Crystal structures of the c-di-AMP synthesizing enzyme CdaA

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Running title: Structures of the di-adenylate-cyclase CdaA

Keywords: second messenger, c-di-AMP, X-ray crystallography, prokaryotic signal-transduction, metal ion-protein interaction

Cyclic di-AMP (c-di-AMP) is the only second messenger known to be essential for bacterial growth. It has been mainly found in gram-positive bacteria, including pathogenic bacteria like *Listeria monocytogenes*. CdaA is the sole di-adenylate cyclase in *L. monocytogenes* rendering this enzyme an attractive target for the development of novel antibiotic compounds. Here we report crystal structures of CdaA from *L. monocytogenes* in the apo-state, in the post-catalytic state with bound c-di-AMP and catalytic Co²⁺ ions, as well as in a complex with AMP. These structures reveal the flexibility of a tyrosine side-chain involved in locking the adenine ring after ATP binding. The essential role of this tyrosine was confirmed by mutation to Ala leading to a drastic loss of enzymatic activity.

Bacteria have the ability to perceive environmental changes leading to rapid and effective adaptation by utilizing different proteins as well as second messengers in order to transduce signals inside the cell. In response to external stimuli the intracellular concentration of second messengers, like cyclic di-nucleotides and linear mono-nucleotides, varies in order to regulate and coordinate cellular processes (1-3). Cyclic di-AMP (c-di-AMP) is the latest discovered bacterial signaling nucleotide, which has been found mostly in gram-positive bacteria up to date. c-di-AMP is involved in different cellular processes like DNA integrity scanning, cell wall metabolism and osmolyte homeostasis (for review see: (4-6)). c-di-AMP is the only essential second messenger in bacteria due to its role in potassium homeostasis. It regulates potassium importers at high intracellular K⁺ concentrations while c-di-AMP is not essential at low K⁺ concentrations (7). Interestingly, c-di-AMP becomes toxic if its degradation is blocked, hence a tightly controlled intracellular c-di-AMP concentration is required for bacterial growth (8).
Proteins containing a di-adenylate cyclase (DAC) domain have been bioinformatically identified mainly in Gram-positive bacteria of the phyla Actinobacteria and Firmicutes, but also in Gram-negative Cyanobacteria, Chlamydiae, Bacteroidetes, Fusobacteria and Deltaproteobacteria and even in archaea of the phylum Euryarchaeota (5). Several DAC domain containing proteins from various bacterial species have also been experimentally proven to produce c-di-AMP. Many of these bacteria are well known pathogens, e.g.: Mycobacterium tuberculosis (9), Staphylococcus aureus (10) and Listeria monocytogenes (11). In total, eight families of di-adenylate cyclases have been identified so far, sharing the highly conserved DAC domain (12). However, the DACs differ in their additional domains and the domain organization, suggesting that DAC enzymes are regulated by different signals (12).

The three-dimensional structure of a DAC domain was first reported for DisA, a multi-domain protein with an N-terminal DAC domain (13). This structure revealed that within the homo-octameric DisA two adjacent and properly positioned DAC domains, each with one ATP bound, are catalyzing the synthesis of c-di-AMP. Based on the homology of all DAC domains it was proposed that DAC domains with bound ATP need to dimerize in a specific arrangement in order to catalyze c-di-AMP formation.

The importance of c-di-AMP for the growth of several pathogenic bacteria is marked by an increased resistance to cell wall targeting antibiotics (10,14). Its absence in humans makes the DAC enzymes an interesting target for the development of novel antibiotics by structure-based drug design. Therefore CdaA, the only DAC of the human pathogen Listeria monocytogenes, was previously biochemically and structurally characterized. The analysis revealed that CdaA is active with Co$^{2+}$ or Mn$^{2+}$ ions as cofactor but is inactive in the presence of Mg$^{2+}$ ions (15). The CdaA crystal structure unveiled the monomeric and catalytically inactive enzyme-substrate complex with bound ATP and Mg$^{2+}$, leaving the structure of a dimeric and active form with bound Co$^{2+}$ or Mn$^{2+}$ cofactor still to be determined. Such a crystal structure could shed light on the role of the metal ion in the catalytic reaction.

