Three-dimensional structure of a mycobacterial oligoribonuclease reveals a unique C-terminal tail that stabilizes the homodimer

Oligoribonucleases (Orns) are highly conserved DnaQ-fold 3’-5’ exoribonucleases that have been found to carry out the last step of cyclic-di-GMP (c-di-GMP) degradation, that is, pGpG to GMP in several bacteria. Removal of pGpG is critical for c-di-GMP homeostasis, as excess uncleaved pGpG can have feedback inhibition on phosphodiesterases, thereby perturbing cellular signaling pathways regulated by c-di-GMP. Perturbation of c-di-GMP levels not only affects survival under hypoxic, reductive stress, or nutrient-limiting conditions but also affects pathogenicity in infection models as well as antibiotic response in mycobacteria. Here, we have determined the crystal structure of MSMEG_4724, the Orn of Mycobacterium smegmatis (Ms_orn) to 1.87 Å resolution to investigate the function of its extended C-terminal tail that is unique among bacterial Orns. Ms_orn is a homodimer with the canonical RNase-H fold of exoribonucleases and conserved catalytic residues in the active site. Further examination of the substrate-binding site with a modeled pGpG emphasized the role of a phosphate cap and “3’ OH cap” in constricting a 2-mer substrate in the active site. The unique C-terminal tail of Ms_orn aids dimerization by forming a handshake-like flip over the second protomer of the dimer. Our thermal and denaturant-induced unfolding experiments suggest that it helps in higher stability of Ms_orn as compared with Escherichia coli Orn or a C-terminal deletion mutant. We also show that the C-terminal tail is required for modulating response to stress agents in vivo. These results will help in further evaluating the role of signaling and regulation by c-di-GMP in mycobacteria.

Cyclic-di-GMP (bis-3’-5’ cyclic dimeric GMP or c-di-GMP) is one of the most widely distributed cyclic dinucleotide signaling molecules in bacteria (1–4). It was first reported as an allosteric activator of membrane-bound cellulose synthase in Komagataeibacter xylinus (formerly known as Gluconacetobacter xylinus) in 1987 (5). Since then, c-di-GMP has been discovered as a key bacterial secondary messenger, regulating several signaling networks viz., motility and biofilm formation in Pseudomonas aeruginosa (6–8), Pseudomonas fluorescens (9), Salmonella typhimurium (10), and Listeria monocytogenes (11); control of cell cycle progression and development in Caulobacter crescentus (12, 13) and Streptomyces venezuelae (14) and affects virulence and pathogenicity of several animal and plant pathogens like Vibrio cholerae (15, 16), Xanthomonas campesiter (17, 18), Klebsiella pneumoniae (19), and Mycobacterium tuberculosis (20).

Alteration of c-di-GMP levels in Mycobacterium smegmatis affects long-term survival of the bacterium under carbon-limiting conditions (21, 22) and response to antibiotics because of modulation of lipid biosynthesis (23, 24). In M. tuberculosis, increased levels of c-di-GMP reduces bacterial dormancy and decreases infectivity in human THP-1 cells and mouse infection models (20). In addition, binding of c-di-GMP to EthA (a TetR-like transcription regulator) is found to enhance binding of EthR to ethA promoter and represses the transcription of monoxygenase EthA. This results in resistance of M. tuberculosis to ethionamide, a second-line anti-tuberculosis drug, by preventing activation of the prodrug by EthA (25), further emphasizing the role of this important signaling molecule in mycobacterial cells.

In order to maintain optimal intracellular levels of c-di-GMP in the cell, a distinct set of enzymes are required. A diguanylate cyclase (DGC) that harbors a glycine-glycine-aspartate-glutamate-phenylalanine (GGDEF) domain has been shown to be required for biosynthesis of c-di-GMP in several bacteria. Degradation of c-di-GMP, may however, vary in different bacteria (1–4). In P. aeruginosa (26) and X. campesiter (27, 28), histidine-aspartate-glycine-tyrosine-proline (HD-GYP) domain containing proteins degrade c-di-GMP fully to GMP. By contrast, in Escherichia coli (29), P. aeruginosa (6, 30), and C. crescentus (31), glutamate-alanine-leucine (EAL) domain containing phosphodiesterases (PDEs) catalyze the asymmetric hydrolysis of c-di-GMP to yield linear di-GMP (pGpG), which must be further catabolized to GMP either by HD-GYP domain containing proteins or by specific exoribonucleases termed oligoribonucleases (Orns) (32–34). In mycobacteria, a bifunctional DGC having both GGDEF and EAL domains brings about biosynthesis or degradation of c-di-GMP to pGpG through the respective domains (21, 35). Degradation of c-di-GMP to pGpG may also...
Structure and stability features of Orn of M. smegmatis

