Structural and Biochemical Insight into the Mechanism of Rv2837c from *Mycobacterium tuberculosis* as a c-di-NMP Phosphodiesterase*

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The intracellular infections of *Mycobacterium tuberculosis*, which is the causative agent of tuberculosis, are regulated by many cyclic dinucleotide signaling. Rv2837c from *M. tuberculosis* is a soluble, stand-alone DHH-DHHA1 domain phosphodiesterase that down-regulates c-di-AMP through catalytic degradation and plays an important role in *M. tuberculosis* infections. Here, we report the crystal structure of Rv2837c (2.0 Å), and its complex with hydrolysis intermediate 5′-pApA (2.35 Å). Our structures indicate that both DHH and DHHA1 domains are essential for c-di-AMP degradation. Further structural analysis shows that Rv2837c does not distinguish adenine from guanine, which explains why Rv2837c hydrolyzes all linear dinucleotides with almost the same efficiency. We observed that Rv2837c degraded other c-di-NMPs at a lower rate than it did on c-di-AMP. Nevertheless, our data also showed that Rv2837c significantly decreases concentrations of both c-di-AMP and c-di-GMP in vivo. Our results suggest that beside its major role in c-di-AMP degradation Rv2837c could also regulate c-di-GMP signaling pathways in bacterial cell.

*Mycobacterium tuberculosis* is an intracellular pathogen that causes tuberculosis, which remains one of the world’s deadliest communicable diseases. There were an estimated 9.0 million people developed tuberculosis in 2013, and 1.5 million people died from the infection (1). The infections of *M. tuberculosis* are regulated by many signaling molecules, in which c-di-NMPs act as triggers for innate immune during tuberculosis infection (2).

c-di-NMPs (c-di-AMP, c-di-GMP, and c-GAMP) have been known to get involved in the regulation and coordination of many cellular processes (3–5). c-di-GMP regulates processes such as biofilm formation, motility, virulence, cell cycle, and type I interferon stimulation (6–9). c-di-AMP regulates many cellular processes like sporulation, fatty acid synthesis, cell wall homeostasis, DNA damage response, potassium transport, and virulence in a wide variety of organisms (10–15). Cellular c-di-AMP is generated by diadenylate cyclases containing DisA_N domain (PFam accession PF02457) that produce c-di-AMP from two molecules of ATP or ADP. Specific phosphodiesterases (PDE) associated with DHH-DHHA1 or HD domain hydrolyze c-di-AMP into pApA or AMP (16–18). Recently, many proteins with PDE activity have been identified. One group is GdpP-like protein family, which usually contains a tandem PAS domain, a degenerate GGDEF domain, and a catalytic DHH-DHHA1 domain (19). The other group is recently discovered PgpH protein family, which contains an extracellular 7TM receptor-like domain and a cytoplasmic HD domain (18). They all hydrolyze c-di-AMP into linear dinucleotide 5′-pApA. There is also a PDE family that only contains a stand-alone DHH-DHHA1 domain, they hydrolyze c-di-AMP in a two-step process: first to linear 5′-pApA, which is subsequently hydrolyzed into two 5′-AMP molecules (16, 17, 20). All types of PDE enzymes require ions for catalytic activity. The degradation mechanism of HD domain has been well studied, but how DHH-DHHA1 domain degrades c-di-AMP is not clarified.

*M. tuberculosis* only harbors a single cytosolic c-di-AMP phosphodiesterase Rv2837c, which contains a stand-alone DHH-DHHA1 domain. It does not encode GdpP homologous proteins or HD domain proteins. Rv2837c exhibits bifunctional 3′-5′ exonuclease, as well as CysQ-like phosphatase (pAp) activity (21). It hydrolyzes different RNA substrates or single-stranded DNA in the 3′-5′ direction. The 2-mer oligonucleotides are preferred substrates of Rv2837c (21, 22). It has been reported that deletion of Rv2837c led to reduced virulence in *M. tuberculosis* infections (23). Rv2837c hydrolyzes c-di-AMP efficiently in vitro, but it has low activity on c-di-GMP (15). It is clear that Rv2837c plays an important role in maintaining c-di-AMP homeostasis. Because *M. tuberculosis* has very few enzymes for c-di-GMP metabolism (24), there is the possibility that Rv2837c also plays a role in c-di-GMP homeostasis.

In this study, we first present the structure of Rv2837c at 2.0 Å resolution. We then obtained the structure of Rv2837c in complex with the c-di-AMP hydrolysis intermediates 5′-pApA.
by soaking Rv2837c crystal with c-di-AMP. The complex structure presents a precise enzymatic model that the DHH domain has the PDE activity catalytic core and the DHHA1 domain contributes to substrate recognition and substrate stabilization. Structure analyses combined with biochemistry studies allow us to propose a two-metal ion catalytic mechanism for c-di-NMP hydrolysis. Moreover, with the support of in vivo data, we speculate that Rv2837c may also regulate c-di-GMP signaling pathways beside its major in down-regulating intracellular c-di-AMP concentration.

