Solution structure of the *Mesorhizobium loti* K1 channel cyclic nucleotide-binding domain in complex with cAMP

Sven Schünke^{1,2}, Matthias Stoldt^{1,2}, Kerstin Novak³, U. Benjamin Kaupp^{3,4} & Dieter Willbold^{1,2+}

¹Institut für Strukturbiologie und Biophysik, Strukturbiochemie (ISB-3), Forschungszentrum Jülich, Jülich, Germany, ²Institut für Physikalische Biologie, Heinrich-Heine-Universität, Düsseldorf, Germany, ³Institut für Strukturbiologie und Biophysik, Zelluläre Biophysik (ISB-1), Forschungszentrum Jülich, Jülich, Germany, and ⁴Center for Advanced European Studies and Research (caesar), Molekulare Neurosensorik, Bonn, Germany

Cyclic nucleotide-sensitive ion channels, known as HCN and CNG channels, are crucial in neuronal excitability and signal transduction of sensory cells. HCN and CNG channels are activated by binding of cyclic nucleotides to their intracellular cyclic nucleotide-binding domain (CNBD). However, the mechanism by which the binding of cyclic nucleotides opens these channels is not well understood. Here, we report the solution structure of the isolated CNBD of a cyclic nucleotide-sensitive K⁺ channel from *Mesorhizobium loti*. The protein consists of a wide anti-parallel β -roll topped by a helical bundle comprising five α -helices and a short 3₁₀-helix. In contrast to the dimeric arrangement ('dimer-of-dimers') in the crystal structure, the solution structure clearly shows a monomeric fold. The monomeric structure of the CNBD supports the hypothesis that the CNBDs transmit the binding signal to the channel pore independently of each other.

Keywords: NMR solution structure; MloK1; ion channels; HCN; CNG
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INTRODUCTION

Ion channels activated by cyclic nucleotides are crucial in neuronal excitability and the signalling of visual and olfactory neurons. They belong to two sub-families: cyclic nucleotide-gated (CNG) channels, and hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels (Kaupp & Seifert, 2001, 2002;

Robinson & Siegelbaum, 2003). Both types of channel share a carboxy-terminal cyclic nucleotide-binding domain (CNBD). HCN channels are activated by hyperpolarization and their activity is modulated by cyclic nucleotides. In contrast, CNG channels are voltage independent and require cyclic nucleotides to open them; binding of cyclic nucleotides promotes the opening of the channel. Thus, a conformational change in the CNBD is likely to be propagated to the pore.

Recently, a prokaryotic cyclic nucleotide-sensitive K⁺-channel, designated MloK1, has been identified in *Mesorhizobium loti* (Nimigean *et al*, 2004; Nimigean & Pagel, 2007). MloK1 contains six transmembrane domains (S1–S6), a 'GYG' signature sequence for K⁺ selectivity and a conserved CNBD connected through a short, C-linker to S6 (Heginbotham *et al*, 1994; Nimigean *et al*, 2004; Fig 1A). The longer C-linker (~80 residues) of mammalian CNG channels is important for relaying the binding signal to the channel gate (Gordon & Zagotta, 1995; Zong *et al*, 1998; Paoletti *et al*, 1999; Wang *et al*, 2001; Johnson & Zagotta, 2001; Zhou & Siegelbaum, 2007). Crystal structures of mammalian HCN channel CNBDs revealed that neighbouring C-linkers contribute virtually all contacts between subunits in the tetrameric protein (Zagotta *et al*, 2003; Flynn *et al*, 2007). The crystal structure of the isolated CNBD of MloK1 suggested that subunits are organized as dimers. The dimer interface formed by the short linker has been proposed to be involved in channel gating (Clayton *et al*, 2004). However, an electron microscopy structure of the complete channel reveals a fourfold symmetry of subunit arrangement (Chiu *et al*, 2007). The CNBDs appear as independent domains separated by discrete gaps, suggesting that CNBDs are not interacting with each other. Furthermore, the MloK1 channel and the isolated CNBD bind to cyclic AMP (cAMP) with similar affinity in a non-cooperative manner (Cukkeman *et al*, 2007). High-resolution studies of the MloK1 transmembrane regions confirm the fourfold symmetry and reveal a flower-like arrangement of the four subunits, with the pore region at the centre and the S1–S4 domains at the periphery (Clayton *et al*, 2008). Here, we study the solution structure of the