be affected by an associated PDE (Rv1357c) in M. tuberculosis (20). However, there is no HD-GYP domain containing PDE in mycobacteria, emphasizing the role of Orn to bring about the final step of degradation of pGpG to GMP.

Orns are highly conserved DnaQ-fold 3'-5' exoribonucleases with a DEDDh active site motif that degrade short 2- to 5-mer oligoribonucleotides in 3'-5' direction and release monoribonucleotides as reaction products (32, 36–39). Deletion of orn led to accumulation of oligonucleotides in E. coli (40), mispriming of transcripts in P. aeruginosa (41) and affects the viability of bacterial cells (40). Deletion of orn in P. aeruginosa also exhibited reduced susceptibility toward antibacterial drugs (42–44) with reduced pathogenesis in mouse infection models (44). In mycobacteria, although effect of orn knockout has not been studied so far, dgc or pde mutant strains exhibited altered colony morphology and altered growth profile in M. smegmatis (21, 35) and reduced pathogenicity in M. tuberculosis (20).

In M. smegmatis, MSMEG_4724 is classified as the ortholog of Orn of M. tuberculosis (Rv2511), sharing an overall sequence similarity of nearly 80%. Interestingly, while bacterial Orns are usually 170 to 180 residues long, mycobacterial Orns are longer with an extended 30- to 35-residue long C-terminal tail of unknown function. However, the role of this unique extended C-terminal tail of Orn of M. smegmatis (Ms_orn), in maintaining the structure, function, or stability, has not been determined thus far. In this study, we have structurally characterized MSMEG_4724 to delineate the main structural characteristics of this mycobacterial Orn and its unique C-terminal tail in particular. Overall, this work shows that the C-terminal tail is required for higher stability of Ms_orn and in modulating response to stress agents in vivo and will help in better understanding cyclic dinucleotide-mediated signaling in mycobacteria.

Results

Protein purification and key sequence features

Ms_orn was purified to homogeneity and its purity, oligomeric state and monodispersity were confirmed on 12% SDS-PAGE, by size-exclusion chromatography (SEC) and light scattering measurements (Fig. 1). Ms_orn eluted as a single peak on a SEC column, corresponding to a dimer when compared with calibrated gel filtration data of molecular weight standards (shown in inset) ((i) bovine serum albumin, 66 kDa; (ii) ovalbumin, 45 kDa; (iii) chymotrypsinogen, 25 kDa; (iv) cytochrome c, 12.5 kDa. Ms_orn is indicated in red)).

In M. smegmatis, MSMEG_4724 is classified as the ortholog of Orn of M. tuberculosis (Rv2511), sharing an overall sequence similarity of nearly 80%. Interestingly, while bacterial Orns are usually 170 to 180 residues long, mycobacterial Orns are longer with an extended 30- to 35-residue long C-terminal tail of unknown function. However, the role of this unique extended C-terminal tail of Orn of M. smegmatis (Ms_orn), in maintaining the structure, function, or stability, has not been determined thus far. In this study, we have structurally characterized MSMEG_4724 to delineate the main structural characteristics of this mycobacterial Orn and its unique C-terminal tail in particular. Overall, this work shows that the C-terminal tail is required for higher stability of Ms_orn and in modulating response to stress agents in vivo and will help in better understanding cyclic dinucleotide-mediated signaling in mycobacteria.