Experimental Procedures

Cloning and Site-directed Mutagenesis—The genes encoding full-length Rv2837c and the Rv2837c DHH domain which contains amino acids 1–206 were amplified by PCR from the M. tuberculosis H37Rv genomic DNA. To get the crystal, the Rv2837c fragment which contains amino acids 10–336 with an additional amino acids sequence (LTRAPPPPPLRSGC) in the C-terminal (designated “Rv2837c-C”) was also PCR-amplified. These PCR products were cloned into the BamHI and XhoI sites of pET-28a-SUMO vector. With Rv2837c-p-pET-28a-SUMO as the template, all mutants were produced using the QuikChange site-directed mutagenesis and were confirmed by DNA sequencing. All constructed vectors were overexpressed in Escherichia coli strain BL21 (DE3).

Protein Expression and Purification—For protein purification, the transformed cells were incubated in LB medium at 37 °C to an A600 of 0.8. Following overnight induction with 0.12 mM isopropyl-β-D-thiogalactopyranoside at 16 °C, the cells were harvested at 6000 × g for 15 min at 4 °C. The pellet was resuspended in 25 mM Tris-HCl buffer at pH 8.0 containing 200 mM NaCl and was lysed by sonication. After ultracentrifugation at 28,370 × g for 45 min at 4 °C, the supernatant was loaded onto nickel-nitrilotriacetic acid affinity resin (Qiagen) for affinity chromatography of the N-terminal His-SUMO-tagged protein and was eluted with 25 mM Tris-HCl buffer at pH 8.0 containing 100 mM NaCl and 250 mM imidazole. The eluted protein was incubated with the protease UlpI at a 100:1 ratio (w/w) and dialyzed against the reaction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl) at 4 °C overnight. The cleavage mixture was loaded onto nickel-nitrilotriacetic acid affinity resin (Qiagen) for affinity chromatography of the N-terminal His-SUMO-tagged protein and was eluted with 25 mM Tris-HCl buffer at pH 8.0 containing 100 mM NaCl and 250 mM imidazole. The eluted protein was incubated with the protease UlpI at a 100:1 ratio (w/w) and dialyzed against the reaction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl) at 4 °C overnight. The cleavage mixture was loaded onto nickel-nitrilotriacetic acid affinity resin (Qiagen) for affinity chromatography of the N-terminal His-SUMO-tagged protein and was eluted with 25 mM Tris-HCl buffer at pH 8.0 containing 100 mM NaCl and 250 mM imidazole. The eluted protein was incubated with the protease UlpI at a 100:1 ratio (w/w) and dialyzed against the reaction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl) at 4 °C overnight. The cleavage mixture was loaded onto nickel-nitrilotriacetic acid affinity resin (Qiagen) for affinity chromatography of the N-terminal His-SUMO-tagged protein and was eluted with 25 mM Tris-HCl buffer at pH 8.0 containing 100 mM NaCl and 250 mM imidazole. The eluted protein was incubated with the protease UlpI at a 100:1 ratio (w/w) and dialyzed against the reaction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl) at 4 °C overnight. The cleavage mixture was loaded onto nickel-nitrilotriacetic acid affinity resin (Qiagen) for affinity chromatography of the N-terminal His-SUMO-tagged protein and was eluted with 25 mM Tris-HCl buffer at pH 8.0 containing 100 mM NaCl and 250 mM imidazole. The eluted protein was incubated with the protease UlpI at a 100:1 ratio (w/w) and dialyzed against the reaction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl) at 4 °C overnight. The cleavage mixture was loaded onto nickel-nitrilotriacetic acid affinity resin (Qiagen) for affinity chromatography of the N-terminal His-SUMO-tagged protein and was eluted with 25 mM Tris-HCl buffer at pH 8.0 containing 100 mM NaCl and 250 mM imidazole. The eluted protein was incubated with the protease UlpI at a 100:1 ratio (w/w) and dialyzed against the reaction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl) at 4 °C overnight.

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the measurements (all at 25 °C). Far UV spectra were recorded from 250 to 190 nm (1-nm bandwidth, 1-nm step, and 0.5-s interval) Rv2837c and its mutants were diluted in Superdex buffer (10 mM Tris-HCl, pH 8.0, with 100 mM NaCl) to a final concentration of 1.0 mg ml\(^{-1}\). During data processing, the spectrum of the “protein-free solution” was subtracted from other spectra as buffer background.