¹Institut für Strukturbiologie und Biophysik, Strukturbiochemie (ISB-3), Forschungszentrum Jülich, 52425 Jülich, Germany

²Institut für Physikalische Biologie, Heinrich-Heine-Universität, 40225 Düsseldorf, Germany

³Institut für Strukturbiologie und Biophysik, Zelluläre Biophysik (ISB-1), Forschungszentrum Jülich, 52425 Jülich, Germany

⁴Center for Advanced European Studies and Research (caesar), Molekulare Neurosensorik, 53175 Bonn, Germany

+Corresponding author. Tel: +49 2461 612100; Fax: +49 2461 612023; E-mail: dieter.willbold@uni-duesseldorf.de

Fig 1 | Solution structure of the isolated cyclic nucleotide-binding domain. (A) Subunit topology and assembly of the full-length MloK1 cyclic nucleotide-gated K^+ channel. MloK1 consists of four subunits; each subunit encompasses six transmembrane segments S1–S6 (yellow) and an intracellular cyclic nucleotide-binding domain (CNBD; shown in ribbon representation). (B) Superposition of the backbone traces including all cyclic AMP (cAMP) atoms of the family of 15 nuclear magnetic resonance structures with the lowest CYANA target function. Backbone atoms of the amino- and carboxy-terminal ends (residues Q216–V218 and A351–A355) are not shown and were not used for least-square superposition of the structures. (C) Ribbon representation of the CNBD structure with the lowest target function. The cAMP ligand is shown as a stick model; secondary structure elements are labelled.

the MloK1 CNBD (supplementary Fig S1 online). From the ^{15}N longitudinal and transverse relaxation rates of $1.03 \pm 0.05 \text{ s}^{-1}$ and $14.24 \pm 0.10 \text{ s}^{-1}$, respectively, for residues with $[^1\text{H}]^{15}\text{N}$ nuclear Overhauser effect (NOE) values greater than 0.65, a rotational correlation time of 8.5 ns at 298 K for isotropic rotational diffusion was derived. This value is consistent with the MloK1 CNBD being present as a monomer in solution and is clearly below the value expected for a dimer. The linewidths in the $(^1\text{H}-^{15}\text{N})$ -heteronuclear single quantum coherence (HSQC) spectra and transverse relaxation rates suggest that the protein exists in a single conformation, consistent with size-exclusion chromatography, which shows that the protein is monomeric (Clayton *et al*, 2004; Cukkemane *et al*, 2007).

The CNBD of MloK1 shows a high affinity for cAMP ($K_D = 107 \text{ nM}$; Cukkemane *et al*, 2007). This agrees well with the observation that the CNBD is saturated with $[U-^{15}\text{N}, ^{13}\text{C}]$ -labelled cAMP because resonance signals arising from cAMP-free CNBD were absent. Nearly all of the cAMP hydrogens were assigned using two- and three-dimensional heteronuclear through-bond correlation and heteronuclear-edited nuclear Overhauser enhancement spectroscopy (NOESY) experiments. Only the HN6 resonance signal of the purine amide group could not be assigned owing to proton exchange. The ^1H , ^{13}C and ^{15}N chemical-shift assignments of CNBD were almost complete (99% of the backbone and 94% of the side chain CH) using multidimensional heteronuclear NMR spectroscopy with $[U-^{15}\text{N}, ^{13}\text{C}]$ -labelled CNBD and cAMP (see experimental procedures). Resonance assignments have been published previously (Schünke *et al*, 2007). The NOE cross-peak assignments were obtained by an iterative procedure using a combination of manual and automatic approaches. For the structural calculations, a total of 2,388 intramolecular NOE distance constraints, including 820 long-range NOEs, were evaluated. Another 215 dihedral constraints were derived from chemical-shift data using the program TALOS and included in the calculation of the final structure. A final ensemble of 15 NMR structures with the lowest CYANA target functions were used to characterize the structure of the CNBD–cAMP complex. None of the 15 structures violated NOE distances more than 0.019 nm. No dihedral-angle constraint was violated more than 5° . Most of the residues (86.8%) were found in the most favoured regions of the Ramachandran plot. The root-mean-square (r.m.s.) displacement of the 15 structures compared with the average structure was 0.025 nm for the backbone and 0.068 nm for all heavy atoms (superposition of all residues