In this study we report two new crystal structures of CdaA from L. monocytogenes at 2.0 Å and 2.8 Å resolution, representing the enzyme in its apo form and the post-catalytic homo-dimeric enzyme-product complex, respectively. The structure of CdaA with bound c-di-AMP was obtained by co-crystallization of CdaA in the presence of ATP and Co$^{2+}$ ions. Comparison of the CdaA structure in the apo state with the ligand bound forms of CdaA (ATP, AMP or c-di-AMP) revealed conformational changes of a tyrosine residue present in the active site. Mutation of this tyrosine to alanine abolishes c-di-AMP formation and thus demonstrates its functional importance. Furthermore, we confirmed that CdaA is active in the presence of Mn$^{2+}$ or Co$^{2+}$ ions, with significantly higher activity in the case of Mn$^{2+}$, but it is inactive in the presence of Mg$^{2+}$ ions.

These new CdaA structures could serve as an important starting point for future rational drug design.

**Results**

Structure-based development of novel antibiotic drugs requires high resolution
three-dimensional structures of the targeted enzyme and enzyme-inhibitor complexes. CdaA of the human pathogen L. monocytogenes appears to be an attractive target, as c-di-AMP synthesis is essential for bacterial growth and CdaA is the only DAC in this pathogenic bacterium, while there are no DACs in humans. For this study truncated ∆100CdaA, missing the N-terminal trans-membrane helices (TM) and the 20 amino acids linking the TM to the DAC domain, was used, since the trans-membrane helices hamper the solubility of the recombinant full-length protein. Previously we had demonstrated that this truncated ∆100CdaA has preserved its enzymatic activity with a higher enzymatic activity for Co2+ in comparison to Mn2+, but no activity for of Mg 2+ (15). While in this previous study the in vitro activity was measured by LC-MS/MS, we now applied a direct fluorescence-based measurement of c-di-AMP formation by its binding to coralyne (16). In contrast to the results obtained with the LC-MS/MS method, a more efficient c-di-AMP synthesis was observed in presence of Mn2+ in comparison to Co2+ (Fig. 1A).

Structure of apo CdaA

One approach for the identification of potential inhibitors is crystallographic fragment screening, which desires crystals of CdaA in its apo state. Therefore ∆100CdaA was crystallized in the absence of ATP and divalent metal ions. Crystals of apo-CdaA were obtained and belong to space group P2_12_1, containing two ∆100CdaA molecules per asymmetric unit. The phase problem was solved by means of Molecular Replacement using the monomeric ∆100CdaA structure of L. monocytogenes (PDB code 4RV7) as a search model. The resulting crystal structure of apo-CdaA was determined at 2.0 Å resolution (Table 1). The CdaA monomer is composed of a slightly twisted central β-sheet, made up of seven mixed-parallel and antiparallel β-strands (β1–β7), flanked on both sides by five α-helices (α1–α5) in total (Fig. 2). The two ∆100CdaA molecules in the asymmetric unit are structurally very similar as indicated by the r.m.s.d. (root-mean-square deviation) of 1.19 Å between all Ca positions.

The structure of apo-CdaA closely resembles that of CdaA with bound ATP (PDB code 4RV7) as they exhibit an r.m.s.d. of 1.56 Å, but a few differences are seen in a loop region (residues 137-140) and the C-terminal residues. Careful inspection of the difference electron density map revealed a small molecule bound to the surface of one out of two CdaA molecules in the asymmetric unit (Fig. S1). This electron density was interpreted as a sucrose molecule originating from the utilized cryo-protectant solution.

In the apo-CdaA crystal structure the active site is accessible from solvent channels, hence this crystal form of apo-CdaA appears to be suitable for a fragment screen.

