Abstract

The leptospira cbiC encodes the enzyme catalyzing the methyl rearrangement reaction of the cobalamin biosynthesis pathway. The protein has been cloned and overexpressed as a His-tagged recombinant protein in Escherichia coli. The crystal structures have been solved in two crystal forms (P4_{2}2_{1}2 and P3_{2}12) diffracting to 3.0 and 2.3 Å resolution, respectively. The structures are similar to the precorrin-8x methyl mutase (CobH), an enzyme of the aerobic pathway to vitamin B12.

Keywords: Leptospira interrogans; Leptospirosis; Vitamin B12; Precorrin isomerase; Crystal structure

1. Introduction

Cobalamin (vitamin B12) is one of the most structurally complex protein cofactors of the nature. Most prokaryotic organisms as well as animals (including humans) and protists have enzymes that require cobalamin as cofactor, whereas plants and fungi are thought not to use it. Among the cobalamin-utilizing organisms, only some bacterial and archaeal species are able to synthesize cobalamin de novo (Rodionov et al., 2003). Studies over the past decade have demonstrated that there are at least two quite distinct routes for cobalamin synthesis, representing oxygent-dependent (aerobic) and oxygen-independent (anaerobic) pathways (Scott and Roessner, 2002). The two pathways differ in their timing of cobalt insertion and the requirement for molecular oxygen (Raux et al., 1999; Schubert et al., 1999).

All the intermediates between uro’gen III and cobinamide are known for the aerobic pathway, in contrast, few intermediates on the anaerobic pathway are known. The genes encoding the aerobic pathway enzymes are prefixed Cob, whereas the genes encoding the anaerobic pathway are prefixed Cbi (Roper et al., 2000). Many of the aerobic, Cob, enzymes share a high degree of similarity with the anaerobic, Cbi, suggesting that although independent, the two pathways are broadly similar (Roth et al., 1993).

Leptospira interrogans is most common in tropical areas and infects animals. Infection can cause mild symptoms or more serious disease, known as leptospirosis. Here, we report the crystal structure of the L. interrogans putative CbiC (CbiC_LEPIN) with two different crystal forms, P4_{2}2_{1}2 and P3_{2}12. CbiC, a cobalt-precorrin 8 isomerase, catalyzes cobalt-precorrin 8 to cobyricin acid by methyl rearrangement in anaerobic pathway (Roessner et al., 1992; Roth et al., 1993). The specific homology of CbiC in aerobic pathway is CobH, which shows high similarity to CbiC despite the divergence between the two pathways. Structure analysis suggests that CbiC_LEPIN are highly similar with CbiC/CobH from other sources in fold and has small differences in surrounding of cobalt-precorrin 8 binding. The CbiC_LEPIN structure will provide clues for understanding the function of CbiC/CobH family in the biosynthesis pathway.
2. Materials and methods

2.1. Protein expression and purification

The gene *cbiC* from *L. interrogans* was cloned into a pET-22b (Novagen) expression plasmid to yield pET-His-cbiC. The plasmid was transformed into *Escherichia coli* strain BL21 (DE3) for protein expression. Five milliliter aliquots of an overnight culture were subcultured into 400 ml fresh Luria–Bertani medium (10 g Bacto tryptone, 5 g yeast extract, and 10 g NaCl per liter of solution) containing ampicillin (50 μg/ml) and allowed to grow at 310 K until OD$_{600}$ = 0.6. At this stage isopropyl-β-D-thiogalactoside was added to a final concentration of 0.2 mM and the cells were grown for a further 20 h at 289 K. Then cells were harvested by centrifugation at 8000 rpm for 10 min. The cell pellet was resuspended in binding buffer (20 mM Tris/HCl, pH 7.5, 0.2 M NaCl) and sonicated using a JY92-II sonic dismembrator. The solution was centrifuged at 277 K. Drops containing equal volumes (1 l) of protein (15 mg/ml) and reservoir solution were equilibrated against 0.1 M Tris, pH 8.5. More suitable crystals for diffraction experiment were obtained in [15%(v/v) ethanol and 0.1 M Tris, pH 8.0] after optimizing the second condition. Preliminary diffraction data were collected on a MAR Research image-plate system with a local X-ray source at 100 K with 1.5418 Å wavelength. All data were processed and scaled with the DENZO and SCALEPACK (Otwinowski and Minor, 1997). The processing statistics of the different crystal forms are summarized in Table 1.

