20,000 × g for 15 min at 4 °C. Affinity chromatography was performed with gravity-flow Strep-Tactin Superflow high-capacity columns of 1.5 mL, as described (17), with buffer W. For purification of LarE in the presence or CoA, 0.5 mM CoA was added to all buffers. Protein concentrations were measured by Bradford assay (18).

LarB was purified from 1.5 L of overnight E. coli culture expressing pGIR026. The cells were collected, and the pellet was washed twice in buffer W, resuspended in buffer W with 1 g L⁻¹ lysozyme, incubated for 30 min at 37 °C, and cooled for 10 min on ice. This treatment was followed by sonication at maximum amplitude (Branson Sonifier 450) using three series of 10 one-second pulses with intervening 2-min pauses on ice. Clear supernatant was obtained after centrifugation at 12,000 × g for 30 min at 4 °C. Affinity
chromatography was performed with gradient-flow Strept-Tactin Superflow high-capacity columns of 1.5 mL, as described (17), with buffer W.

In Vitro Activation of LarB by Lysates from LarB-Containing Cells or by Purified LarB, LarC, and LarE. We incubated a mixture of LarL (5 μM), LarC (5 μM), ATP (5 mM), MgCl₂ (20 mM), and CoA (1 mM) in Tris-HCl buffer (100 mM; pH 8) with 10 μL of lysates from lar-free cells or cells expressing larB in a final volume of 20 μL. After 1 h of incubation at room temperature (RT), 5 μL of the assay mixture was diluted into 45 μL of-lactate (45 mM) supplemented with LarA₅₆ apoprotein (0.8 μM) in Hepes buffer (100 mM; pH 7). The reaction was incubated for 5 min at 50 °C and then stopped by heat treatment at 90 °C. The resulting l-lactate concentration was measured by enzymatic lactate oxidation to pyruvate using a l-lactid acid-lactid acid commercial test (Megazyme), as previously described (3). The NADH absorbance was monitored at 340 nm with a SpectraMax M5 (Molecular Devices) (SI Appendix, Fig. S5). For optimization of MgCl₂, ATP, and CoA concentrations, the concentration of one component was varied with the other components held static. For analyses of LC fractions, the lysates were replaced by LarB reaction products (control) or by LC-separated fractions of LarB reaction products (SI Appendix, Fig. S2).

To examine the purified LarB reaction, we replaced the lysate in the previous experiment with LarB (1 μM) and incubated it with NaMN (0.5 mM) or NaAD (0.5 mM), NaHCO₃ (0.1 mM), MgCl₂ (4 mM), and ATP (5 mM) in Tris-HCl buffer (100 mM; pH 8) and heat-treated for 50 °C. The reaction solutions were used in Tris or NaAD (0.5 mM) along with NaHCO₃ (10 mM) and NiCl₂ (0.1 mM). After 2 h products were held static. For analyses of LC fractions, the lysates were replaced by LarB (1 μM) and incubated it with NaMN (0.1 mM), NaAD (0.2 mM), MgCl₂ (4 mM), and ATP (5 mM) in Tris-HCl buffer (100 mM; pH 8) and heat-treated for 50 °C. The resulting D-lactate concentration was measured by enzymatic lactate oxidation to pyruvate using a l-lactid acid-lactid acid commercial test (Megazyme), as previously described (3). The NADH absorbance was monitored at 340 nm with a SpectraMax M5 (Molecular Devices) (SI Appendix, Fig. S5). For optimization of MgCl₂, ATP, and CoA concentrations, the concentration of one component was varied with the other components held static. For analyses of LC fractions, the lysates were replaced by LarB reaction products (control) or by LC-separated fractions of LarB reaction products (SI Appendix, Fig. S2).

LC-ESI-MS of Proteins and Peptides. For analysis of the different forms of LarL (Fig. 3A and SI Appendix, Figs. S8 and S11), LarE (10 μM) was incubated with ATP (5 mM), MgCl₂ (20 mM), and 50% volume of LarB reaction (as described for LC-ESI-MS of LarB product) for 0, 15, 30, and 45 min and analyzed by LC-ESI-MS; 10-μL aliquots were injected into the UPLC system coupled to the QToF mass spectrometer. Separations were performed on a BetaBasic CN (10 mm × 1 mm; 5-μm particle size) column with an aqueous phase of 0.1% formic acid and a gradient of increasing acetonitrile at 30 °C. The run time for each sample was 15 min. MS/MS spectra were generated by using argon as the collision gas and a collision cell potential of 40 V.

