

The nickel-pincer coenzyme of lactate racemase: A case study of uncovering cofactor structure and biosynthesis

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Methods in Enzymology

ISBN 0076-6879

<https://doi.org/10.1016/bs.mie.2023.03.006>

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Abstract

Cofactors are essential components of numerous enzymes, therefore their characterization by structural, biophysical, and biochemical approaches is crucial for understanding the resulting catalytic and regulatory mechanisms. In this chapter, we present a case study of a recently discovered cofactor, the nickel-pincer nucleotide (NPN), by demonstrating how we identified and thoroughly characterized this unprecedented nickel-containing coenzyme that is tethered to lactate racemase from *Lactiplantibacillus plantarum*. In addition, we describe how the NPN cofactor is biosynthesized by a panel of proteins encoded in the *lar* operon and describe the properties of these novel enzymes. Comprehensive protocols for conducting functional and mechanistic studies of NPN-containing lactate racemase (LarA) and the carboxylase/hydrolase (LarB), sulfur transferase (LarE), and metal insertase (LarC) used for NPN biosynthesis are provided for potential applications towards characterizing enzymes in the same or homologous families.

1. Introduction

Lactate racemase (EC:5.1.2.1, Lar) and related proteins contain a nickel-pincer nucleotide (NPN) coenzyme (Fig. 1A) that was discovered in 2015 (Desguin et al., 2015). The pincer part of the name derives from planar tri-coordinating ligands of metal ions that are well known in the field of inorganic chemistry (van Koten et al., 2020), but in biology pincer-like compounds are limited to the NPN cofactor and pyrrolo-quinoline quinone metal complexes (Nevarez et al., 2020). The structure of the lactate racemase cofactor is a pyridinium-3,5-bisthiocarboxylate mononucleotide with σ -bonds to nickel from C4 of the pyridinium

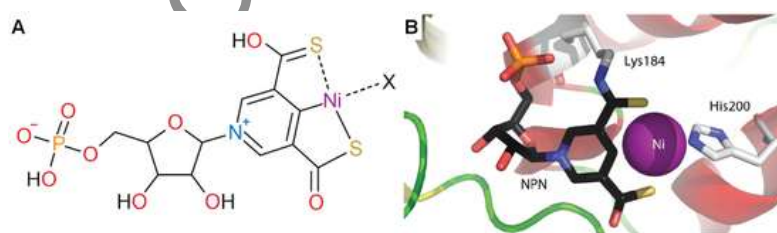


Fig. 1 The nickel-pincer nucleotide (NPN) cofactor. (A) Chemical structure. (B) View of the NPN cofactor at the active site of lactate racemase (Lar) from *L. plantarum* (PDB: 5H UQ).

ring and a thiocarboxylate sulfur. When associated with the protein, a histidine residue completes the four-coordinate planar geometry of the metal. In the enzyme from some sources such as *Lactiplantibacillus* (formerly *Lactobacillus*) *plantarum*, the cofactor is covalently bound by a thioamide linkage between one thiocarboxylate and a lysine residue (Fig. 1B).

This chapter first describes a commonly utilized method for assaying lactate racemase and highlights some of the enzyme properties. It then details a series of approaches that led to the discovery and structural identification of the NPN cofactor. Next, techniques used to uncover the NPN biosynthetic pathway are summarized along with procedures to further characterize these enzymes. Finally, methods to reveal other enzymes containing the NPN cofactor are discussed. This information can serve as a case study for devising experiments to identify and characterize other enzyme cofactors.

2. Lactate racemase assay

A spectroscopic assay for Lar activity uses two coupling enzymes, an L- or D- lactate specific lactate dehydrogenase (LDH) (L-LDH or D-LDH; EC:1.1.1.27 or EC:1.1.1.28, respectively) and D-glutamate-pyruvate transaminase (GPT, EC:2.6.1.2) (Fig. 2). The sample to be assayed is incubated in a defined buffer for selected timepoints with one lactate isomer, and the amount of corresponding product is monitored by mixing the aliquots with a solution containing the appropriate LDH, GPT, nicotinamide adenine dinucleotide (NAD⁺), and D-glutamate. The pyruvate generated by the LDH is converted to D-alanine by GPT while D-glutamate is transformed to 2-oxoglutarate. The amount of NADH generated is quantified based on the absorbance at 340 nm. Kits for measuring L- or D-lactate are commercially available (*e.g.*, from Megazyme/Neogen) and the manufacturers provide details on buffers, conditions, and calculations. When used for measuring Lar activity, it is important to emphasize that the racemase equilibrates the two enantiomers so all kinetics studies should be carried out using timepoints that result in conversion of a small proportion of the original substrate. In addition to the fixed-timepoint assay, the Lar activity can be monitored continuously by using the coupling enzymes to assess NADH generation as the lactate isomer is produced, but only if the lactate oxidation reaction is much faster than the lactate

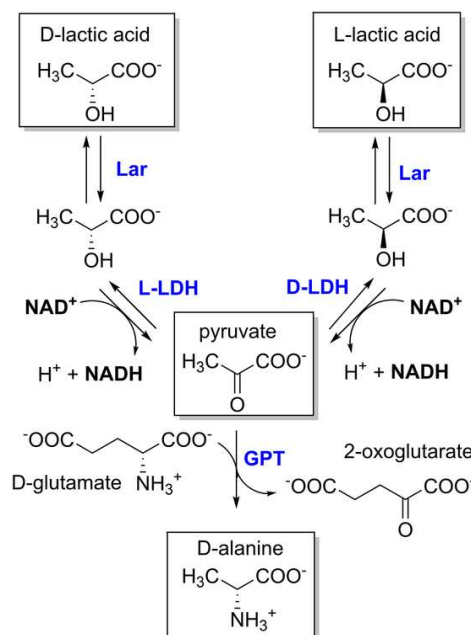


Fig. 2 Summary of a spectroscopic assay for lactate racemase (Lar) activity. The enzyme is incubated with the D- or L-isomer of lactic acid and the amount of product is quantified by reacting with the appropriate lactate dehydrogenase (LDH) that is specific to the product while driving the reaction by consuming the resulting pyruvate using D-glutamate-pyruvate transaminase (GPT).

racemase reaction. A limitation of such a continuous assay is the reaction mix pH, which should be at least at 9 to approach the optimum of pH 10 for the lactate oxidation reaction.

Using this assay, the pH optima, temperature dependences, and kinetic parameters were defined for several purified Lar enzymes (Desguin et al., 2014, 2020; Hiyama et al., 1968). The optimum activity was observed using a pH 6.5 buffer for the *L. plantarum* and *Thermoanaerobacterium thermosaccharolyticum* Lar activities, whereas pH 5.8–6.2 and pH 7.5 were optimal for *Latilactobacillus* (formerly *Lactobacillus*) *sakei* and *Isosphaera pallida*, respectively. Maximal activity was detected at 35–40 °C and 45–50 °C for Lar from *L. plantarum* and *I. pallida*, whereas 50 °C was used for measuring Lar activity in the enzyme from *T. thermosaccharolyticum*. The temperature dependence of

the *L. sakei* enzyme was not reported. The kinetic parameters associated with these enzymes are shown in Table 1.

The Lar assay also was used to measure the stability of Lar activity. LarA loses activity over time accompanied by oxidation of the NPN cofactor and loss of nickel. The addition of L-ascorbic acid and nickel minimally stabilizes the activity, whereas sulfite, a strong reversible inhibitor of LarA, prolongs the Lar activity for several days (Fig. 3) (Desguin et al., 2014, 2015).

3. Identification of the NPN cofactor-associated genes

Genetic and biochemical studies were used to distinguish the genes associated with the NPN cofactor in *L. plantarum*. This microorganism contains D-lactate at the peptide termini of its peptidoglycan precursors, and the D-isomer is produced even in mutant cells with a disrupted gene for D-lactate dehydrogenase (Ferain et al., 1996). Production of D-lactate in these cells uses Lar, an enzyme that was shown to be expressed in the presence of L-lactate (Goffin et al., 2005). DNA microarray studies initially revealed a six-gene operon (denoted here as *larA*, *larB*, *larC1*, *larC2*, *glpF1*, and *larE*) that is induced 18-fold by the addition of L-lactate. Later investigations uncovered an adjacent L-lactate-induced operon containing four genes (*larR*, *larMN*, *larQ*, and *larO*) that is oppositely oriented from *larABC1C2DE* (Desguin et al., 2014). The region of DNA linked to lactate racemase is illustrated in Fig. 4.