Structure of CdaA - c-di-AMP complex

To gain more insight into the structure and function of CdaA, we crystallized ∆100CdaA also in the presence of ATP and the cofactor Co2+. The obtained crystals belong to a different space group (H3 2) than the previously determined structure, but also contain two CdaA molecules in the asymmetric unit. The newly obtained crystal structure was determined at 2.8 Å resolution. The two CdaA molecules in the asymmetric unit superpose very well as the r.m.s.d. calculated between all Ca positions amounts to 0.65 Å. Analysis of the protein
contact surfaces in the crystal revealed that one of the two CdaA molecules in the asymmetric unit forms a dimer with a symmetry mate related by a crystallographic 2-fold axis (Figure 3A). This CdaA homo-dimer corresponds to the catalytically active DAC-domain dimers seen in the DisA homo-octamer. The calculated r.m.s.d between superimposed CdaA and DisA dimers amounts to 1.72 Å (198 matched Ca positions, Fig S4). The CdaA-CdaA dimer interface buries about 605 Å² of the accessible surface area (7.3%) and is stabilized by six hydrogen bonds and two salt bridges. However, additional interactions between the monomers are mediated by the ligand bound to the active site (see below).

Surprisingly, the difference electron density map clearly revealed the presence of a c-di-AMP molecule and of two metal ions bound in the active site of the CdaA crystallographic dimer (Fig. S2A). As only ATP and Co²⁺ were added to the protein right before it was subjected to crystallization, the c-di-AMP must have been formed during or after crystallization droplets were set up. It appears very likely that the bound metal ion is a Co²⁺, as no other catalytic metal cation was present in the crystallization solution. The Co²⁺ is coordinated by the phosphate moiety and the carboxylate group of Glu224 as well as the carboxylate group of the Asp171 and the imidazole ring nitrogen of His170 of the symmetry related subunit (Fig. 3B and C). The metal-oxygen distances of 2.1 Å for Asp171 and Glu224, and 2.3 Å for phosphate correspond to distances observed in other proteins containing a Co²⁺ ion (17). Elongated distances observed between Co²⁺ and the imidazole ring of His170 (3.1 Å) and the c-di-AMP 3’OH group (3.8 Å) indicates that this complex corresponds to the post-catalytic state. In order to fulfill its catalytic role the metal ion must be shifted. Only then, it can act as Lewis acid to increase the nucleophilicity of the metal-activated 3’ hydroxyl group of ATP and to enhance the electrophilicity of the phosphorus atom of the adjacent ATP molecule.

The asymmetric unit of the crystal contains a second CdaA molecule which also accommodates a nucleotide bound in the active site, but no bound metal ion (Fig. 4). Based on the observed difference omit electron density map (Fig. S2B) the nucleotide was identified as AMP. Since previous crystal structures of DisA and CdaA with bound ATP or 3’-dATP showed well-defined electron density for the β and γ phosphates, it appears likely that the second CdaA molecule has indeed AMP bound, which must have been formed out of ATP during the crystallization process. The ATP hydrolysis also explains the presence of another difference electron density map peak which has been interpreted as a free phosphate. This phosphate ion is bound in the vicinity of the c-di-AMP molecule and could potentially mark an exit route of the pyrophosphate molecule on the surface of CdaA.

Conformational rearrangements of the active site induced by ligands

The comparison of CdaA in the apo state to CdaA complexed with AMP or c-di-AMP unveils different orientations of the Tyr187 side-chain, which is located in close proximity to the adenine base. In the CdaA apo state this tyrosine side chain is rotated outwards from the active site leading to an opening of the binding site for the adenine base (Fig. 5). In the monomeric CdaA-AMP complex the tyrosine is rotated inwards the active site and stacks on the adenine in an
almost coplanar orientation. In contrast, in the dimeric c-di-AMP complex the tyrosine side chain is flipped outwards, as the Thr202 side chain of the other subunit packs against the adenine ring (Fig. 3B).

In order to investigate whether Tyr187 plays an important role in c-di-AMP formation, a Tyr187Ala mutant was generated. This mutation led to a significant reduction (about 80%) in activity, confirming the functional impact of Tyr187 (Fig. 1B). In order to exclude that the Tyr187Ala mutation perturbed the fold of CdaA, the crystal structure of Δ100CdaA_Y187A was determined as well (Table 1). Comparison with the structure of wildtype Δ100CdaA demonstrates no structural changes caused by the mutation.

