Biosynthesis of the nickel-pincer nucleotide cofactor of lactate racemase requires a CTP-dependent cyclometallase

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Benoît Desguin, Matthias Fellner, Olivier Riant, Jian Hu, Robert P. Hausinger, Pascal Hols, and Patrice Soumillion

From the Institute of Life Sciences and Institute of Condensed Matter and Nanosciences, Université Catholique de Louvain, B-1348 Louvain-La-Neuve, Belgium and the Departments of Biochemistry and Molecular Genetics, Chemistry, and Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824

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Bacterial lactate racemase is a nickel-dependent enzyme that contains a cofactor, nickel pyridinium-3,5-bisthiocarboxylic acid mononucleotide, hereafter named nickel-pincer nucleotide (NPN). The LarC enzyme from the bacterium Lactobacillus plantarum participates in NPN biosynthesis by inserting nickel into pyridinium-3,5-bisthiocarboxylic acid mononucleotide. This reaction, known in organometallic chemistry as a cyclometalation, is characterized by the formation of new metal–carbon and metal–sulfur σ bonds. LarC is therefore the first cyclometallase identified in nature, but the molecular mechanism of LarC-catalyzed cyclometalation is unknown. Here, we show that LarC activity requires Mn2+ -dependent CTP hydrolysis. The crystal structure of the C-terminal domain of LarC at 1.85 Å resolution revealed a hexameric ferredoxin-like fold and an unprecedented CTP-binding pocket. The loss-of-function of LarC variants with alanine variants of acidic residues leads us to propose a carboxylate-assisted mechanism for nickel insertion. This work also demonstrates the in vitro synthesis and purification of the NPN cofactor, opening new opportunities for the study of this intriguing cofactor and of NPN-utilizing enzymes.

Lactate racemase (LarA), the ninth discovered nickel-dependent enzyme (1), was shown to contain a newly identified cofactor, nickel pyridinium-3,5-bisthiocarboxylic acid mononucleotide, hereafter named nickel-pincer nucleotide (NPN), covalently attached to an active-site lysine residue (2). Synthesis of NPN occurs through a pathway involving three proteins encoded in the lar operon, i.e. LarB, LarC, and LarE (3). A bioinformatics study revealed that these genes are found in almost one-quarter of the analyzed prokaryotic genomes, showing the wide distribution of the NPN biosynthetic pathway (3). In 15% of the genomes, the genes encoding the NPN biosynthetic proteins were even found without any LarA homolog gene (3), indicating that other enzymes probably use the NPN cofactor as well.

NPN biosynthesis begins with LarB, a carboxylase/hydrolase that produces pyridinium-3,5-biscarboxylic acid mononucleotide (P2CMN) from nicotinic acid adenine dinucleotide (NaAD) (4). P2CMN is converted into pyridinium-3,5-bisthiocarboxylic acid (P2TMN) by LarE through two successive sacrificial sulfur transfer reactions (5). Finally, LarC inserts nickel and forms the final NPN cofactor with its five-membered metallacycle structure (Fig. 1a) (4) in which the metal is σ-bonded to a carbon atom and a heteroatom (a metallacycle). We therefore call LarC a cyclometallase. In organometallic chemistry, cyclometalation refers to the transition metal-mediated and heteroatom-assisted activation of a C–R or C–H bond to form a metallacycle (6). Cyclometalation has become one of the most popular organometallic reactions, providing a straightforward method for activating strong C–R or C–H bonds and creating a metal–carbon bond. This reaction has been successfully applied in organic transformations, catalysis, and for the stabilization of reactive intermediates (6).

In Lactobacillus plantarum, two open reading frames, larC1 and larC2, encode the full-length LarC protein through a programmed ribosomal frameshift (PRF) (Fig. 1b) (7). The reason for this PRF is unknown, but only 8% of larC genes show this feature, and the PRF can be bypassed by gene fusion without affecting the activity (3). So, this process is not an essential requirement for LarC activity. Consistent with a nickel-dependent function, a His-rich region is present in its N-terminal region, and nickel is found associated with the purified LarC (3). The LarC sequence shows no homology with any protein of known function, and its catalytic activity remains unexplored. Here, we show that LarC hydrolyzes CTP to CMP while inserting nickel into P2TMN, forming the metallacycle NPN that is stabilized in solution by the presence of thiols. The CTPase activity requires Mn2+ or Mg2+. We determined the crystal structure of LarC2, the C-terminal domain of LarC, revealing a...
**LarC is a CTP-dependent cyclometallase**

![Diagram](image)

**Figure 1. Cell lysates and CTP have similar effects on NPN cofactor biosynthesis.** a, NPN cofactor biosynthesis starts with NaAD that is carboxylated and hydrolyzed by LarB forming P2CMN. Two LarE proteins each catalyze a sacrificial sulfur transfer reaction to synthesize P2TMN from P2CMN. LarC inserts nickel into P2TMN, forming the NPN cofactor containing a metallacycle (bold blue lines) with Ni- and Ni-S-S bonds. b, schematic representation of the larC gene. LarC1 and LarC are the two open reading frames that code for LarC. c, effect of 10% (v/v) *L. lactis* cell lysates on in vitro NPN cofactor biosynthesis by LarB, LarE, and LarC. Lar activity with cell lysates after 30 min was set to 100%. d, effect of 10% cell lysates, 10% cell lysates deproteinized with heat treatment (lysate HT), or 1 μM nucleotides on the in vitro NPN cofactor biosynthesis by LarB, LarE, and LarC after 30 min. Lar activity with cell lysates was set to 100%. b and c, NPN cofactor biosynthesis was assessed after addition of LarA, apoprotein and l-lactate by assaying for d-lactate production. The lines indicate the mean values, and the points indicate the individual data points.

hexameric ferredoxin-like fold and an unprecedented CTP-binding pocket involving three subunits, establishing LarC2 as a new member of the GlnB-like superfamily. Extensive mutagenesis studies suggested that LarC1, the N-terminal part of LarC, likely catalyzes the cyclometalation reaction somehow assisted by the CTPase activity of LarC2.

**Results**

**CTP enhances NPN cofactor biosynthesis**

We overproduced LarC in *Lactococcus lactis* cells grown in the presence of divalent nickel and purified the nickel-containing protein. Prior studies had shown LarC to be required in 2-fold excess over LarE to achieve maximum NPN cofactor biosynthesis *in vitro* (4), suggesting that LarC activity was not optimal in these conditions. We observed that inclusion of cell lysates strongly enhanced NPN cofactor biosynthesis by LarB, LarE, and LarC as shown by the >50-fold increase in LarC activity after 1 min of incubation preceding addition of LarA, i.e., LarA apoprotein from *Thermoanaerobacterium thermosaccharolyticum* (Fig. 1c). Because removal of the proteins by heat treatment and centrifugation of the lysates did not abolish the stimulation (Fig. 1d), the involvement of a small molecule was suspected. Several small organic molecules were tested with CTP showing a similar effect to cell lysates, whereas other nucleotides had no effect (Fig. 1d), thus indicating that LarB, LarE, or LarC activity is CTP-dependent.