LarA₅₆ Apoprotein in LarB Reaction. We incubated LarB (1 μM), LarE (10 μM), NaAD (0.5 mM), ATP (5 mM), MgCl₂ (20 mM), CoA (1 mM), NaHCO₃ (10 mM), and NiCl₂ (0.1 mM) in Tris-HCl buffer (100 mM; pH 8) in a final volume of 25 μL for 2 h at RT. LarA₅₆ in vitro activation was started by adding apoprotein (6 μM) in Tris-HCl buffer (100 mM; pH 8) in a final volume of 50 μL. The sample was activated for 30 min at RT, and 3 μL was diluted into 47 μL of 45 mM l-lactate in Hepes (100 mM; pH 7) buffer to measure Lar activity. For the time-course experiment (Fig. 4B), we used LarA₅₆ in Hepes buffer (100 mM; pH 7), and NaCeO₃ (10 mM) was added when needed. The assay was incubated for 0, 30, 60, 90, and 120 min at RT, and 3 μL was diluted into 47 μL of 45 mM l-lactate in Mes (100 mM; pH 6) buffer. The reaction was incubated for 5 min at 35 °C and stopped by heat treatment at 90 °C, and the Lar activity was measured as described above. For LC-ESI-MS analysis, 250 μL of the LarA in vitro activation mixture (incubated for 90 min at RT) was directly analyzed (SI Appendix, Fig. S12A) or washed three times with AB buffer (pH 8); trypsin-digested as described previously (1), and analyzed (SI Appendix, Fig. S12B and S13).

ACKNOWLEDGMENTS. We thank the Michigan State University Mass Spectrometry and Metabolomics Core for data collection. This work was supported by National Science Foundation Grant CHE-1516126 (to R.P.H.), Michigan State University (R.P.H.), the Belgian National Fund for Scientific Research (FRS) (P.H. and P.S.), and the Université catholique de Louvain-Fonds Spéciaux de Recherche (P.H. and P.S.).

Nickel-pincer cofactor biosynthesis involves LarB-catalyzed pyridinium carboxylation and LarE-dependent sacrificial sulfur insertion

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Fig. S1. In vitro activation of LarA using lysates from LarB-containing cells. (A) Lar activity generated upon addition of LarA apoprotein to solutions incubated for 1 h with the LarE (5 µM), LarC (5 µM), MgCl₂ (20 mM), ATP (5 mM) and CoA (1 mM) in Tris-HCl buffer (100 mM, pH 8) along with 50% V/V lysates from lar-free cells (X), lysates from cells expressing larB (B), or ultrafiltered lysates from cells expressing larB (B₁). Concentration
dependence of (B) MgCl₂, (C) ATP, and (D) CoA in the in vitro LarA activation reaction with 50 % V/V lysates from cells expressing larB. 100% Lar activity is that obtained for a LarE + LarC reaction containing 12 mM MgCl₂, 1 mM ATP and 20 mM CoA, respectively. Error bars represent the SD (N=3).
Fig. S2. LC of the LarB product. (A) Retention time of the m/z 380 species (top) and m/z 381 species (bottom) of the LarB in vitro reaction with NaHCO₃ (top) and NaH¹³CO₃ (bottom) in positive ionization mode. The ordinate represents the abundance relative to the highest peak in %. (B) Relative Lar activity of LarA in vitro activation with the 0.5 min-6.5 min fractions of the LarB in vitro reaction after LC as in A. 100% activity is the Lar activity of the sample before injection (Co). Error bars represent the SD (N=4).
Fig. S3. MS/MS Fragmentation of P2CMN. LC-ESI-MS/MS fragmentation in positive (A) and negative (B) ionization mode of the 379 Da (top) and 380 Da (bottom) species from the \textit{in vitro} LarB reaction with NaHCO$_3$ (top) and NaH$^{13}$CO$_3$ (bottom). The ordinate represents the abundance relative to the highest peak in %. Possible structures of the fragments are drawn next to the corresponding peaks of the spectra. A “*” indicates the presence of $^{13}$C, while “(*)” indicates a 50% probability of $^{13}$C. The species at $m/z$ 335.03 also contains the +1 isotopic species of the species at $m/z$ 334.03, which explains why the species at $m/z$ 335.03 is slightly more intense than the species at $m/z$ 334.03.
Fig. S4. Properties of the LarB *in vitro* carboxylation reaction as assessed by Lar activity. Concentration dependence of (A) NaAD, (B) MgCl₂, and (C) NaHCO₃ in the *in vitro* LarB reaction followed by *in vitro* activation of LarA. The non-variable concentrations for NaAD, MgCl₂, and NaHCO₃ are 200 µM, 4 mM, and 50 mM, and 100% Lar activity is that obtained for a LarB reaction containing these concentrations. Symbols represent the average of four replicates; fitted curves are calculated by non-linear least squares regression using the equation $v_0 = \frac{v_{max}[S]}{K_m+[S]}$. 
Fig. S5. Properties of the LarB in vitro hydrolysis reaction as assessed by AMP release.