Discussion

The synthesis of c-di-AMP requires the dimerization and proper orientation of two DAC domains, each with one ATP bound and accompanied by the metal ion cofactor. In DisA this is achieved permanently by the homo-octameric oligomerization state (13). The first structure of CdaA of L. monocytogenes had shown that the DAC domain crystallized as monomer even though ATP was bound to the active site (15). However, for the previous and as well this study a truncated CdaA was used. Up to know the influence of the missing transmembrane domain on oligomerization and catalytic activity is unknown.

Here, a new crystal form of CdaA was obtained which contains two CdaA molecules with different nucleotides bound. One CdaA molecule forms a catalytically active dimer with a symmetry mate in the crystal. This dimer contains a c-di-AMP molecule and two metal ions in the active site, hence it closely resembles the dimer arrangement of DAC domains seen in DisA (Fig. S4).

The c-di-AMP must have been formed during crystallization as only ATP and Co²⁺ ions were added to CdaA. This complex corresponds to the enzyme-product complex, which is supposed to have a lower stability. However, the c-di-AMP mediates multiple contacts between the monomers therewith increasing the interaction surface area between the monomers, and the catalytically active dimer appears to be caught in the crystalline lattice.

Conserved active site residues of DisA and CdaA directly involved in substrate binding and catalysis were previously identified (15,18). Each of the mutations in DisA (D75N, R130A, RHR (108-110)AAA, T107V+T111V), and in CdaA (D171N, G172A, and T202N) led to a reduction or complete loss of enzymatic activity. However, by analyzing the structure of the monomeric CdaA in the asymmetric unit with bound AMP we realized that the Tyr187 side chain might also be involved in substrate binding as it stacks on the adenine ring, but it is rotated outwards in the structure of apo CdaA (Fig. 5). Hence, it appears likely that upon binding of ATP to monomeric CdaA Tyr187 rotates toward the adenine moiety and locks the ATP in the active site by a π-
π-stacking interaction, as it has been observed for many other ATP binding proteins (19). However, upon CdaA dimerization the tyrosine side chain gets replaced by the side chain of Thr202 of the other subunit, which then stabilizes the bound ATP. The replacement of the Tyr by a side chain from the other subunit might facilitate the product release after catalysis, as upon dimer dissociation the product can be released more easily. By mutation of Tyr187 to Ala, which strongly reduced the
activity in vitro, we demonstrate that Tyr187 indeed plays an essential role in c-di-AMP formation by CdaA. Notably, this Tyr187 is conserved in most CdaA enzymes, but not in other DAC proteins like DisA suggesting a slightly different mechanism of substrate binding between different classes of DACs.

A remarkable difference between DisA and CdaA concerns the metal ion specificity in the catalytic center. While DisA appears to be active with Mg$^{2+}$ and Mn$^{2+}$ (20), CdaA is not active in the presence of Mg$^{2+}$. Such unexpected differences in metal ion preferences have been also observed for other protein families, e.g.: metal-dependent serine/threonine phosphoprotein phosphatase (PPP) family (21). Since the catalytic mechanism of PPP enzymes, as well as of DAC proteins, does not require the redox potential of Mn$^{2+}$ to carry out the catalyzed reaction, it isn't clear why some members of the DAC family would prefer Mn$^{2+}$ or other divalent cations over Mg$^{2+}$ (22). Hence, the observed strict dependence of CdaA on Mn$^{2+}$ or Co$^{2+}$ ions raised the question concerning its structural basis. The observed metal ion dependency is most likely related to different chemical properties of the cation, e.g. ionic radius, and to the amino acid composition of the active site.

Comparing the DisA and CdaA structures reveals significant differences in the metal ion coordination. In the catalytically active dimer of DisA with bound ATP, the Mg$^{2+}$ ion is coordinated by three phosphate groups and the Asp carboxylate. In CdaA more protein residues contribute to the metal binding resulting in a more crowded active site. In addition to Asp171 (which corresponds to Asp75 of DisA), the side chains of Glu224 and of His170 coordinate the Co$^{2+}$ ion. These two residues are not structurally conserved in DisA, as there is an Arg instead of the Glu, and a Met instead of the His. The side chains of both, Arg and Met, are rotated outwards from the metal binding site making it less crowded. Hence, the major difference appears to be presence of the His. While Mg$^{2+}$ strongly prefers a coordination by Asp and Glu, the transition metal ions Mn$^{2+}$ and Co$^{2+}$ are bound as well by His, as deduced from the analysis of all metal binding sites in known protein structures (23).

