LarC cyclometallase

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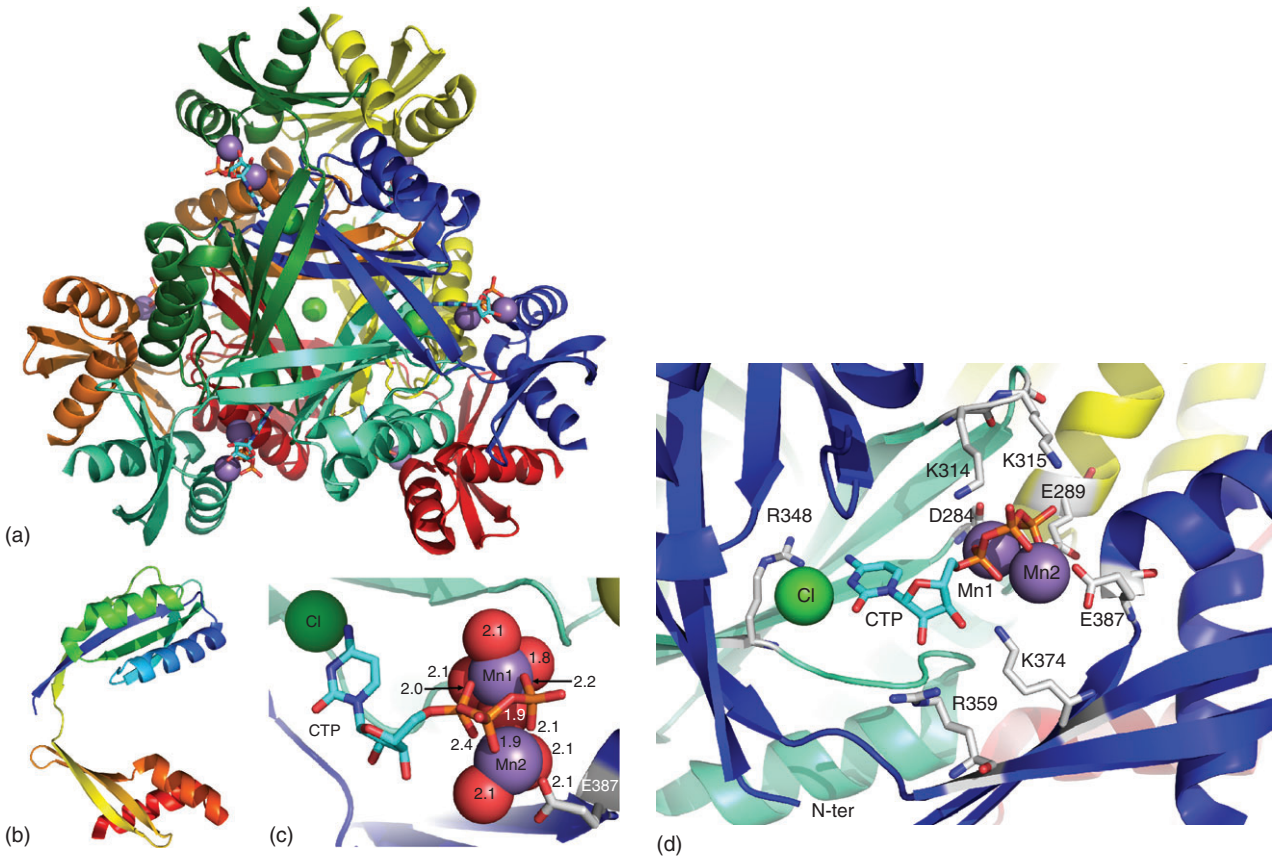
FUNCTIONAL CLASS

Enzyme, nickel: pyridinium-3,5-bisthiocarboxylic acid mononucleotide cyclometallase; catalyzes nickel-carbon and nickel–sulfur bond formation; proposed EC classification: EC 6.7.1

LarC catalyzes the last step of the biosynthesis of the nickel-pincer nucleotide (NPN) cofactor of lactate racemase (Figure 1) by CTP-dependent nickel insertion into the precursor pyridinium-3,5-bisthiocarboxylic acid mononucleotide (P2TMN).[2,3] In organometallic chemistry, the NPN product is defined as a nickelacycle and the metal insertion reaction is known as a cyclometalation.



