Crystal structure of cyclic nucleotide-binding-like protein from *Brucella abortus*

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ABSTRACT

The cyclic nucleotide-binding (CNB)-like protein (CNB-L) from *Brucella abortus* shares sequence homology with CNB domain-containing proteins. We determined the crystal structure of CNB-L at 2.0 Å resolution in the absence of its C-terminal helix and nucleotide. The 3D structure of CNB-L is in a two-fold symmetric form. Each protomer shows high structure similarity to that of cGMP-binding domain-containing proteins, and likely mimics their nucleotide-free conformation. A key residue, Glu17, mediates the dimerization and prevents binding of cNMP to the canonical ligand-pocket. The structurally observed dimer of CNB-L is stable in solution, and thus is likely to be biologically relevant.

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1. Introduction

Brucella abortus is a Gram-negative bacterium found in cattle populations [1] and is the cause of brucellosis which often leads to the premature abortion of cattle fetus. Its potential infection to humans is a serious health concern in ranch areas [2]. Therefore, significant efforts have been made for the development of new therapeutics against *B. abortus*. Cyclic nucleotide monophosphate (cNMP)-binding proteins are one of such potential drug targets due to their essential roles in many cellular functions [3,4]. Some cNMP analogs have been used to study the functions of cyclic nucleotide binding (CNB) proteins and have showed promising application prospect [5–7].

The CNB-like (CNB-L) protein (GenBank ID: AE017223.1) identified from *B. abortus* is a putative cNMP-binding protein for its conserved CNB domain. In both prokaryotes and eukaryotes, CNB domains are the critical components of cellular machineries that regulate multiple inner cellular processes [8]. For examples, these protein domains have been found in several protein families, such as cAMP receptor proteins (CRP), cAMP- and cGMP-dependent protein kinases (*e.g.* PKA and PKG) [9], and the ether-a-go-go (EAG) channel [10]. CNB domains are responsible for the binding of 3′, 5′-cyclic adenosine monophosphate (cAMP) and 3′, 5′-cyclic guanosine monophosphate (cGMP), two universal secondary messengers. Generally, a CNB domain consists of approximately 120 amino acid residues, forming an eight-stranded antiparallel β-barrel which accommodates the cyclic nucleotide molecule and is flanked by a variable number of α-helices [11]. A signature motif referred to as the phosphate binding cassette (PBC) is situated between β6 and β7 strands [12]. Upon ligand binding, the flexible α-helix subdomain usually undergoes a dramatic structural transformation to deliver the allosteric signal [13].

In the current work, we report the crystal structure of *B. abortus* CNB-L protein. It is in a homo-dimeric form, resembling the nucleotide-free form of PKG CNB domains. However, the nucleotide binding site is blocked by a residue in the dimer interface, suggesting that *B. abortus* CNB-L dimer does not function as a signaling molecule.

2. Material and methods

2.1. Cloning

The target gene of CNB-L was amplified from *B. abortus* bv. 1 strain 9-941 by the polymerase chain reaction (PCR) with forward primer 5′-CGGAA TTCAT GGCGC TAG-3′ and reverse primer 5′-CCGCT CGAGT CAGTC GCGGT TT-3′. The PCR product was digested with *Eco*RI and *Xho*I and subsequently cloned into the expression vector pGEX-6P-1 (Qiagen, USA). The construct was verified by DNA sequencing and then transformed into *E. coli* strain BL21 (DE3) cell for protein expression.

2.2. Overexpression and purification

The seleno-methionine (SeMet) protein was overexpressed in *E. coli* BL21 (DE3) strain at 16 °C for 12 h, containing 100-μg/ml ampicillin. The cell was induced with 0.5 mM IPTG when OD600 reached 0.8 and was grown further for 18 h at 16 °C. The cells were harvested by centrifugation at 4000 g for 15 min. Then, the cell pellets were resuspended in buffer A (20 mM Tris–HCl (pH 8.0), 400 mM NaCl, and 10% glycerol) and lysed using high pressure breaking method (EF-C3, from Avestin) at 15,000 psi. The lysate was centrifuged at 22,000 g for 30 min at 4 °C to remove unbroken cells and debris, and the supernatant was collected. A slurry of Gluta-thione Sepharose 4B (GE Healthcare) pre-equilibrated with buffer A was added into the supernatant and agitated by end-over-end rotation for 1 h at 4 °C. Then the mixture was transferred to a 10 ml column and washed extensively with buffer A to remove contaminant proteins. To remove the GST tag, the slurry was incubated with Prescission protease in buffer A at an enzyme:-protein ratio of 1:20 (w/w) for 4 h at 4 °C. The flow-through containing the target protein was collected and subjected to size-exclusion chromatography (SEC) (Superdex 200, GE Healthcare) in buffer B (20 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 5% glycerol). Peak fractions were collected (monitored by 280 nm absorption) and concentrated to ~20 mg/ml for crystallization. Purity of the protein sample was estimated to be 98% by using SDS-PAGE.

