Hydrolysis of diadenosine polyphosphates. Exploration of an additional role of *Mycobacterium smegmatis* MutT1

S.M. Arif, U. Varshney, M. Vijayan

*Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India*

*Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560 012, India*

**ABSTRACT**

Diadenosine polyphosphates (Ap$_n$A, $n$ = 2–6), particularly Ap$_4$A, are involved in several important physiological processes. The substantial sequence identity of the Nudix hydrolase domain (domain 1) of *Mycobacterium smegmatis* MutT1 (MsMutT1) with a known Ap$_4$A hydrolase suggested that MsMutT1 could also hydrolyse diadenosine polyphosphates. Biochemical experiments yielded results in conformity with this suggestion, with Ap$_4$A as the best among the substrates. ATP is a product in all experiments; small amounts of ADP were also observed in the experiments involving Ap$_4$A and Ap$_6$A. Hydrolysis was inhibited by fluoride ions in all cases. The mechanism of action and its inhibition in relation to Ap$_n$A were explored through the X-ray analysis of the crystals of the MsMutT1 complexes with Ap$_5$A; Ap$_5$A and MnCl$_2$; Ap$_4$A; ATP; and ATP.NaF.MgCl$_2$. The aggregation pattern of molecules in the first four crystals is similar to that found in a majority of MsMutT1-NTP crystals. Substrate molecules occupy the primary binding site and ATP occupies a site at an intermolecular interface, in the first two. ATP occupies both the sites in the third and fourth crystal. The protein-ligand interactions observed in these crystal structures lead to an explanation of the molecular mechanism of hydrolysis of Ap$_n$A by MsMutT1. The fifth crystal exhibits a new packing arrangement. The structure of the complex provides an explanation for the fluoride inhibition of the activity of the enzyme. It would thus appear that MutT1 has a major role involving the hydrolysis of diadenosine polyphosphates, which could be elucidated at the molecular level.

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1. Introduction

Nudix hydrolase superfamily, with members found in all kingdoms of life, consists mainly of pyrophosphohydrolases that catalyse the hydrolysis of substrates with a general structure nucleoside diphosphate linked to any other moiety X, (NDP-X), into NMP and P-X in the presence of divalent cations (preferably Mg$^{2+}$ or Mn$^{2+}$) (Bessman et al., 1996; McLennan, 2006; Mildvan et al., 2005). The enzymes are known to serve a house cleaning purpose, with some having the ability to degrade potentially mutagenic substrates, while others implicated in maintaining the physiological homeostasis by controlling the levels of signalling molecules and potentially toxic metabolites (Bessman et al., 1996). The Nudix substrates utilized by these enzymes include NTPs and dNTPs (both canonical and non-canonical), dinucleoside polyphosphates (N$_p$nN), nucleotide sugars and alcohols, dinucleotide coenzymes and capped RNAs. However, hydrolase activity towards non-Nudix substrates such as NDPs, diphosphoinositol polyphosphates (DIPs), dihydronorotperin triphosphate (DHNTP) and thiamine pyrophosphate (TPP) have also been reported (Fisher et al., 2004; Klaus et al., 2005; Lawhorn et al., 2004; Safrany et al., 1999). Despite such substrate diversity, the enzymes share a common αβ/α sandwich scaffold in their structures and a conserved 23 amino acid Nudix motif (GX$_5$EX$_7$REUXEEXGU, where U is preferably a bulky hydrophobic residue and X is any residue), implicated in metal binding and catalysis.

Ap$_4$A, a member of diadenosine polyphosphates (Ap$_n$A, where n = 2–6), is among the substrates utilized by Nudix hydrolases. The intracellular Ap$_4$A is an inevitable byproduct of cellular metabolism. It, along with other diadenosine polyphosphates, is synthesised *in vitro* (and presumably *in vivo*) predominantly by a side reaction of an aminoacyladenylate with an acceptor nucleotide, catalysed by various aminoacyl-tRNA synthetases (Goerlich et al., 1982; Plateau and Blanquet, 1982; Plateau et al., 1981; Randerath et al., 1966; Zamecnik et al., 1966). Ap$_4$A is formed by addition of an aminoacyl-adenylate to an ATP molecule. Although the synthesis of Ap$_4$A seems to be non specific, it is degraded

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[Corresponding author.
E-mail address: mv@mbu.iisc.ernet.in (M. Vijayan).](mailto:mv@mbu.iisc.ernet.in)
specifically by a symmetrically acting ApA hydrolase, cleaving it into two ADP molecules (Guranowski et al., 1983), an asymmetrically acting ApA hydrolase cleaving it into ATP and AMP or by an ApA phosphorylase producing ATP and ADP (Baxi and Vishwantha, 1995; McLennan, 2000). In addition, ApA is also degraded by phosphodiesterase, luciferase and nucleotidyl transferase.