Sequence analyses along with a range of biochemical and cellular studies were used to clarify the roles of the proteins encoded by the *lar* gene clusters. The sequence of LarR showed it to be a member in the Crp-Fnr family of transcription regulators (Goffin et al., 2005). Later, it was demonstrated to bind the inducer L-lactate and the antagonist D-lactate to transcriptionally control expression of both *lar* operons (Desguin et al., 2015). LarMN, LarQ, and LarO most closely resembled in sequence three components of an ATP-binding cassette high affinity nickel transporter (Desguin et al., 2014). This function for Lar(MN)QO was confirmed by deleting *larQO* in *L. plantarum* cells and demonstrating a lack of Lar activity, but with the capability restored by addition of nickel ions (Desguin et al., 2014). LarD was annotated as a glycerol uptake facilitator (Goffin et al., 2005) and later shown to be an aquaporin capable of transporting D- and L-lactic acid (Bienert et al., 2013). The se-

Table 1 Kinetic parameters of purified Lar proteins.

Source	Substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($\text{s}^{-1} \text{mM}^{-1}$)
<i>L. plantarum</i>	L-lactate	4750 ± 500	46 ± 20	103 ± 50
	D-lactate	1300 ± 150	11 ± 4	121 ± 56
<i>T. thermosaccharolyticum</i>	L-lactate	990 ± 90	8 ± 3	110 ± 45
	D-lactate	550 ± 100	3 ± 2	180 ± 120
<i>L. sakei</i>	L-lactate	6.7	80	0.084
	D-lactate	16.7	17	1.0
<i>I. pallida</i>	L-lactate	4.7 ± 0.3	0.15 ± 0.04	31 ± 10
	D-lactate	8.1 ± 0.5	0.56 ± 0.10	14 ± 4

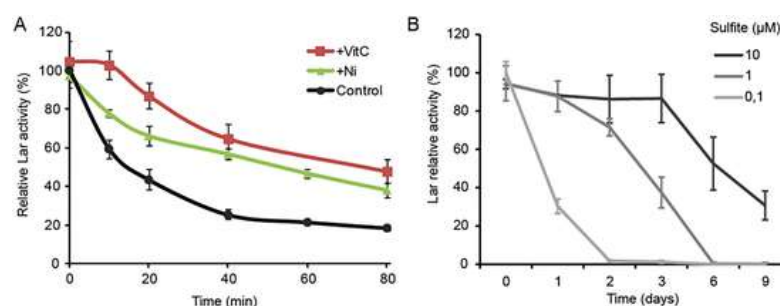


Fig. 3 Stability of Lar activity. (A) Minimal stabilization of Lar activity by 10 mM nickel (+Ni) or 10 mM L-ascorbic acid (+VitC). (B) Stabilization of Lar activity by several concentrations of sulfite. Panel (A) from Desguin, B., Goffin, P., Viaene, E., Kleerebezem, M., Martin-Diaconescu, V., Maroney, M. J., Declercq, J.-P., Soumilion, P., & Hols, P. (2014). Lactate racemase is a nickel-dependent enzyme activated by a widespread maturation system. *Nature Communications*, 5, 3615. Panel (B) from Desguin, B., Zhang, T., Soumilion, P., Hols, P., Hu, J., & Hausinger, R. P. (2015). A tethered niacin-derived pincer complex with a nickel-carbon bond in lactate racemase. *Science*, 349, 66–69.



Fig. 4 Gene clusters associated with Lar activity in *L. plantarum*.

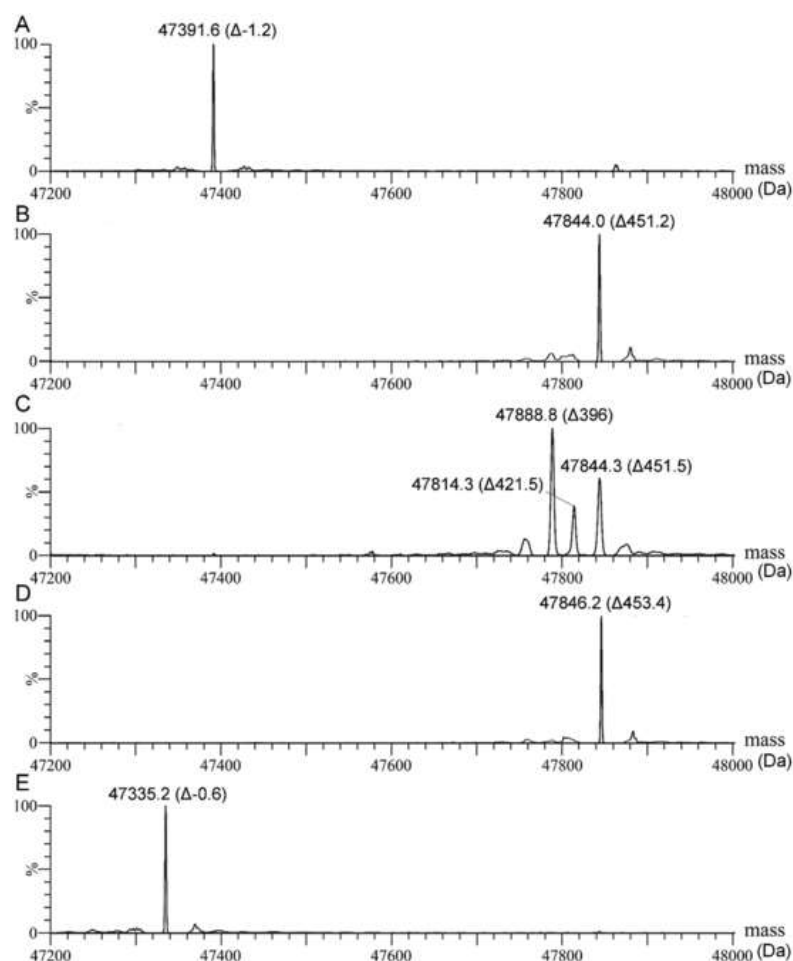
quence of LarA identified an uncharacterized protein within the COG3875 of the Cluster of Orthologous Genes database (Galperin et al., 2021; Tatusov et al., 1997). The LarB sequence was related to PurE-like proteins (COG1691) that exhibit *N*⁵-carboxyaminoimidazole ribonucleotide mutase activity. The N- and C-terminal portions of LarC, designated LarC1 and LarC2, correspond to a single uncharacterized protein (COG1641) in most other LarC-containing organisms, with the *L. plantarum* genome containing a –1 frameshift and a stop codon at the junction that accounts for the dual open reading frames. Finally, LarE is a member of the PP-loop ATPases (COG1606).

By expressing the *L. plantarum* *larABC1C2DE* genes in *Lactococcus lactis*, a microorganism that natively lacks the *lar* genes, and making individual gene deletions, each of LarA, LarB, LarC, and LarE were established as being needed for Lar activity whereas LarD is nonessential. Furthermore, by creating individual gene fusions to a sequence encoding the StrepII tag, the Lar activity was shown to be associated with purified

LarA protein (Desguin et al., 2014). The StrepII-tagged LarC1 protein copurified with a LarC1-LarC2 fusion protein derived by a programmed ribosomal frameshift, and when the two genes were purposely joined into a single reading frame the fused LarC functioned to generate Lar activity. The nickel contents of the individual proteins were determined by inductively coupled plasma atomic absorption spectroscopy and by reactivity with the chromophore 4-(2-pyridylazo)resorcinol; LarA, LarC, and LarE all contained this metal ion, but the nickel bound to LarA was labile and its loss correlated to a decrease in Lar activity (Desguin et al., 2014). In combination, these results indicated that lactate racemase has some type of nickel cofactor bound to LarA, with activity also requiring LarB, LarC, and LarE. The Lar(MN)QO transporter is used to provide nickel, LarD allows for influx/efflux of lactate, and LarR regulates the entire system.

4. Structural characterization of the NPN cofactor

Liquid chromatography (LC)-electrospray ionization (ESI)-mass spectrometry (MS) was used to demonstrate that LarA has a covalently tethered nickel cofactor (Desguin et al., 2015). LarA was purified from *L. lactis* cells expressing either *larABCDE* or just *larA* from *L. plantarum*, revealing an enzyme mass of 47,844.0 Da and cofactor-free protein mass of 47,391.6 Da (matching the LarA sequence missing its N-terminal methionine), a difference of 451.2 Da (Fig. 5A and B). For enzyme incubated for a day prior to performing LC-ESI-MS, additional species with masses of 47,788.8 and 47,814.3 Da (losses of 56 and 30 Da) were observed (Fig. 5C), consistent with the known lability of Lar activity. *L. lactis* cells (expressing *L. plantarum larABCDE*) that were grown on minimal medium required supplementation with nicotinic acid to detect Lar activity, consistent with this vitamin being a precursor of the cofactor. Indeed, by purifying LarA from such a culture that was provided tetradeuterated nicotinic acid LC-ESI-MS revealed an enzyme mass of 47,846.2 Da (Fig. 5D). Thus, the NPN cofactor is derived from nicotinic acid and retains two of the hydrogen atoms from this precursor. To identify the site of covalent attachment of the cofactor, the enzyme was isolated from cells grown with ^2H -labeled or nonlabelled nicotinic acid, digested with trypsin, and examined by LC-ESI-MS. Four species exhibiting a 2 Da mass increase in the labeled sample were all derived