monomeric CNBD in complex with cAMP by using nuclear magnetic resonance (NMR) spectroscopy and compare it with the structure in the dimer.

RESULTS AND DISCUSSION

Solution structure of the cAMP–CNBD complex

All NMR spectra of the CNBD showed a single set of resonance signals. Nitrogen-15 relaxation experiments were performed to characterize the dynamics and the apparent molecular weight of

except four amino-terminal and five carboxy-terminal residues). This shows that the structure is well defined (Fig 1B). A summary of the experimental constraints and structural statistics is given in the supplementary information (supplementary Table S1 online).

The solution structure of the CNBD–cAMP complex shows a compact fold (Fig 1C), which is similar to the typical fold of other CNBDs (Weber & Steitz, 1987; Su *et al*, 1995; Diller *et al*, 2001; Rehmann *et al*, 2003; Zagotta *et al*, 2003, Flynn *et al*, 2007). The protein core consists of eight antiparallel β -strands (β 1: R252–V256; β 2: V261–C263; β 3: R271–E277; β 4: V280–A283; β 5: V288–L290; β 6: F295–G297; β 7: T310–S312; β 8: V317–H323), five α -helices (α 1: G221–A231; α 2: P241–V248; α 3: M299–I302; α 4: S324–S333; α 5: P335–G350) and a short 3_{10} -helix (L235–K238). The eight β -strands form an antiparallel β -roll topped by a helix bundle (α 1– α 5), whereas the short 3_{10} -helix is located between the α 1- and α 2-helices of the N-terminus.

Interactions between cAMP and CNBD

The cAMP molecule is bound in a pocket formed by the β -roll, the α 3-helix and the α 5-helix. The C-terminal α 5-helix is placed like a lid above the binding pocket (Fig 2A). A total of 25 intermolecular NOE-distance constraints between CNBD and cAMP were derived. An example of the quality of the NOE spectra used to extract intermolecular distance constraints is shown in Fig 2B. The absence of a strong H1'/H8 NOE, as well as the absence of purine base H2 NOEs to S308, indicates that cAMP is bound in the *anti* conformation.

Residues R307, A300 and S308 interact with the cyclic phosphate through electrostatic and hydrogen-bonding interactions, as seen in all members of the obtained structure ensemble. The amide of G297 and the carboxy group of E298 undergo additional hydrogen-bonding interactions with the 2'-hydroxy group of the ribose. Residues V282, V288, L290, A309 and V311, which are located below the purine base of cAMP, show tight van der Waals contacts with the purine base. The side chain of R348 reaches across the purine base and undergoes close polar contacts with residues F295 and E298. In the crystal structure of the HCN2 channel CNBD, the N6 amine of cAMP forms a hydrogen bond with the backbone carbonyl oxygen of R632. In contrast, the corresponding R348 backbone carbonyl of the MloK1 CNBD is too far away to allow any specific interaction with cAMP.