Adenosine polyphosphates have been a topic of considerable interest for years owing to their multiple biological functions, some advantageous and some deleterious. They have been shown to be involved in the initiation of DNA replication (Bambara et al., 1985; Baril et al., 1985; Grummt, 1978) either by binding to protein complexes of DNA polymerase α (Grummt et al., 1979; Rapaport et al., 1981) or by functioning as an acceptor for ADP-ribose in a histone H1 dependent reaction catalysed by poly (ADP-ribose) polymerase (Yoshihara and Tanaka, 1981). They are also implicated in coupling DNA replication to cell proliferation (Grummt, 1978; Nishimura, 1998; Nishimura et al., 1997). In addition, they function as neurotransmitters, cell-signalling molecules in response to oxidative stress or heat shock (“alarmones”) (Bochner et al., 1984; Fuge and Farr, 1993; Johnstone and Farr, 1991; Lee et al., 1983; Pietrowska-Borek et al., 2011), effectors of the cardiovascular system (Flores et al., 1999), etc.

Reports have shown that regulation of intracellular levels of ApA is necessary in bacteria. For instance, increase in the intracellular level of ApA, due to lack of hydrolysases responsible for its degradation, affects the mobility and cell metabolism in E. coli (Farr et al., 1989). Moreover, decrease in the level of ApA has been related to the reduction in the intracellular invasion capacity of many pathogenic and non pathogenic bacteria (Badger et al., 2011; Murillo et al., 2007; Terwilliger et al., 2003). In particular, one of the genes encoding the enzyme involved in the degradation of ApA, Rv2613c, has been shown to be essential in M. tuberculosis and its product has been recognized as potential target for anti-tuberculosis drugs (Raman et al., 2008; Sassetti et al., 2003).

Assuming a functional role of ApA in mycobacterial pathogenesis, various enzymes involved in the regulation of intracellular level of ApA can be targeted for the development of inhibitors/drugs. An understanding of the structural basis underlying the mechanism of these enzymes might be helpful towards achieving the same.

We have carried out detailed biochemical and structural studies on Mycobacterium smegmatis MutT1 (MsMutT1) with a view to understanding the mechanism of the known role of the enzyme in sanitizing the nucleotide pool in the cell (Arif et al., 2017), in pursuance on a long range program on the structural biology of mycobacterial proteins (Arif and Vijayan, 2012; Arif et al., 2015; Chandran et al., 2017; Chetnani et al., 2010; Prabu et al., 2009; Roy et al., 2008; Selvaraj et al., 2007; Singh et al., 2016; Vijayan, 2005) as part of a national and international effort (Arora et al., 2011; Murillo et al., 2007; Terwilliger et al., 2003). MsMutT1 belongs to Nudiva hydrolase superfAMILY of enzymes, a family which encompasses members with substrate multiplicity (McLennan, 2013). The enzyme is made up of an N-terminal Nudiva hydrolase domain (MsMutT1-NTD) and a C-terminal histidine phosphatase domain (MsMutT1-CTD). A sequence comparison showed that the Nudiva hydrolase domain has a sequence identity of 32% with an ApA hydrolase from Aquifex aeolicus (Jeyakanthan et al., 2010). This indicated that MsMutT1 might also have an ApA hydrolase activity. That prompted the initiation of an investigation on this activity of the M. smegmatis enzyme. While this effort was in progress, a report of similar activity of the corresponding M. tuberculosis enzyme, Rv2985 (Peipei Zhu, M. S. degree thesis, 2015, Rochester Institute of Technology) came to our notice. However, no structural studies on the M. tuberculosis enzyme have been reported. Therefore, we continued with our efforts on MsMutT1, the results of which are reported here. The results lead to the elucidation of a plausible mechanism of the hydrolysis of ApA and ApA, with ATP as the common product, and that of ATP with ADP as the product. The investigation also results in an understanding, at the molecular level, of the mechanism of the fluoride inhibition of MsMutT1.

2. Materials and methods

2.1. Enzyme assay

MsMutT1, MsMutT1-NTD and MsMutT1-CTD, were purified as described previously (Arif et al., 2017) and dialyzed against a buffer consisting of 25 mM Tris–HCl pH 7.5, 50 mM NaCl, 2% (v/v) glycerol, 2 mM β-mercaptoethanol, ApA, ApA, ApA, ApA and ATP, all obtained from Sigma Aldrich, were tested for their hydrolysis. MsMutT1, MsMutT1-NTD and MsMutT1-CTD were incubated separately with the nucleotides (~100 μM) in a 10 μL reaction mixture containing MutT buffer (25 mM Tris–HCl pH 7.5, 8 mM MgCl2, 50 mM NaCl, 5 mM DTT, 2% glycerol), as used previously (Arif et al., 2017), for 60 min at 37 °C. MsMutT1 was used at a final concentration of approximately 0.54 μM (0.02 mg/mL) while MsMutT1-NTD and MsMutT1-CTD were used at final concentrations of approximately 2.12 μM (0.04 mg/mL) and 1.92 μM (0.04 mg/mL), respectively. 10 μL of 0.1% SDS was added to the mixtures to terminate the reactions. The nucleotide components of the reaction mixtures were then separated by HPLC (UltiMate 3000) using a DNApac column (DNApac PA200 analytical, 4 × 250 mm). The solvent system used for the separation consisted of 25 mM Tris–HCl pH 9.0, for equilibration, and 1 mM lithium chloride gradient (0–50%) for elution. An isocratic flow rate of 0.5 ml/min was maintained for a period of 28 min. Peaks corresponding to adenosine nucleotides were detected at a wavelength of 259 nm using UV light detector.