The reason for this difference between DisA and CdaA most likely is related to the fact that DisA contains stably associated catalytically active dimers, while for CdaA the catalytic dimer might just transiently exist. Therefore, in DisA the Asp171, which belongs to the second DAC domain, is sufficient for binding of the substrate ATP and the metal ion. In CdaA, the ATP and the metal ion are initially bound to the monomeric DAC domain by the Glu224 side chain, and solvent molecules complete the metal coordination sphere. Upon formation of the catalytically active dimer, the metal ion needs to be slightly repositioned in order to be further coordinated by His170 and Asp171 provided by the second monomer (Fig. S3).

Recently, CdaA from Staphylococcus aureus was structurally and biochemically characterized (24). Surprisingly, in contrast to L. monocytogenes CdaA the S. aureus CdaA homolog shows activity not only in the presence of transition metal ions but also in the presence of Mg$^{2+}$. The comparison of the active sites of both available CdaA structures unveils an identical positioning of the amino acids directly involved in c-di-AMP and metal ion coordination (Fig. S5). The largest structural differences can be observed for N
and C-terminal α-helices and a loop connecting β-strand 4 and α-helix 4. The latter is located in the close vicinity of phosphate moiety of ATP and could indirectly alter the metal binding preferences or even metal catalytic efficiency. This could serve as a potential explanation of the metal ion promiscuity of CdaAs showing a strict conservation of three residues His170, Asp 171 and Glu 224 directly involved in metal binding as revealed by sequence alignment of ten bacterial CdaAs (Fig. S6). The chemical properties of these three residues are most likely the structural basis for the observed metal ion promiscuity of CdaAs, which has been also observed for other enzymes, e.g.: mannosylglycerate synthase (25). The observed capability of utilizing several ions by one enzyme is still one of many not well understood marvels of enzymology which requires a further investigation.

Experimental procedures
Bacterial Strains and Growth Conditions
For cloning procedures and protein overexpression Escherichia coli strains DH5α and BL21(DE3) were used. The E. coli strains were cultivated in 2YT medium whereas transformed cells were selected on LB plates containing ampicillin (100 µg/ml).

Plasmid construction
For purification procedures the DAC type CdaA was equipped with a GST-tag. CdaA is known to be a transmembrane protein. The Δ300cdaA allele was amplified, which lacks the TM domain, using the primer pairs JH004 forward (5’-CCGGATCTATGGATCAGAATTGC CG-3’) / JH005 reverse (5’ GGCTCGAG TCATTGCTTTTGCCCTCCTTTCC-3’). As template the plasmid pBP33 was used (15). The resulting PCR products were cloned in the pGEX-6P-1 (GE Healthcare) expression vector using the restriction sites XhoI and BamHI leading to the plasmid pGEXpBP33, which encodes for the truncated Δ100CdaA protein with a N-terminal GST-tag.

Site-directed mutagenesis
Δ100CdaA mutants were generated with site-directed mutagenesis to identify amino acid residues that undertake an important function in the catalytic reaction mechanism. CdaA mutant Y187A was created by polymerase chain reaction using the mutagenesis primer pairs JH_Y187A_Forward (5’-CAGGAAGTGCCTTGCCA CTTTCAGATAGCCGTTTATCCAA AGAAC-3’) and JH_Y187A_Reverse (5’- GTGGCAAGGCACTTGCTGCGATGC AATTTGTTTCTTTAATAAATCG C-3’) resulting in the plasmid encoding the truncated mutant variant Δ100CdaA_Y187A.