3D Structure Ribbon representation of LarC2 chain A (PDB code: 6BWQ). (a) Hexamer of LarC2, each monomer is in a different color. CTP is in sticks representation. The Cl and the Mn ions are in sphere representation in green and violet, respectively. (b) Monomer of LarC2, spectrum of color from blue (N-terminus) to red (C-terminus). (c) metal binding site of LarC2. Same color code as is (a). In addition, the water molecules interacting with the Mn ions are represented by red spheres and residue E387 are in sticks representation with the (c) in white. The distances with the Mn ions are indicated in Å. (d) CTP binding site of LarC2. Same color code as is (a). In addition, the residues interacting with CTP are in sticks representation with the (c) in white. The 3D structure was generated with PyMol.[1]

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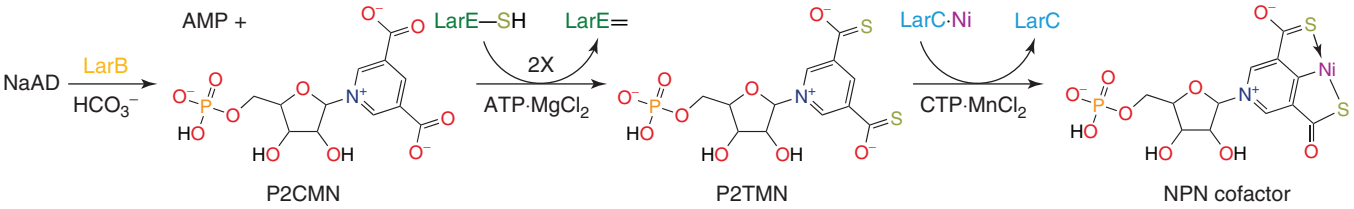

Figure 1 NPN biosynthetic pathway. NaAD, nicotinic acid adenine dinucleotide; P2CMN, pyridinium-3,5-biscarboxylic acid mononucleotide; P2TMN, pyridinium-3,5-bisthiocarboxylic acid mononucleotide.

OCCURENCE

The gene encoding LarC was first reported in the lactate racemization operon in *Lactobacillus plantarum*, a lactic acid bacterium which produced both lactate isomers by fermentation. Together with a carboxylase (LarB) and a sacrificial sulfur insertase (LarE), LarC participates in the synthesis of the NPN from nicotinic acid adenine dinucleotide (NaAD) (Figure 1). NPN is the cofactor of lactate racemase (LarA).[4]

A bioinformatics study of over 1000 genomes showed the presence of the NPN biosynthetic genes (LarBCE) in 24% of the studied bacterial and archaeal genomes and their occurrence in the same operon in 35% of these genomes. This suggests that LarBCE function, and thus LarC, is strictly linked to NPN biosynthesis. Furthermore, the NPN biosynthetic genes together with genes encoding homologs of LarA are only found in around 10% of the studied genomes, the biosynthetic genes were found without any gene encoding a LarA homolog in the remaining 14% of the genomes harboring LarBCE, suggesting that other enzymes may also use the NPN cofactor in order to catalyze yet undiscovered reactions.[5]

BIOLOGICAL FUNCTION

LarC's role in the cyclometalation of P2TMN (Figure 1) is its only known function. LarC does not bind its product, NPN, strongly, as suggested by the fact that the cofactor was observed in solution when LarC was thermally denatured in presence of thiols. LarC activity was observed *in vitro* in presence of Mn.CTP and is reduced by approximately 175-fold when magnesium is used instead of manganese. Stoichiometric hydrolysis of CTP to CMP is associated with the formation of NPN, yet the mechanistic origin of this coupling is mysterious. *In vitro*, LarC purified from cells grown in presence of nickel showed around 90% nickel content and could catalyze one single turnover, even in presence of added nickel and Mn.CTP.[3]

The NPN cofactor is used by LarA for the racemization of lactate and also by other LarA homologs for the isomerization of other α-hydroxyacids, such as 2-hydroxybutyrate, phenyllactate or D-mannonate.[6] Other reactions catalyzed by NPN-dependent enzymes, probably involving hydride chemistry, as the LarA reaction was shown to proceed by proton-coupled hydride transfer,[7] yet remain to be discovered.