The CNBD–cAMP complex is rigid

Large values of average local displacement relative to the calculated mean structure indicate either local flexibility of the respective residues or a lack of sufficient experimental data for this region. Only the very N- and C-terminal residues of the MloK1 CNBD (Q216 to R219 and A351 to A355) show increased values of average local displacement and a decreased number of NOE-derived distance constraints (supplementary information S2 online). Heteronuclear $[^1\text{H}]-^{15}\text{N}$ steady-state NOE values are sensitive to the dynamics of the local environment, and values close to 0.8 indicate that residues are highly immobile on the pico- to nanosecond time scale (Kay *et al*, 1989). Rapid internal motion is revealed by lower heteronuclear NOE values that might become negative for residues with large-amplitude motions on a subnanosecond time scale (Yao *et al*, 2001). Most residues of CNBD yielded heteronuclear NOEs close to 0.8, indicating a

very rigid compact fold (supplementary information S1 online). Only the very N- and C-terminal residues (Q216–R219 and A351–A355) show lower heteronuclear NOE values indicating increased flexibility.

Comparison with the crystal structure

The solution structure is very similar to the structure of a monomer in the dimer crystal (Clayton *et al*, 2004). A comparison of all CNBD backbone coordinates (residues V218–G350) between solution and crystal structures results in an r.m.s. displacement value of 0.21 nm. The conformations of cAMP in the solution and the crystal structures are also very similar—that is, the glycosyl torsion angles exist in an anti conformation with averaged χ values of -141° and -123° for the NMR and the crystal structure, respectively. However, the coordinates for the N-terminal residues (V218 to P241)—which represent the α 1-helix, 3_{10} -helix and associated loop regions—differ remarkably. The α 1-helix region (G221–A231) in the solution structure is a straight helix without bending (Fig 3). This is directly supported by the helix-typical NOEs within α 1. Their homogeneous intensities are not in agreement with any bending within this helix. In the crystal structure, however, residues R220–N226 of α 1 are bent and form the dimer interface. A comparison of backbone coordinates between the solution and crystal structures, excluding these N-terminal residues (V218–P241), results in an r.m.s. displacement value of only 0.12 nm. In addition, the positions of the short 3_{10} -helix (L235–K238) and the associated loop regions between the α 1 and α 2 helices are displaced towards the top of the helical bundle in the solution structure.

The two most important insights of our study are that (i) the CNBD, even at the high concentration required for NMR measurements, is a monomer, and that (ii) the solution structure, except for the N-terminal C-linker region, is similar to the monomer structure in the dimer crystal structure. The much longer C-linker region of vertebrate CNG and HCN channels is involved in intra- and intersubunit contacts, and contributes virtually all contacts between the subunits in the tetrameric crystal structures of CNBDs from two different HCN channels (for reviews, see Zagotta *et al*, 2003; Craven & Zagotta, 2006; Flynn *et al*, 2007). The formation of dimers and tetramers from monomeric CNBDs requires cAMP (Zagotta *et al*, 2003), suggesting that rearrangement of the C-linker interface represents an important gating event. Thus, the MloK1 channel seems to be set apart from its vertebrate counterparts by the lack of a full-blown C-linker that coordinates intersubunit contacts. This conclusion is supported by an electron microscopic study of the complete MloK1 channel (Chiu *et al*, 2007; Taraska & Zagotta, 2007). An important feature of the electron microscopic structure is that the four CNBDs are separated by discrete gaps and that four isolated CNBDs could be modelled into the electron density map. This structure predicts that binding sites act independently. The C-linker contact observed in the crystal structure (Clayton *et al*, 2004) was possibly enforced by the packing of dimers in the crystal, and these contacts seem to be functionally irrelevant. In fact, cAMP and several analogues bind non-cooperatively to the monomeric CNBD and the tetrameric full-length MloK1 with a high affinity that is virtually identical (Cukkemane *et al*, 2007). Together with the presented solution structure, this shows that the MloK1 cyclic nucleotide binding sites are functionally independent of each other.

Fig 2 | Interactions between cyclic AMP and the cyclic nucleotide-binding domain. (A) Stereo view of the binding pocket. The binding pocket consists of highly conserved residues known as the phosphate-binding cassette (PBC). The PBC includes G297, E298, A300, R307 and S308, which form polar contacts (shown as yellow dotted lines) with the cyclic nucleotide. The purine of cAMP is positioned towards the exit of the binding pocket. (B) Strips of various ^{13}C -edited HSQC-NOESY and ^{15}N -edited NOESY-HSQC spectra containing intermolecular NOEs between CNBD and cAMP. The respective NOE is characterized by the cAMP proton labelled at the bottom of each strip (schematic shown in the upper right corner) and the CNBD proton labelled to the right of each cross-resonance within the respective strip. CNBD, cyclic nucleotide-binding domain; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy.