Adapted from Desguin, B., Zhang, T., Soumilion, P., Hols, P., Hu, J., & Hausinger, R. P. (2015). A tethered niacin-derived pincer complex with a nickel-carbon bond in lactate racemase. *Science*, 349, 66–69.

from the same peptide ($K^{184}SVLPGLASYK^{194}$, 1162 Da) due to modification with molecules of 448.9, 425.0, 409.0, and 393.0 Da (*i.e.*, the bound cofactor and its degradation products). LC-MS/MS fragmentation analyses confirmed the peptide sequence and showed that the cofactor was bound to Lys184. Substitution of this residue with alanine, by mutagenesis of the corresponding codon, resulted in detection of only the

apoprotein (47,335.2 Da) by LC-ESI-MS (Fig. 5E). Additional scrutiny of the mass fragments for the tryptic peptide from *L. plantarum* LarA supported the structure assignment of the NPN cofactor (Fig. 1) linked to the lysyl group, *i.e.*, a pyridinium-3-thioamide-5-thiocarboxylic acid mononucleotide nickel pincer. Notably, the isotopic distribution of this species demonstrated the presence of nickel (68% ^{58}Ni and 26% ^{60}Ni). This structure of the LarA cofactor was confirmed by solving the enzyme crystal structure in a functional state (Desguin et al., 2015). To crystallize this redox-sensitive enzyme, 50 μM of sulfite was provided in the sample throughout purification and crystallization to prevent nickel loss, and the crystallization conditions were optimized so that the protein crystals grew to full size within 3 days with the aid of microseeding. Anomalous signals were generated to unambiguously locate nickel in the active site, establishing the Ni—C and Ni—S covalent bonds in the NPN cofactor.

5. Elucidation of the NPN cofactor biosynthesis pathway

At least two hypotheses can be considered for how LarB, LarC, and LarE participate in synthesis of the covalently bound NPN cofactor: they could generate a protein adduct that is further modified or they could act sequentially on small molecule precursors with the completed NPN cofactor attaching to LarA. The finding that heterologous expression in *L. lactis* of *larA* from *T. thermosaccharolyticum* (encoding LarA_T) along with *larBCE* from *L. plantarum* yields Lar activity (Desguin et al., 2014) suggests that assembly of the cofactor on the protein

Fig. 5 LC-ESI-MS analysis of *L. plantarum* LarA samples. (A) Inactive LarA apoprotein. (B) Active LarA enzyme. (C) Enzyme that was incubated for a day prior to analysis. (D) LarA enzyme from cells grown with tetradeutero-nicotinic acid. (E) K184A variant of LarA. In each case, 10- μL aliquots were injected into a Waters Acquity ultra performance liquid chromatography (UPLC) system coupled to a Waters Xevo G2-S QToF mass spectrometer. Separations were performed at 30 °C on a BetaBasic CN column (1 mm $\times$ 10 mm, 5 μm particle size) with an aqueous phase of 0.1% formic acid and using a gradient of increasing acetonitrile. The run time for each sample was 15 min. The masses were calculated from the ESI-MS spectra using an advanced maximum entropy (MaxEnt)-based procedure included in the Micromass MassLynx software package.

is unlikely. More compelling evidence against this proposal is the demonstration that the cofactor does not covalently attach to LarA_{Tl} as shown by solving the crystal structure which reveals an apoprotein (Desguin et al., 2014). Furthermore, Lar activity is generated by incubation of the accessory proteins with appropriate additives followed by addition of LarA_{Tl} (Desguin et al., 2016). Thus, LarB, LarC, and LarE function prior to LarA modification. A reasonable supposition using chemical logic is that nicotinic acid mononucleotide (NaMN) or similar compound is carboxylated at C5 of the pyridinium ring, the two carboxyl groups are converted to thiocarboxylic acids, and nickel is added. The homology between PurE and LarB is consistent with the latter protein functioning in the carboxylation reaction. In a similar manner, the observation that LarE is a PP-loop ATPase is consistent with this protein activating a substrate by adenylation followed by conversion to product with release of AMP. Such chemistry is known to occur during the transformation of carboxyl groups to thiocarboxyl groups. LarC would then function as the nickel insertase.

Lar assays and LC-ESI-MS were used to establish the order in which the auxiliary proteins function during NPN cofactor biosynthesis. The first enzyme of the *L. plantarum* pathway is LarB as shown by using a membrane filter to generate protein-free extracts of LarB-containing cells, mixing the metabolites with purified LarC and LarE, incubating with Mg-ATP, adding LarA_{Tl} apoprotein, and assaying Lar activity (Desguin et al., 2016). The results demonstrated that a small molecule generated by LarB was converted by LarC and LarE to the NPN cofactor that provided activity to LarA_{Tl}. Using such an assay, the substrate of LarB was identified as nicotinic acid adenine dinucleotide (NaAD), rather than NaMN. Bicarbonate/CO₂ (these are in equilibrium) was shown to stimulate the reaction. LC-ESI-MS was used to identify the product of purified LarB. This compound has a mass of 380.04 Da when using unlabeled bicarbonate or 381.04 when using ¹³C-labeled bicarbonate. The fragmentation pattern of the product demonstrated that the pyridinium ring is carboxylated, and when using the ¹³C-labeled sample the resulting two carboxyl groups were equally susceptible to loss indicating a symmetric compound. LarB catalyzes both carboxylation and phosphoanhydride hydrolysis of NaAD to generate pyridinium-3,5-biscarboxylic acid mononucleotide (P2CMN) and AMP (Fig. 6).

LC-ESI-MS and Lar assays also were utilized to characterize the *L. plantarum* LarE reaction. P2CMN and its ¹³C-labeled version were syn-

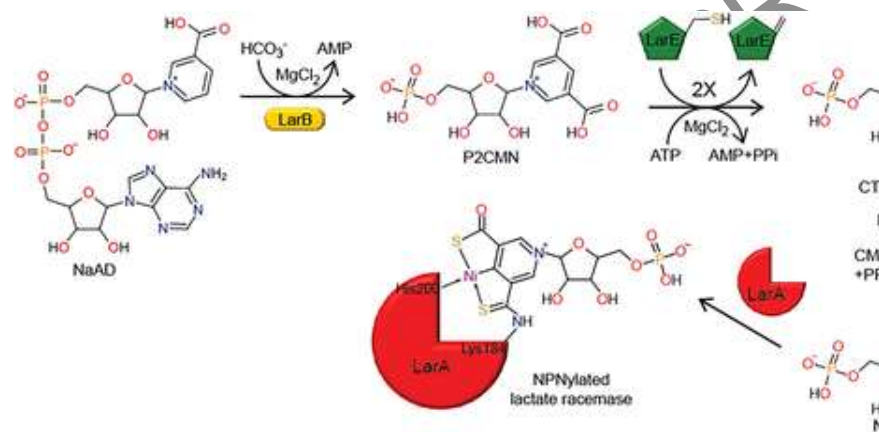


Fig. 6 The NPN biosynthetic pathway in *L. plantarum*. See text for details.

thesized by LarB, the samples were incubated with purified LarE and Mg·ATP, and products with masses of 396.02/397.02 and 411.99/413.00 Da were detected (Desguin et al., 2016). Analyses of the fragments were consistent with formation of pyridinium-3-carboxy-5-thio-carboxylic acid mononucleotide (PCTMN) and pyridinium-3,5-bisthiocarboxylic acid mononucleotide (P2TMN) along with their ^{13}C -labeled species (Desguin et al., 2016). The products were incubated with LarC, LarA_{Tl} was added, and Lar assays showed the formation of active enzyme (Desguin et al., 2018). Although it was clear that LarE catalyzed the two sulfurization reactions to generate these products, the source of added sulfur was unclear. LC-ESI-MS analysis of LarE or its tryptic fragments resolved this question; specifically, *L. plantarum* LarE sacrifices the sulfur atom of residue Cys176 to form a dehydroalanine (Dha) residue and shifts from a mass of 31,550 Da (for StrepII tagged LarE missing its N-terminal methionine) to 31,516 Da, or 34 Da smaller (with values of 894.4 and 860.5 Da for the corresponding peptide). Also noteworthy is the transient formation of a 31,911 Da species (or 31,912 Da when using ^{13}C -labeled P2CMN) corresponding to the P2CMN-LarE enzyme adduct as an intermediate in the reaction (Fig. 7). LarE thus reacts

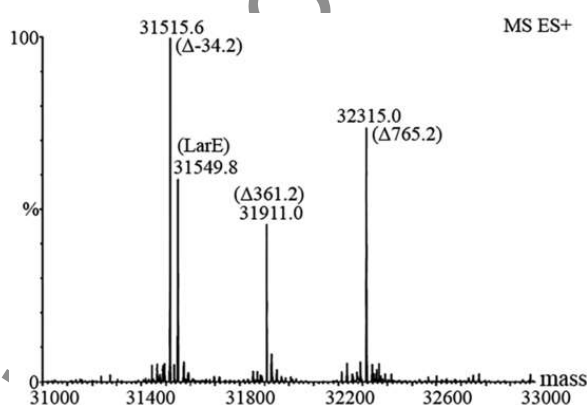


Fig. 7 LC-ESI-MS profile of coenzyme A-bound LarE (32,315 Da) 15 min after addition of Mg·ATP and P2CMN. The samples were analyzed as described in Fig. 5. From Desguin, B., Soumillon, P., Hols, P., & Hausinger, R. P. (2016). Nickel-pincer cofactor biosynthesis involves LarB-catalyzed pyridinium carboxylation and LarE-dependent sacrificial sulfur insertion. *Proceeding of the National Academy of Science of the United State of America*, 113, 5598–5603.

with substrate (presumably activated by adenylylation) and catalyzes a single turnover sulfur transfer reaction under *in vitro* conditions (Fig. 6).