**LarC is a CTP-dependent enzyme**

To analyze the activity of LarC, we developed a method to purify its substrate, P2TMN, by reacting NaAD with LarB and LarE (Fig. 1a). After the combined LarB/LarE reaction, the proteins were heat-denatured and removed by centrifugation with the supernatant loaded onto an anion-exchange column. P2TMN was eluted using a NaCl gradient, and its identity was confirmed with mass spectrometry (MS) (Fig. 2a). LarC was reacted with P2TMN, MgCl₂, and CTP, followed by addition of LarA, and l-lactate, with Lar activity observed (Fig. 2b). Lactate racemization was not detected when P2TMN or LarC was omitted, but a residual 2 or 15% activity was observed in the absence of CTP or MgCl₂, respectively. These residual activities suggest that some CTP and Mg²⁺ co-purify with LarC. In agreement with this hypothesis, the mass of purified LarC includes an additional peak that is 505 Da larger than the LarC apoprotein, corresponding to the mass of CTP + Mg²⁺ − 2H⁺ (505.4 Da) (Fig. 2c). The absence of bound nickel in the observed spectrum (Fig. 2c) is probably due to its dissociation during the LC step preceding MS. These results demonstrate...
that LarC is a CTP-dependent enzyme that uses P2TMN as substrate.

Hydrolysis of CTP by LarC

The enzymatic properties of LarC were investigated in more detail. The nucleotide specificity for CTP was confirmed using purified P2TMN as a substrate (Fig. 3a). In addition, Mn$^{2+}$ was shown to be 5-fold more efficient than Mg$^{2+}$ to activate LarC and at 1/20th of the concentration (Fig. 3b), indicating that Mn$^{2+}$ is the preferred cation for LarC in vivo. This result was surprising but not unexpected as *Lb. plantarum* accumulates approximately 30 mM Mn$^{2+}$ in its cytoplasm (8). *Lc. lactis*, however, does not accumulate Mn$^{2+}$ in its cytoplasm (8), which explains why Mg$^{2+}$ was found instead of Mn$^{2+}$ in LarC purified from *Lc. lactis* cells (Fig. 2c). To test whether and how LarC hydrolyzes the triphosphate, we performed thin-layer chromatography (TLC) of the LarC reaction products that form using $[^{32}\text{P}]$CTP. When P2TMN and MgCl$_2$ were added to the reaction, CMP accumulated over time (Fig. 3c) showing that LarC hydrolyzes CTP to CMP. In the absence of divalent cation, aberrant hydrolysis took place with CDP formed instead of...
LarC is a CTP-dependent cyclometallase

Figure 4. Characterization of the LarA activation by NPN. a, activation of LarA\textsubscript{Tt} apoprotein. b, time-dependent activation of LarA\textsubscript{Tt} and LarA\textsubscript{Lp} apoproteins by in vitro-synthesized NPN, with or without (Ctrl) heat denaturation of LarB, LarC, and LarE and with or without addition of cysteine (+Cys) and β-mercaptoethanol (+BME) (1 or 10 mM in a and 10 mM in b). LarA was incubated with in vitro-synthesized NPN for 1 min in a and for the indicated time in b. Lar activity was assessed after addition of ω-lactate by assaying for ω-lactate production. The lines indicate the mean values, and the points indicate the individual data points. c, LarC reaction summary.

CMP (Fig. 3c). Thus, LarC is a CTP-hydrolyzing nickel cyclometallase.

Soluble NPN produced by LarC directly activates LarA

The expected product of the LarC reaction, NPN, may be released as the free cofactor or it may require a protein that transfers and perhaps activates it for binding to LarA\textsubscript{Tt}, i.e., LarA from \textit{Lb. plantarum}. LarA\textsubscript{Tt} forms a covalent thioamide bond with NPN, which is why its activation is time-dependent, in contrast to LarA\textsubscript{Lp} that interacts noncovalently with NPN (4). To address this question, we obtained NPN by reaction of NaAD with LarB, LarE, and LarC, the proteins were heat-denatured and removed by centrifugation, and the supernatant was tested for its ability to generate Lar activity in the presence of LarA\textsubscript{Tt} (Fig. 4a). Heat treatment of the LarB, LarE, and LarC reaction strongly decreased activation, but the activation could be restored, or even enhanced, when free thiols such as cysteine or β-mercaptoethanol (BME) were added before heat treatment (Fig. 4a). This observation suggests that free thiols stabilize NPN in solution, possibly by serving as a fourth ligand in the square-planar nickel coordination sphere.

The activation of LarA\textsubscript{Lp} was then compared with LarA\textsubscript{Tt} activation. LarA\textsubscript{Lp} activation was lower and much slower, compared with LarA\textsubscript{Tt} (Fig. 4b), as expected by the requirement to form a covalent bond between LarA\textsubscript{Lp} and NPN. LarA\textsubscript{Lp} activation was still observed, and even enhanced three times, after heat denaturation of LarB, LarE, and LarC when BME was added (Fig. 4b), demonstrating that the thiol-stabilized free NPN activates Lar better without the synthetic proteins LarB, LarE, and LarC (Fig. 4c). The binding of the cofactor to a biosynthetic protein may slow down the activation of LarA.

The supernatant of the combined LarB, LarE, and LarC reactions was chromatographed on an anion-exchange column in the presence of 10 mM BME, and the identity of the purified NPN was confirmed by MS (Fig. 5a). The optical spectra of purified P2TMN and NPN show broad absorbance peaks around 260 and 450 nm, respectively (Fig. 5b). The NPN peak at 450 nm was observed previously in purified LarA holoprotein (2).

LarC behaves as a single-turnover enzyme hydrolyzing one CTP per nickel insertion

We next examined whether LarC is capable of catalyzing multiple turnovers. This protein does not contain nickel when purified from cells grown in the absence of this metal, and previous studies had shown that subsequent Ni\textsuperscript{2+} addition had only a slight positive effect on LarC activity (4). When LarC was limiting and the reaction time extended, added nickel could only slightly activate LarC and only at stoichiometric concentrations. Higher nickel concentration showed no effect (Fig. 6a). Moreover, nickel-devoid LarC cannot be activated by adding Ni\textsuperscript{2+} to the solution (Fig. 6a). In summary, added nickel enhances NPN biosynthesis when LarC is already nickel-loaded but cannot activate the LarC apoprotein. These results show that LarC cannot catalyze multiple nickel insertions into P2TMN using the conditions of the \textit{in vitro} test.

Given that the LarC purified from nickel-replete cells was ~90% loaded with nickel, the P2TMN concentration could be
LarC is a CTP-dependent cyclometallase

estimated based on the amount of LarC needed to completely react with all of the substrate (Fig. 6b). This P2TMN concentration could then be compared with the estimated CTP hydrolysis based on densitometry measurement of the TLC autoradiograph. The amount of CTP hydrolyzed equaled the amount of P2TMN added (Fig. 6c). This one-to-one ratio showed that CTP hydrolysis is stoichiometric with NPN biosynthesis.