Protein expression and purification
E. coli BL21(DE3) was used for expression of the fusion protein GST-Δ100CdaA. The cells were grown in 1 L 2YT medium at 37 °C. Protein expression was induced after the culture reached an optical density (OD600) of ~ 0.6 by addition of 1 mM IPTG and incubated at 16 °C for 18 h. After harvesting and subsequent to cell disruption with a microfluidizer (M-110S Microfluidizer, Microfluidics) and centrifugation at 15.600 xg for 30 min to get rid of cell debris, the lysate was loaded onto a Glutathione Sepharose column (GE Healthcare) in 300 mM NaCl, 20 mM Tris/HCl pH 7.5, 10 mM EDTA. The target protein GST-Δ100CdaA was eluted from
the column with 40 mM reduced glutathione. The eluate was incubated overnight with PreScission protease (1:100 (w/w)) in a cellulose tubing placed in dialysis buffer (100 mM NaCl, 20 mM Tris/HCl pH 7.5) at 4 °C to remove the high glutathione concentration and to dissect the GST-tag from Δ100CdaA. In order to remove the cleaved-off tag from the truncated CdaA a second glutathione sepharose purification step was included.

Crystallization and cryo protection

For crystallization the sitting-drop vapour-diffusion method was applied. Initial crystallization trials were performed at 20 °C using Δ100CdaA at a concentration of 4.0 mg/mL supplemented with 500 µM CoCl₂ and 500 µM ATP. Rectangular shaped crystals grew after ca. 48 h in a 2 µL droplet composed of the aforementioned protein solution mixed with reservoir in a 1:1 ratio. The reservoir has been composed of 0.2 M Ca(CH₃COO)₂, 0.1 M Na-HEPES pH 7.5 and 10 % (w/v) PEG8000. Crystals were cryo-protected by soaking them in a reservoir solution supplemented with 25 % PEG8000.

For crystallization of the apo form and the Tyr187Ala variant of Δ100CdaA a protein concentration of 6.0 mg/mL was used keeping 2 µL droplet size and 1:1 protein to reservoir ratio. In order to facilitate crystal growth micro seeding has been performed in combination with small alterations of NaCl concentration. Thin crystal plates were obtained after ca. 18 h in a salt concentration ranging between 3.7-4.5 M NaCl, 0.1 M Na-HEPES pH 8.5. Crystals were cryo-protected by soaking them in a saturated sucrose solution obtained by solubilizing sucrose in reservoir solution.

X-ray data collection and processing

Diffraction images were collected at PETRA III EMBL beamlines P13 and P14 (DESY, Hamburg, Germany) and processed with the XDS package (26,27). Data collection and processing statistics are summarized in Table 1. A trigonal lattice with unit cell parameters of $a = b = 121.90$ Å, $c = 141.59$ Å was determined for the crystals containing CdaA-c-di-AMP complex. The cell content analysis indicated the presence of two CdaA molecules occupying the asymmetric unit ($V_m = 2.89 \, \text{Å}^3/$Da, corresponding solvent content of 57.4 %). The crystals of apo CdaA and Tyr187Ala mutant exhibit an orthorhombic lattice and the unit cell parameters of $a = 42.96$ Å, $b = 64.67$ Å, $c = 129.75$ Å and $a = 46.49$ Å, $b = 65.13$ Å, $c = 131.33$ Å. The Matthews coefficient ($V_m = 2.55 \, \text{Å}^3/$Da, corresponding solvent content of 51.78 %) implicates two molecules occupying the asymmetric unit.

Structure determination and refinement

The crystallographic phase problem was solved by molecular replacement with PHASER (28) using the structure of the DAC Δ100CdaA from L. monocytogenes (PDB code: 4RV7) as search model. Manual model building was implemented with Coot (29), and the structure was refined with Refmac (30) and PHENIX (31). For monitoring the $R_{free}$ 5.0 % of the reflections were randomly selected and excluded from refinement. During the refinement process the coordination distance for Co²⁺ ion in the ligand bound structure was restrained to 2.1 Å for Glu224 and Asp171 side chains and to 2.3 Å for the cyclic di-AMP phosphate. The final structure of the CdaA-c-di-AMP complex has been refined at a resolution of 2.8 Å to $R_{work}$ of 18.7 % and $R_{free}$ of 23.4 %. The
apo-CdaA has been refined at a resolution of 2.0 Å to R_work of 18.6 % and R_free of 22.5 %. The structure of Tyr187Ala mutant has been determined at 2.32 Å resolution and refined to 17.5 % and 22.0 % for R_work and R_free, respectively.