AMINO ACID SEQUENCE INFORMATION

L. plantarum, 420 amino acids, UniProtKB – F9UST1, LarC sequence harbors a minus-1 Programmed Ribosomal Frameshift (PRF)[8] after residue 263, which occurs in 8% of LarC sequences.[5] The role of this frameshift is unknown, but it can be removed without affecting activity. The PRF site separates LarC in two domains: LarC1 (1 to 263) and LarC2 (264 to 420).

PROTEIN PRODUCTION, PURIFICATION, AND MOLECULAR CHARACTERIZATION

LarC was produced and expressed in *Lactococcus lactis*, a lactic acid bacterium without any lactate racemase gene. An artificial in-frame fusion between *larC1* and *larC2* ORFs resulted in the expression of only the full-length LarC protein, which did not affect Lar activity. *L. lactis* was grown in M17 broth supplemented with 0.5% glucose at 28 °C without agitation. $NiSO_4$ (1 mM) and chloramphenicol (10 mg l^{-1}) was added to cultures expressing LarC, nisin A was added during the early exponential phase (OD_{600} = 0.3–0.4) at a concentration of 1 μg l^{-1} and the cells were collected after 4 h.[5] For determining the apoprotein structure, *L. lactis* cells were grown following the above mentioned protocol, except that no $NiSO_4$ was added. For the selenomethionine containing crystal from *L. lactis*, we grew the cells in chemically defined medium containing selenomethionine instead of methionine.[3]

For the CTP-Mn and Ni bound structures, LarC was expressed in *Escherichia coli* Rosetta 2(DE3) cells. Rosetta 2 cells were grown with agitation at 37 °C in lysogenic broth containing ampicillin (100 mg l^{-1}) until exponential phase (OD_{600} = 0.5–0.6). The culture was transferred to 16 °C, induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside and grown for ~20 h with agitation.[3]

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LarC was fused to a StrepII-tag at its N-terminus and the recombinant protein was purified by affinity chromatography using a Strep-Tactin column. The buffers are devoid of ethylenediaminetetraacetic acid, which would otherwise strip LarC of its nickel. Purified LarC protein is monomeric in solution, according to gel filtration analysis (unpublished results). The mass of LarC after purification corresponds to the mass of its apoprotein (without its N-terminal methionine) and a smaller peak corresponds to LarC copurifying with Mn and CTP. All bound nickel is probably loss during the liquid chromatography step preceding mass spectrometry analysis.[3]

X-RAY STRUCTURE

Crystallization

Crystallization of full length LarC was attempted but the crystals obtained were always crystals of LarC2, generated by spontaneous cleavage of LarC into LarC1 and LarC2. Protein crystals were obtained by mixing 5 µl of ~10 mg ml^{-1} LarC (50 mM Tris(hydroxymethyl) aminomethane-HCl pH 7.5, 150 mM NaCl) with 5 µl of reservoir solution. Sitting drop reservoir contained 100 µl of 10% polyethylene glycol 8000, 100 mM Tris(hydroxymethyl)aminomethane-HCl pH 7.0, and 200 mM $MgCl_2$. In total, over 25 different crystallization conditions were confirmed to be only LarC2 crystals. LarC2 crystals grew between 1 week and 3 months and varied in space group with $P4_32\,2$ (one copy in the asymmetric unit), P231 (two copies) and I4 (six copies) observed. The $P2_13$ crystals were highly twinned, but using the twinlaw l,-k,h these crystals resulted in the highest quality datasets. Each asymmetric unit of the $P2_13$ datasets showed the unit cell parameters $a = b = c = 97.2$ Å and contains two copies of LarC2 (chain A and B) which are not 100% identical with an rmsd of ~0.7 Å. Comparison between apoprotein (PDB code: 6BWO) and CTP-Mn^{2+} bound (PDB code: 6BWQ) structures did not reveal any major structural changes upon CTP binding, with an rmsd of ~0.5 Å between corresponding chains.[3]