Crystal structure of the CTP-binding domain of LarC

To gain better insight into the LarC catalytic mechanism, we attempted to crystallize the full-length (N-terminal StrepII-tagged) fused LarC protein (Table 1), first purified from Lc. lactis and later from Escherichia coli. In both cases, we obtained only LarC2 crystals (without the eight N-terminal residues and with the last five C-terminal residues being disordered). To investigate the cleavage of the full-length protein into LarC1 and LarC2 over time, we incubated the protein in purification buffer at ~1 mg ml⁻¹ at room temperature for 2 weeks. The precipitate was separated from the supernatant, which was subjected to MS analysis. The supernatant only contained LarC2 with three MS peaks of 17,286, 17,074, and 16,804 Da (Fig. 7), corresponding to LarC2 with 4, 6, or 8 residues missing at the N terminus. The precipitate appeared to contain the full-length LarC and the degraded LarC1. This result shows that the full-length protein is cleaved at three different positions over time when purified from either Lc. lactis or E. coli, resulting in a very stable LarC2 that is crystallizable. In contrast, the remaining LarC1 and the full-length protein are less stable, precipitate with time, and do not form crystals. We overexpressed just LarC1 in Lc. lactis and in E. coli, but no soluble protein was produced or LarC1 purified as an aggregate according to gel-filtration analysis, respectively, so we focused on the LarC2 structure.

We solved the LarC2 structure under multiple crystallization conditions, which always revealed the same hexamer composed of a dimer of trimers (Fig. 8a). Each LarC2 unit comprises two distinct domains (Fig. 8b) as follows: the N-terminal domain 1 (residues 272–356) with a ferredoxin-like fold (β1α1β2β3α2β4); and the C-terminal domain 2 (residues 357–412) containing three successive β-strands (β5–7) followed by two α-helices (α3–4). Domain 1 forms a symmetric trimer with orthogonally packed β-sheets, reminiscent of the typical fold of the GlBN-like superfamily. Two domain 1 trimers stack vertically and share the 3-fold symmetry axis, forming the central part of the hexamer. Domain 2, which is extended from the C terminus of domain 1, forms a protrusion from the central hexamer and contributes to the deep CTP-binding pocket (see below). The substantial differences for β2 and β3 compared with known family members and the unique C-terminal extension (domain 2) establish LarC as a new member of GlBN-like superfamily (Fig. 8c). A three-dimensional protein structure similarity search using the Dali server (9) and a sequence search in the Protein Data Bank only resulted in one similar structure...
Table 1
Strains, plasmids, and primers

| Strain, plasmid, or primer | Characteristic(s) or sequence | Source or Ref. |
|---------------------------|-------------------------------|---------------|
| Strain | Characteristic(s) or sequence | Source or Ref. |
| Le. lactis | MG1363 derivative | 33 |
| N3900 | | |
| E. coli | DH10B F’ endA1 recA1 galE16 galK16 mprG pslp ΔlacX74 Δphi80lacΔZΔM15 araD139 Δ[araE1::kan]7697 mcrA Δ(mrr-hsdRMS-mcrBC) Δ3 | Invitrogen |
| | DH5α | fhua2 lacU169 phoA1 glnV4 Δphi80lacZΔM15 gyrA96 recA1 relA1 endA1 thi-1 hsdR7 | Invitrogen |
| | ArticExpress | Contains Cpm60 and Cpm10 from Oleispyra antarctica | Agilent |
| | Rosetta 2 | F’ amp Tn3Δs(tra m<5’) gld dcm (DE3) pRARE2 (Cam<5’) | Novagen |

Plasmid

| Prime | Characteristic(s) or sequence | Source or Ref. |
|-------|-------------------------------|---------------|
| pNZ8048 | Cm<5’, P<5’ | 34 |
| pGIR012 | Cm<5’; pNZ8048 with a 1.31-kb insert after P<5’ containing larA<5’ translationally fused to DNA encoding the StreptII-tag | LarA<5’ purification |
| pGIR082 | Cm<5’; pNZ8048 with a 1.31-kb insert after P<5’ containing larA<5’ translationally fused to DNA encoding the StreptII-tag | LarA<5’ purification |
| pGIR26 | Em<2; pGIZ660<2 with DNA encoding the StreptII-tag translationally fused at the 5’-end of the larB ORF | LarB purification |
| pGIR31 | Cm<5’; pNZ8048 with a 1.30-kb insert after P<5’ containing DNA encoding the StreptII-tag translationally fused to larC | LarC purification |
| pGIR41 | Cm<5’; pNZ8048 with a 0.79-kb insert after P<5’ containing DNA encoding the StreptII-tag translationally fused to larC1 | LarC1 purification |
| pGIR51 | Cm<5’; pNZ8048 with a 1.30-kb insert containing the translational fusion between P<5’ and Strept-tag--larC fusion with an 1 bp insertion between larC1 and larC2 | LarC<5’ purification |
| pGIR43 | Amp<5’; pET22b with a 0.79-kb insert containing DNA encoding the StreptII-tag translationally fused to larC1 | LarC1 purification |
| pGIR53 | Amp<5’; pET22b with a 1.30-kb insert containing DNA encoding the Strept-tag--larC fusion with an 1 bp insertion between larC1 and larC2 | LarC<5’ purification |
| pGIR76 | Amp<5’; pBADHisA with a 0.89-kb insert after P<5’ containing larE translationally fused to the StreptII-tag sequence | LarE purification |
| pGIR301_MutCX | pGIR301 encoding the LarC variant X (see primers for detail) | LarC variants purification |

Primers for pGIR301 modification

| Primer | Characteristic(s) or sequence | Source or Ref. |
|--------|-------------------------------|---------------|
| MutCl_A | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | ΔH75-H86 variant |
| MutCl_B | 3’-TTCGAAACAAATGCATGCTTTACC-5’ | This study |
| MutCl_C | 3’-GAAGGCGAGCGAGGTACGTTTACCAG-5’ | This study |
| MutCl_D | 3’-GGTGTCGCGCGGTGCGTACGTTTACCAG-5’ | This study |
| MutCl_E | 3’-ATCGTGATCAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_F | 3’-CGATGTCGCGCGGTGCGTACGTTTACCAG-5’ | This study |
| MutCl_G | 3’-GGTGTCGCGCGGTGCGTACGTTTACCAG-5’ | This study |
| MutCl_H | 3’-ATCGTGATCAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_I | 3’-TAAGTGCATGCGAGGCATGCGATCATTGAGTGTG-5’ | This study |
| MutCl_J | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_K | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_L | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_M | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_N | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_O | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_P | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_Q | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_R | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_S | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_T | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_U | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutCl_V | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_A | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_B | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_C | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_D | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_E | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_F | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_G | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_H | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_I | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_J | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_K | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_L | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_M | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_N | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_O | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_P | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_Q | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_R | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_S | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_T | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_U | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| MutC_V | 3’-GATCTAGAAGGCGATCATTGAGTGTG-5’ | This study |
| UP_NZ8048 | 3’-ACATGACATGCTTACAGGCGATCATTGAGTGTG-5’ | Sequencing |
| DW_NZ8048 | 3’-ACATGACATGCTTACAGGCGATCATTGAGTGTG-5’ | Sequencing |