Protein contact areas in the crystals were analyzed using “Protein interfaces, surfaces and assemblies” (PISA) services at the European Bioinformatics Institute using standard settings (32).

In vitro DAC activity assay

The di-adenylate cyclase activity was measured with a quantitative fluorescence assay that is based on an increased fluorescence signal due to the specific interaction of the fluorescent dye coralyne with c-di-AMP (16). A 200 µL reaction mixture contained 40 mM Tris/HCl pH 7.5, 100 mM NaCl, 10 mM XCl_2 (X = Mg, Co, Mn or Ca), 100 µM ATP. The reaction was started by adding 10 µM Δ100CdaA and incubated for 1 h at 37 °C. To stop the reaction the reaction mixture was boiled for 5 min and centrifuged for another 5 min at 13,400 xg to remove the precipitated protein. For quantification of the synthesized c-di-AMP 250 mM KBr and 10 µM coralyne were added to the reaction mixture, respectively. After incubating the samples for 30 min in the dark the c-di-AMP concentration was determined by excitation at a wavelength of 420 nm and measuring the fluorescence emission at a wavelength of 475 nm using a microplate reader (VICTOR Nivo Multimode Microplate Reader, Perkin Elmer).

Acknowledgements

We thank the EMBL-Outstation Hamburg (DESY PETRAIII beamlines P13 and P14, Germany) for the allocation of beam time and Isabel Bento, Johanna Hakanpää as well as Saravanan Panneerselvam for their excellent support at the beam line. This work was supported with funding by the DFG (priority program SPP1879 and INST186/1117). Furthermore, we are grateful to Johannes Gibhardt, Fabian Commichau and Jörg Stülke for fruitful discussions.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.
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|                  | $\Delta 100\text{CdaA}$ with AMP | $\Delta 100\text{CdaA}$-APo | $\Delta 100\text{CdaA}_Y187A$-APo |
|------------------|---------------------------------|----------------------------|---------------------------------|
| **Crystallographic data** |                                 |                             |                                 |
| **Beamline**     | Petra III-P14, EMBL, Hamburg   | Petra III-P14, EMBL, Hamburg | Petra III-P13, EMBL, Hamburg   |
| **Wavelength (Å)** | 0.97620                         | 0.97620                     | 0.97625                         |
| **Resolution range (Å)** | 42.27-2.80 (2.90-2.80)          | 45.89-2.00 (2.10-2.00)      | 46.49-2.23 (2.33-2.23)          |
| **Unique reflections** | 9435                            | 24884                       | 19512                           |
| **Redundancy**   | 5.6 (5.7)                       | 7.1 (7.0)                   | 5.8 (4.2)                       |
| **Completeness (%)** | 93.0 (95.4)                    | 99.7 (98.5)                 | 97.1 (79.3)                     |
| **Space group**  | $H32$                            | $P2_12_12_1$                | $P2_12_12_1$                    |
| **a (Å)**        | 121.90                          | 42.69                       | 46.49                           |
| **b (Å)**        | 121.90                          | 64.67                       | 65.13                           |
| **c (Å)**        | 141.59                          | 129.75                      | 131.33                          |
| **$R_{merge}$ (%)** | 10.9 (80.5)                    | 9.4 (119.0)                 | 8.0 (52.0)                      |
| **$I/\sigma(I)$** | 12.4 (1.9)                      | 13.6 (2.0)                  | 15.6 (2.8)                      |
| **CC1/2**        | 99.8 (72.6)                     | 99.9 (77.2)                 | 99.8 (80.5)                     |
| **Refinement statistics** |                                 |                             |                                 |
| **$R_{work}$/$R_{free}$** | 0.1875/0.2337                  | 0.1858/0.2245               | 0.1837/0.2258                   |
| **No. of atoms** | 2453                            | 2610                        | 2740                            |
| **Average $B$ factor (Å²)** | 58.0                           | 47.6                        | 39.8                            |
| **Root mean square deviation** |                                 |                             |                                 |
| **Bonds Å**      | 0.003                           | 0.008                       | 0.006                           |
| **Angles (degree)** | 0.644                          | 1.003                       | 1.258                           |
| **Ramachandran plot** |                                 |                             |                                 |
| **Favoured (%)** | 98.05                          | 98.11                       | 98.79                           |
| **Allowed (%)**  | 1.95                            | 1.57                        | 1.21                            |
| **Outlier (%)**  | 0.00                            | 0.31                        | 0.00                            |
| **PDB codes**    | 6HVHL                           | 6HVM                        | 6HVN                            |