**Synthesis of c-di-AMP**—c-di-AMP was synthesized by 2 mM ATP with 10 \(\mu\)M of the protein DncV in reaction buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, and 10 mM Mg\(\text{Cl}_2\)) at room temperature overnight (31). The reaction was incubated for 10 min at boiling water and centrifuged at 28,370 \(\times\) \(g\) for 30 min to remove protein. The supernatant was separated and purified by HPLC with a C18 column (21.2 \(\times\) 250 mm; Sapphire). The following buffers were used in the gradient program: buffer A (10 mM NH\(_4\)Ac, pH 5.0) and buffer B (100% methyl cyanide). The following protocol was used for separation (the values are times in minutes and percentage of buffer B used): 0.0, 3%; 20.0, 30%; 25.0, 30%; 26.0, 3%; 38.0, 3% at a flow rate of 10 ml min\(^{-1}\); with a diode array detector at 254 nm, the samples containing c-di-AMP were gathered. The organic solvent was removed by distillation, and then pure sample powder was acquired by freeze-thaw cycles.

**Crystallization of c-di-AMP**—The purified c-di-AMP was diluted in 5 mM, the crystal of c-di-AMP was grown in solution containing equal volume of c-di-AMP solution and 3.5 M ammonium chloride, 0.1 M sodium acetate trihydrate, pH 4.6, using the sitting drop method. The details of data collection and structure determination are not shown.

**Motility Assays**—*E. coli* MG1655 overnight cultures grown in LB medium at 42 °C without shaking were diluted to the same concentration. 0.5 \(\mu\)l of each were inoculated directly into LB agar (0.5% agar) and incubated for 10h at 42 °C, and the diameter of the diffuse growth zone was measured.
**Results**

**Rv2837c Is a c-di-AMP Phosphodiesterase**—Size exclusion chromatography analysis indicated that Rv2837c (molecular mass = 34 kDa) forms a stable dimer in solution (Fig. 1A). To directly examine Rv2837c phosphodiesterase activity toward c-di-AMP, we incubated the protein with c-di-AMP under different conditions and analyzed the reactions using HPLC. Our results showed that Rv2837c hydrolyzed both c-di-AMP and 5‘-pApA into 5‘-AMP quickly (Fig. 1B). Co²⁺ or Mn²⁺ is necessary for Rv2837c PDE activity toward c-di-AMP (Fig. 1C), with Co²⁺-associated catalytic activity being as twice high as that of Mn²⁺. In contrast, Streptococcus pneumoniae Pde2 binding Mn²⁺ has the highest efficiency (16). The enzymatic activity of Rv2837c adapts to a broad pH range (Fig. 1D) and peaks at pH 8.5.

**Overall Structure of Rv2837c**—The final model of Rv2837c is refined to 2.0 Å resolution and contains one Rv2837c molecule in an asymmetric unit (Fig. 2A). Each monomer misses 15 residues at the N terminus and has 6 extra residues of the 14-amino acids peptide at the C terminus. Two crystallographic symmetry related monomers make up the biologically active dimer (Fig. 2C). The details of the data collection and refinement statistics are given in Table 1. The dimeric assembly of Rv2837c resembles its homologue, MSMEG-2630, which has the unique subunit packing and large domain interface (22).

A monomer of Rv2837c consists of two distinct domains (Fig. 2A). Its N-terminal DHH and C-terminal DHHA1 domains consist of residues 15–202 and 220–336, respectively. Two domains are linked by a longer flexible loop. The DHH domain has a five-parallel strand β-sheet (β1–5), which is sandwiched by 10 α-helices (with α1, α2, α3, α4, α5, α6, and α7 on one side and α5, α6, and α7 on the other side). Structurally similar to DHH, the DHHA1 domain has an anti-parallel β-sheet (β6–β10) that is sandwiched by five α-helices (with α12–α14 and...
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TABLE 1
X-ray diffraction data collection and refinement statistics

|                      | Rv2837c-C native | Rv2837c-C/5'-pApA | Rv2837c-C SAD |
|----------------------|-----------------|------------------|--------------|
| Data collection      |                 |                  |              |
| Wavelength (Å)       | 0.9791          | 0.9791           | 0.9791       |
| Space group          | P2,2,2          | P2,2,2           | P2,2,2       |
| a, b, c (Å)          | 58.9, 98.6, 55.1| 58.4, 99.3, 108.7| 58.6, 98.2, 54.6|
| α, β, γ (°)          | 90.0, 90.0, 90.0| 90.0, 90.0, 90.0| 90.0, 90.0, 90.0|
| Resolution (Å)*      | 50–2.0 (2.07–2.00)| 50–2.33 (2.43–2.35)| 50–2.10 (2.18–2.10) |
| Total no. of reflections | 160,863        | 148,741          | 213,723      |
| No. of unique reflections | 22,675       | 25,317           | 15,582       |
| Completeness (%)     | 99.9 (100.0)    | 96.1 (100.0)     | 99.5 (100.0) |
| Redundancy*          | 7.1 (7.2)       | 5.9 (6.8)        | 7.4 (7.5)    |
| I/σ(I)*              | 23.94 (5.19)    | 21.03 (16.31)    | 27.76 (18.20) |
| Rmerge (%)           | 8.4 (45.3)      | 8.3 (16.9)       | 12.5 (37.6)  |