P2TMN was generated by LarB and LarE (using NaAD, bicarbonate/CO₂, and Mg·ATP) and used as a substrate for LarC based on its ability to generate Lar activity in LarA_{7t} apoprotein and by its detection using LC-ESI-MS (Desguin et al., 2018). The activation of LarA_{7t} is greatly stimulated by the presence of cell-free extracts that, significantly, could be replaced simply by providing CTP. This molecule, a required substrate of LarC, undergoes hydrolysis to CMP (and presumably pyrophosphate, PP_i) as shown by thin layer chromatographic studies using α -³²P-labeled CTP. A divalent metal ion also is needed, and LarC exhibits greater activity using manganese over magnesium.

6. Methods for the purification of Lar-associated enzymes

The heterologous production of lactate racemase holoenzyme uses a plasmid expressing the *L. plantarum* *larABC1C2DE* genes in *L. lactis*. The addition of 1 mM nickel in the culture medium is required for the maximal Lar activity, given the absence of a nickel transporter in *L. lactis*. Fusion of *larA* with a sequence encoding the StrepII tag allows for the purification of the LarA holoprotein by affinity chromatography (Table 2) (Desguin et al., 2014). The addition of sulfite in all buffers greatly stabilizes the Lar activity and nickel content of the purified protein (Table 2) (Desguin et al., 2015), but this approach is only effective for LarA_{Lp}, not for other LarA homologs. LarE with some NPN attached can be expressed from a similar plasmid encoding the StrepII tag, but LarB and LarC are best purified from plasmids expressing only *larB* or *larC*. The addition of a detergent, Triton X-100, is required for the purification of LarB from *L. lactis*. LarA and LarE apoproteins can also be purified from plasmids containing the *larA* or *larE* genes expressed alone (Table 2) (Desguin et al., 2014).

For the purification of LarB, plasmids were constructed that allow for *larB* expression in *E. coli* (Table 2) (Desguin et al., 2016; Rankin et al., 2021). For the purification of LarE, a plasmid was constructed that allows for high-yield expression of *larE* in an *E. coli* ArticExpress strain (Table 2) (Fellner et al., 2017). For crystallization purposes, LarC_{fused} was overexpressed and purified from *E. coli* cells (Table 3) (Desguin et

Table 2 Plasmids used for the expression of *lar*-associated genes and purification of selecte

Plasmid	Strain	Culture conditions	Purification conditions	Purific
pGIR100 (<i>larABC1C2DE</i>)	<i>L. lactis</i>	M17G + 1 mM nickel	50 mM Tris-HCl, pH 7.5, 150 mM NaCl (W buffer)	–
pGIR112 (<i>larA^{Strep}BC1C2DE</i>)	<i>L. lactis</i>	M17G + 1 mM nickel	W buffer	LarA Ni lo
pGIR112 (<i>larA^{Strep}BC1C2DE</i>)	<i>L. lactis</i>	M17G + 1 mM nickel	W buffer + 50 μ M sulfite	LarA Ni lo
pGIR172 (<i>larABC1C2DE^{Strep}</i>)	<i>L. lactis</i>	M17G + 1 mM nickel	W buffer with 500 mM NaCl	LarE NPN
pGIR182 (<i>larA_{Ti}^{Strep}BC1C2DE</i>)	<i>L. lactis</i>	M17G + 1 mM nickel	W buffer	LarA Ni lo
pGIR012 (<i>larA^{Strep}</i>)	<i>L. lactis</i>	M17G	W buffer	LarA apop
pGIR022 (<i>larB^{Strep}</i>)	<i>L. lactis</i>	M17G	W buffer + Triton X-100	LarB
pGIR031 (<i>larC^{Strep}</i>)	<i>L. lactis</i>	M17G + 1 mM nickel	W buffer	LarC
pGIR031 (<i>larC^{Strep}</i>)	<i>L. lactis</i>	M17G	W buffer	LarC
pGIR051 (<i>larC_{fused}^{Strep}</i>)	<i>L. lactis</i>	M17G + 1 mM nickel	W buffer	LarC Ni)
pGIR072 (<i>larE^{Strep}</i>)	<i>L. lactis</i>	M17G	W buffer with 500 mM NaCl	LarE
pGIR082 (<i>larA_{Ti}^{Strep}</i>)	<i>L. lactis</i>	M17G	W buffer	LarA apop

Plasmid	Strain	Culture conditions	Purification conditions	Purification
pGIR026 (<i>larB^{Strep}</i>)	<i>E. coli</i> TOP10	LB	W buffer	LarB
pBAD:LarB (<i>larB^{Strep}</i>)	<i>E. coli</i> BL21 (DE3)	LB	W buffer	LarB
pET:LarB (<i>larB^{Strep}</i>)	<i>E. coli</i> BL21 (DE3)	LB at 16 °C	W buffer	LarB
pGIR076 (<i>larE^{Strep}</i>)	<i>E. coli</i> Artic Express	LB at 13 °C	W buffer with 300 mM NaCl	LarE
pGIR053 (<i>larC_{fused}^{Strep}</i>)	<i>E. coli</i> Rosetta 2(DE3)	LB at 16 °C	W buffer	LarC apop
pET:LarCMT	<i>E. coli</i> BL21 (DE3)	Autoinduction medium	100 mM Tris-HCl, pH 8.0, 300 mM NaCl	LarC
pGIR210 (<i>lar^{Strep}BC1C2DE</i>)	<i>L. lactis</i>	M17G + 1 mM nickel	W buffer	—
pGIR211 (<i>larAH6^{Strep}BC1C2DE</i>)	<i>L. lactis</i>	M17G + 1 mM nickel	W buffer	LarA Ni)
pGIR212 (<i>larAH7^{Strep}BC1C2DE</i>)	<i>L. lactis</i>	M17G + 1 mM nickel	W buffer	LarA Ni)
pGIR213 (<i>larAH20^{Strep}BC1C2DE</i>)	<i>L. lactis</i>	M17G + 1 mM nickel	W buffer	LarA Ni)
pGIR311 (<i>larAH3^{Strep}</i>)	<i>E. coli</i> TOP10	LB	W buffer	LarA apop
pGIR312 (<i>larAH4^{Strep}</i>)	<i>E. coli</i> TOP10	LB	W buffer	LarA apop

Plasmid	Strain	Culture conditions	Purification conditions	Purification
pGIR313 (<i>larAH5^{Strep}</i>)	<i>E. coli</i> TOP10	LB	W buffer	LarA apop
pGIR314 (<i>larAH8^{Strep}</i>)	<i>E. coli</i> TOP10	LB	W buffer	LarA apop
pGIR315 (<i>larAH10^{Strep}</i>)	<i>E. coli</i> TOP10	LB	W buffer	LarA apop
pGIR316 (<i>larAH12^{Strep}</i>)	<i>E. coli</i> TOP10	LB	W buffer	LarA apop
pGIR317 (<i>larAH14^{Strep}</i>)	<i>E. coli</i> TOP10	LB	W buffer	LarA apop
pGIR318 (<i>larAH19^{Strep}</i>)	<i>E. coli</i> TOP10	LB	W buffer	LarA apop
pGIR320 (<i>larAH23^{Strep}</i>)	<i>E. coli</i> TOP10	LB	W buffer	LarA apop

Abbreviations used in table: ^{Strep}, StrepII tag sequence; LB, Luria-Bertani broth; M17G, M17 broth with 0.5% glucose.

Table 3 Ingredients required for *in vitro* NPN biosynthesis.

Ingredient	Quantitative NPN biosynthesis	Analytical NPN biosynthesis
LarB	10 μ M	1 μ M
LarE	>100 μ M	5 μ M
LarC	>50 μ M (half the amount of LarE)	2.5 μ M
NaAD	200 μ M	20 μ M
CTP	200 μ M	20 μ M
ATP	2 mM	2 mM
MgCl ₂	20 mM	20 mM
MnCl ₂	1 mM	1 mM
NaHCO ₃	50 mM	50 mM
Tris-HCl, pH 7	100 mM	100 mM

al., 2018), but proteolysis led to crystallization of only the inactive LarC2 portion of the protein. The LarC homolog from *Moorella thermoacetica* was purified from *E. coli* cells and showed 3% of activity compared to LarC_{Lp} (Table 2) (Turmo et al., 2022).