2.2. Crystallization

All of the crystallization experiments were performed employing the microbatch-under-oil method at a temperature of 293 K. Soaking experiments including ‘replacement soaking’ (Skarzynski and Thorpe, 2006) as well as co-crystallization were tried for generating crystals of MsMutT1 complexes with ligands. For soaking experiments, crystals of the ligand-free apo MsMutT1 and of its complex with 8-oxo-GTP were obtained as described earlier (Arif et al., 2017). These crystals were then soaked in the respective precipitants supplemented with 5 mM of the ligands (ApA and ApA) for 24 h. Crystals of the ApA complex were again soaked in the same precipitant, but supplemented now with 5 mM MnCl2 for 4 min and frozen in liquid nitrogen. In co-crystallization experiments, MsMutT1 at a concentration of 7 mg.ml⁻¹ and respective ligands (ApA, ApA and ATP) at a molar concentration of approximately 10 times (~2 mM) as that of protein was mixed and incubated for 2 h at 277 K prior to crystallization. A similar mixture supplemented with MgCl2 and NaF, at approximate concentrations of 10 mM each, was also prepared and incubated. The crystallization drops were prepared by mixing equal volumes of the incubated mixtures and the precipitants available in screening kits from Hampton Research. These attempts resulted in crystals of MsMutT1 complexes of ApA, ApA with magnesium ions replaced by manganese ions and ApA by replacement soaking and those of ATP and ATP-Mg,F by co-crystallization (Table 1).
2.3. X-ray data collection, structure solution, refinement and validation

Diffraction data from all crystals but one, were collected at ESRF, X-ray beamline BM14, using a CCD detector. Diffraction data from one of the crystals were collected at a home source using a MAR345 detector mounted on a Bruker-AXS Microstar Ultra II Cu Kα rotating-anode X-ray generator. A temperature of 100 K was maintained throughout the data collection employing liquid nitrogen flow. Glycerol was used as cryoprotectant. Intensity data were processed and merged using iMosflm (Battye et al., 2011) and SCALA (Evans, 2006) of the CCP4 program suite (Winn et al., 2011). Structure-factor amplitudes from intensities were obtained using TRUNCATE of the CCP4 program suite. The structures were refined using REFMAC5 (Winn et al., 2001) and SCALA (Evans, 2006) of the CCP4 program suite (Winn et al., 2011). Structure-factor amplitudes from intensities were obtained using TRUNCATE of the CCP4 program suite. The structures were solved by molecular replacement with Phaser (McCoy et al., 2007) using the co-ordinates of apo MsMutT1 (PDB code 5GG5) as the search model. Iterative cycles of model building using COOT (Cohen, 1997) and subsequent refinement using REFMAC5 (Murshudov et al., 2011) were carried out until convergence of R and Rwite. Alternative side chain conformations for few of the protein residues were assigned during model building. The ligands were placed appropriately into the model based on unambiguous 2Fo – Fc and Fo – Fc difference maps calculated during refinement. The locations of ligands were further confirmed by calculating simulated annealing Fo – Fc OMIT maps for them using CNS v.1.3 (Brunger et al., 1998). Water O atoms were located based on the peaks with heights greater than 3.0 sigma in 2Fo – Fc and 1.0 sigma in 2Fo – Fc maps. At the final stage, anisotropic B-factor refinement was carried out in the case of one crystal structure (crystal 4) which had resolution better than 1.5 Å. The quality of the refined models were evaluated by PROCHECK (Laskowski et al., 1993). The data-collection parameters along with data processing and refinement statistics are summarized in Table 2.

2.4. Analyses of structures

Inter-atomic distances were calculated using CONTACT from the CCP4 program suite. Hydrogen bond assignment was based on the criteria that the distance between the donor (D) and acceptor (A) atom was less than or equal to 3.6 Å and the D–H···A angle was greater than 90°. Structural superpositions were done using ALIGN (Cohen, 1997). Figures were generated using PyMOL (DeLano, 2002).

3. Results and discussion

3.1. Biochemical characterization: Ap4A hydrolase like activity

The enzymatic activities of the full-length MsMutT1, MsMutT1-NTD and MsMutT1-CTD against diadenosine polyphosphates (Ap4A, Ap5A, Ap6A and Ap7A) were analyzed in identical assay conditions as described in Materials and Methods. The full-length MsMutT1 as well as MsMutT1-NTD hydrolysed Ap4A (Fig. 1A), Ap5A (Fig. 1B) and Ap6A (Fig. 1C) asymmetricaly according to following schemes, while both of them failed to hydrolyse Ap7A.

\[ \text{Ap}_4\text{A} \quad \text{Enzyme} \rightarrow \text{ATP} + \text{AMP} \]

\[ \text{Ap}_5\text{A} \quad \text{Enzyme} \rightarrow \text{ATP} + \text{ADP} \]

\[ \text{Ap}_6\text{A} \quad \text{Enzyme} \rightarrow \text{ATP} + \text{ATP} \]

\[ \text{Ap}_7\text{A} \quad \text{Enzyme} \rightarrow \text{No reaction} \]

Thus, like other Ap4A hydrolases, MsMutT1 also seems to require a minimum of four intact phosphate groups for catalysis to occur. Moreover, ATP is always one of the products irrespective of the kind of polyphosphate substrate, while the other product, AMP, ADP or ATP depends, respectively, on whether Ap4A, Ap5A or Ap6A, was used as substrate.