Figure 1. Purification and size estimation of Ms_orn. A, electrophoretic profile of purified Ms_orn on a 12% SDS-PAGE indicates a band of high purity with apparent M_w of 23 kDa. B, SEC profile of Ms_orn on Superdex-75 column. Ms_orn eluted as a single peak at 10.1 mL, corresponding to a dimer when compared with calibrated gel filtration data of molecular weight standards (shown in inset) ((i) bovine serum albumin, 66 kDa; (ii) ovalbumin, 45 kDa; (iii) chymotrypsinogen, 25 kDa; (iv) cytochrome c, 12.5 kDa. Ms_orn is indicated in red). C, SEC–MALLS of Ms_orn showing signal peaks of LS (light scattering), dRI (differential refractive index), and UV (at 280 nm), and a calculated M_w (green) of 42.97 kDa. MALLS, multiangle laser light scattering; Ms_orn, Orn of Mycobacterium smegmatis; SEC, size-exclusion chromatography.
Sequence analysis of Ms_orn using Conserved Domain Database indicated that the protein belongs to DEDDh-type DnaQ-like 3’-5’ Orn family that cleaves RNA in a metal-dependent manner. Sequence comparison of Ms_orn with the other bacterial Orns, whose structures are available in Protein Data Bank (PDB), reveals more than 40% sequence identity across the entire length of the protein sequences with high degree of conservation surrounding the catalytic DEDDh residues (Fig. 2). Significant level of conservation is also seen for residues required in substrate binding or present at dimer interface (discussed later). The C-terminal region of Orns, however, shows a lot of variability and differences in lengths across all sequences. The C-terminal region of mycobacterial Orns is much longer, with a 29-residue long C-terminal tail.

Figure 2. Sequence analysis of Ms_orn. Sequence alignment of Ms_orn and Mycobacterium tuberculosis orn (Mtb_orn: Rv2511) is shown with other bacterial oligoribonucleases (Orns) whose structures are available in Protein Data Bank (PDB). Sequences of Orn of Xanthomonas campestris (Xca_orn; PDB ID: 2GBZ), Colwellia psychrerythraea (Cps_orn; PDB ID: 6A4A), Coxiella burnetii (Cbu_orn; PDB ID: 3TR8), Acinetobacter baumannii (Aba_orn; PDB ID: SCY4), Vibrio cholerae (Vc_orn; PDB ID: 6N6A), and Haemophilus influenzae (Hin_orn; PDB ID: 1J9A) are shown. Ms_orn, Orn of Mycobacterium smegmatis.
Structure and stability features of Orn of M. smegmatis

(residues 181–209) in Ms_orn, in particular (Fig. 2). This extended C-terminal tail of Ms_orn hence appears to be unique among bacterial Orns and any function associated with it remains to be elucidated.

Overall structure of Ms_orn

The structure of Ms_orn in apo form was determined to 1.87 Å resolution to a final Rwork of 17.9% and Rfree of 22.2% (Table 1). There are four molecules of Ms_orn in one asymmetric unit with two homodimers packed in a side-to-side orientation (Fig. 3A). PISA server (45) indicated homodimer as the probable quaternary structure for Ms_orn, which was confirmed by SEC–MALLS (Fig. 1C).

The overall structure of Ms_orn consists of the typical RNase-H fold of DnaQ-like exoribonuclease superfamily of proteins consisting of five central β sheets, β5-β4-β1-B2-β3 (arranged in ↑↑↑↓↑ direction) and nine α helices, along with an additional C-terminal helix, α10 (Fig. 3, B and C). The final model of Ms_orn in each subunit could be traced from residues 1 to 196 of the full-length protein. Although the terminal residues 197 to 209 of Ms_orn lacked any discernible density, a significant part of the unique C-terminal tail of Ms_orn (residues 181–196) could be built and formed the additional helix, α10, through residues 186 to 195. α10 of one subunit packs against the other subunit of the dimer in a “handshake”-like manner, creating a small flap over the other protomer (Fig. 3B) and is unique to Ms_orn among all bacterial Orn structures (Fig. 3D). Average B-factors of the C-terminal tail (residues 181–196) were found to be slightly higher than the rest of the chain (41.58 Å² or 43.56 Å² for the tail in the two subunits versus 33.59 Å² or 36.91 Å² for the N-terminal region, i.e., residues 1–180), indicating slightly larger flexibilities for the tail region in both subunits.