Fig 3 | Comparison of nuclear magnetic resonance and crystal structure. (A) Comparison of the solution structure (blue) and the crystal structure of the cyclic nucleotide-binding domain (CNBD; white). A part of the second domain in the dimer crystal structure that is involved in the dimer interface is shown in green. Alignment of the solution and crystal structure backbones (residues V218–G350) yielded an r.m.s. displacement of 0.21 nm. A comparison of the solution and crystal structures, excluding the amino-terminal residues (V218–P241), results in an r.m.s. displacement value of 0.12 nm. (B) CNBD backbone comparison of the monomeric solution structure (shown in blue) and the backbone of the crystal structure dimer (shown in red).

METHODS

Protein expression and purification. The isolated CNBD was expressed as a fusion protein with glutathione *S*-transferase. Thrombin cleavage yielded the isolated CNBD (Q216–A355) with additional glycine and serine residues at the N-terminus. Details of cloning, expression and purification of isotopically [U - ^{15}N , ^{13}C]-labelled recombinant protein have been described previously (Schünke et al, 2007).

NMR spectroscopy. NMR samples contained 0.5 mM uniformly [U - ^{15}N , ^{13}C]-labelled protein with an equivalent amount of cAMP in aqueous solution (10 mM potassium phosphate, pH 7, 100 mM potassium chloride, 100 μM EDTA, 5% (v/v) $^2\text{H}_2\text{O}$ and 0.02% (w/v) sodium azide). All NMR experiments were carried out at 298 K on a Varian ^1H INNOVA spectrometer equipped with a 5 mm cryogenic Z-axis PFG- ^1H [^{13}C , ^{15}N] triple resonance probe at a proton frequency of 800 MHz.

Backbone and side chain assignments of CNBD were carried out as reported previously (Schünke et al, 2007). The resonances of cAMP were identified and assigned using a combination of the following experiments: aliphatic and aromatic 2D (^1H – ^{13}C)-HSQC (Kay et al, 1992), 2D ct-(^1H – ^{13}C)-HSQC (Santoro & King, 1992; Vuister & Bax, 1992), 3D aliphatic ^{13}C -edited HSQC-NOESY (100 ms mixing time), 3D aromatic ^{13}C -edited HSQC-NOESY (140 ms mixing time; Norwood et al, 1990)

and ^{15}N -edited NOESY-HSQC (120 ms mixing time; Zuiderweg & Fesik, 1989). Adenine H2 resonance was distinguished from other aromatic resonances and assigned by the unique chemical shift of the attached C2 carbon, and the H8 resonance was respectively assigned. Proton resonances of the ribose were successively identified by using the unique H1' resonance as a starting point for interpretation of the NOE experiments. Structural constraints were derived from 3D ^{15}N -edited NOESY-HSQC (120 ms mixing time), aliphatic ^{13}C -edited HSQC-NOESY (100 ms mixing time) and aromatic ^{13}C -edited HSQC-NOESY (140 ms mixing time) experiments. Aliphatic ^{13}C -edited HSQC-NOESY (100 ms mixing time) and aromatic ^{13}C -edited HSQC-NOESY (140 ms mixing time) experiments were also used for structural constraints with protein in buffer after replacement of H_2O by D_2O .