Overall structure of LarC2

LarC2 structure revealed a hexamer composed of a dimer of trimers (3D Structure (a)). Each LarC2 unit comprises two distinct domains (3D Structure (b)) – 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. Two domain 1 trimers stack vertically and share the threefold 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 (3D Structure (a)).[3]

Metal binding site

Crystal soaking with CTP-Mn revealed the CTP binding site containing one CTP and two Mn^{2+} ions. The first Mn ion (Mn1) is coordinated with two phosphates of CTP and four water molecules and the second Mn ion (Mn2) is coordinated with three phosphates of CTP, two water molecules, and Glu387 in chain A (3D Structure (c)). The distance between Mn2 and the α-phosphate oxygen is longer than for the other interactions (3D Structure (c)), and this interaction is lost in chain B. This loss of interaction hypothetically represents a conformational state relevant for catalysis from CTP to CMP.

Catalytic site

CTP-Mn is sandwiched by domain 1 and domain 2 of one subunit, with the participation of additional residues from the domain 1 of two other LarC2 subunits, one from the same trimer, and one from another trimer (3D Structure (d)). Site-directed mutagenesis of conserved residues of LarC2 involved in CTP binding yielded the variants K314A, R348A, R359A, K374A, and E387A (3D Structure (d)), which showed only 1% residual LarC activity, suggesting that these residues are important for catalysis. Moreover, a decreased binding affinity for CTP was shown for two variants, D284A and K315A (3D Structure (d)). These data confirmed that the observed binding site is the native CTP binding site and show that CTP binding and hydrolysis is required for LarC activity.[3]

A chloride ion is observed between residue Arg348 and the cytosine base of CTP (3D Structure (d)), which is probably involved in the recognition specificity for CTP, as other triphosphates were not recognized by LarC.[3]

LARC1 STRUCTURAL MODEL

As LarC1 and the full-length protein are less stable, precipitate with time and did not form crystals,[3] the structure of LarC1 was modelled *de novo* using evolutionary information (Figure 2).[9] The structure model is composed of nine alpha helices and seven beta strands arranged in an original fold (β1α1α2β2β3α3-6β4-6α7α8β7α9, Figure 2(a)). The structure model shows many loops without secondary structure (Figure 2(b)), which is probably due to the inaccuracy of the modelling. The comparison with the whole PDB database using the VAST algorithm[10] did not yield any structural homolog, suggesting that LarC1 shows a new fold.

The most conserved residues of LarC1 are mostly on one face of the protein (Figure 2(b)), which suggests that they delimitate the catalytic site. Several acidic residues of LarC1 were mutated to alanine and yielded the variants E124A, E139A and D144A (Figure 2(b)), which showed

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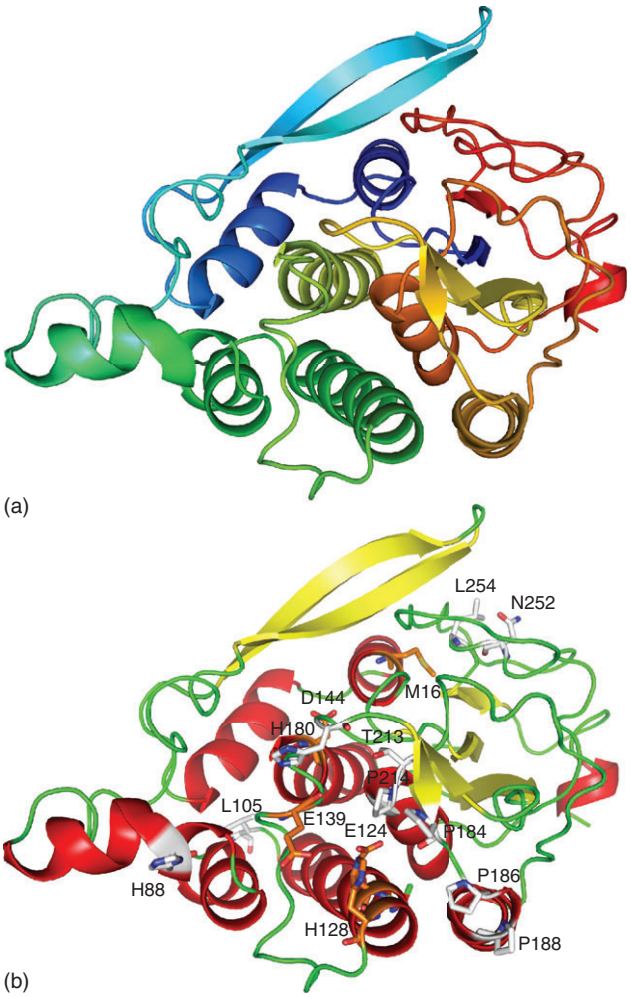