2.3. Crystallization

Initial crystallization experiments were carried out using commercial crystallization screening kits from Hampton Research (US) and sitting drop method. Crystals appeared in 4–6 weeks under multiple conditions. Hanging-drop vapor-diffusion method was further used to optimize crystallization conditions by mixing 1 μl protein solution with 1 μl reservoir solution and equilibrating against 200-μl reservoir solution at 20 °C. The crystals used for data collection were grown in 0.2 M potassium sodium tartrate (pH 7.4), 0.1 M KCl, and 20% (w/v) polyethylene glycol 3350. Crystals grew to full size (150 × 150 × 200 μm³) in 30–40 d.

2.4. Data collection and structure determination

The crystals were cryo-protected with 20% (v/v) glycerol in reservoir solution and flash-cooled in a liquid nitrogen stream at 100 K. The single-wavelength anomalous diffraction (SAD) data were collected at beamline BL17U of Shanghai Synchrotron Radiation Facility (SSRF) with the ADSC Quantum 315r CCD detector. X-ray diffraction data were collected from single crystals over a range of 360° with an oscillation angle of 1° at the wavelength of 0.9762 Å. The data were processed and scaled with the program package of HKL2000 [14]. The initial phase calculation, density modification, and partial model building were carried out with Phenix.autosolve [15]. The structure was completed manually with COOT [16], and the refinement was carried out with Phenix.refine [15]. Statistics of data collection and refinement are summarized in Table 1. All the structure figures were prepared with Pymol [17].

2.5. Truncation and site-directed mutagenesis

A plasmid to express a truncated form of CNB-L (tCNB-L) was constructed by removing nucleotides coding for the last 31 amino acid residues from the C-terminus of the full-length CNB-L (flCNB-L) [18]. Then, based on tCNB-L a site-directed mutation of Glu17 into tryptophan (tCNB-L/E17W) was constructed. Both tCNB-L and tCNB-L/E17W were over-expressed and purified with the same method as flCNB-L. The purified protein samples were analyzed using SEC.

2.6. Isothermal titration calorimetry

ITC experiments were performed at 25 °C using the iTC200 (MicroCal, Northampton, MA, USA). Protein and cyclic nucleotides were prepared in the same buffer, i.e. ITC buffer containing 10 mM Tris (pH 8.0) and 100 mM NaCl. The protein was placed in the sample cell at a concentration of 0.4 mM in the ITC buffer. Cyclic nucleotides were placed in the injection syringe at a concentration of 10 mM. The injection volume was 2 μl and the interval between injections was 2 min. All data were corrected using the heat changes arising from injection of the cyclic nucleotides into the buffer. Data were processed using the Origin software with a manufacturer-supplied custom-add-on ITC sub-routine. The reported results were repeated in at least duplicate.

3. Results

3.1. Overall structure of CNB-L

The structure of CNB-L was resolved at 2.0 Å resolution in space

Table 1
Statistics of data collection and refinement.

Data	Se (peak)
Data Processing	
Wavelength (Å)	0.9762
Space group	I222
Cell dimensions *a*, *b* and *c* (Å)	56.1, 82.9, 103.9
Resolution (Å)	34.6–2.0 (2.1–2.0)[a]
Completeness (%)	98.8 (100)
R_{merge} (%)[b]	8.5 (38.1)
I/σ(I)	45.1 (3.0)
Unique reflections	16,773 (1519)
Redundancy	20 (15)
Refinement	
Resolution (Å)	24.6–2.0
No. of reflections (test)	16,665 (833)
R_{work}/R_{free} (%)[c]	18.5/23.3
Number of atoms	1798
Water	171
Average *B* factor (Å²)	28
Water	44
Root-mean-square-deviation	
Bond lengths (Å)	0.007
Bond angles (°)	0.73
Ramachandran plot (%)[d]	
Favored region	99.13
Allowed region	0.87

[a] Values in parentheses are for the highest resolution shell.

[b] $R_{merge} = \sum_{hkl} \sum_i |I_i - (I)|/|(I)|$, where I_i is the intensity for the *i*-th measurement of an equivalent reflection with indices h, k and l.

[c] $R = \sum |F_o - F_c|/\sum |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

[d] Calculated using MolProbity.