LarC is a CTP-dependent cyclometallase.
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By aligning seven divergent LarC sequences, we found that most of the above-cited residues are substantially invariant (Fig. 10). Arg-354 is not absolutely conserved, but an Arg residue is most prevalent at this position. To examine their importance, these conserved residues were substituted by Ala, and the in vitro activity of the LarC variants was evaluated. Most LarC variants, except R317A, showed reduced activity compared with the WT enzyme (WT) (Fig. 11a). Two LarC variants, D284A and K315A, showed around 10% activity compared with the WT, and the last five LarC variants, K314A, R348A, R359A, K374A, and E387A, showed only residual activity (Fig. 11a). It is interesting to note that for two variants with only residual LarC activity, K314A and K374A, a lysine residue pointing toward the α-phosphate of CTP was substituted (Fig. 9a), suggesting that these residues could be involved in catalysis. The LarC variant E387A has probably lost its ability to chelate metal ion (Mn2), which suggests that the metal ion coordinated by Glu-387 is necessary. The reduced, but not completely abolished, activity of D284A might indicate that the Asp-284–facilitated Mn2+-coordinating water molecules within a well-ordered hydrogen-bond network. The triphosphate of CTP is further stabilized by Lys-374 from domain 2 electrostatically associating with the α-phosphate, making it the most ordered portion of CTP. In contrast, the ribose is the least ordered part with only Arg-359 (domain 2) being within hydrogen-bond distance. Arg-354 (on the linker between domain 1 and 2) forms hydrogen bonds with the cytidine, implying a role in determining the specificity toward CTP. Near the cytidine, an atom with about the same electron density peak height as found for sulfur or phosphates atoms in the structure is surrounded by nitrogen atoms from CTP, Arg-354, and Arg-348 (domain 1’), and therefore we interpreted it as a chloride ion. Comparison between apoprotein (PDB code 6BWO) and CTP-Mn2+-bound (PDB code 6BWQ) structures did not reveal any major structural changes upon CTP binding, with an r.m.s.d. of ~0.5 Å between corresponding chains.

Site-directed mutagenesis in LarC2 confirms its CTP-binding site

By aligning seven divergent LarC sequences, we found that most of the above-cited residues are substantially invariant (Fig. 10). Arg-354 is not absolutely conserved, but an Arg residue is most prevalent at this position. To examine their importance, these conserved residues were substituted by Ala, and the in vitro activity of the LarC variants was evaluated. Most LarC variants, except R317A, showed reduced activity compared with the WT enzyme (WT) (Fig. 11a). Two LarC variants, D284A and K315A, showed around 10% activity compared with the WT, and the last five LarC variants, K314A, R348A, R359A, K374A, and E387A, showed only residual activity (Fig. 11a). It is interesting to note that for two variants with only residual LarC activity, K314A and K374A, a lysine residue pointing toward the α-phosphate of CTP was substituted (Fig. 9a), suggesting that these residues could be involved in catalysis. The LarC variant E387A has probably lost its ability to chelate metal ion (Mn2), which suggests that the metal ion coordinated by Glu-387 is necessary. The reduced, but not completely abolished, activity of D284A might indicate that the Asp-284–facilitated Mn2+-coordinating water molecules within a well-ordered hydrogen-bond network. The triphosphate of CTP is further stabilized by Lys-374 from domain 2 electrostatically associating with the α-phosphate, making it the most ordered portion of CTP. In contrast, the ribose is the least ordered part with only Arg-359 (domain 2) being within hydrogen-bond distance. Arg-354 (on the linker between domain 1 and 2) forms hydrogen bonds with the cytidine, implying a role in determining the specificity toward CTP. Near the cytidine, an atom with about the same electron density peak height as found for sulfur or phosphates atoms in the structure is surrounded by nitrogen atoms from CTP, Arg-354, and Arg-348 (domain 1’), and therefore we interpreted it as a chloride ion. Comparison between apoprotein (PDB code 6BWO) and CTP-Mn2+-bound (PDB code 6BWQ) structures did not reveal any major structural changes upon CTP binding, with an r.m.s.d. of ~0.5 Å between corresponding chains.

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binding, two LarC variants, D284A and K315A, were tested for their CTP-binding affinity (Fig. 11b). The initial rate of LarC reaction was measured as a function of CTP concentration, allowing the estimation of the CTP concentration required to attain 50% of the maximum LarC activity ($K_{50}$). As shown in Fig. 11b, this $K_{50}$ is around 0.05 mM for the WT and increased to around 0.5 mM in both variants. The large effect of the substitutions on the affinity for CTP shows that the CTP-binding site is affected by these substitutions and confirms that the crystal structure revealed the native CTP-binding site.

**Essential Asp/Glu residues in LarC1**

With the knowledge that LarC2 is involved in CTP binding, we hypothesize that LarC1 functions in binding nickel and
LarC is a CTP-dependent cyclometallase

**Figure 10. Alignment of LarC homologs from** *Lb. plantarum* NCIMB 8826, *Staphylococcus epidermidis* ATCC 12228, *T. maritima* MS88, *Thermosinus carboxydivorans* NOR1, *Desulfotomaculum carboxydivorans* CO-1-SRB, *Synechocystis* sp. PCC 6803, and *Methanosarcina acetivorans* C2A.

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P2TMN. To test the involvement of the His-rich region and conserved negatively-charged residues of LarC1 in nickel binding or other functions of LarC, several variants were analyzed. The LarC variant with a deletion of the entire His-rich region showed decreased LarC activity and nickel content (WT, Δ75–86, Fig. 11, c and d), indicating that the His-rich region binds nickel but is not strictly required for LarC activity, as also suggested by the low conservation of this sequence (Fig. 10). However, the three alanine variants of highly conserved acidic residues E124A, E139A, and D144A (Fig. 10) exhibited complete loss of LarC activity and variable nickel content (Fig. 11, c and d), potentially indicating a functional role. Thus, several carboxylic acid or carboxylate residues are critical for activity.

To rule out LarC2 binding of nickel, we soaked LarC2 crystals in a high concentration of this metal ion. The structure revealed four nickel-binding sites (Fig. 12), but none of the chelating residues are conserved (Fig. 10), and the sites were not observed in both trimers.

Discussion

We described here a previously uncharacterized CTP-hydrolyzing nickel cyclometallase, LarC. Perhaps the closest functionally-related enzyme is HcgD, a Nif3-like protein that is thought to act as iron chaperone in the biosynthesis of the iron-guanalylypyridinol (FeGP) cofactor of [Fe]-hydrogenase (10). In the case of the FeGP cofactor, the enzymatic system involved in the iron–acyl formation is unknown, but an Fe(0) or Fe(I)–carbonyl complex is thought to be the precursor of the acyl–iron bond formation and has been used for the chemical synthesis of FeGP cofactor model compounds (11). By contrast,
LarC is a CTP-dependent cyclometallase

LarC inserts nickel in the +2 oxidation state, without the need for reduction.