For the purification of LarA homologs (LarAHs), plasmid pGIR210 was constructed. This plasmid allows for the overexpression of any LarAH in frame with the StrepII tagged sequence in an operon containing *larBC1C2DE*. LarAHs were overexpressed either in *L. lactis* using a plasmid derived from the pGIR210, allowing for the purification of LarAHs partially loaded with NPN, or in *E. coli* as the apoproteins (Table 2).

7. *In vitro* NPN cofactor biosynthesis

The NPN cofactor can be generated *in vitro* by methods that have been detailed (Desguin et al., 2018). Generation of the cofactor requires large amounts of purified LarE and LarC, as these proteins only react once *in vitro*. Briefly, LarE and LarB were produced in *E. coli* ArcticExpress (DE3) and *E. coli* TOP10 or BL21 expression strains, respectively (Table 2). LarC was produced in *L. lactis* in the presence of 1 mM nickel in the medium. All enzymes were purified by affinity chromatography with Strep-Tactin resin. Table 3 lists the ingredients for the synthesis of

either a quantitative amount of NPN for the study of NPN-dependent enzymes or for the synthesis of analytical amounts of NPN for characterization of the NPN biosynthetic pathway.

The biosynthesized NPN was assayed by incubating the assay mixture with LarA_{TL} apoprotein, supplementing with L-lactate, and measuring the resulting D-lactate (or providing D-lactate and measuring the L-lactate produced). The biosynthesized NPN was used directly or stored at -80 °C in 10% glycerol for further use. Deproteination was achieved by adding 10 mM 2-mercaptoethanol and heat precipitating the proteins at 80 °C for 5 min, as thiols appear to stabilize the enzyme-free product. The NPN was purified by anion-exchange chromatography using thiol-containing buffers, as described (Desguin et al., 2018). Deproteinized NPN is capable of binding to LarA from *L. plantarum* and forming the thioamide linkage, but it must be used immediately, as the cofactor is unstable in these conditions, even at -80 °C (Desguin et al., 2018). The purified deproteinized NPN was analyzed by LC-ESI-MS and a compound (with masses of 465.90 and 467.89 Da in an approximate 2:1 ratio) was identified, consistent with expectations for the nickel-containing NPN cofactor (Fig. 8).

8. Methods for mechanistic analyses of the Lar-associated enzymes

This section briefly describes some of the key methods that were used to characterize the catalytic mechanisms of the NPN biosynthetic enzymes and Lar itself.

8.1 LarB

An indirect LarB assay incorporated the downstream LarE and LarC enzymes to carry out *in vitro* NPN cofactor biosynthesis, the coenzyme was incubated with LarA apoprotein, and the generated Lar activity was monitored, varying the amounts of LarB or the reaction conditions of the reaction mix (Desguin et al., 2016). Later, a capillary electrophoresis assay for the LarB reaction was developed using a fused silica capillary and 0.1 M sodium borate, pH 8.5, as running buffer. This assay allowed for simultaneous analysis of substrate consumed and products generated by directly measuring the amounts of P2CMN, AMP, and NaMN (Fig. 9) (Rankin et al., 2021).

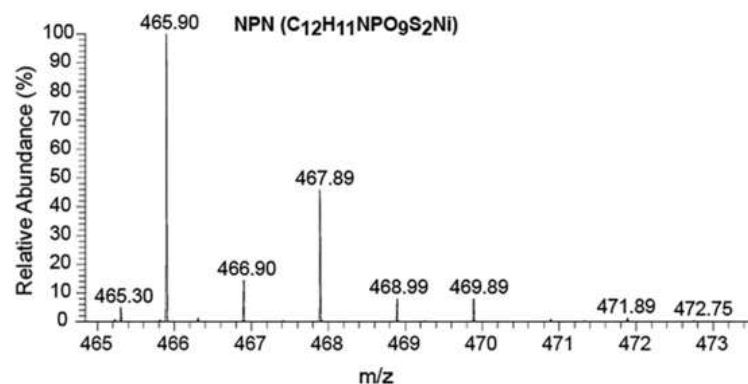


Fig. 8 LC-ESI-MS analysis of *in vitro* synthesized NPN. 10- μ L aliquots were injected into an Accela HPLC system coupled to a Q-Exactive (ThermoFisher) mass spectrometer. Separations were performed using a Kinetex C18 column (150 mm $\times$ 2.1 mm) in 0.1% formic acid with a gradient of acetonitrile. The run time for each sample was 25 min. From Desguin, B., Fellner, M., Riant, O., Hu, J., Hausinger, R.P., Hols, P., & Soumilion, P. (2018). Biosynthesis of the nickel-pincer nucleotide cofactor of lactate racemase requires a CTP-dependent cyclometallase. *Journal of Biological Chemistry*, 293, 12303–12317.

A radioisotope incorporation assay was used to define whether CO₂ or bicarbonate is the substrate of LarB's carboxylation reaction (Rankin et al., 2021). The enzyme was added to a solution containing NaAD with an equilibrated ¹⁴C-labeled bicarbonate/CO₂ mixture, and after 1 min the sample was vigorously mixed with unlabeled gaseous CO₂. Aliquots were taken every 5 s, terminated with acetic acid, and assessed for radioactivity by scintillation counting (Sudhani et al., 2015). Under these conditions, the only radioactivity remaining in the sample was due to its incorporation into P2CMN. Over the first min, a linear increase in radioactivity was observed (Fig. 10A). Upon mixing with unlabeled CO₂, the amount of radioactivity in the sample remained unchanged for ~30 s as the newly introduced cold CO₂ competed with the ¹⁴CO₂ that was already present. The incorporation of radioactivity then increased to a reduced linear rate as most of the cold CO₂ reacted with water to form bicarbonate. This result indicated that CO₂, not bicarbonate, is the authentic substrate of LarB. When carbonic anhydrase was included in the mixture, the pattern was altered: upon mixing with unlabeled CO₂, the

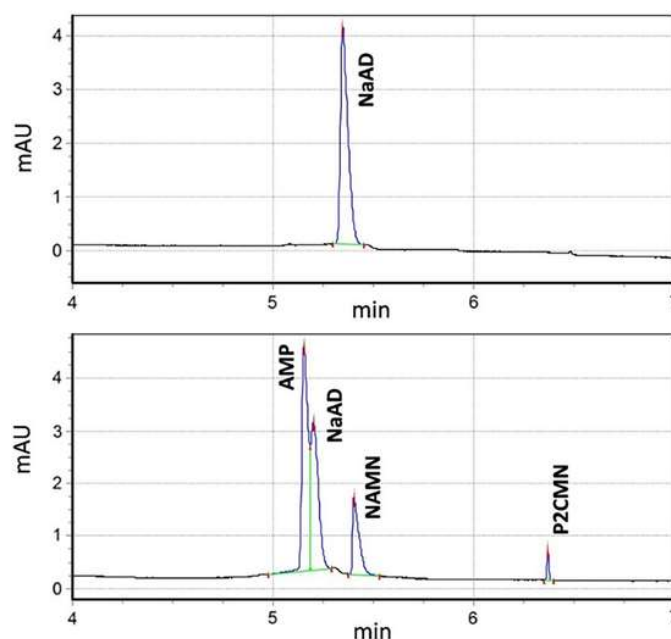
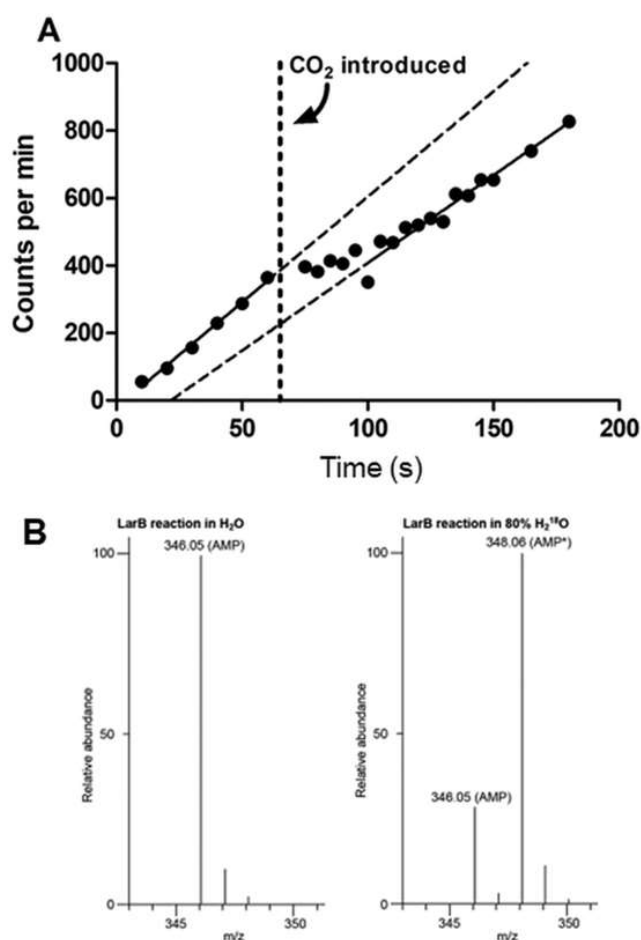


Fig. 9 Electrochromatograms of the LarB reaction substrate and its products analyzed using a 48 cm fused silica capillary run at 25 KV with 0.1 M sodium borate, pH 8.5, as running buffer and detection at 254 nm. 1 mM NaAD before (top) and 60 min after the addition of 1 μ M LarB (bottom). From Rankin, J. A., Chatterjee, S., Tariq, Z., Lagishetty, S., Desguin, B., Hu, J., & Hausinger, R. P. (2021). The LarB carboxylase/hydrolase forms a transient cysteinyl-pyridine intermediate during nickel-pincer nucleotide cofactor biosynthesis. *Proceeding of the National Academy of Science of the United State of America*, 118, e2106202118.

initial linear incorporation of radioactivity immediately shifted to the decreased linear rate of radiolabeling observed in the prior study.

