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Autoinhibitory mechanism and activity-related structural changes in a mycobacterial adenylyl cyclase

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ABSTRACT

An adenylyl cyclase from *Mycobacterium avium*, Ma1120, is a functional orthologue of a pseudogene Rv1120c from *Mycobacterium tuberculosis*. We report the crystal structure of Ma1120 in a monomeric form and its truncated construct as a dimer. Ma1120 exists as a monomer in solution and crystallized as a monomer in the absence of substrate or inhibitor. An additional α-helix present at the N-terminus of the monomeric structure blocks the active site by interacting with the substrate binding residues and occupying the dimer interface region. However, the enzyme has been found to be active in solution, indicating the movement of the helix away from the interface to facilitate the formation of active dimers in conditions favourable for catalysis. Thus, the N-terminal helix of Ma1120 keeps the enzyme in an autoinhibited state when it is not active. Deletion of this helix enabled us to crystallize the molecule as an active homodimer in the presence of a P-site inhibitor 2′,5′-dideoxy-3′-ATP, or pyrophosphate along with metal ions. The substrate specifying lysine residue plays a dual role of interacting with the substrate and stabilizing the dimer. The dimerization loop region harbouring the second substrate specifying residue, an aspartate, shows significant differences in conformation and position between the monomeric and dimeric structures. Thus, this study has not only revealed that significant structural transitions are required for the interconversion of the inactive and the active forms of the enzyme, but also provided precise nature of these transitions.

1. Introduction

Adenylyl cyclases (ACs) play pivotal roles in mediating diverse physiological processes by converting ATP into the universal second messenger, cAMP, which amplifies external signals through activation of cAMP-dependent protein kinases. ACs are classified into six types based on their sequences (Danchin, 1993). Class III enzymes are present in eukaryotes, bacteria and archaebacteria, and are hence referred to as the universal class (Barzu and Danchin, 1994). Class III ACs are further divided into four subclasses, IIIa to IIId, each with distinct sequence features (Linder and Schultz, 2003). A well-characterised member of Class IIIa is the mammalian AC (mAC) that has nine membrane bound (tmAC) and one cytosolic (soluble adenylyl cyclase-sAC) isoforms. tmACs are made of 12 transmembrane helices and two similar but non identical cytoplasmic catalytic domains C1 and C2. Crystal structures (Zhang et al., 1997; Tesmer et al., 1997) show that the C1 domain contributes two metal binding aspartates and C2 contributes nucleotide binding residues (lysine and aspartate) and transition state stabilizing residues (asparagine and arginine) to the single active site formed at the dimeric interface of a head-to-tail pseudo heterodimer (Tesmer et al., 1999). The ACs require two Mg^{2+} ions for catalysis. Mg^{2+}–ATP acts as a substrate and free Mg^{2+} is essential for catalysis. Both C1 and C2 domains contribute critical residues for catalysis. Thus, C1 and C2 domains provide complementary residues to form a single catalytic site.

In contrast to mammalian enzymes, those from bacteria exist as homodimers with two active sites formed at the interface. Crystal

Abbreviations: AC, adenylyl cyclase; mAC, mammalian adenylyl cyclase; tmAC, transmembrane mammalian adenylyl cyclase; sAC, soluble adenylyl cyclase; 2′,5′-dd-3′-ATP, 2′,5′-dideoxy-3′-adenosine triphosphate; PP_i, pyrophosphate; cAMP, cyclic adenosine monophosphate.

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structures of three adenylyl cyclases from *Mycobacterium tuberculosis* strain H37Rv are available: Rv1900c, Rv1264 and three forms of inactive Rv1625c. Rv1900c, Rv1264 as well as Ma1120 belongs to Class IIIc ACs which are characterised by short dimerization arm regions. Rv1900c is composed of an N-terminal α/β-hydrolase domain and a C-terminal cyclase homology domain. Crystal structure of the cyclase domain shows that it forms a homodimer with asymmetry between the two active sites (PDB code: 1YBU) (Sinha et al., 2005). pH dependent structural transition activates Rv1264 with its N-terminal regulatory (pH sensor domain) and C-terminal catalytic domains interacting in different ways in the active and inhibited states (Tews et al., 2005). Rv1625c, studied extensively in our lab shows sequence similarity with the mammalian ACs and GCs. Rv1625c predominantly elutes as a monomer with a minor dimeric fraction during gel filtration. Both the fractions crystallized as domain-swapped inactive dimers devoid of proper catalytic sites due to the formation of a new head-to-head arrangement of the subunits. Thus, this domain-swapped inactive dimer exhibits a new inhibitory form that was not observed previously in this class of enzymes (Barathy et al., 2014). Single and triple mutants of this enzyme crystallized as inactive monomers (Barathy et al., 2014; Ketkar et al., 2006). Structures of Rv1625c and two of its mutants revealed that the dimerization arm and the N-terminal region are vital for determining the oligomeric state of the enzyme, which in turn regulate its activity.