We showed that LarC activity is energy-dependent and requires hydrolysis of the rarely used nucleotide CTP (Fig. 1d). The function of CTP hydrolysis remains unclear. Perhaps the nickel is brought into close proximity to the P2TMN through a CTP-dependent conformational change. Alternatively, the P2TMN thioacid may be activated by cytidylylation, although our attempts to accumulate this covalent intermediate using LarC WT and variants were unsuccessful. A direct binding of CTP or CMP to nickel at some stage of the reaction also cannot be excluded. This hypothesis may relate to the basis of LarC’s specificity for CTP; cytidine is the only pyrimidine not protonated at N3 at physiological pH and, hence, would be available for metal ion binding (12).

The observation that LarC behaves as a single-turnover enzyme in vitro may reflect the fact that LarC may not need to catalyze multiple turnovers in vivo. Indeed, LarC acts downstream of LarE, which is itself a single-turnover enzyme (Fig. 1d). Moreover, the limited amount of NPN synthesized will prevent the exhaustion of the NaAD precursor, which is required for NAD biosynthesis. Moreover, as NPN is a catalyst derived from a precursor of NAD, the physiological need in biosynthesis must be low and overproduction may be toxic.

We identified several residues involved in LarC function. First, we showed that the previously identified His-rich sequence (3) is a probable region for nickel binding (Fig. 11d), although not strictly required for LarC activity (Fig. 11c). This site may be used for nickel storage before its transfer to the active site and its insertion into P2TMN. Three conserved carboxylic residues, Glu-124, Glu-139, and Asp-144, are required for LarC activity (Fig. 11c). The essentiality of these residues leads us to postulate a mechanism for LarC activity involving carboxylate groups (Fig. 11e). In this hypothesis, two carboxylate residues, presumably Glu-124 and/or Glu-139 and/or Asp-144, coordinate a nickel ion close to P2TMN. This binding might be unstable and might only take place temporarily during catalysis, which would explain why some of these variants show a higher nickel content than the WT (Fig. 11d). A pericyclic reaction involving the concerted movement of six electrons would then result in nickel replacing the hydrogen at the C4 position of the pyridinium ring. Such carboxylate-assisted C–H activation is a classical mechanism in organometallic cyclometalations (6). Asp and Glu residues have also been suggested to be involved in the transfer of iron into ferrochelatase (13) and in transmetalation of porphyrin (14).

The structure of the C-terminal domain of LarC (LarC2) was solved in the presence and absence of CTP, and the binding site of CTP was identified at a deep cleft at the interface of three LarC2 units (Figs. 8 and 9). The importance of CTP binding and catalysis was demonstrated by mutagenesis and activity assays (Fig. 11, a and b). Interestingly, the LarC sequence is devoid of known nucleotide-binding motifs according to the nucleotide-binding database (15). We note that the PII signaling transduction proteins of the GlnB-like superfamily also bind a nucleotide, but in that case it is ATP. ATP is not hydrolyzed, but its binding induces a conformational change that appears to be the structural basis of PII activation for its nitrogen-sensing role in bacteria (16, 17). The conserved ATP-binding pocket in the PII signaling transduction proteins only partially overlaps with the CTP-binding site of LarC2, and significant differences can be identified through side-by-side structural comparison (Fig. 13). Particularly, domain 2 of LarC2, which is absent in known members in the GlnB-like superfamily, provides key residues in CTP binding, supporting the notion that LarC2 has evolved to constitute a critical part of the unique nucleotide-binding pocket. Because CTP binding does not lead to any meaningful conformational change of LarC2, it is unlikely that CTP acts as a regulator of LarC as found for ATP in activation of the PII signaling transduction proteins. Instead, we have shown that CTP is a substrate of LarC, indicating that the function of LarC2 is to bind and present CTP to LarC1.

LarC is the first identified cyclometallase catalyzing the formation of a stable nickel–carbon bond. We have unveiled some characteristics of this intriguing enzyme, including that it is a CTP-hydrolyzing enzyme. We also performed the first in vitro
**LarC is a CTP-dependent cyclometallase**

synthesis and purification of the NPN cofactor, which will open the way to the direct investigation of this particular cofactor in vitro.

**Experimental procedures**

**Materials and growth conditions**

Bacterial strains and plasmids used in this study are listed in Table 1. Chemicals were purchased from Sigma. We grew *Lc. lactis* in M17 broth supplemented with 0.5% glucose at 28 °C. NiSO₄ (1 mM) was added to cultures expressing pGIR031 (3), and chloramphenicol (10 mg ml⁻¹) was provided when expressing plasmids were derived from pNZ8048. For the induction of genes under control of the nisA expression signals, nisin A was added during the early exponential phase (A₆₀₀ = 0.3–0.4) at a concentration of 1 μg l⁻¹, and the cells were collected after 4 h. *E. coli* DH10B was grown with agitation at 37 °C in LB containing erythromycin (200 mg l⁻¹). For LarE expression in *E. coli* ArticExpress, 10 ml of an overnight culture in LB with ampicillin (200 mg l⁻¹) and gentamycin (40 mg l⁻¹) was transferred to 1 liter of LB and grown for 3 h at 30 °C with agitation, and induction was started by addition of 1-thiogalactopyranoside (0.2%) and grown at 13 °C for 24 h with agitation.

For determining the apoprotein structure, *Lc. lactis* cells containing pGIR051 were grown following the above-mentioned protocol, except that no NiSO₄ was added. For the selenomethionine-containing crystal from *Lc. lactis*, we grew the cells in chemically defined medium containing selenomethionine instead of methionine, as described elsewhere (18). For the CTP-Mn and nickel-bound structures, pGIR053 was utilized in *E. coli* Rosetta 2(DE3) cells. Rosetta 2 cells were grown with agitation at 37 °C in LB containing ampicillin (100 mg l⁻¹) until exponential phase (A₆₀₀ = 0.5–0.6). The culture was transferred to 16 °C, induced with 0.1 mM isopropyl β-D-1-thio-galactopyranoside, and grown for ~20 h with agitation.

For investigation of LarC1 without LarC2, construct pGIR041 was introduced in *Lc. lactis* as described above for pGIR031. In addition, the LarC1 construct carrying pGIR043 was introduced in *E. coli* Rosetta 2 cells as mentioned above.

**DNA techniques**

We carried out general molecular biology techniques according to standard protocols (19). Transformation of *E. coli* and *Lc. lactis* was performed by electroporation (20, 21). PCR amplifications used Phusion high-fidelity DNA polymerase (New England Biolabs). The primers used in this study were purchased from Eurogentec and are listed in Table 1. Plasmids bearing variants of larC for expression in *Lc. lactis* were derived from pGIR031 (3) and provided the 13 variant forms of LarC.

For each construction, pGIR031 was first methylated by Dam methylase using *E. coli* DH5α and provided the 13 variant forms of LarC. For construction of pGIR043 and pGIR053 in *E. coli*, we amplified, digested, and ligated the larC1 gene, including the N-terminal Strep-II tag into the pET22b vector, similar to the description above. Transformation first in *E. coli* DH5α and then *E. coli* Rosetta 2(DE3) cells followed standard protocols. Constructs were confirmed via sequencing.