Role of C-terminal helix, α10, at the dimeric interface

In order to obtain better understanding of the additional C-terminal helix, α10, in Ms_orn, the structure of Orn of Escherichia coli (Eco_orn) with the canonical Rnase-H fold was also determined for comparative structural analysis. The final structure of Eco_orn could be traced from residues 2 to 181 of the polypeptide chain. Eco_orn was also identified as a dimer by gel filtration, SEC–MALLS and PISA (Fig. S1). The structure refinement statistics for the final model are given in Table 1. Superposition of Eco_orn structure over Ms_orn indicates overall structural similarity with rmsd of 1.134 Å across 347 Cα-atoms of the dimer but lacking the C-terminal tail of Ms_orn (Fig. S1).

As the C-terminal helix, α10, of Ms_orn packs against the opposite protomer and appears to aid the dimeric packing of the two subunits, the structures of Ms_orn and Eco_orn were first analyzed for differences in the dimer interface through PISA (45). Ms_orn buries 22.2% of total solvent-accessible area comprising nearly 2550 Å² in the dimer interface (Fig. 4A). The dimer interface of Ms_orn is stabilized by two salt bridges (B: Arg128 [NH1]–A: Glu137 [OE2] and B: Glu137 [OE2]–A: Arg128 [NH1]) (Fig. 4B) and an additional set of 16 H-bonds.

Eco_orn, on the other hand, buries only 17.2% of total solvent-accessible area comprising nearly 1650 Å² in the dimer interface. The residues involved in dimer formation in Ms_orn are also conserved in Eco_orn, with the dimer interface in Eco_orn being stabilized by the conserved salt bridges between B: Arg130 [NH1]–A: Glu139 [OE2] and B: Glu139 [OE2]–A: Arg130 [NH1]) (Fig. 4B) and an additional set of 16 H-bonds.

Table 1

| Parameter | Ms_orn | Eco_orn |
|-----------|--------|---------|
| PDB ID | 7WI1 | 7VH4 |
| Beam source | Synchrotron | Synchrotron |
| Beamline | Beamline ID29 | Beamline ID29 |
| Wavelength (Å) | 0.9686 | 0.9686 |
| Resolution (Å) | 81.07–1.87 (1.90–1.87) | 59.63–2.30 (2.38–2.30) |
| Space group | P2₁2₁2₁ | P4₁2₁2 |
| Unit cell dimensions | a, b, c (Å) 0.265, 97.26, 146.73 | 100.57, 100.57, 147.83 |
| a, β, γ (%) | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| No. of unique reflections | 71,290 (3483) | 34,389 (3274) |
| Completeness (%) | 99.5 (99.8) | 99.8 (98.6) |
| Multiplicity | 4.6 (4.3) | 7.8 (8) |
| Rmerge (%) | 6.2 (69.4) | 7.4 (53.3) |
| CC1/2 | 0.09 (0.803) | 0.98 (0.925) |
| Rwork (%) | 15.9 (20.0) | 11.7 (2.9) |
| Rfree (%) | 17.92 | 19.35 |
| Wilson B-factor (Å²) | 22.24 | 24.47 |
| rmm | 33.637 | 55.09 |
| Bond length (Å) | 0.009 | 0.008 |
| Bond angles | 1.461 | 1.423 |
| Ramachandran plot | Favored (%) 99 | 98 |
| Allowed (%) 1 | 2 |
| Outliers (%) 0 | 0 |

*Numbers in parentheses correspond to the highest resolution shell.

**Rmerge = Σ|Ii−|0|I|/ΣI, where I is the integrated intensity of a given reflection.

***<(|Ii−|0|I|)/> = mean h₀ over the standard deviation of the mean hᵢ averaged over all reflections in a resolution shell.

**Rwork = Σ|Fᵢ|–|Fᵢ|/Σ|Fᵢ|, where |Fᵢ| is the observed structure factor amplitude and |Fᵢ| is the calculated structure factor amplitude.