While *M. tuberculosis* H37Rv encodes 16 cyclase genes, *Mycobacterium avium* harbours 12 cyclase genes. Of these 12 cyclases, Ma1120 has the highest sequence identity of about 80% with the first 145 residues of the catalytic domain of Rv1120c of *M. tuberculosis*. However, Rv1120c has no adenylyl cyclase activity due to the deletion of a single nucleotide that leads to a frame shift after residue 145 and premature termination beyond residue 165. Ma1120 was shown to have AC activity and also cross-reacted with an antibody raised against Rv1120c. Hence Ma1120 was called the functional orthologue of the pseudogene product Rv1120c (Shenoy et al., 2005). This is the first, and until now the only biochemically characterised AC of *M. avium*. The pure enzyme shows adenylyl cyclase activity at pH and temperature optima of 7.5 and 37 °C, respectively. One of the P-site inhibitors, 2′,5′-dd-3′-ATP, inhibits the enzyme more potently than 2′-d-3′-AMP and tyrphostinA25, similar to other Class III ACs (Shenoy et al., 2005). The only structure of the enzyme available in complex with 2′,5′-dd-3′-ATP is that of the heterodimeric tmAC (Tesmer et al., 2000). The structure provided insights into the inhibition mechanism of the cyclase activity by P-site inhibitors. Here, we report crystal structures of a monomer of Ma1120 and dimers of a deletion mutant of the enzyme in complex with 2′,5′-dd-3′-ATP and with pyrophosphate in the presence of metals. The resolution of these structures varies from 1.95 to 1.38 Å. Our study reveals autoinhibition of the enzyme by an extra N-terminal helix and a large movement of the dimerization arm harbouring one of the substrate specifying residues, induced by activation.

2. Materials and methods

2.1. Cloning, protein expression and purification

pPRO-Ma1120 was cloned as described previously (Shenoy et al., 2005). Structural analysis showed that the N-terminal helix occupies the active site of Ma1120-Wt enzyme. We therefore cloned a construct deleting the first 53 residues (Ma1120-ΔN53) by using inverse PCR. Primers used for inverse PCR are Fwd: CGGGTGGTGATCCTGTTCACCGAC, Rev: GGATCCCATGGCGCCCTGA AAATACAG. pPRO-Ma1120-ΔN53 was transformed and expressed in *Escherichia coli* BL21 cells in Luria–Bertani (LB) medium containing 100 μg ml^{-1} ampicillin at 37 °C till OD reached 0.6. After induction for 3 h with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (Calbiochem) cells were harvested at 4000*g* for 20 min. The harvested cells were resuspended in lysis buffer (50 mM Tris pH 8.2, 100 mM NaCl, 2 mM PMSF, 5 mM β-mercaptoethanol) and lysed by sonication, after which the lysate was centrifuged at 14,000*g* for 1 h. The supernatant was passed through nickel–nitrilotriacetic acid column (Sigma) pre-equilibrated with lysis buffer. The column was then washed with wash buffer (50 mM Tris pH 8.2, 500 mM NaCl, 2 mM PMSF, 5 mM β-mercaptoethanol, 20 mM imidazole) and eluted with elution buffer (lysis buffer with 500 mM imidazole and 10% glycerol). Eluted protein was desalted using HiTrap Desalting Columns (GE Healthcare Life Science) with desalting buffer (50 mM Tris pH 8.2, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol). The eluted fractions were loaded on SDS–PAGE. The concentrated protein was further purified by gel filtration. Gel filtration was performed using Superdex 75 10/300 GL (Amersham Pharmacia Biotech) equilibrated with desalting buffer on Biorad Duoflow fast protein liquid chromatography (FPLC) system at a flow rate of 0.25 ml min^{-1}. To determine the oligomeric state, 200 μl of the purified protein was injected into the column. The column was calibrated with protein markers: bovine γ-globulin (158 kDa), bovine serum albumin (67 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B12 (1.3 kDa). Ma1120 ΔN53 construct was purified similar to wild type enzyme except for NaCl concentration, which was changed to 500 mM in all the buffers including desalting buffer, as the protein tends to precipitate in lower NaCl concentration.

2.2. Crystallization

The single monomeric peak obtained after gel filtration for both Ma1120-Wt and the deletion mutant, Ma1120-ΔN53, were concentrated to 5–6 mg ml^{-1} for setting up crystallization using the kits from Hampton Research. Crystallization was set up using microbatch plates layered with paraffin and silicon oil (1:1) with 2 μl protein solution and 2 μl condition and kept at 291 K. The protein separately and mixed with (i) 2′,5′-dd-3′-ATP and Mn^{2+} (ii) 2′,5′-dd-3′-ATP and Mg^{2+} and (iii) combinations of ATP, PP_i and Ca^{2+}, was set up for crystallization. The protein, ligand and the metals were added in 1:1:10 M ratio. Crystals were obtained in many screening conditions and those used for data collection are shown in Supplementary Fig. 1.

2.3. Adenylyl cyclase assay

Adenylyl cyclase assays were carried out with 200 nM of Ma1120-Wt and Ma1120-ΔN53 protein in 50 mM Tris–Cl pH 8.0, 50 mM NaCl, 10% glycerol and 10 mM free Mn^{2+}. For the substrate dose experiments, indicated amounts of MnATP were used. The mixture was incubated at 25 °C for 10 min. The reaction was stopped with 50 mM sodium acetate buffer (pH 4.75) and samples were boiled for 10 min. Cyclic AMP levels were estimated by radioimmunoassay. Data analysis and curve fitting of enzyme kinetics and inhibition were done using GraphPad Prism software. All assays included substrate and enzyme blanks as controls. A similar experiment was set up to determine the effect of Ca^{2+} and PP_i by addition of either 0.1 mM Ca^{2+} or 0.1 mM PP_i to the reaction mixture containing 2.5 mM MnATP. The experiments were performed with two biological and two technical replicates each ($n = 4$).

2.4. Data collection and structure determination

Data were collected at 100K on beamline 14, European Synchrotron Radiation Facility (ESRF), Grenoble with an oscillation