Refinement

|                      | Rwork/Rfree (%) |
|----------------------|-----------------|
| No. atoms            | Rwork/Rfree (%) |
| Protein              | 19.60/22.90     |
| 5'-pApA              | 2.425           |
| Mn*                  | 34.78           |
| Water                | 28.01           |
| Average B-factors (Å²)| 38.00           |
| Protein              | 175             |
| 5'-pApA              | 34.78           |
| Mn*                  | 28.01           |
| Water                | 38.00           |
| Root mean square deviations |
| Bond lengths (Å)     | 0.007           |
| Bond angles (°)      | 1.070           |
| Ramachandran plot (%)| 96.32           |
| Allowed              | 3.68            |
| Generally allowed    | 0               |

α15–α16 on both sides, respectively). The high average B-factor of the DHHA1 domain suggests that it is more flexible than DHH domain (Fig. 2B). The two active site-binding Mn2+ ions of the enzyme were observed in DHH domain.

A DALI search (32) for the homologues of Rv2837c was performed against the Protein Data Bank (PDB). The output includes a number of DHH superfamily proteins, with the closest homologue being MSMEG-2630 (Z score = 39.6, root mean square deviation = 4.9 Å for 319 Ca atoms, PDB entry 4LS9). Structural comparison of Rv2837c with MSMEG2630 indicates that the high root mean square deviation comes from domain shift (Fig. 3A). Structure analysis shows that the space between the DHH and DHHA1 domain in MSMEG-2630 is much larger than that in Rv2837c. Previous studies have shown that MSMEG-2630 has unique subunit packing that leads to the ability to bind many different substrates (22). Rv2837c is also a versatile enzyme that can hydrolyze various substrates, such as nanoRNA, single-stranded DNA, pAp, and c-di-AMP (15, 21, 22, 33). The long linker region between DHH and DHHA1 domains may give Rv2837 flexibility to bind various substrates.

Two-metal Ion Active Site of Rv2837c—The active site of Rv2837c is characterized by two Mn2+ ions (Mn1 and Mn2). A water molecule (Wat1) serves as a bridge to connect these two Mn2+ ions. Both Mn1 and Mn2 are present in an octahedral geometry with six coordination states. Mn1 is coordinated by Asp47, Asp106, His131, Asp181, Wat1, and Wat3. Mn2 is coordinated by His24, Asp45, Asp106, Wat1, and Wat4 (Fig. 2D). The six chelating residues of Rv2837c are all conserved in the DHH-DHHA1 domain proteins (Fig. 4). We have mutated these residues into alanine and found the mutations had greatly reduced the enzymatic activity of Rv2837. Mutations on Mn12+ ligands, D47A, D106A, H131A, and D181A completely abolished the catalytic activity, indicating their importance in maintaining the enzymatic activity. In contrast, mutations on Mn22+ ligands, such as H41A and D45A, do not eliminate Rv2837c activity completely. The above-mentioned results indicate that Mn1 could be more important than Mn2 in performing Rv2837c enzymatic activity (Fig. 3B).

The 13DHH132 motif of Rv2837c has been found highly conserved in all DHH superfamily members, the right conformation of the 13DHH132 loop, which enables His131 to coordinate Mn2+. Accordingly, both D130A and H132A completely lose their catalytic activity (Fig. 3B). CD spectra of these mutants indicate that the losses of catalytic activity are not due to misfolding of Rv2837c (Fig. 3C). The structure and our biochemical data also suggest that Rv2837c follows a two-metal ion catalytic mechanism.

Structure of Rv2837c in Complex with 5'-pApA—The structure of Rv2837c in complex with 5'-pApA (Table 1) reveals a dimer similar to the previous structure (Fig. 2C), with an exception that the side chain of Arg112 shifts dramatically to stabilize 5'-pApA. In addition, two water molecules, Wat2 and Wat3 in coordination with the two Mn2+ ions in the substrate-free structure are replaced by two oxygens of phosphate-2 from a 5'-pApA molecule (Fig. 3D). The 5'-pApA molecule, an intermediate of the hydrolysis of c-di-AMP to AMP, was unambig-
uously identified by the difference electron density. It sits in a large tunnel between the DHH and DHHA1 domains (Fig. 5A) and is stabilized by several kinds of interactions. The nonbridging oxygen of the esterase phosphate group of 5′-pApA directly chelates two Mn$^{2+}$ ions. The two adenine bases of 5′-pApA molecule are perpendicular to each other, with nucleotide A1 being sandwiched by the 309GGGH312 motif and Arg$^{112}$. The adenine base of A1 is readily oriented by the cation–π interaction with Arg$^{112}$. The phosphate group of A1 forms H-bonds with Ser$^{292}$ and the conserved Arg$^{294}$. The adenine base of nucleotide A2 is positioned at one end of substrate binding tunnel and forms a π-π interaction with the benzene group of His$^{312}$, the signature residue of the conserved 309GGGH312 motif (Fig. 5A). Notably, His$^{312}$ forms an H-bond with the phosphate group of the phosphodiester bond of 5′-pApA. The two ribose groups of 5′-pApA have no interaction with protein.