"Rfree = Rmerge based on 5% of the data excluded from refinement."
An additive effect of weak van der Waal interactions appears to play a role in packing the two subunits. Interactions with modeled pGpG in the active site

The active site of Ms_orn and additional structural features in the substrate-binding pocket were next analyzed. The catalytic Asp9, Glu11, Asp110, and Asp162 along with the general base His157, which are characteristic of DEDDh family of exoribonucleases, are conserved in the active site of Ms_orn, as expected (Fig. 5A). In the absence of a bound substrate in the apo Ms_orn structure, His157 of Ms_orn was found to be disordered in one of the subunits. Interestingly, this active site histidine is present in a flexible loop and was observed to be disordered in several other apo Orn structures (46, 47), including Eco_orn. The carboxylate side chains in the active site of Orns usually coordinate two divalent metal ions for substrate binding and catalysis. In the apo form of Ms_orn, we observed only one monovalent cation, K⁺ ion, that was coordinated by Asp9 and Glu11 in all the chains (Fig. 5A).

In order to understand the structural basis of substrate binding in Ms_orn, pGpG was modeled in the active site by superposing the structure of V. cholerae Orn (Vc_orn)–pGpG complex (PDB ID: 6N6A) (46). Vc_orn superposes very well with Ms_orn with an rmsd of 0.908 Å for 348 aligned residues allowing the placement of pGpG into the Ms_orn active site and providing a template for further analysis of interactions of pGpG with active site residues of Ms_orn. The 5’ phosphate group of modeled pGpG was found to overlay a bound sulphate ion of Ms_orn, whereas the 3’ OH of modeled pGpG overlays a bound glycerol molecule, suggesting equivalent binding site for the substrate in the two structural homologs (Fig. 5B). The 5’ phosphate of modeled pGpG interacts with Ser106 and Ser133 of one subunit and Tyr127 and Arg128 of the other subunit and constricts the active site of Ms_orn (Fig. 5B). A similar set of residues has earlier been reported to cap the 5’ end of pGpG substrate in Vc_orn, thereby constricting the active site and preventing binding of substrates longer than diribonucleotides and was termed as a

(Figs. 2 and 3), an additive effect of weak van der Waal interactions appears to play a role in packing the two subunits.

Figure 3. Crystal structure of Ms_orn. A, two homodimers of Ms_orn (one dimer in different hues of blue and other in magenta) in side-to-side orientation in the asymmetric unit (a.s.u.). B, biological unit of Ms_orn homodimer with the extended C-terminal helix of one protomer packing against the other in a “handshake”-like manner. A close-up view of the C-terminal tail showing the interactions of the residues in the tail with the main domain of the other subunit is shown. A 2Fₒ–Fᵦ map contoured at 1.5 sigma level for the C-terminal chain is also plotted. C, ribbon diagram of Ms_orn protomer (chain A) showing the nine α-helices surrounding the five central β-sheets along with the extended helix, α₁₀. D, homodimers of bacterial Orns (strain names and Protein Data Bank IDs are mentioned in Fig. 2) clearly indicate that the C-terminal helical flap is unique only to Ms_orn. Ms_orn, Orn of Mycobacterium smegmatis.
“phosphate-cap” or P-cap (46). We examined the Orn sequences and found that the P-cap residues are conserved among them (Fig. 2) and hence may be a common structural feature among all Orns. Fitting of a longer oligonucleotide substrate, for example, a 3-mer in Ms_orn active site showed steric clashes of the additional nucleotide at the -1 position, with P-cap residues, Tyr127 and Arg128 (Fig. 5C). The catalytic pocket of Ms_orn is also lined by Glu11, His63, Val59, and Met12, which prevent accommodation of oligonucleotides that are longer on the 3’ end of pGpG, as suggested earlier (47). Notably, Glu11, Met12 and His63 are conserved among Orn sequences (Fig. 2), suggesting conservation of this “3’-OH capping” mechanism as well in the bacterial enzymes and limiting the binding site access to a 2-mer substrate only.

Additional interactions with the bound pGpG substrate in Vc_orn are through stacking interactions of the 5’ and 3’ nucleotide bases with a Tyr and Trp residue, respectively, with a conserved Leu forming a wedge between the two bases (46–48). In Ms_orn, Tyr127 and Leu15 were identified with the respective roles at equivalent positions although the Trp is replaced by Val58. Although the presence of a nonpolar aliphatic group in place of the bulky Trp slightly increases the pocket size, the hydrophobicity of the substrate-binding pocket is maintained by additional compensatory Val56 and Met62 substitutions in Ms_orn replacing Asp59 or Thr65 of Eco_orn, respectively (Fig. 5D).