Isotope labeling also proved useful in characterizing the hydrolytic reaction catalyzed by LarB (Rankin et al., 2021). The enzyme was incubated in buffer prepared with ^{18}O -labeled water and containing NaAD. The products of the reaction were investigated by LC-ESI-MS and the label was shown to be associated with AMP (Fig. 10B), not P2CMN. This result implies that the hydrolytic water molecule attacks the AMP-associated phosphate of the phosphoanhydride.

Further mechanistic analysis of LarB included structural and mutagenesis studies. The structure of *L. plantarum* LarB was solved in com-



From Rankin, J. A., Chatterjee, S., Tariq, Z., Lagishetty, S., Desguin, B., Hu, J., & Hausinger, R. P. (2021). The LarB carboxylase/hydrolase forms a transient cysteinyl-pyridine intermediate during nickel-pincer nucleotide cofactor biosynthesis. *Proceeding of the National Academy of Science of the United State of America*, 118, e2106202118.

plex with the substrate analog NAD⁺ (and Mg²⁺) (Rankin et al., 2021). Notably, NAD⁺ forms an enzyme adduct in which Cys221 covalently bonds to C4 of the pyridinium ring, which deforms to a boat structure consistent with its reduction (Fig. 11A). This reaction would enhance the nucleophilicity of C5 and, in the case of NaAD, lead to its carboxylation.

The only potential general base in the vicinity, Glu180, is located on a separate protein chain (the active site of carboxylation is at the interface of three subunits). Abstraction of the proton at C5 would lead to rearomatization of the ring and expulsion of Cys221. The complete carboxylation reaction mechanism is shown in Fig. 11B. The Mg^{2+} in the LarB structure associates with the phosphoanhydride of NAD^+ . The newly formed carboxylate on NaAD is proposed to bind to the metal ion and activate a bound water molecule that reacts to form the P2CMN and AMP products. An AMP-bound structure reveals that both AMP and Mg^{2+} overlap the positions of the corresponding components in the $\text{Mg}^{2+}\cdot\text{NAD}^+$ structure (Rankin et al., 2021). Mutagenesis methods were used to substitute several of the residues at the active site to confirm the importance of Cys221 and Glu180 as well as several side chains that function in hydrogen-bonding or aromatic stacking interactions with the substrate.

8.2 LarE

The activity of LarE was assessed indirectly by a four-step process using purified proteins and assaying for Lar activity. The first step was the production of P2CMN with LarB, the second step involved LarE synthesis

Fig. 10 Isotopic investigation of LarB. (A) Carbon dioxide, not bicarbonate, is the substrate of LarB. Short-term incorporation of ^{14}C into substrate using radiolabeled CO_2 /bicarbonate, with unlabeled gaseous CO_2 added using vigorous mixing at 65 s for isotope dilution. The conditions were 50 mM MOPS-NaOH, pH 7.5, containing 4 mM MgCl_2 , 1 mM NaHCO_3 , 0.2 μCi $\text{NaH}^{14}\text{CO}_3$, and 0.2 mM NaAD in a volume of 100 μL . The reactions were incubated at 21 $^\circ\text{C}$ and quenched by addition of 10 μL of glacial acetic acid. Samples were dried in a vacuum centrifuge, dissolved in 100 μL of 1% v/v acetic acid, and dried again. The solids were dissolved in 100 μL of water and added to 5 mL of Safety-Solve scintillation solution (RPI). $\text{NaH}^{14}\text{CO}_3$ specific activity was measured using an alkaline scintillation fluid and analyzed using a Beckman-Coulter LS6500 liquid scintillation counter. (B) LarB incorporates ^{18}O from labeled solvent into AMP during NaAD hydrolysis. The figure depicts the mass spectra of LarB-generated AMP derived from NaAD in unlabeled water (left) and ^{18}O -labeled water (right). The 10- μL aliquots were injected into an Accela HPLC system coupled to a Q-Exactive (ThermoFisher) mass spectrometer. Separations were performed using a Kinetex C18 column (150 mm $\times$ 2.1 mm) in 0.1% formic acid with a gradient of acetonitrile. The run time for each sample was 25 min.

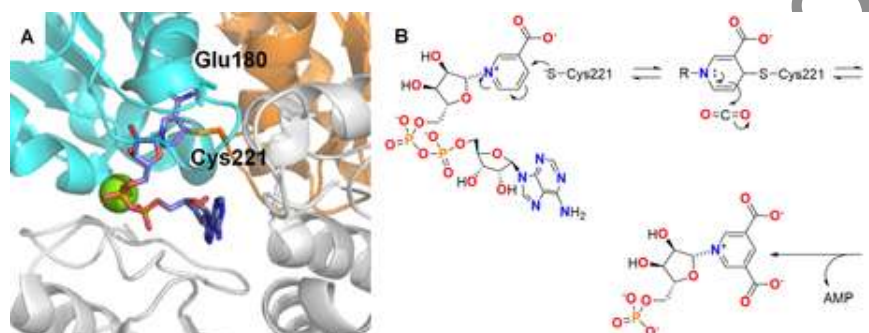


Fig. 11 Structure and mechanism of LarB. (A) Three subunits of octameric LarB from *L. plantarum* (illustrated interacting with the substrate analog NAD⁺ (depicted as blue sticks) and a magnesium ion (green sphere) (PDE tributes much of the AMP-binding site, the orange subunit provides the Cys221 nucleophile, and the cyan subunit mononucleotide portion of the ligand and includes the Glu180 general base (shown truncated due to disorder of forms a covalent bond with C4 of the pyridinium group. (B) The proposed reaction mechanism of LarB involves the pyridinium ring, carboxylation on C5, proton abstraction by Glu180, ring rearomatization and elimination of the phosphoanhydride by Mg-coordinated water to release AMP and P2CMN.

of P2TMN. The third step involved synthesis of the NPN cofactor, generation of active LarA, and incubation with D-lactate. The final step was to measure L-lactate with an enzymatic kit (Fellner et al., 2018).

The crystal structures were solved for ligand-free *L. plantarum* LarE as well as its complexes with bound Mg-ATP, AMP, and nicotinamide mononucleotide (an analog of P2CMN) (Fellner et al., 2017). A view of the latter structure is shown (Fig. 12A). The structure supports the proposed mechanism (Fig. 12B) involving P2CMN activation by adenylation, formation of the P2CMN-LarE adduct by attack of Cys176 and release of AMP, sacrificial sulfur transfer to form PCTMN while generating Dha in the protein, then repeating these steps at another subunit to form P2TMN and creating another protein chain possessing Dha. The predominant form of LarE purified from *L. lactis* expressing *larABCDE* has a mass of 31,156 Da indicating loss of its cysteinyl sulfur atom and suggesting that the Dha species cannot be recycled in those cells (Desguin et al., 2016). The Dha-containing form of LarE was incubated with various additives to restore the native sulfur-containing version of the protein. The persulfide of coenzyme A (CoA) generated a mixed disulfide with LarE that could be reduced to yield the native 31,550 Da species (Fellner et al., 2018). In contrast, supplements including sulfide, cysteine desulfurase (IscS) plus L-cysteine, and the persulfides of L-cysteine or glutathione were much less effective *in vitro*. Prior studies had shown that CoA stabilizes *L. plantarum* LarE (Desguin et al., 2016) and the crystal structure of the complex of CoA bound to LarE (Fellner et al., 2018) showed that the thiol group of CoA is proximal to Cys176. Together, these results suggest that the CoA persulfide might be used to recycle the Dha-containing form of LarA in wild-type *L. plantarum* cells, but this supposition has not been experimentally confirmed. Support for many aspects of the LarE mechanism was obtained by mutagenesis that substituted key residues.