Interestingly, during the hydrolysis of Ap5A and Ap6A by full-length enzyme, small amounts of ADPs (panels ii in Fig. 1A and C), in addition to the usual products, were also observed which required further investigation. In a separate experiment in assay conditions identical to the previous one, with ATP as reactant, it was observed that the full length enzyme hydrolysed ATP into ADP to some extent (panel ii in Fig. 1D). This explains the presence of additional ADPs in the reaction mixtures with Ap4A and Ap6A as substrates, which could have been formed by further hydrolysis of ATP.

The additional ADP molecules were either not formed or present in undetectable amounts (if formed) when the assays were done with MsMutT1-NTD (panel iii in Fig. 1D). This suggests that in the absence of the C-terminal domain, the enzyme either could not hydrolyse ATP further or became very slow in doing so. This is in consonance with our earlier finding that in the absence of C-terminal domain the enzyme hydrolyses the nucleoside triphosphates (8-oxo-dGTP/8-oxo-GTP) at a slower rate than the full length enzyme does (Arif et al., 2017). MsMutT1-CTD alone, on the other hand, did not hydrolyse any of the polyphosphates or the triphosphate (panels iv in Fig. 1A–D).

The relative efficiencies of Ap4A and Ap5A towards the hydrolysis by MsMutT1 were also examined by performing partial hydrolysis at a single common concentration of both the substrates. It turns out that the enzyme hydrolyses Ap4A at a faster rate than Ap5A. For instance, the percentages of Ap4A and Ap5A hydrolysed by the enzyme were, 43.2 and 11.5, respectively, after an incubation period of 10 min.

A comparative study of the effect of magnesium and manganese ions upon hydrolysis by MsMutT1 was carried out as both of them have been shown to be cofactors in other Ap4A hydrolases. Ap4A only was used in this study. The enzyme hydrolysed 83.0% of the substrate in the presence of magnesium, while only 37% was hydrolysed in the presence of manganese, in identical experimental conditions by MsMutT1 which had resolution better than 1.5 Å. The quality of the refined models were evaluated by PROCHECK (Laskowski et al., 1993). The data-collection parameters along with data processing and refinement statistics are summarized in Table 2.

### Table 1

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<th>Crystal No.</th>
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<td>ATP</td>
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<td>Ap6A + MgCl2</td>
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<td>–</td>
<td>ATP-Mg-F-PO₄</td>
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* Crystal with two molecules in the asymmetric unit (ASU).

** Site A in molecule A of the ASU.

b Site A in molecule B of the ASU.
Table 2
Details of X-ray data collection and refinement.

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Refinement and model statistics

| R factor (%) | 17.4 | 17.8 | 16.2 | 12.3 | 16.6 |
| Rfree (%) | 20.9 | 22.8 | 20.4 | 16.0 | 20.5 |
| No. of non-hydrogen atoms | 2374 | 2347 | 2314 | 2424 | 4608 |
| Protein atoms | 70 | 70 | 62 | 62 | 62 |
| Ligands (Ap₅/ATP) | 351 | 302 | 305 | 308 | 513 |
| Solvent atoms | 29.1 | 31.2 | 30.0 | 27.6 | 32.7 |
| Average B factors (Å²) | 70 | 70 | 62 | 62 | 62 |
| Protein atoms | 34.1 | 37.9 | 40.6 | 29.4 | 33.5 |
| Ligands (Ap₅/ATP) | 40.4 | 40.3 | 40.9 | 41.9 | 41.2 |
| Solvent atoms | 40.7 | 40.3 | 40.7 | 41.2 | 4.1 |
| R.M.S. deviation from ideal bond lengths (Å) | 0.013 | 0.016 | 0.017 | 0.012 | 0.017 |
| Bond angles (°) | 1.79 | 1.87 | 1.89 | 1.66 | 1.85 |
| Ramachandran plot statisticsb (Residues in %) | 91.9 | 91.9 | 91.1 | 91.6 | 92.0 |
| Favored region | 8.1 | 8.1 | 8.9 | 8.4 | 8.0 |
| Allowed region | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Generously allowed region | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Disallowed region | 5XD1 | 5XD2 | 5XD3 | 5XD4 | 5XD5 |
| PDB code | | | | | |

Values in parenthesis are those for the highest resolution shell.

aRmerge = \sum_{i=1}^{\text{all}} \sum_{hkl} |I_{i hkl} - \langle I_{hkl} \rangle| / \sum_{i=1}^{\text{all}} \sum_{hkl} I_{i hkl}, where I_{i hkl} is the ith observation of reflection hkl and \langle I_{hkl} \rangle is the weighted average intensity for all i observations of reflection hkl.

bCalculated for non-glycine and non-proline residues using PROCHECK.

conditions. Thus, manganese is a less efficient cofactor than magnesium for hydrolysis by MsMutT1. Since Ap₅A hydrolases are known to be inhibited by fluoride ions (Guranowski, 1990), the hydrolysis of Ap₅A by MsMutT1 in the presence of fluoride ions was also examined. As illustrated qualitatively in Fig. 1E, presence of fluoride ions in the reaction mixture resulted in the inhibition of Ap₅A hydrolysis by MsMutT1. The extent of inhibition was roughly estimated by carrying out the reaction for a small time period using a constant concentration of Ap₅A and the enzyme, in the absence and the presence of NaF (50 μM and 100 μM). While the enzyme converted 35.4% of Ap₅A in the absence of NaF, it converted only 7.0% and 2.6% in the presence of 50 μM and 100 μM, respectively, of NaF, in identical assay condition. Thus, in the context of fluoride inhibition as well, the behaviour of MsMutT1 is similar to that of other Ap₅A hydrolases.