Concentration dependence of (A) NaAD, (B) MgCl₂, and (C) NaHCO₃ in the in vitro LarB reaction as followed by using an assay for AMP. The non-variable concentrations for NaAD, MgCl₂, and NaHCO₃ are 200 µM, 4 mM, and 50 mM. The observed NaAD hydrolysis rate
constants were estimated by dividing the rate of AMP appearance by the concentration of the LarB protein. Symbols represent the average of three replicates; fitted curves in A and B are calculated by non-linear least squares regression using the equations $k_{\text{obs}} = \frac{k_{\text{cat}}[S]}{K_m+[S]} + k_1[S]$ and $k_{\text{obs}} = \frac{k_{\text{cat}}[S]}{K_m+[S]}$, respectively. (D) Scheme depicting the potential reaction kinetics of LarB as measured by Lar activity and AMP release where X is a nucleophile of the enzyme.
Fig. S6. MS/MS Fragmentation of PCTMN. LC-ESI-MS/MS fragmentation in positive (A) and negative (B) ionization mode of the 395 Da (top) and 396 Da (bottom) species after tryptic digestion of the \textit{in vitro} reaction of LarE with P2CMN (top) and P2CMN* (bottom). The ordinate represents the abundance relative to the highest peak in %. Possible structures of the fragments are shown next to the corresponding peaks of the spectra. A “*” indicates the presence of $^{13}$C, while “(*)” indicates 50% probability of $^{13}$C.
Fig. S7. MS/MS Fragmentation of P2TMN. LC-ESI-MS/MS fragmentation in positive (A) and negative (B) ionization mode of the 411 Da (top) and 412 Da (bottom) species after tryptic digestion of the *in vitro* reaction LarE with P2CMN (top) and P2CMN* (bottom). The ordinate represents the abundance relative to the highest peak in %. Possible structures of the fragments are shown next to the corresponding peaks of the spectra. A “**” indicates the presence of $^{13}$C, while “(*)” indicates 50% probability of $^{13}$C.
Fig. S8. LC-ESI-MS profiles of LarE prior to and during its in vitro reaction. (Top row) Masses of LarE purified without (LarE -CoA) and with CoA (LarE +CoA) as observed in positive ionization mode. (Middle 2 rows) LarE masses as observed by LC-MS-ESI in positive ionization mode examined at 0, 15, 30, and 45 min after addition of the optimized in vitro LarB reaction solution (0, 15, 30 and 45 min). (Bottom row, left) LarE masses 15 min after addition of the in vitro LarB reaction solution prepared with NaH$^{13}$CO$_3$ (+P2CMN*). (Bottom row, right) LarE masses 30 min after addition of the LarB reaction solution following treatment with apyrase (-ATP). In each case, the ordinate represents the abundance relative to the highest peak in %. The numbers in parentheses indicate the mass difference from LarE (StrepII-tagged and minus the N-terminal methionine).
Fig. S9. MS and MS/MS analysis of the CoA-containing tryptic peptide in LarE. (A) LC-ESI-MS in negative (left) and positive (right) ionization modes of a doubly-charged species from the trypsin digestion of LarE that was purified in the presence of CoA. The mass of the modified peptide (1659.51 Da) corresponds to the mass of the peptide VASCSVSSR (894.41 Da) plus the mass of CoA (767.12 Da) minus the mass of two hydrogens (2.02 Da). (B) LC-
ESI-MS/MS fragmentation of the modified peptide in negative (left) and positive (right) ionization modes. The structures of possible fragments are shown next to the corresponding peaks or below the spectra. The ordinate represents the abundance relative to the highest peak in %.