The Binding of c-di-AMP to Rv2837c Based on Molecular Docking—The structure of 5′-pApA binding Rv2837c provides very important information for us to understand the second step of the catalytic reaction. However, to clarify the whole process of the reaction, we need to know how c-di-AMP reacts with the enzyme. Because it is difficult to get the structure of Rv2837 in complex with c-di-AMP because of its fast transition into 5′-pApA, we modeled the interaction of c-di-AMP with Rv2837 by the AUTODOCK program (version 4.2) (34), based on the structure of Rv2837c in complex with 5′-pApA (Fig. 5B).

In the calculated model of c-di-AMP-Rv2837 complex, the central ring of c-di-AMP compared with that of 5′-pApA, turns 180° horizontally around the center of the molecule. At this moment, the 5′-phosphate group of nucleotide A1 in the c-di-AMP has not been cut off from the nucleotide A2 and is bound to two Mn$^{2+}$ ions. Similar to the 5′-phosphate group in the
5′-pApA, the other phosphate group is stabilized by Ser292 and Arg294. Two adenine groups of c-di-AMP are sandwiched by the same residues as those in the Rv2837c/5′-pApA complex and the two ribose groups of c-di-AMP do not show any major interaction with their surrounding residues.

Because c-di-AMP has two symmetrical phosphodiester bonds, it has to take two steps to hydrolyze both phosphodiester bonds to produce AMP finally. Our molecular docking result, in combination with the structure of Rv2837c/5′-pApA, suggests that a flip should happen to the substrate between the two reaction steps: a c-di-AMP first binds to the large tunnel of Rv2837c. One phosphodiester bond in contact with two Mn2+/H11032 ions is then hydrolyzed and results in the linear 5′-pA1pA2. This intermediate product will flip its ribose and phosphate groups to make the second phosphodiester bond contact the catalytic center of Rv2837c, which was captured in the Rv2837c/pApA complex structure. Finally, the second phosphodiester bond breaks and releases two AMP molecules (Fig. 5).

**Molecular Mechanism of PDE Activity of Rv2837c**—To evaluate the proposed reaction catalyzed by Rv2837c from c-di-AMP into AMP, we have conducted a series of mutagenesis study on residues involved in the catalytic process. Our data showed the mutants that affect substrates/intermediates binding usually impaired enzymatic activity. Mutations of H292A, R294A, G309R, and H312A decrease catalytic capability of Rv2837c dramatically. Mutations of R112A, W187A/H312A, G309R/G310R/G311R, and deletion of the DHHA1 domain revoked PDE activity (Fig. 6A). Intriguingly, mutation of W187A resulted in a doubled catalytic activity compared with WT protein. Because Trp187 interacts with the A2 adenine group, W187A may produce a spacious room to expedite the turnover of 5′-pApA.

Because 5′-pApA is a perfect nanoRNA, the complex structure of Rv2837c/5′-pApA also provides an excellent model of Rv2837c hydrolyzing nanoRNA. To test whether this model also applies to other nonoRNA, we use 5′-pGpG as a nanoRNA substrate to test the catalytic activity of Rv2837c (Fig. 6B). It is indicated that the hydrolysis of 5′-pGpG by Rv2837c is quite similar to the hydrolysis of c-di-AMP by Rv2837c in vitro. This suggests that Rv2837c degrades c-di-AMP and other nanoRNA with the same catalytic mechanism. The W187A mutant did not show any difference in hydrolyzing linear 5′-pGpG compared with Rv2837c WT, because 5′-pGpG, like 5′-pApA need not flip during the hydrolysis process.
The structure of Rv2387c-5'-pApA complex has several important structural features: First, Arg112 and the 309GGGH312 motif stabilize the binding of 5'-pApA through cation-π, H-bonds, and π-π interactions. Second, Mn$^{2+}$ ions are directly chelated by the side chains of residues His41, Asp45, Asp47, Asp106, His131, and Asp181, as well as the two oxygens of 5'-pApA phosphate-2. Third, the catalytic water molecule WAT1, bonding with both Mn$^{2+}$ ions, is only 3.0 Å from the phosphate group. Finally, the carboxyl group of Asp181 interacts with both Mn$^{2+}$ and WAT1. The important role of Asp181 in activating the WAT1 has been confirmed by the destructive effect of its mutation D181A on the catalytic activity of Rv2837c (Fig. 3B).