3.2. Preparation of crystals

The plan of the present structural studies involved, to start with, the X-ray crystallographic analysis of the complexes of the enzyme with Ap₅A, Ap₅A and ATP, which is a product of the hydrolyses of both the substrates, with no externally added magnesium in the milieu. Also envisaged was the preparation of a complex with a ligand involving ATP, Mg²⁺ and F⁻ as it has been shown earlier that X-MgF²⁻-O-ATP can function as a substrate/transition state analogue in Ap₅A hydrolysis (Mildvan et al., 2005). Earlier studies on MsMutT1 had indicated that the crystal structure always contained a limited number of magnesium ions which presumably came along with the enzyme during preparation. The location of magnesium ions could be identified with reasonable confidence, but it was important to confirm them with the aid of a heavier ion. This was sought to be achieved through the structure analysis of a complex of the enzyme with Ap₅A, which is a poorer substrate of MsMutT1 than Ap₅A, in which magnesium is replaced by manganese which mimics, but is catalytically much less efficient cofactor than magnesium.

Attempts at co-crystallization succeeded in the preparation of the complex with ATP. Co-crystallization of MsMutT1 with ATP, MgCl₂ and NaF, to obtain a complex with ATP-Mg-F, did not result in diffraction quality crystals. However, quality of the crystals was retained when ATP was replaced by Ap₅A during the crystallization.
Fig. 1. HPLC chromatograms showing the separation of the nucleotides (the substrates and/or the products) from the reaction mixtures. Nucleotide products resulting from the action of full length \textit{Mm} MutT1, \textit{Ms} MutT1-NTD and \textit{Ms} MutT1-CTD on (A) Ap$_4$A, (B) Ap$_5$A, (C) Ap$_6$A and (D) ATP. (E) The effect of fluoride on the hydrolysis of Ap$_4$A by \textit{Mm} MutT1. The first chromatogram in each figure is the control. In all the chromatograms, the X-axis represents the retention time (min) of nucleotides and the Y-axis represents intensity of the nucleotide peak in milli-absorbance unit (mAU) at a wavelength (WVL) of 259 nm.
Efforts to soak the ligands into apo MsMutT1 crystals also failed to yield complexes with Ap₅A and Ap₃A. These complexes were eventually prepared using ‘replacement soaking’ in which crystals of the MsMutT1-8-oxo-GTP complex, described earlier (Arif et al., 2017), were soaked in solutions containing appropriate ligands. The complex involving Ap₅A was soaked in 5 mM MnCl₂ for 4 min to replace magnesium by manganese. As subsequent structural analysis confirmed, the ligands in the MsMutT1-8-oxo-GTP complex were replaced by new ligands in all the three cases. Structures of the five new complexes thus prepared were determined and refined at resolutions ranging from 1.78 Å to 1.47 Å (Table 2).

3.3 Overall structural features

Four of the five crystals belong to the same form (form IV) to which the MsMutT1-8-oxo-GTP complex belongs. This was expected in the case of crystals 1–3 from the way they were prepared. The complex prepared by co-crystallization with ATP (crystal 4) also belongs to this form. The complex formed by co-crystallization with Ap₅A, MgCl₂, and NaF (crystal 5) belongs to an altogether new form (form VII). The molecules in all the crystals have well defined electron-density except for 20–22 N-terminal residues and residues 35–46 in a loop. Crystal 5 contains two molecules with nearly identical geometries in the crystal asymmetric unit. Each of the other crystals contains one crystallographically independent molecule. All the crystallographically independent molecules have nearly the same structure. This structure is in turn similar to those reported earlier in the crystals of apo MsMutT1 and its complexes with 8-oxo derivatives of guanoisine nucleotides (Arif et al., 2017).

The disposition of molecules in crystals 1–4 is similar to that in a majority of the crystal structures reported earlier and involves a head-to-tail arrangement in which domain 1 of each molecule is always in contact with domain 2 (trans domain 2) of a neighbouring molecule (Arif et al., 2017) (Fig. 2A). These two domains together constitute the functional unit (Fig. 2B), although they belong to two different molecules. No such specific aggregation pattern exists in crystal 5.

The disposition and the structure of nucleoside binding sites in crystals 1–4 are, understandably, very similar to those of the binding sites in the complexes of MsMutT1 with 8-oxo-(d)NTPs, which exhibit head-to-tail arrangement in their crystal structures. This is particularly true about site A which is almost entirely made up of residues from domain 1. In the relevant 8-oxo-(d)NTP complexes, two sites, namely, sites B and C, which are contiguous and had residues common to both, occurred at the interface between domain 1 and trans domain 2. The contiguity was such that a nucleotide bound at site B would sterically prevent the access of another nucleotide to site C. In these complexes, site C was occupied by a nucleotide only in cases where no nucleotide was bound at site B. In crystals 1–4, reported here, a nucleotide is invariably bound at site B and therefore site C does not exist as an accessible binding site. Site B is generated on account of the particular mutual disposition of domain 1 and trans domain 2, obtained through the head-to-tail arrangement found in crystals 1–4. This arrangement does not occur in crystal 5. Therefore, only site A is available for nucleotide binding in the two crystallographically independent molecules in crystal 5.