An alternative reaction mechanism (not depicted) was demonstrated for LarE of *Thermotoga maritima* (Chatterjee et al., 2022). This enzyme possesses an oxygen-labile [4Fe-4S] cluster bound by three cysteine residues, with one iron atom having an open coordination site. LC-ESI-MS was used to show that incubation of IscS with L-cysteine yielded the persulfide version of the sulfur transferase and that subsequent incubation with *T. maritima* LarE decreases the persulfide content. Furthermore, native MS of the LarE sample suggested that it accepts the sulfane sulfur atom. The iron atom with the open coordination site within the

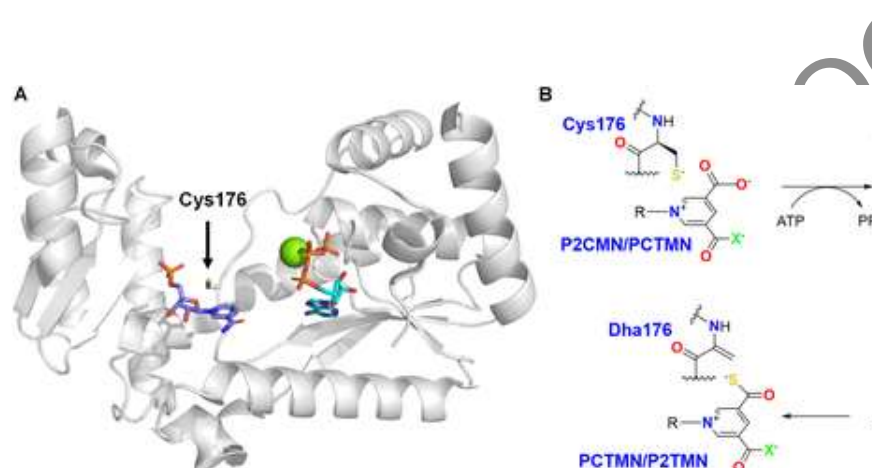


Fig. 12 Structure and mechanism of LarE. (A) One subunit of homohexameric LarE from *L. plantarum* (illustrated interacting with the substrate analog NMN (depicted as dark blue sticks) (PDB:5UDR) superimposed with 1 sticks) from another structure (PDB:5UDS). The Cys176 side chain is shown in stick view. (B) The proposed mechanism involves adenylation of the substrate (P2CMN or PCTMN, where X represents O or S, respectively) with release of an activated carboxyl group leading to adduct formation and release of AMP, deprotonation of the backbone α carbon sulfur atom, and release of the product PCTMN or P2TMN while leaving the modified side chain as a Dha residue.

[4Fe—4S] cluster is proposed to bind sulfide and transfer it to the activated carboxyl group of P2CMN or PCTMN.

8.3 LarC

The activity of LarC was assessed indirectly by a four-step process using purified proteins and assaying for Lar activity, similarly to LarE. The first step was the production of P2TMN with LarB and LarE, the second step involved LarC synthesis of the NPN cofactor, the third step involved generation of active LarA and incubation with D-lactate, and the final step was to measure L-lactate with an enzymatic kit (Desguin et al., 2018). Surprisingly, the activity of LarC reflected, at maximum, a single turnover reaction. Using conditions of P2TMN excess, the Lar activity was shown to be strictly dependent on the concentration of LarC and was independent of P2TMN concentration (Fig. 13). Perhaps associated with this behavior, LarC must be produced in cells that were provided with nickel ions; this requirement could not be overcome by inclusion of nickel in the assay solution.

The structure was obtained only for the LarC₂ portion of the *L. plantarum* enzyme (Desguin et al., 2018). This domain binds CTP as shown

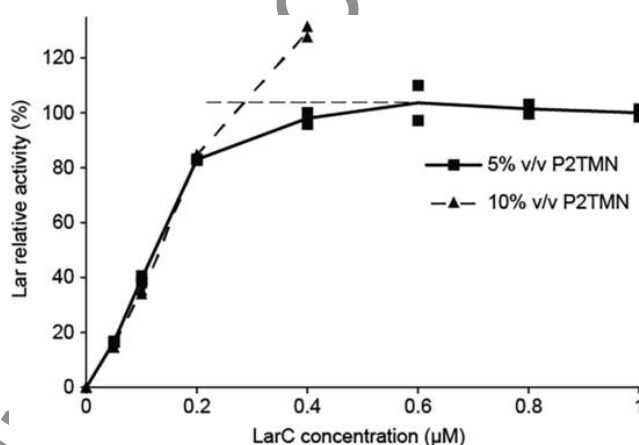


Fig. 13 Lar activity from activated LarA_{Ti} depends on the LarC concentration during NPN cofactor biosynthesis. From Desguin, B., Fellner, M., Riant, O., Hu, J., Hausinger, R.P., Hols, P., & Soumillon, P. (2018). Biosynthesis of the nickel-pincer nucleotide cofactor of lactate racemase requires a CTP-dependent cyclometallase. *Journal of Biological Chemistry*, 293, 12303–12317.

by structure determination of the complex (Fig. 14A). Substitutions of several residues that interact with CTP led to significant reductions in the ability of LarC to activate LarA_{Ti} and two of these variants exhibit greatly increased values of K_M for CTP. The LarC1 region of the protein likely binds the P2TMN substrate and possesses a histidine-rich region that could bind nickel. Mutagenesis studies suggest that several carboxylate residues and the histidine-rich region in LarC1 are important for the nickel transfer.

LC-ESI-MS was used to discern the function of CTP by using LarC from *M. thermoacetica* (Turmo et al., 2022). During nickel insertion into P2TMN by *M. thermoacetica* LarC, a transient metabolite of 715.02 Da was identified. Fragmentation analysis of this species revealed it to be the CMP-P2TMN adduct where the two species are linked by a phosphoanhydride. The sequential reactions of CMPylation, nickel insertion, and hydrolysis to form CMP and NPN are suggested to position the pincer portion of the intermediate near the site of nickel binding, so it is appropriately oriented for metal insertion (Fig. 14B).

UV-vis spectroscopy also can be used to directly follow the reaction catalyzed by LarC, as the spectral properties of NPN are different from those of P2TMN (Fig. 15A). Notably, NPN has an absorbance peak around 450 nm, which is also observed in LarA holoprotein (Fig. 15B) (Desguin et al., 2018, 2015).

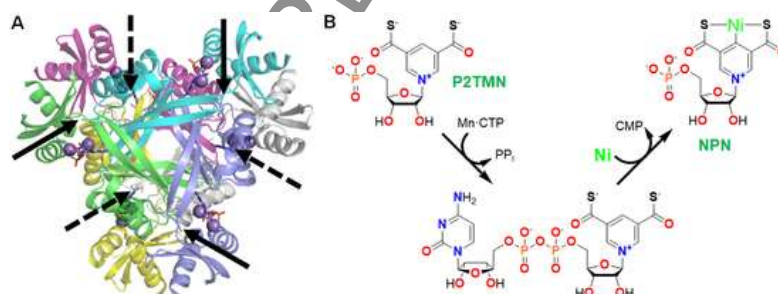


Fig. 14 Structure and mechanism of LarC. (A) The homo-hexamer of LarC2 from *L. plantarum* (illustrated in cartoon mode with each subunit in a different color) is shown interacting with Mn_2 -CTP (depicted as purple spheres and dark blue sticks) (PDB:6BWQ). The amino terminal domain, LarC1, connects to these subunits were indicated (solid arrows in front and dashed arrows behind). (B) The proposed reaction mechanism of LarC involves cytidinylation of the substrate (P2TMN) with release of PP_i , insertion of nickel, and hydrolysis to release CMP and the NPN cofactor.