Based on the structure of Rv2837c-5'-pApA complex and biochemical data, we propose a simplified catalytic mechanism for phosphodiester bond hydrolysis by Rv2837c: the WAT1 molecule is activated by Asp181, together with two Mn$^{2+}$ ions, generating a nucleophile to attack the phosphorus atom of 5'-pApA$_2$. Then it attacks the 3'-phosphate-ester bond. After the reaction, the 3'-hydroxyl of nucleotide A2 is the leaving group and is likely to be protonated by a solvent water molecule. Finally, two AMP molecules are released (Fig. 3).

Rv2837c Specifically Targets 3'-5' Phosphodiester Bond—Our structure shows that Rv2837c interacts with the adenine base of 5'-pApA mainly through cation-π, H-bonds, and π-π interactions. Because guanine and adenine are structurally similar, we speculate that Rv2837c may not distinguish between 5'-pApA and 5'-pGpG or between c-di-AMP and c-di-GMP during hydrolysis. This hypothesis was first supported by molecular docking results. Both 5'-pGpG and c-di-GMP fit well into the active site Rv2837c just like 5'-pApA and c-di-AMP, respectively (Fig. 5, D and E). We then tested the PDE activities of Rv2837c toward 5'-pApA and 5'-pGpG and found that Rv2837c hydrolyzed them with all most the equivalent efficiencies (Fig. 8A).

Based on the activities of Rv2837c toward 5'-pApA and 5'-pGpG, we infer that Rv2837c may hydrolyze other c-di-NMPs as it does c-di-AMP. The relevant experiments show that Rv2837c breaks down c-di-AMP very fast and produces AMP as the final product. It can also degrade other c-di-NMPs.
but with much lower activity (Fig. 8A). Importantly, Rv2837c hydrolyzes c-di-GMP and 3′,3′-cGAMP, producing single nucleotides as the final products, whereas it can only degrade 2′,3′-cGAMP into pGpA (2′-5′), a linear dinucleotides (Fig. 6, C–E). Because c-di-AMP, c-di-GMP, and 3′,3′-cGAMP all have two 3′-5′ phosphodiester bonds and 2′,3′-cGAMP only has one, it is obvious that Rv2837c specifically targets 3′-5′ phosphodiester bond.

**FIGURE 6. Hydrolysis activities of Rv2837c and mutants for different substrates analysis.** A, effects of mutants in the DHH and GGGH motif and other residues involved in 5′-pApA binding on c-di-AMP hydrolysis. B, effects of Rv2837c mutants involved in 5′-pApA binding on 5′-pGpG hydrolysis. Different mutagenesis containing the amino acid substitutions as indicated above each lane. C–E, the same amount of Rv2837c was incubated with c-di-GMP (C), 3′,3′-cGAMP (D), and 2′,3′-cGAMP (E) 1, 1, and 2 h, respectively. The bottom panels in C–E are the diagrams showing the cleavage bonds of each c-di-NMP by Rv2837c.

Rv2837c Hydrolyzes Both c-di-AMP and c-di-GMP in Vivo—Rv2837c has long been known as a c-di-AMP regulator. However, our data show that it can also degrade c-di-GMP although with a lower activity. This raises the question of whether Rv2837c also plays a role in c-di-GMP signaling in vivo. To explore the c-di-NMP phosphodiesterase activity of Rv2837c in vivo, we overexpressed Rv2837c in *M. smegmatis* of the MSMEG_2630 deletion mutant strain. Intracellular c-di-AMP and c-di-GMP levels of ΔMSMEG_2630 and overexpression Rv2837c (OERv2837c) strains were determined by LC-MS/MS.

According to our results, intracellular c-di-AMP concentration declined very significantly (~10-fold) that it is hard to detect it in OERv2837c strain. The c-di-GMP concentration also declined significantly (~4-fold) compared with ΔMSMEG_2630 strain (Fig. 9A). This indicates that Rv2837c can hydrolyze both c-di-AMP and c-di-GMP considerably in *M. smegmatis*. Although the decline in c-di-GMP concentration is smaller than the decline in c-di-AMP concentration, it still could be large enough to produce a physiological effect. However, the potential physiological effect of c-di-GMP cannot be easily distinguished from that of c-di-AMP.