3.4 Occupancy of binding sites

Crystals 1 and 2 have the same structure except that the magnesium ions in 1 are replaced by manganese ions in 2. There is clear electron density in both the crystals at site A for Ap₅A (Fig. 3A). The other terminal nucleoside points to the solvent region and is presumably disordered. Site B is occupied by an ATP molecule (Fig. 3B). It would thus appear that the crystals represent a situation when the catalysis is only partially complete, presumably on account of the limited supply of magnesium. The substrate is seen at site A and the product at site B. Crystals 3 and 4 also have the same structure except for a somewhat inconsequential difference in a short peptide stretch in domain 2. In crystal 4, which was prepared by co-crystallization of the enzyme with ATP, site A as well as site B is occupied by ATP. In crystal 3 also, both sites are occupied by ATP (Fig. 3B and Fig. 3C), although the crystal of the complex was prepared by soaking a preformed crystal in a solution containing Ap₃A, just as crystals 1 and 2 were prepared by soaking in solutions of Ap₅A. However, Ap₅A is a better substrate of the enzyme than Ap₃A and the limited quantity of available magnesium ions is sufficient to complete the hydrolysis of Ap₅A, unlike in the case of Ap₃A. The product now occupies both the sites.

Crystal 5 represents a somewhat different picture. Only site A exists and is occupied in both the crystallographically independent molecules. Presumably, part of the Ap₅A in solution is cleaved into ATP and AMP during co-crystallization. ATP so formed combines with magnesium and fluoride ions and occupies the binding site, thus preventing further binding and hydrolysis of Ap₅A. ATP along with magnesium and fluoride ions are seen at the binding site of one molecule (molecule A) (Fig. 3D); in addition, a phosphate, perhaps that of the AMP resulting from the initial cleavage of Ap₅A, is seen at the binding site of the other molecule (molecule B) (Fig. 3E). Admittedly, it is difficult to distinguish between fluoride ions and water molecules. The crystal structures of the complex of RhoA with GDP-Mg-F (Graham et al., 2002) and that of diphosphoinositol phosphatase 1 with inositol-6-phosphate-Mg-F (Thorsell et al., 2009) were substantially relied upon for identifying the fluoride ions. The other appropriate peaks in the neighbourhood were identified as water molecules as they are in the proximity of residues with acidic side chains and were therefore unlikely to correspond to fluoride ions. It is also interesting that each fluoride ion interacts with at least two magnesium ions (see later). Thus, the identification of fluoride ions and water molecules in the ligand binding region makes eminent chemical sense and is in consonance with the results of earlier investigations on similar structures.

3.5 Protein-ligand interactions

The interactions of Ap₃A at site A with the protein in crystal 1 are illustrated in Fig. 4. Those in crystal 2 are exactly the same except for the replacement of the three magnesium ions by manganese ions. The basic side-chains of Arg55, Lys65, Lys67 and Lys108 and the side-chain hydroxyls of Tyr58 and Tyr101 interact with the phosphate tail. The side-chain hydroxyl of Tyr145 is hydrogen-bonded to O2’ of the sugar moiety while the aromatic side-chain of Tyr58 is involved in hydrophobic interaction with it. The nitrogen base, sandwiched between the aromatic side-chains of Tyr101 and Tyr145, has stacking interaction with them. No residue from trans domain 2 directly interacts with the ligand. However, water bridges occur between the main-chain O atoms of Gly272 and Lys273 of trans domain 2 on the one hand and the sugar moiety of the ligand at site A on the other hand. These water bridges are absent in crystal 5 which does not exhibit domain1-trans domain 2 proximity of the kind found in other crystals. However, the Pro124–Val128 stretch and in particular Glu127, in this crystal, is oriented close to the phosphate tail of the nucleotide. As will be seen later, this residue, along with others, has role in catalysis. The environment of ATP at site A in crystals 3 and 4 (Fig. 5) is very similar to that of the ATP component of Ap₅A in crystals 1 and 2. Mg₂⁺ in the latter is now replaced by a water molecule. The site occupied by Mg₃⁺ which interacts with the terminal phos-
phosphates of ATP, and those of two flanking water molecules are vacant in crystals 3 and 4.

Interactions of ATP at site B with the protein are almost same in crystals 1–4 (Fig. 6). Here again, the major interactions of the bound nucleotides with the protein is through their phosphate tails. The basic side-chains of Arg169, His170, Arg176, Arg186, Arg218 and Lys297 are involved in interactions with the phosphate tail. The side-chain N atom of Gln271 and the main-chain O atom of Gly272 also interact with the phosphate tail. The base has stacking interaction with the planar guanidinium group of Arg176. The base also has hydrogen-bonded interactions with the main-chain O atoms of Ser177 and Tyr179 and side-chain of Lys273. Interestingly, the short 144–146 stretch in domain 1, part of which interacts with ligand at site A, is close to the nucleoside moiety at site B.
The side chain of Gln144 forms a hydrogen bond with a hydroxyl group of the sugar. The side-chains of Tyr145 and Pro146 of this stretch form a hydrophobic wall on one side of the nucleoside.