**Cell lysate preparation**

The cells from 1.5 liters of *Lc. lactis* cultures expressing LarC from pNZ8048 derivatives were collected by centrifugation (5000 × g for 10 min) and washed twice with 50 ml of 100 mM Tris-HCl, pH 7.5, buffer containing 150 mM NaCl (W buffer). 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 FastPrep-24 cell disruptor (MP Biomedical) twice for 1-min periods, with 5 min cooling on ice between the runs. After lysis, the soluble fractions were collected after centrifugation at 20,000 × g for 15 min at 4 °C. For obtaining denatured cell lysates, the soluble fractions were incubated at 80 °C for 10 min, and following protein precipitation, the supernatants were collected after centrifugation at 20,000 × g for 5 min at room temperature.

**Protein purification**

LarA₁₅₁, LarA₁₇₂, LarC, and LarC variants were purified from 1.5 liters of the appropriate *Lc. lactis* cultures (containing plasmids that are listed in Table 1). Cell lysates were obtained as described above. Affinity chromatography was performed with gravity flow Strep-Tactin® Superflow® high-capacity columns of 1.5 ml, as described (23), with W buffer. Protein concentrations were measured by the Bradford assay (24) and confirmed by the absorbance at 280 nm for LarC (within 10% range). Only for crystallization purposes, W buffer contained 300 mM NaCl.

LarB and LarE were purified from 1.5 liters of *E. coli* culture transformed with pGIR026 or pGIR076. The cells were collected, and the pellet was washed twice in W buffer, resuspended in W buffer with 1-glutathione lysozyme, incubated for 30 min at 37 °C, and cooled for 10 min on ice. This treatment was followed by sonication at maximum amplitude with a Vibra Cell 75022 (Bioblock Scientific) using three series of 10 1-s 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 gravity flow Strep-Tactin® Superflow® high-capacity columns of 1.5 ml, as described (23), with W buffer. 300 mM NaCl was used for LarE purification instead of 150 mM.

LarC and LarC1 were prepared for crystallization purposes from *E. coli* cultures transformed with pGIR043 and pGIR053. After centrifugation, cells were resuspended in W buffer with 300 mM NaCl and lysed via sonication on ice (40% amplitude, 5-s pulses, 10-s pauses for 12 total min). Centrifugation and purification followed the mentioned LarB and LarE steps with 1.0-ml columns.

**In vitro activation of LarA by purified LarB, LarC, and LarE and lysates**

We incubated a mixture of LarE (5 μM), LarC (5 μM), LarB (1 μM), NaAD (0.1 mM), ATP (2 mM), MgCl₂ (20 mM), and NaHCO₃ (50 mM) in Tris-HCl buffer (100 mM, pH 8) with 2-μl
lysates from *lar*-free cells (when indicated) and 1 mM nucleotides (when indicated) in a final volume of 20 μl. After incubation at room temperature for 30 min (or other incubation time, when indicated), 5 μl of the assay mixture was diluted into 45 μl of l-lactate (45 mM) supplemented with LarA₂₉, 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 d-lactate concentration was measured by enzymatic lactate oxidation to pyruvate using a d-lactic acid/d-lactic acid commercial test (Megazyme), as described previously (3). The NADH absorbance was monitored at 340 nm with an Infinite 200 PRO plate reader (Tecan).

**Purification of P2TMN and the NPN cofactor**

For purification of P2TMN, we incubated 1 ml of a mixture of LarE (200 μM), LarB (10 μM), NaAD (0.2 mM), ATP (2 mM), MgCl₂ (20 mM), and NaHCO₃ (50 mM) in Tris-HCl buffer (100 mM, pH 7). For purification of the NPN cofactor, we incubated 1 ml of a mixture of LarE (100 μM), LarC (50 μM), LarB (10 μM), NaAD (0.2 mM), CTP (0.2 mM), ATP (2 mM), BME (10 mM), MgCl₂ (20 mM), and NaHCO₃ (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 °C for 10 min, and the supernatant was collected by centrifugation at 20,000 × g for 5 min and loaded onto a 5-ml HiScreen Q HP column (GE Healthcare) using an Akta Purifier (GE Healthcare), washed with Tris-HCl buffer (30 mM, pH 8), and eluted 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 BME was added to all buffers. P2TMN and NPN peaks were identified by their absorbance at 330 and 450 nm, respectively.

**P2TMN and NPN cofactor analysis**

For analysis of P2TMN and the NPN cofactor by MS, 10-μl aliquots were injected into an Accela HPLC system coupled to a Q-Exactive (ThermoFisher Scientific) mass spectrometer. Separations were performed using a Kinetex C18 column (150 × 2.1 mm) in 0.1% formic acid with a gradient of acetonitrile. The run time for each sample was 25 min. For analysis of the UV-visible spectrum of P2TMN, a Cary 50 BIO (SpectroVarian) was used.

For nickel quantification, we used 4-(2-pyridylazo)resorcinol (PAR) (25). The protein sample was denatured for 10 min at 80 °C prior to a 2-min incubation at room temperature with 100 μM PAR in 100 mM Tris-HCl buffer, pH 7.5. The absorbance was read at 496 nm with an Infinite 200 PRO plate reader (Tecan).

**In vitro activation of LarA by purified LarC and P2TMN**

We incubated a mixture of LarC (2.5 μM), P2TMN (50% v/v), MgCl₂ (10 mM) (or MnCl₂, when indicated), BME (10 mM), and CTP (0.1 mM) (or other nucleotides, when indicated) in MES buffer (100 mM, pH 6) using a final volume of 10 μl. After incubation at room temperature for 1 min, the reaction was stopped by heat treatment at 80 °C for 10 min, and 5 μl of the assay mixture was diluted into 45 μl of l-lactate (45 mM) supplemented with LarA₂₉, 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 d-lactate concentration was measured as described above.

For optimization of MgCl₂, MnCl₂, and P2TMN concentrations, the concentration of one component was varied with the other components held static. For the effect of nickel on the LarC reaction, nickel-devoid LarC was purified from cells expressed in the absence of added nickel. For the quantification of P2TMN concentration, the CTP concentration was 1 mM and the incubation time was 5 min.

**TLC**

We incubated a mixture of LarC (2.5 μM), P2TMN (50% v/v), MgCl₂ (10 mM), and CTP (5 μM) containing [α-³²P]CTP (1 μCi) in MES buffer (100 mM, pH 6) using a final volume of 10 μl. The reaction was stopped by the addition of 10 μl of 8 M urea, and 5 μl of the sample was spotted on a plate of PEI-cellulose F. The plate was washed twice with deionized water and then eluted with 1 N acetic acid/ethanol (80:20, v/v) containing 0.5 mM lithium chloride. The plate was dried, revealed with an Imaging Screen-K (Bio-Rad, Belgium), and read on a Pharus FX Plus (Bio-Rad, Belgium).

**Mass spectrometry of proteins**

10-μl aliquots were injected into the UPLC system coupled to the QToF MS. Separations were performed on a BetaBasic CN (10 × 1-mm, 5-μm particle size) column with an aqueous phase of 0.1% formic acid and using a gradient of increasing acetonitrile at 30 °C. The run time for each sample was 15 min. The masses were calculated from the ESI-MS spectrum using the advanced maximum entropy (MaxEnt)-based procedure included in the Micromass MassLynx software package.