Data evaluation and structure calculation. On the basis of the almost complete assignment of ^1H , ^{13}C and ^{15}N resonances of CNBD, NOE cross-peak assignments were obtained by an iterative procedure using a combination of manual and automatic steps. As an initial step, the program CARRA (Keller, 2004) was used to evaluate NOE spectra and to manually assign nearly all of the apparently unambiguous NOEs. NOE cross-peak intensities were classified as strong, medium or weak, corresponding to upper limit distance constraints of 2.7, 3.8 and 5.5 Å, respectively. The cross-peak intensities of NOEs between protons of known distances

were used for calibration. For NOEs involving methyl groups, upper limit distance constraints of 2.9, 4.0 and 5.7 Å for strong, medium or weak interactions, respectively, were used. To perform the structural calculation with simulated annealing in torsion angle space, the program CYANA version 1.1 (Güntert *et al*, 1997) was used. All of the 25 intermolecular NOEs between the protein and cAMP were manually assigned. According to the manually assigned NOEs, an initial fold of the protein was calculated. CYANA runs were performed according to the protocol for simulated annealing with 100 randomly generated starting conformations, 25,000-steps torsion angle dynamics and 2,000 conjugate gradients minimization steps. With subsequent use of the ATNOS/CANDID version 1.1 software package in combination with CYANA, additional NOEs were automatically assigned in an iterative approach. The package incorporates the functionalities of the following two algorithms: ATNOS (Herrmann *et al*, 2002a) for automated NOE peak picking, and NOE signal identification in 2D homonuclear- and 3D heteronuclear-resolved [¹H,¹H]-NOE spectra, and CANDID (Herrmann *et al*, 2002b) for automated NOE assignment. The input consisted of nearly complete assignments of ¹H, ¹³C and ¹⁵N resonances, the previously assigned NOEs and the three mentioned NOE spectra. The standard protocol with seven cycles of peak picking, NOE assignment and subsequent structural calculation with CYANA was applied (Güntert *et al*, 1997; Herrmann *et al*, 2002a,b). In the final step, dihedral angle restraints for the backbone ϕ and ψ angles were derived from H^α, C^α, C^β, C' and N chemical shifts using the program TALOS (Cornilescu *et al*, 1999). Restraints were applied for the 111 high-confidence predictions found by the program using the calculated range ± 10°. For further refinement, CYANA runs were performed according to the protocol for simulated annealing with 100 randomly generated starting conformations, 35,000-steps torsion angle dynamics and 2,000 conjugate gradients minimization steps. A final bundle of 15 NMR structures with the lowest target function that did not show any distance constraint violations of more than 0.019 nm were used for further analysis. Geometry of the structures, structural parameters and secondary structural elements were analysed and visualized using the following programs: MOLMOL (Koradi *et al*, 1996), PyMOL (DeLano, 2002), WHATIF (Vriend, 1990) and PROCHECK (Laskowski *et al*, 1993).

For the characterization of overall and internal motions, ¹⁵N longitudinal (*R*₁) and transverse (*R*₂) relaxation rates, together with the steady-state [¹H]¹⁵N NOE, were recorded at 298 K on a uniformly [¹⁵N]-labelled protein sample using standard methods at 800 MHz proton frequency. Peak integral values were obtained by fitting signals to an adjustable 'peak model' shape using the program CARRA. A superposition of the Gauss and Lorentz functions was used, and adjusted manually and independently for both spectral dimensions. For ¹⁵N *R*₁ measurement, relaxation delay values of 11, 60, 140, 240, 360, 530, 750 and 1,150 ms were applied. For *R*₂, delays of 10, 30, 50, 70, 90 and 110 ms were used. Data of *R*₁ and *R*₂ relaxation experiments were fitted to a mono-exponential decay using the program CURVEFIT (A.G. Palmer, Columbia University, USA). The correlation time was determined for an isotropic tumbling model using the TENSOR2 package (Dosset *et al*, 2000). [¹H]-¹⁵N NOE-TROSY spectra (Farrow *et al*, 1994; Pervushin *et al*, 1997; Zhu *et al*, 2000) were acquired with a 2.5 s proton saturation.

Database depositions. Resonance assignments and the atomic coordinates for the resulting 15 NMR structures with the lowest target function are available at the BioMagResBank and at the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (codes 15249 and 2K0G), respectively.

Supplementary information is available at *EMBO reports* online (<http://www.emboports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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