The interactions of ATP-Mg-F at site A in molecule A (Fig. 7A) and molecule B (Fig. 7B) in crystal 5 are of particular interest in relation to the fluoride mediated inhibition of Ap4A hydrolysis by MsMutT1. Interactions of ATP with the enzyme are substantially the same in crystal 5 and in crystals 1–4. Those involving the terminal phosphate group and the magnesium and fluoride ions in crystal 5 are of special interest. In both the molecules, the locations of the magnesium ions are the same as those in the complex of the enzyme with Ap2A. A fourth magnesium ion, Mg4, now appears at the binding site. It interacts directly with the terminal phosphate of ATP. The water molecule that bridges Mg2 and Mg3 in the complex with Ap2A is now replaced by a fluoride ion (F2). Another fluoride ion, F1, is situated at the middle of a triangle formed by Mg1, Mg2 and Mg4. The location of this ion is close to a water molecule that coordinates to Mg1 in the ATP complex. Yet another fluoride ion, F3, bridges Mg3 and Mg4. There are a couple of differences between molecules A and B in the coordination of Mg3 and Mg4. Side-chain OD1 of Asp182 from domain 2 coordinates to Mg4 while OD2 of the same residue interacts with Mg3, in molecule A. A phosphate O atom of (presumably) AMP coordinates to Mg4, in molecule B. A water molecule now occupies the position of OD2. Yet another small difference is the presence of an additional water molecule in the coordination sphere of Mg4 in molecule A.
These differences lead to somewhat different coordination geometries of Mg4 in the two molecules. While that in molecule A is roughly octahedral, that in molecule B can be described as trigonal bipyramidal.

Locations of the ligands at site A in different crystals are remarkably similar (Fig. 8). The ATP moiety common to all of them superpose well. P4 of the fourth phosphate of Ap5 in crystals 1 and 2 superpose on Mg4 associated with molecules A and B of crystal 5. F1 and F3 occur at the O atom positions of this phosphate. The fifth phosphate has a location similar to that of the free phosphate group (probably part of AMP) in molecule B of crystal 5. F2 superposes on the functionally important water molecule, W1, of crystals 1 and 2. F1 is close to water molecule W2 of crystals 3 and 4. It is also very interesting to compare the locations of nucleotides at the two binding sites in the present structures with those in complexes involving 8-oxo-guanosine nucleotides (Fig. 9). At site A, the phosphate tail and the sugar of 8-oxo-GTP and ATP in their respective complexes, superpose almost exactly. The bases also occupy nearly the same region, except that 8-oxo-guanine is in the sym conformation while adenine is in the anti conformation. ATP occupies site B in the present structure. The nucleotide that occupies in the other set of complexes is 8-oxo-dGDP (or 8-oxo-GDP). The γ- and β-phosphates of ATP superpose on the β- and α-phosphates, respectively, of 8-oxo-dGDP. The bases of the two nucleotides are close to each other, with a small lateral shift and a change in orientation between them. The extra (γ-) phosphate in ATP is accommodated in the binding site by the molecule adopting a bent conformation. Consequently, this phosphate and sugar moiety do not superpose on any region of 8-oxo-dGDP.

3.6. Mechanism of action

The structure of the MsMutT1-Ap5A complex (crystal 1) suggests a plausible mechanism of the hydrolysis of Ap5A to yield ATP as the common product. At site A in the structure (Fig. 4), Glu127 and Glu81 bridge Mg1 and Mg2, and Mg2 and Mg3, respectively. A water molecule (W1) held between Mg2 and Mg3 is positioned appropriately for an in-line nucleophilic attack on P4 (P5) of the substrate and could be activated by the two metal ions and deprotonated by Glu84 (general base) before nucleophilic attack. This water is at a contact distance of 3.3 Å from P5 and forms an angle (W1-P5-O3G) of 170.5°, consistent with the criteria for associative mechanism (Mildvan, 1997). Counterparts of Glu81, instead of Glu84, in other Ap5A hydrolases have been suggested to be involved in such activation of a water or a hydroxide nucleophile (Mildvan et al., 2005). Since both the O atoms of Glu81 are involved in bridging Mg2 and Mg3, it is unlikely but not impossible that this residue acts as an activator of a water or a hydroxide nucleophile. Arg80, held in its position by Glu72, might orient the general base Glu84/Glu127 correctly to facilitate the activation of the attacking water. Mg1, coordinated by the main-chain O atom of Lys65 and a side-chain O atom of Glu85, may promote the cleavage of sessile bond by first stabilizing the negatively charged phosphonate intermediate and later by neutralizing the developing negative charge on the phosphonyl group of the leaving product (ATP) before translocation to site B in a manner suggested previously for NDP product (Arif et al., 2017). The negative charge on the product (ATP) might be further neutralized by the positively charged side-chains of Arg55, Lys65 and Lys108, all oriented to interact with the γ-phosphate. Lys67, with its side-chain NZ positioned nearest to the leaving O atom (O3G), might be the candidate for proton donor or Lewis acid. Mg2, coordinated by Glu81, Glu85 and Glu127 might have a role similar to that of Mg1 in stabilizing the intermediate, in addition to holding the water nucleophile in position and promoting its nucleophilicity. Mg3, held in position by Glu81, interacts with the fourth and the fifth phosphate of the Ap5A moiety. It is possible that this metal ion assists the departure of the second product (AMP, ADP or ATP) from the opposite end after the breakage of the sessile bond, in addition to holding and promoting the nucleophilicity of the water. This is analogous to the role of a second metal ion in the release of pyrophosphate product as Mg2PPI in the reaction mechanism of E. coli MutT (Mildvan et al., 2005).