**Protein crystallization**

Phases were solved with a selenomethionine-containing crystal obtained from selenomethionine-substituted LarC purified from *L. lactis*. Protein crystals were obtained by mixing 5 μl of ~10 mg·ml⁻¹ selenomethionine-substituted LarC (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) with 5 μl of reservoir solution. Sitting drop reservoir contained 100 μl of 10% PEG 8000, 100 mM Tris-HCl, pH 7.0, and 200 mM MgCl₂. The crystals grew within 2 weeks and were of space group P4₁. In total, over 25 different crystallization conditions, with crystal growth between 1 week and 3 months, were confirmed to be only LarC2 crystals. The crystals varied in space group with P4₁ (0.7 Å) (three copies), and I4 (six copies) observed. The P2₁ crystals were highly twinned, but using the twinlaw l-k-h, these crystals ultimately resulted in the highest quality datasets. Each asymmetric unit of the P2₁ data sets contains two copies of LarC2 chain A and B, which are not 100% identical with a r.m.s.d. of ~0.7 Å. For the apoprotein structure, 0.13 μl of ~13 mg·ml⁻¹ LarC (100 mM Tris-HCl, pH 7.5, 300 mM NaCl) was mixed with 0.13 μl of reservoir solution. The sitting drop reservoir contained 100 μl of 0.15 M DL-malic acid and 20% (w/v) PEG 3350. Crystals grew within a week. The crystal was soaked for 1 min in 25% PEG 400, 75% reservoir solution before freezing. For the CTP-Mn structure, 0.34 μl of ~8 mg·ml⁻¹ LarC (100 mM Tris-HCl, pH 7.5, 300 mM NaCl) were mixed with 0.17 μl of reservoir solution. The sitting drop

**LarC is a CTP-dependent cyclometallase**

We incubated a mixture of LarC (2.5 μM), P2TMN (50% v/v), MgCl₂ (10 mM), and CTP (5 μM) containing [α-³²P]CTP (1 μCi) in MES buffer (100 mM, pH 6) using a final volume of 10 μl. The reaction was stopped by the addition of 10 μl of 8 M urea, and 5 μl of the sample was spotted on a plate of PEI-cellulose F. The plate was washed twice with deionized water and then eluted with 1 N acetic acid/ethanol (80:20, v/v) containing 0.5 mM lithium chloride. The plate was dried, revealed with an Imaging Screen-K (Bio-Rad, Belgium), and read on a Pharus FX Plus (Bio-Rad, Belgium).

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reservoir contained 100 μl of 0.1 M MES monohydrate, pH 6.5, and 12% (w/v) PEG 20,000. Crystals grew within 2 months. The crystal was soaked in ~15 mM CTP, ~54 mM MnCl₂, and reservoir solution for 28 min and then for 2 min in 20% PEG 400, 80% reservoir solution. CTP binding was only observed in the presence of magnesium or manganese with no binding or only very weak binding when these salts were substituted for NaCl, NaBr, LiCl, KNO₃, or no salt. For the nickel structure, 0.5 μl of ~5.5 mg/ml LarC (100 mM Tris-HCl, pH 7.5, 300 mM NaCl) were mixed with 0.5 μl of reservoir solution. The hanging drop reservoir contained 100 μl of 0.2 M LiCl, 0.1 M Tris, pH 8.0, 20% (w/v) PEG 6000. Crystals grew within 2 weeks. The crystal was soaked in 16 mM NiCl₂ and reservoir solution for 30 min and then for 1 min in 20% PEG 400, 80% reservoir solution.

Diffraction data collection, structure determination, and analysis

X-ray diffraction data were collected at the Advanced Photon Source LS-CAT beamlines (21-ID-D, 21-ID-F, and 21-ID-G) and at beamline 23-ID-B GM/CA. Datasets were processed with xdsapp2.0 (26), and merging and scaling were done using aimless (27). The phase of a selenomethionine-substituted crystal was solved using single wavelength anomalous dispersion in Phenix (28) at 2.5 Å, and molecular replacement was used for all subsequent datasets. Modeling and refinement were conducted in COOT (29) and Phenix (28). Statistics for the datasets are listed in Table 2. Structure figures were created with UCSF Chimera (30) and LigPlot+ (31).

Table 2
Crystal statistics for the LarC2 structures

| LarC2 crystals | Apo | MnCTP | Nickel |
|----------------|-----|-------|--------|
| Data collection | LS-CAT 21-ID-D | GM/CA 23-ID-B | LS-CAT 21-ID-D |
| Beamline | 1,127 | 1,033 | 1,033 |
| Wavelength (Å) | 150 | 250 | 170 |
| Detector distance (mm) | 56 at 1° | 151 at 0.5° | 166 at 0.5° |
| Space group | P2₁ | P₂₁ | P₂₁ |
| Unit cell a, b, c (Å) | 97, 97, 97 | 97, 97, 97 | 97, 97, 97 |
| Resolution* (Å) | 43.33–2.03 (2.08–2.03) | 48.61–1.85 (1.89–1.85) | 48.32–1.81 (1.85–1.81) |
| Unique reflections | 19,861 (1440) | 26,499 (1623) | 27,744 (1665) |
| Redundancy* | 6.3 (6.2) | 8.4 (8.5) | 9.4 (9.4) |
| Completene** (%) | 99.7 (97.8) | 100.0 (99.4) | 99.9 (99.7) |
| I/σ(I) | 12.0 (2.2) | 11.7 (2.1) | 19.4 (2.2) |
| Rmerge, a,b,c | 0.095 (0.833) | 0.105 (0.974) | 0.073 (1.050) |
| Rmerge, a,b,c | 0.060 (0.540) | 0.057 (0.509) | 0.037 (0.527) |
| CC | 0.997 (0.553) | 0.997 (0.615) | 0.999 (0.537) |
| Data refinement | | | |
| Protein atoms | 2280 | 2260 | 2272 |
| CTP molecules | 2 | 2 | 2 |
| Manganese or nickel atoms | 6 | 4 | 4 |
| Chloride atoms | 3 | 3 | 3 |
| H₂O molecules | 98 | 140 | 148 |
| Rwork/Rmerge | 0.140/0.194 | 0.144/0.192 | 0.139/0.192 |
| B-factors (Å²) | 34.9 | 28.1 | 28.7 |
| Protein | 34.8 | 27.8 | 28.3 |
| CTP molecules | | | |
| Manganese or nickel atoms | 32.8 | 34.3 | |
| Chloride atoms | | | 41.4 |
| H₂O | | | 34.1 |
| Root mean square deviation in bond lengths (Å) | 0.009 | 0.011 | 0.009 |
| Root mean square deviation in bond angles (°) | 0.911 | 0.986 | 0.937 |
| Ramachandran plot (%) favored | 99.30 | 98.58 | 99.30 |
| Ramachandran plot (%) outliers | 0 | 0 | 0 |
| Rotamer outliers | 0 | 0 | 0 |
| PDB code | 6BWO | 6BWQ | 6BWR |
| *Highest resolution shell is shown in parentheses. |
| **Rmerge = Σ(|Fobs(i)|–|Fcalc(i)|)/Σ|Fobs(i)|, where F is the intensity of reflection. |
| Rmerge = Σ(|Fobs(i)|–|Fcalc(i)|)/Σ|Fobs(i)|, where N is the redundancy of the dataset. |
| dCC devices is the cross-validation R factor for the test set of reflections (10% of the total) omitted in model refinement. |