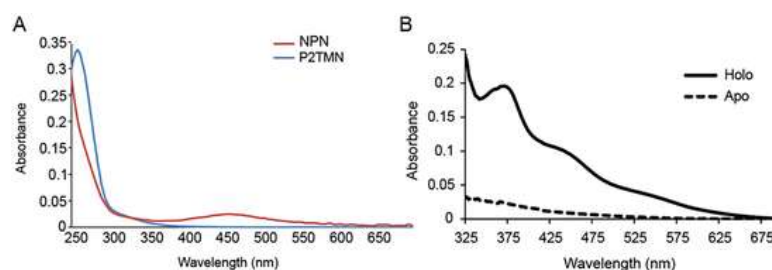


Fig. 15 UV-vis properties of P2TMN, NPN and LarA. (A) P2TMN and NPN were purified by loading samples on a 5-ml HiScreen Q HP column (GE Healthcare), washing with Tris-HCl buffer (30 mM, pH 8), and eluting with a gradient of NaCl (0 to 1 M) in Tris-HCl buffer (30 mM, pH 8). For purification of the NPN cofactor, 10 mM 2-mercaptoethanol was added to all buffers. The P2TMN-containing sample was obtained by incubating a 1 ml mixture of LarE (200 μ M), LarB (10 μ M), NaAD (0.2 mM), ATP (2 mM), $MgCl_2$ (20 mM), and $NaHCO_3$ (50 mM) in Tris-HCl buffer (100 mM, pH 7). Similarly, the NPN-containing fraction was obtained by incubating 1 ml of a mixture of LarE (100 μ M), LarC (50 μ M), LarB (10 μ M), NaAD (0.2 mM), CTP (0.2 mM), ATP (2 mM), 2-mercaptoethanol (10 mM), $MgCl_2$ (20 mM), and $NaHCO_3$ (50 mM) in Tris-HCl buffer (100 mM, pH 7). The reactions were incubated for 1 h at room temperature and heat-treated at 80 $^{\circ}C$ for 10 min, then the supernatant was collected by centrifugation at 20,000g for 5 min and loaded onto the resin. The P2TMN and NPN peaks were identified by their absorbances at 330 and 450 nm, respectively. (B) The spectra of the purified LarA apoprotein (Apo) and holoprotein (Holo) were measured in a quartz cuvette using a UV-2401PC spectrometer (Shimadzu). The concentrations of samples were adjusted to have an absorbance of 1.0 at 280 nm for both spectra. Panel (A) from Desguin, B., Fellner, M., Riant, O., Hu, J., Hausinger, R.P., Hols, P., & Soumillion, P. (2018). Biosynthesis of the nickel-pincer nucleotide cofactor of lactate racemase requires a CTP-dependent cyclometallase. *Journal of Biological Chemistry*, 293, 12303–12317. Panel (B) from Desguin, B., Zhang, T., Soumillion, P., Hols, P., Hu, J., & Hausinger, R. P. (2015). A tethered niacin-derived pincer complex with a nickel-carbon bond in lactate racemase. *Science*, 349, 66–69.

8.4 LarA

Biochemical studies were used to demonstrate that the NPN cofactor facilitates the racemization of lactate *via* a proton-coupled hydride transfer mechanism (Fig. 16). For *L. plantarum* LarA, either His108 or His174 (depending on the isomer of lactic acid) abstracts the proton from the C_2 -

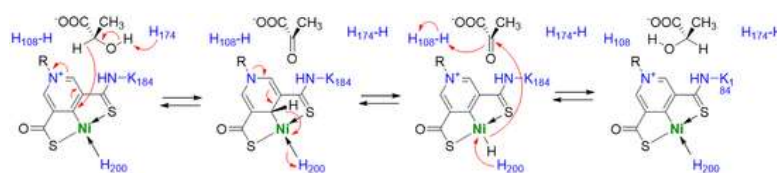


Fig. 16 A proposed proton-coupled hydride-transfer reaction mechanism for LarA with two sites for hydride interaction with NPN – one involving the pyridine ring and the other on the nickel.

hydroxyl group, the C₂-hydrogen atom transfers as a hydride to the co-factor while forming the intermediate pyruvate, and the hydride then reduces the intermediate by attack on either face (Rankin et al., 2018). Evidence for this mechanism derives from substrate isotope effects that reveal cleavage of the C₂—H bond as the rate limiting step in the reaction when using ²H-labeled lactate, detection of the pyruvate intermediate, observation of chromophoric changes associated with cofactor reduction, and computational studies. Substitution of the key residues by mutagenesis of the corresponding codons also supports this mechanism (Desguin et al., 2015). The proposal for two hydride interaction sites on the NPN cofactor offers a reasonable mechanism to account for racemization (Fig. 16), but has not been established.

9. Related NPN cofactor-containing enzymes

Lactate racemase is a member of a highly diverse superfamily of enzymes, classified as the COG3875 cluster of enzymes (Galperin et al., 2015). These enzymes are found in 15% of bacteria and 22% of archaea, and some are even found in lower eukaryotes. The diversity of this superfamily and of their genomic contexts suggest that many different enzymatic reactions are catalyzed by these enzymes. Lactate racemase homologs from different bacterial and archaeal species were purified and tested for other NPN-dependent epimerase and racemase activities. Two malate racemases, a 2-hydroxyglutarate racemase, two D-glucuronate 2-epimerases, a short-chain aliphatic 2-hydroxyacid racemase, and a phenyllactate racemase were identified among these lactate racemase homologs, and many are probably still to be discovered (Desguin et al., 2020).

These LarA-like enzymes were either purified from *L. lactis* by inserting the corresponding gene into the operon with the *larBC1C2DE* genes, for production of partial holoenzyme, or from *E. coli*, for production of apoenzyme, and then activated with biosynthetic NPN (Table 2). Different assays were set up for each of these enzymes. The racemization of L-malate into D-malate was assayed using a decarboxylating D-malate dehydrogenase that generates pyruvate and CO₂ from malate and reduces NAD⁺ to NADH (Fig. 17A, left). The conversion of D-malate to L-malate can be assayed with L-malate dehydrogenase, which generates oxaloacetate while reducing NAD⁺ to NADH, and glutamate-oxaloacetate transaminase, which consumes the oxaloacetate in the presence of glutamate (Fig. 17A, right). The hydroxyglutarate racemase was assayed with D-hydroxyglutarate dehydrogenase, which generates 2-oxoglutarate while reducing NAD⁺ to NADH, and glutamate-pyruvate transaminase, which consumes the 2-oxoglutarate in the presence of D-alanine (Fig. 17B). The epimerization of D-mannonate into D-gluconate was assayed with gluconate kinase, which phosphorylates D-gluconate to form D-gluconate-6-phosphate in the presence of ATP, and D-gluconate-6-phosphate dehydrogenase, which oxidizes D-gluconate-6-phosphate to D-ribulose-6-phosphate while reducing NADP⁺ to NADPH (Fig. 17C). The racemization of other short-chain 2-hydroxyacids, *e.g.*, 2-hydroxyisovalerate or 2-hydroxyisocaproate, was assayed by using a wide spectrum D-hydroxyacid dehydrogenase, which generates the corresponding 2-keto acid while reducing NAD⁺ to NADH (Fig. 17D). The formation of NADH is coupled with a reaction involving a diaphorase, which transforms iodonitrotetrazolium into formazan, a red compound, to pull the reaction to completion. The racemization of phenyllactate was assayed by chiral capillary electrophoresis. Briefly, the sample was loaded onto a polyvinyl alcohol-coated capillary and run with 50 mM phosphate buffer, pH 6, as background electrolyte and with 125 mM β-cyclodextrin as chiral separator. Products were detected at 190 nm (Desguin et al., 2020).

Acknowledgments

We thank the members of our laboratories who carried out the studies described here and for their comments on the text. The funding that supported these studies was obtained from the National Institutes of Health (Grant GM128959 to R.P.H. and J.H.) and Fonds de la Recherche Scientifique (FNRS) (to B.D.).

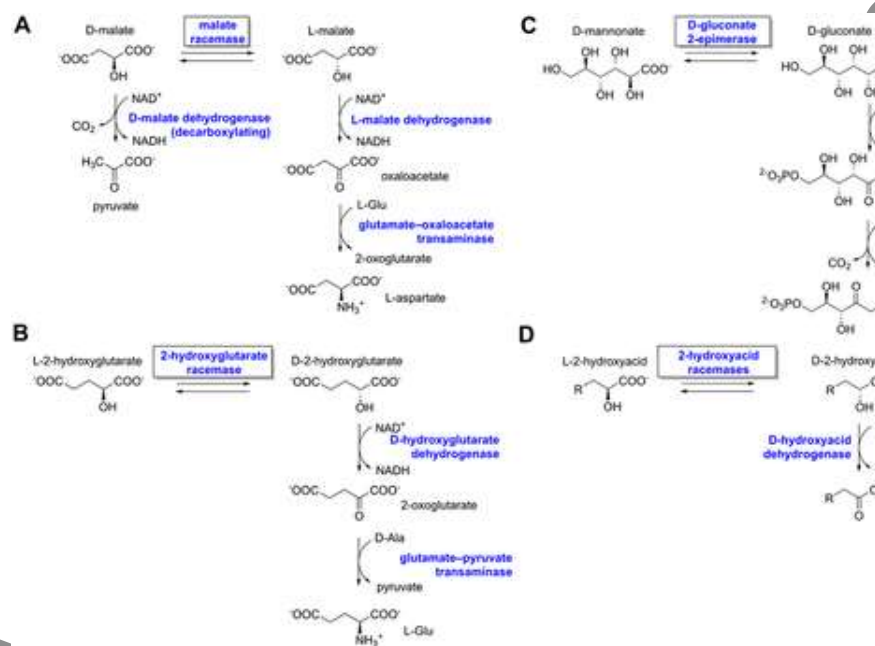


Fig. 17 Schemes for assaying selected other NPN-containing enzymes. (A) Malate racemase. (B) 2-Hydroxyglutamate 2-epimerase. (C) D-gluconate 2-epimerase. (D) Other 2-hydroxyacid racemases.

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