The structure of and interactions at site A in crystal 3 (and crystal 4) suggest a mechanism for the hydrolysis of ATP into ADP and phosphate. This mechanism is similar to that proposed earlier for the hydrolysis of 8-oxo-(d)GTP to 8-oxo-(d)GDP by MsMutT1 (Arif et al., 2017). The lone magnesium, Mg1, coordinated by a γ-phosphoryl O atom of ATP, the backbone O atom of Lys65 and the side-chain of Glu85, holds a water molecule (W2) in a position suitable for nucleophilic attack on Pγ of the ATP (Fig. 5). Glu81, which interacts with Arg80, could deprotonate and hence activate this water for the nucleophilic attack and thus might be the general base. Arg80, in turn is fixed at its location by Glu72. Arg55, positioned appropriately to donate a proton to the leaving O atom (O3B), might be the candidate for Lewis acid. The basic side-chains of Lys65, Lys67 and Lys108 might stabilize the leaving product (ADP) by neutralizing the developing charge on it after bond breakage.

The structure of crystal 5 provides an explanation for the role of ATP-Mg-F as a mimic/transition state analogue of Ap5A or as an inhibitor of Ap5A hydrolysis. As mentioned earlier, Mg4 occupies the same location as P4 in Ap5A and fluoride ions occupy the positions of the two O atoms attached to P4. Interestingly, what appears to be the phosphate group of the leaving AMP in molecule B of crystal 5 occupies a location close to the fifth phosphate in Ap5A. This could conceivably represent an intermediate stage of hydrolysis of Ap5A by MsMutT1. The water molecule (W1) involved in the nucleophilic attack leading to the generation of ATP is now replaced by a fluoride ion (F2). W2, which appears to take part in the hydrolysis of ATP, does not exist in the ATP-Mg-F complex. Instead a fluoride ion (F1) is located in the neighbourhood. Therefore the reaction that results in ATP as well as the one that leads to hydrolysis of ATP is inhibited by fluoride ions. The fact that comparable configurations of magnesium and fluoride ions leading to inhibition have been reported in other enzymes including Ap5A hydrolases, (Coleman et al., 1994; Fletcher et al., 2002; Graham et al., 2002; Park et al., 1999) provides added confidence to the above interpretation.
4. Conclusions

Mycobacteria encounter immense oxidative stress inside the macrophage. Oxidative damage can be harmful to DNA and RNA as it can lead to mutations. Moreover, intracellular level of diadenosine polyphosphates (Ap₄A) has been shown to increase during oxidative stress (Bochner et al., 1984; Lee et al., 1983). Increased level of Ap₄A has been associated with reduction in the invasion capacity of many pathogenic bacteria (Ismail et al., 2003; Mitchell and Minnick, 1995). A similar possibility in the case of mycobacteria cannot be ruled out. The pathogen must evolve and develop an efficient mechanism to deal with such detrimental consequences of oxidative stress. Protective role of mycobacterial Nudix hydrolases against the consequences of oxidative stress has been suggested and proved earlier. For instance, gene knockout studies have shown that MutT1, that hydrolyses the mutagenic oxidized nucleotide, 8-oxo-dGTP, has antimutator role (Dos Santos studies have shown that MutT1, that hydrolyses the mutagenic oxidized nucleotide, 8-oxo-dGTP, has antimutator role (Dos Vultos et al., 2006). A concerted action of MutT1 and another Nudix hydrolase, RV1700 from M. tuberculosis, has been shown to provide maximal rescue to MutT deficient E. coli by decreasing A to C mutations and thus, is suggested to have a crucial role in survival of the bacteria against oxidative stress (Patil et al., 2013). More recently, RenU (MutT3), another Nudix hydrolase from mycobacteria, has been shown to be necessary for the survival of the pathogen in oxidative stress environment inside macrophages (Wolff et al., 2015). The present elucidation of the activity of MsMutT1 against Ap₄A and other diadenosine polyphosphates extends the understanding of the involvement of MutT1 beyond an antimutator role as proposed earlier. The high level of homology between MsMutT1 and MtMutT1 strengthens the relevance of studies of the M. smegmatis enzyme to pathogenicity. It might strengthen the consideration of MutT1 as a potential target for the development of inhibitors/drugs. Furthermore, the presence of an additional domain, which binds to the product only, differentiates the mycobacterial enzyme from the corresponding human enzyme and widens the scope of exploration of specific sites to target.

In the context of the above observations, it is interesting that the biochemical and structural studies presented here provide a plausible explanation of the catalytic activity of mycobacterial MutT1 against diadenosine polyphosphates. In particular, the work leads to a detailed characterization of the binding sites of the enzyme. The elucidation of the molecular details of the interaction of ATP-Mg-F with enzyme might also provide supplementary information for the design of inhibitors against mycobacterial MutT1.

Accession numbers

Coordinates and structure factors of the five crystal structures reported in this manuscript have been deposited in the Protein Data Bank with accession numbers 5XD1, 5XD2, 5XD3, 5XD4 and 5XD5.

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