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References
1. Boer, J. L., Mulrooney, S. B., and Hausinger, R. P. (2014) Nickel-dependent metalloenzymes. Arch. Biochem. Biophys. 544, 142–152 CrossRef Medline
2. Desguin, B., Zhang, T., Soumillion, P., Hols, P., Hu, J., and Hausinger, R. P. (2015) A tethered niacin-derived pincer complex with a nickel-carbon bond in lactate racemase. Science 349, 66–69 CrossRef Medline
3. Desguin, B., Goffin, P., Viaene, E., Kleebezem, M., Martin-Diaconescu, V., Maroney, M. J., Declercq, J. P., Soumillion, P., and Hols, P. (2014)
Lactate racemase is a nickel-dependent enzyme activated by a widespread maturation system. *Nat. Commun.* **5**, 3615 CrossRef Medline

4. Desguin, B., Soumillon, P., Hols, P., and Hausinger, R. P. (2016) Nickel-pincer cofactor biosynthesis involves LarB-catalyzed pyridinium carboxylation and LarE-dependent sacrificial sulfur insertion. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 5596–5603 CrossRef Medline

5. Fellner, M., Desguin, B., Hausinger, R. P., and Hu, J. (2017) Structural insights into the catalytic mechanism of a sacrificial sulfur insertion of the N-type ATP pyrophosphatase family, LarE. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 9074–9079 CrossRef Medline

6. Albrecht, M. (2010) Cyclometalation using d-block transition metals: fundamental aspects and recent trends. *Chem. Rev.* **110**, 576–623 CrossRef Medline

7. Caliskan, N., Peske, F., and Rodnina, M. V. (2015) Changed in translation: mRNA recoding by –1 programmed ribosomal frameshifting. *Trends Biochem. Sci.* **40**, 265–274 CrossRef Medline

8. Archibald, F. (1986) Manganese: its acquisition by and function in the lactic acid bacteria. *Crit. Rev. Microbiol.* **13**, 63–109 CrossRef Medline

9. Holm, L., and Rosenström, P. (2010) Dali server: conservation mapping in 3D. *Nucleic Acids Res.* **38**, W545–W549 CrossRef Medline

10. Fujishiro, T., Ermler, U., and Shima, S. (2014) A possible iron delivery function of the dinuclear iron center of HcgD in [Fe]-hydrogenase cofactor biosynthesis. *FEBS Lett.* **588**, 2789–2793 CrossRef Medline

11. Fujishiro, T., Kahnt, J., Ermler, U., and Shima, S. (2015) Protein-pyridinol thioester precursor for biosynthesis of the organometallic acyl-iron ligand in [Fe]-hydrogenase cofactor. *Nat. Commun.* **6**, 6895 CrossRef Medline

12. Sigel, R. K., and Sigel, H. (2007) *Nickel and Its Surprising Impact in Nature*, pp. 109–180, John Wiley & Sons, Ltd., Chichester, UK

13. Leerof, D., Fodje, M. N., Alvarez León, R., Olsson, U., Hansson, A., Sigfridsson, E., Ryde, U., Hansson, M., and Al-Karadaghi, S. (2003) Metal binding to *Bacillus subtilis* ferrochelatase and interaction between metal sites. *J. Biol. Inorg. Chem.* **8**, 452–458 CrossRef Medline

14. Orzel, L., Fiedor, L., Wolak, M., Kania, A., van Eldik, R., and Stochel, G. (2008) Interplay between acetate ions, peripheral groups, and reactivity of the core nitrogens in transmetallation of tetrapyrroles. *Chemistry* **14**, 9419–9430 CrossRef Medline

15. Zheng, Z., Gonccarencio, A., and Berezovsky, I. N. (2016) Nucleotide binding database NDBB—a collection of sequence motifs with specific protein-ligand interactions. *Nucleic Acids Res.* **44**, D301–D307 CrossRef Medline

16. Kamberov, E. S., Atkinson, M. R., and Ninfa, A. J. (1995) The *Escherichia coli* PII signal transduction protein is activated upon binding 2-ketoglu-tarate and ATP. *J. Biol. Chem.* **270**, 17797–17807 CrossRef Medline

17. Palanca, C., and Rubio, V. (2017) Effects of T-loop modification on the PII-signalling protein: structure of uridylylated *Escherichia coli* GlnB bound to ATP. *Environ. Microbiol. Rep.* **9**, 290–299 CrossRef Medline

18. Zhang, G., Mills, D. A., and Block, D. E. (2009) Development of chemically defined media supporting high-cell-density growth of lactococci, enterococci, and streptococci. *Appl. Environ. Microbiol.* **75**, 1080–1087 CrossRef Medline

19. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

20. Holo, H., and Nes, I. F. (1989) High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl. Environ. Microbiol.* **55**, 3119–3123 Medline

21. Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* **16**, 6127–6145 CrossRef Medline

22. Zheng, L., Baumann, U., and Reymond, J. L. (2004) An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res.* **32**, e115 CrossRef Medline

23. Schmidt, T. G., and Skerra, A. (2007) The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. *Nat. Protoc.* **2**, 1528–1535 CrossRef Medline

24. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254 CrossRef Medline

25. McCaff, K. A., and Fierke, C. A. (2000) Colorimetric and fluorometric assays to quantitate micromolar concentrations of transition metals. *Anal. Chem.* **284**, 307–315 CrossRef Medline

26. Sparta, K. M., Krug, M., Heinemann, U., Mueller, U., and Weiss, M. S. (2016) Xdsapp2.0. *J. Appl. Crystallogr.* **49**, 1085–1092 CrossRef Medline

27. Evans, P. R., and Murshudov, G. N. (2013) How good are my data and what is the resolution? *Acta Crystallogr. D Biol. Crystallogr.* **69**, 1204–1214 CrossRef Medline

28. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 213–221 CrossRef Medline

29. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 486–501 CrossRef Medline

30. Petterson, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605–1612 CrossRef Medline

31. Laskowski, R. A., and Swindells, M. B. (2011) LigPlot+: multiple ligand–protein interaction diagrams for drug discovery. *J. Chem. Inf. Model.* **51**, 2778–2786 CrossRef Medline

32. Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2002) Multiple sequence alignment using ClustalW and ClustalX. *Curr. Protoc. Bioinformatics* **2002**, Chapter 2, Unit 2.3 CrossRef Medline

33. de Ruyter, P. G., Kuipers, O. P., and de Vos, W. M. (1998) Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl. Environ. Microbiol.* **62**, 3662–3667 Medline

34. Kuipers, O. P., de Ruyter, P. G., Kleerebezem, M., and de Vos, W. M. (1998) Quorum sensing-controlled gene expression in lactic acid bacteria. *J. Biotechnol.* **64**, 15–21 CrossRef