2.2. Crystallization and preliminary data collection

Preliminary crystallization conditions were screened using Crystal Screen I and II (Hampton Research, CA, USA) with the hanging-drop vapour-diffusion technique at 277 K. Drops containing equal volumes (1 μl) of protein (15 mg/ml) and reservoir solution were equilibrated against 400 μl reservoir solution. Two conditions ([10%(v/v) PEG6000, 2.0 M sodium chloride] and [20%(v/v) ethanol, 0.1 M Tris, pH 8.5]) produced CbiC_LEPIN protein crystals with *P*4$_2$12 and *P*3$_2$1 crystal forms, respectively. More suitable crystals for diffraction experiments were obtained in [15%(v/v) ethanol and 0.1 M Tris, pH 8.0] after optimizing the second condition. Preliminary diffraction data were collected on a MAR Research image-plate system with a local X-ray source at 100 K with 1.5418 Å wavelength. All data were processed and scaled with the DENZO and SCALEPACK (Otwinowski and Minor, 1997). The processing statistics of the different crystal forms are summarized in Table 1.

2.3. Structure determination

The crystal structure of CbiC_LEPIN with the *P*4$_2$12 crystal form was solved using Molrep (Vagin and Teplyakov, 1997). The monomer structure of Precorrin-8x Methyl Mutase from *Thermus thermophilus* (PDB code: 1V9C) was used as a search model for the molecular replacement. The model consisting of a dimer was refined in 20–3.0 Å resolution range by using CNS (Brunger et al., 1998) with maximum-likelihood amplitude targets and manually adjusted and rebuilt of the model using the program O (Jones et al., 1991) with 2*F$_o$ – *F$_c$ and *F$_o$ – *F$_c$ electron-density maps as references. Water molecules were added to the model and individual atomic B factor was refined at latter stage of refinement. NCS restraints were applied through all stages of refinement. The final *R*$_{cryst}$ and *R*$_{free}$ factors are 20.4 and 25.1%, respectively. A monomer structure of this refined model was used as a search model to find solutions for the *P*3$_2$12 crystal form with one molecule per asymmetric unit. The refinement strategy...
of the P321 structure is similar to the P4222 structure with the final $R_{\text{cryst}}$ and $R_{\text{free}}$ factors 22.4 and 28.4%, respectively, in 50–2.3 Å resolution range. The stereochemical qualities of the final models of two crystal forms were checked by PROCHECK (Laskowski et al., 1993), and the final refinement statistics and geometry are listed in Table 1. The relatively high $R_{\text{free}}$ factor value of the P321 structure is mainly due to existing obvious ice rings during data collection.

3. Results and discussion

3.1. Protein preparation and crystallization

The recombinant protein of CbiC_LEPIN was overexpressed at 310 K at first, but the protein was insoluble. Various expression conditions were tried to increase solubility of the protein. Finally, the recombinant protein was expressed solubly in higher yield under the following conditions: 289 K, 0.2 mM IPTG, 20 h induction in strain BL21 (DE3). The protein was purified using the nickel-affinity column and the sample was monitored by SDS–PAGE after purification and concentration. The purified His-tagged putative CbiC protein was used for crystallization. After an initial screen, two crystal forms of the protein were observed. The polyhedral crystal form was only obtained a 7–8 Å resolution data at first. After optimization and using a cryoprotectant solution (10% glycerol) before flash freezing the crystal to liquid nitrogen, the resolution was improved greatly. The maximum resolution was 2.3 Å and the space group was hexagonal P321, with unit-cell parameters $a = b = 55.89$ Å, $c = 142.55$ Å, $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$. The rod-shaped crystal form was obtained a 3.0 Å resolution data set. The space group was tetragonal P4222, with unit-cell parameters $a = b = 113.08$ Å, $c = 114.50$ Å, $\alpha = \beta = \gamma = 90^\circ$.