Finally we overexpressed Rv2837c in *E. coli* K-12 MG1655 strain to see whether Rv2837c can trigger a physiological effect through degrading c-di-GMP. The results showed that the intracellular c-di-GMP level of OERv2837c declined by 45% compared with wild type *E. coli* MG1655 (Fig. 9B). Because c-di-GMP is known to modulate swimming motility in various bacterial species (35), we further examined the impact of Rv2837c on this phenotype of *E. coli* MG1655. Indeed, overexpression of Rv2837c resulted in a significant increase in the swimming zone of *E. coli* MG1655 (Fig. 9C). Taken together, these observations suggest that Rv2837c may play a role in both c-di-AMP and c-di-GMP signaling in vivo.
Discussion

Rv2837c Hydrolyze Its Substrate with a Two Metal-based Mechanism—Rv2837c is a soluble, stand-alone DHH-DHHA1 phosphodiesterase from *M. tuberculosis* that hydrolyzes c-di-AMP to AMP efficiently (15). In this study, we have solved the crystal structure of Rv2837c and its complex with hydrolysis intermediate 5′-pApA. We have conducted structural and biochemical characterization of Rv2837c from *M. tuberculosis*. Our data lead to a two-metal-based mechanism for the DHH-DHHA1 to hydrolyze c-di-NMP to pNpN and finally to NMP. The two-metal ion mechanism could be conserved in the DHH-DHHA1 superfamily (20). Although few enzymatic analysis are available for the MSMEG-2630 mutants, the residue conservation of the active site makes us believe that MSMEG-2630 would also need two Mn$^{2+}$ ions in its active site. The Mn$^{2+}$ in MSMEG-2630 could be lost during the process of purification and crystallization.

Rv2837c Primarily Adopts the Flipping Mechanism—c-di-AMP has two phosphodiester bonds. According our structures, there is no way for Rv2837c to cleave the two phosphodiester bonds simultaneously. Therefore Rv2837c needs to sequentially catalyze the hydrolysis of phosphodiester bond twice before reaching the final product AMP. This raises the question of whether Rv2837c would complete the two-step reaction and produce AMP once c-di-AMP binds to its active site or release the intermediate product 5′-pApA first and finish the reaction the next time when 5′-pApA binds back to the active site (Fig. 8B). The first mechanism requires intermediate 5′-pApA to flip before it can get the remaining phosphodiester bond close to the two Mn$^{2+}$, whereas the second does not. The small amount of intermediate product (5′-pApA) observed during the reaction seems favor the second mechanism (Fig. 1B). However, the low concentration of 5′-pApA cannot simply exclude the first mechanism without quantitative study.

To clarify the catalytic mechanism, we perform a kinetic study of the c-di-AMP hydrolysis by Rv2837c. The idea is that using 5′-pApA as substrate we can measure the time evolution of both 5′-pApA and AMP and calculate the $k_m$ and the catalytic rate constant. Then with the $k_m$ and the catalytic rate constant value for 5′-pApA and the 5′-pApA concentration at a given instant in the reaction of c-di-AMP, we can calculate the theoretical reaction rate for AMP production. Suppose the second mechanism is right and the first wrong; then the theoretical reaction rate for AMP production should be comparable to the observed value. If not, that means the first mechanism also happens. The difference between the theoretical value and the observed value reflects how much c-di-AMP takes the first mechanism or the second mechanism. The detailed kinetic analysis for two mechanisms as follows. The second mechanism shows that c-di-AMP and 5′-pApA would compete for enzyme to degrade. In the reaction system, we have
c-di-AMP (Eq. 5)

\[
\begin{align*}
\text{E} + \text{c-di-AMP} & \rightleftharpoons \text{E} \cdot \text{c-di-AMP} \\
\text{E} + 5'-\text{pApA} & \rightleftharpoons \text{E} \cdot 5'-\text{pApA} \\
\end{align*}
\]

Substituting the \( k_{\text{cat}} \) and \( k_m \) values for c-di-AMP and 5'-pApA from Fig. 8A, we have

\[
\begin{align*}
\frac{v_1}{v_2} &= \frac{\left[ \text{c-di-AMP} \right] \cdot k_{\text{cat}} \cdot k_{\text{cat} \cdot \text{AMP}}}{\left[ 5'-\text{pApA} \right] \cdot k_{\text{mol}} \cdot k_{\text{mol} \cdot \text{AMP}}} \\
&= \frac{0.672 \text{ nmol} \times 129.88 \text{ mM} \times 0.23 \text{s}^{-1}}{0.068 \text{ nmol} \times 30.89 \text{ mM} \times 0.87 \text{s}^{-1}} = 10.98 \quad \text{(Eq. 7)}
\end{align*}
\]

According to c-di-AMP time evolution during the degradation (Fig. 8C), we can get the observed values for \( V_1 \) and \( V_2 \) according to c-di-AMP reduction and AMP production.

\[
\begin{align*}
\frac{v_{1,\text{obs}}}{v_{2,\text{obs}}} &= \frac{-d[\text{c-di-AMP}]}{d[t]} = \frac{1.64 \times 10^{-7} \text{ nmol} \times \text{min}^{-1}}{1.56 \times 10^{-2} \text{ nmol} \times \text{min}^{-1}} = 10.67 \quad \text{(Eq. 8)}
\end{align*}
\]

The great difference between the observed value and the calculated value strongly suggests that Rv2837c primarily adopts the flipping mechanism.