*Values for the data in highest resolution shell are shown in parentheses*
**Figure 1:** *In vitro* di-adenylate cyclase activity of Δ100CdaA. Presented is a histogram displaying three independent measurements. Control measurement has been performed using wt Δ100CdaA without addition of any divalent metal cations. The histogram represents the divalent metal cation preferences of wt Δ100CdaA. The highest amount of c-di-AMP was formed in the presence of MnCl₂, while in the presence of CoCl₂ the amount of the product is significantly reduced. In case of MgCl₂ and CaCl₂ production of c-di-AMP could not be confirmed as it was within the range of the control. Additionally, it represents the importance of Tyr187 on catalysis. The mutant of Tyr187Ala causes a significant reduction (5-fold) of di-adenylate cyclase activity, confirming its essential role in c-di-AMP synthesis.
Figure 2: Crystal structure of Δ100CdaA in the apo state refined at 2.0 Å resolution. The fold of the CdaA DAC domain consists of seven β-strands forming a central β-sheet, which is surrounded by five α-helices. The positions of residues forming the active site are highlighted in red.
Figure 3: The active site of dimeric CdaA with bound c-di-AMP. (A) The catalytically active Δ100CdaA homo-dimer is depicted as ribbon cartoon and the bound reaction-product c-di-AMP is shown as balls and sticks (carbon in pale blue and blue, phosphate in orange, oxygen in red and nitrogen in dark blue). The two Δ100CdaA monomers are coloured according to the c-di-AMP in pale blue and blue, respectively. Co$^{2+}$ ions are depicted as pale red spheres. (B) A detailed view of the CdaA active site. Amino acids involved in binding the c-di-AMP molecule (coloured and depicted as in (A)) are shown as sticks (carbon in pale blue and blue, oxygen in red and nitrogen in dark blue). The Co$^{2+}$ ions are coloured and depicted according to (A). For simplicity only one half of the 2-fold symmetric CdaA active site is shown. (C) A detailed view of the Co$^{2+}$ binding site and its coordination sphere.
Figure 4: Structure of the CdaA-AMP complex. (A) CdaA monomer (cartoon, pale green) with a bound AMP molecule depicted as balls and sticks. (B) A detailed view of the active site showing the amino acids (sticks, carbon in pale green, oxygen in red and nitrogen in blue) that are involved in AMP binding. The bound AMP is shown as ball-and-stick model (carbon in wheat, phosphate in orange, oxygen in red and nitrogen in dark blue).
Figure 5: Conformational switch of Tyr187 during c-di-AMP synthesis. For convenience only the Δ100CdaA monomer (grey) with bound AMP (carbon in wheat, phosphate in orange, oxygen in red and nitrogen in dark blue) and Tyr187 (wheat) is shown. The Tyr187 and AMP are depicted in ball and stick mode. The side chain of Tyr187 (pale blue) of the Δ100CdaA - c-di-AMP complex structure is superimposed. Upon ATP binding to monomeric CdaA the Tyr187 stacks parallel on the adenine base (π–π interaction) and stabilizes the protein-substrate complex. Upon homo-dimer formation the side chain of Tyr187 rotates outwards as it gets replaced by the Thr202 side chain of the second monomer in the catalytically active homo-dimer.
Crystal structures of the c-di-AMP synthesizing enzyme CdaA
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J. Biol. Chem. published online May 22, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA119.009246

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