3.2. Structure overview

The final models contain 215 residues in one molecule of P321 crystal form and 434 residues (217 residues each molecule) in two molecules (forming a dimer) of P4222. N-terminal residues, Met1–Gln5 (in P321) and Met1–Asp3 (in P4222), the C-terminal residues, Glu222–Arg223 (in P321) and Gly221–Arg223 (in P4222), as well as the His_tag were not visible in electron-density maps. The three monomer structures (one in P321 and two in P4222) are almost identical with the average root-mean-square deviation (rmsd) about 0.46 Å (for 215 Cα between P321 and any one of P4222 monomer structures) or about 0.26 Å (for 217 Cα between two P4222 monomer structures). The CbiC_LEPIN structure contains 11 α-helices and a six-stranded mixed β-sheet in which the five strands ($\beta_2$–$\beta_6$) are parallel and the edge strand $\beta_1$ run anti-parallel to $\beta_6$. The β-sheet is surrounded by four α-helices in one side and seven in another (Fig. 1A).

CbiC_LEPIN forms a dimer in both of the two crystal forms with a crystallographic (in P321 crystal) or a non-crystallographic (in P4222 crystal) 2-fold symmetry between the two monomers (Fig. 1B). The dimer is formed by both hydrophobic and hydrophilic interactions (including some salt bridges.) The dimerization form of CbiC_LEPIN is in agreement with other CbiC/CobH structures. It suggests that dimerization is necessary for having biological function in CbiC/CobH family. Two identical cavities formed by two CbiC/CobH molecules in a dimer are also essential for their substrates binding (Shipman et al., 2001).

3.3. Comparison with other CbiC/CobH

Sequence and structural alignment reveal that CbiC_LEPIN is fairly similar to other CbiC/CobH (Figs. 2A and B). Farther structural analyses show that CbiC/CobH

![Fig. 1. The ribbons diagram of the monomer and the dimer structure of CbiC_LEPIN. (A) The protein is color-coded by secondary structure types. (B) The two monomers are related by a crystallographic (in P321 structure) or a non-crystallographic (in P4222 structure) 2-fold screw axis where is showed as “2.” This figure and Fig. 2B were prepared with RIBBONS (Carson, 1997).](image-url)
family members also have high similarity in shape of the two identical cavities (Fig. 2B) and centralized distributions of conserved residues on and around the surface of the cavities (Figs. 2A and C). Recently, the complex structure of *Pseudomonas denitrificans* CobH (CobH_PSEDE) binding with hydrogenobyrinic acid (HBA), which is the product of the reaction catalyzed by CobH, has been solved (Shipman et al., 2001). This structure shows that the cavities supply the binding surface for the substrates. And comparing with the complex structure, conformation of residues on the cavities of the CobH_PSEDE native structure does not show obvious changes. Thus, although without substrates binding, the similarity of the cavities in CbiC/CobH family suggests that cobalt-precorrin 8,
which is the substrate of CbiC, is highly similar to the substrate of CobH, precorrin 8.

Although the residues that composed the cavities are mostly conserved, there are still some non-conserved residues distribute on the surface of the cavities. These non-conserved residues might respond for different substrates (cobalt-precorrin 8 or precorrin 8) binding. In addition, N-terminal helices functioning to cap the active site of cavity in CbiC/CobH structures are probably disordered in a precorrin-8x methylmutase related protein from Thermoplasma acidophilum (Fig. 2B), which is a putative CbiC/CobH family member.

There is little biochemical characterization of CbiC_LE-PIN and consequently the information that can be derived from the structure reported here is limited. Whether binding mode of cobalt-precorrin 8 is highly similar to that of precorrin 8 and what the cobalt-precorrin 8 is, need to be further investigated.

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References