The Oligomeric State of c-di-NMPs Affects Rv2837c Activity—Rv2837c is a 3’-5’ PDEase for many c-di-NMPs. Notably, its activity on c-di-AMP is much higher than on c-di-GMP and other c-di-NMPs, because Rv2837c hydrolyzes 5'-pApA and 5'-pGpG at almost the same rate (Fig. 8A). The difference in the activities on different substrates in vitro may come from their first step of the reactions. Our structure shows that Rv2837c is unlikely to distinguish between c-di-AMP and c-di-GMP (or cGAMP) rigorously during hydrolysis. Thus we speculate that the difference may derive from the particular properties of different substrates.

It has been reported that c-di-GMP forms heterogeneous oligomers (including monomer, dimer, tetramer, and octamer) in solution (36). Because only the monomer of c-di-NMP can enter the active site, we further tested whether the difference in
reaction rate for c-di-AMP and c-di-GMP is related to the effective diffusion of their monomers into the active site of Rv2837c. We determined the crystal structure of c-di-AMP and compared it with that of c-di-GMP (37, 38). Crystal structure shows that two monomers of c-di-GMP form an interlocked dimer in crystal structure, whereas two monomers of c-di-AMP have parallel matching with each other (Fig. 10). The structure clearly shows that c-di-GMP tends to form dimer at high concentration, whereas c-di-AMP forms monomer. These data suggest that c-di-AMP is more prone to the isolated monomers than c-di-GMP in solution. In vivo all c-di-NMPs tend to exist as monomers because of their low physiological concentrations (<10 μM). The difference between hydrolysis rates for c-di-AMP and c-di-GMP would then be smaller. Indeed, the ratio of degradation of c-di-AMP to that of c-di-GMP, catalyzed by Rv2837c, significantly dropped at their diluted concentrations (Fig. 10B). Consequently, we guess Rv2837c could also play a role in c-di-GMP signaling regulation in addition to its function in c-di-AMP signaling because intracellular c-di-NMPs generally have a low concentration.

Rv2837c May Regulate Both c-di-AMP and c-di-GMP Signaling Pathways in M. tuberculosis—Both c-di-AMP and c-di-GMP are important intracellular signaling molecules, and their homeostasis processes are regulated by many proteins in bacteria cells (3–5). c-di-GMP is down-regulated by EAL domain and HD-GYP domain, resulting in 5′-pGpG and GMP, respectively (6, 39), whereas c-di-AMP is broken down by stand-alone DHH-DHHA1 domain and membrane-bound DHH-DHHA1, as well as HD domain to AMP and 5′-pApA, respectively (16, 18, 40). M. tuberculosis does not have HD-GYP domain protein and seems lack an essential link in c-di-GMP metabolism (41). On this issue, the ability of Rv2837c to hydrolyze 5′-pGpG effi-
c-di-GMP efficiently may fill this important link. What is more, Rv2837c can also use c-di-GMP as substrate with a lower activity, and our in vivo data have indicated that it decreases the intracellular c-di-GMP significantly enough to produce obvious physiological phenotype. Because there is no membrane-bound DHH-DHHA1 or HD domain protein for c-di-AMP regulation discovered so far in M. tuberculosis. Rv2837c, with its activity to down-regulate both c-di-AMP and c-di-GMP, is of central importance in regulating both signaling pathways in M. tuberculosis. Moreover, the ability of Rv2837c to degrade 2’3’-cGAMP may raise a possibility that Rv2837c works as an innate immune system suppressor during the infection of M. tuberculosis.

Author Contributions—Q. H., F. W., and L. G. designed the study. Q. H., F. W., S. L., H. C., D. Z., F. G., H. W., Z. L., J. L., and L. G. performed experiments. Q. H., F. W., J. L., and L. G. analyzed the data. Q. H. and F. W. wrote the original draft. Q. H., F. W., B. L., Z. L., J. L., and L. G. edited the paper. All authors reviewed the results and approved the final version of the manuscript.

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FIGURE 10. The oligomeric state of c-di-AMP and c-di-GMP affect Rv2837c’s activity. A, overview of the crystal structures of dimeric c-di-AMP and c-di-GMP. B, the different ratios of reaction rate of c-di-AMP to that of c-di-GMP by Rv2837c in high (200 μM) and low (10 μM) concentrations.

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