Fig. S10. Fragmentation of the LarE tryptic peptide composed of residues 173-181 prior to and after the LarE reaction. The \( m/z \) 50-750 (A) and \( m/z \) 460-800 (B) ranges of the LC-ESI-MS/MS fragmentations are shown in positive ionization mode for the doubly-charged \( m/z \) 448.2 peak from trypsin digested LarE (top) and the \( m/z \) 431.2 peak from trypsin digested LarE 30 min after addition of the \textit{in vitro} LarB reaction solution (bottom). The y axis is the relative abundance in \%. (C) Schematic representations of the fragmented peptides.
Fig. S11. LC-ESI-MS and activity of LarE. (A) Masses of D30A LarE (top left), C176A LarE (top right), and LarE purified from cells expressing the larA-E operon (bottom) purified with CoA observed in positive ionization mode. The ordinate represents the abundance relative to the highest peak in %. The numbers in parentheses indicate the mass difference from LarE (StrepII-tagged and minus the N-terminal methionine). (B) Relative Lar activity after in vitro activation of LarA by D30A LarE and C176A LarE variants. 100% Lar activity is that obtained for wild-type LarE. Error bars represent the SD (N=3). (C) Lar activity generated upon addition of LarA apoprotein (from 0 to 6 µM) to solutions incubated for 1 h.
with LarE (5 µM), LarC (5 µM), MgCl$_2$ (20 mM), LarB (1 µM), NaAD (0.5 mM), NaHCO$_3$ (10 mM), NiCl$_2$ (0.1 mM) ATP (5 mM), CoA (1 mM) and cell lysate (20% volume) in Tris-HCl buffer (100 mM, pH 8). Error bars represent the SD (N=4). The dashed lines represent limiting amounts of LarA (LarA/LarE ratio $\leq 0.25$) and the line of maximal Lar activity (LarA/LarE ratio $\geq 1$), their intersection represents the optimal LarA/LarE ratio of 0.6±0.1 (or 1.7 LarE per LarA cofactor).
Fig. S12. LC-ESI-MS analysis of in vitro activated LarA. (A) LarA<sub>Lp</sub> masses as observed by LC-ESI-MS in positive ionization mode after purification (LarA<sub>Lp</sub>) and after 90 min of in vitro activation with 10 µM sulfite and in vitro-generated P2TMN (LarA<sub>Lp</sub> activated) and in vitro-generated P2TMN* (LarA<sub>Lp</sub> activated*). The numbers in parentheses indicate the mass differences from the mass of the LarA apoprotein minus the N-terminal methionine (Apo). (B) LC-ESI-MS in negative ionization mode of two versions of a modified peptide from the trypsin digestion of LarA<sub>Lp</sub> that was activated with in vitro-generated P2TMN (top) and in vitro-generated P2TMN* (bottom). The 1554 Da and 1570 Da species are degradation products of the Lar cofactor (1). The y axis is the abundance relative to the highest peak in %.
Fig. S13. LC-ESI-MS/MS analysis of a tryptic peptide generated from *in vitro* activated LarA. The ranges from *m/z* 50-290 (A), *m/z* 300-650 (B), and *m/z* 655-900 (C) of the LC-ESI-MS/MS fragmentation in positive ionization mode are shown for the doubly-charged 1554 Da tryptic peptide species of LarA$_{Lp}$ that was activated with *in vitro*-generated P2TMN (top) and *in vitro*-generated P2TMN* (bottom). The numbers in violet indicate the peaks corresponding to y fragments of the peptide K$^{184}$SVLPGIASYK. The numbers in blue indicate the peaks corresponding to the a and b fragments of the modified peptide minus ribose phosphate. The y axis is the abundance relative to the highest peak in %.
Table S1. Strains, plasmids, and primers.

<table>
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<th>Strain, plasmid, or primer</th>
<th>Characteristic(s) or sequence</th>
<th>Source or reference</th>
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<tr>
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<td>pGIR026</td>
<td>Em(^r) Ap(^r); pGIZ660(^p) with DNA encoding the StrepII-tag translationally fused at the 3′-end of the lar(_B) ORF Lar(_B) purification</td>
<td>This study</td>
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