Figure 2 **Ribbon representation of LarC1 structural model.** (a) Monomer of LarC1, spectrum of color from blue (N-terminus) to red (C-terminus). (b) Monomer of LarC1 with the α-helices in red, β-strands in yellow, and loops in green. The most conserved residues are in sticks representation. The residues with the C in orange are the residues which abolish activity when mutated. The Figure was generated with PyMol.[1]

complete loss of activity.[3] Two other variants also showed loss of activity: M16A and H128A (Figure 2(b)) (unpublished results), yet their role in catalysis is unknown. These results show that the LarC1 part of LarC is responsible for the cyclometalation reaction.

CATALYTIC MECHANISM

LarC inserts nickel into P2TMN forming a C—Ni and a S—Ni bond. Ni becomes then part of 5-membered ring, a metallacycle, and LarC is therefore called a cyclometallase. This reaction requires the hydrolysis of CTP, probably in order to catalyze a conformational change favoring the cyclometalation reaction. The binding of nickel into NPN is higher in energy than the binding of nickel into LarC and thus the energy released from CTP hydrolysis is probably required for binding of nickel to P2TMN.

It must be noted that the N-terminus of LarC2, which should be nearby the C-terminus of LarC1, lies at the top of the LarC2 trimer, close to the CTP (3D Structure (d)). This suggests that the CTP lies close to LarC1, and that some interaction may take place between CTP and LarC1. The structure of the complete LarC will certainly be helpful for determining how the LarC1 and LarC2 domains interact.

Several acidic residues where shown to be indispensable for LarC activity. These residues could participate into the cyclometalation reaction by abstracting the proton at the C4 position of the P2TMN pyridinium ring and replacing it by nickel via a six-membered transition state, similarly to acetate effect observed in the cyclometalation by palladium acetate.[11]

The single turnover LarC reaction was demonstrated *in vitro*, when the enzyme was preloaded with Ni *in vivo*. This may reflect the fact that LarC do not need to catalyze multiple turnovers *in vivo*. Indeed, LarC acts downstream of LarE, which is itself a single turnover enzyme.[2] Moreover, this would prevent the consumption of too much NaAD, which is required by the cell for NAD biosynthesis. Alternatively, it is also possible that an unknown metallochaperone is required for loading LarC with nickel, which would be necessary for multiple turnovers. Nevertheless, this hypothesis requires that the chaperone is not specific, as LarA was activated *in vivo* when heterologously expressed in *L. lactis*,[5] which is devoid of any nickel-dependent enzyme.

The only enzyme which possibly catalyzes a similar reaction to LarC is HcgD, which is proposed for catalyzing the insertion of iron into the iron-guanylylpyridinol (FeGP) cofactor of [Fe]-hydrogenase.[12] HcgD is homolog to the ubiquitous Nif3-like protein family, and is thus not related to LarC.

Undoubtedly, LarC is a unique enzyme which still holds a lot of surprises.

ACKNOWLEDGEMENT

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RELATED ARTICLE

Lactate Racemase

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