The overall structural features of Ms_orn, residues at the active site and potential interactions with substrates hence appear to be highly conserved. The primary difference identified in the structure is the additional C-terminal helix, α10, that provides additional interactions at the dimer interface and appears to have no direct role in substrate binding. The role of this C-terminal helix in activity and/or stability was next investigated.

**Role of C-terminal tail in protein stability**

Any possible role of the extended C-terminal tail on stability was investigated by comparison of thermodynamic parameters of Ms_orn and its deletion mutant (lacking the C-terminal tail) to thermal and chemical (guanidium hydrochloride [Gdm-HCl])–induced denaturation and measuring changes in their secondary structure. First, a C-terminal deletion mutant of Ms_orn, lacking residues 181 to 209, was constructed and termed as Ms_ornΔC. However, Ms_ornΔC could not be obtained in soluble form. Ms_ornΔC (lacking residues 186–209) was hence alternately expressed and could be obtained in soluble form as a dimer (Fig. S3) for further experiments.

The secondary structures of Ms_orn, Ms_ornΔC and Eco_orn were first analyzed using far-UV CD spectra measurements in wavelength range of 195 to 250 nm at 25 °C. Figure 6A shows characteristic spectra of α/β-type proteins with negative peaks at 208 and 225 nm. Although both Ms_orn and Eco_orn are dimers and have similar structural content, Ms_orn has a slightly more negative far-UV CD signal than Eco_orn. Interestingly, the far-UV CD signal for Ms_ornΔC is similar to that of Eco_orn, possibly because of loss of the additional helical signal of the C-terminal helix, α10.

The Orns were then subjected to heat in the temperature range of 20 to 85 °C to monitor their thermal denaturation profiles. No change in [θ]222 nm in any of the proteins was observed as a function of temperature although Ms_orn shows a slight change in [θ]222 nm between 40 °C and 60 °C (Fig. 6B). There is a heat-induced increase in [θ]222 nm up to 50 °C, possibly because of partial unfolding but again appears to retain a state that exhibits [θ]222 nm signals similar to the pretransition state (Fig. 6B). Reversibility of the spectra was monitored by measuring [θ]13 nm of all three Orns at 20 °C after first heating the proteins to 85 °C. The spectra were fully reversible and trace the spectra as obtained under native buffer conditions (Fig. 6A), suggesting high conformational stability for the orn dimers.

Since the proteins appear to be thermostable under the aforementioned conditions, we next performed thermal denaturation experiments in the presence of small amount of a denaturant to enhance the unfolding of proteins. For Eco_orn, a sharp transition in temperature range 42 to 60 °C was observed, which is indicative of a major conformational change suggesting unfolding of the protein with an estimated T_m of 50 °C. Thermal unfolding of Ms_ornΔC was also observed with 0.3 M Gdm-HCl, and the T_m estimated to be 50 °C with no intermediate transitions at lower temperatures.

In case of Ms_orn, however, again an intermediate transition from 36 to 55 °C similar to that of its thermal denaturation curve in the absence of Gdm-HCl was observed, followed
by the major unfolding transition with an estimated $T_m$ of 70 °C (Fig. 6C). The change in secondary structure content of Ms_orn at the intermediate transitions was estimated by K2D2 program of Dicroweb server. Under the native buffer conditions, a small change in alpha-helical content between 40–50 °C (corresponding to the observed intermediate transition of 36–55 °C) was observed and the helical content is restored at temperatures >55 °C to that observed at 25 °C (Fig. S4). Similarly, helical content of Ms_orn (with 0.3 M Gdm-HCl) is also first marginally reduced suggesting a transient local unfolding between 35 and 45 °C, followed by a slight increase in helical content at 50 °C before unfolding completely. In contrast to thermal unfolding under native conditions, thermal denaturation in the presence of 0.3 M Gdm-HCl (but not in its
absence) was found to be irreversible (Figs. 6C and S2), suggesting that the low amount of denaturant was sufficient to perturb local structure to enable unfolding.

**Gdm-HCl induced denaturation of Ms_orn and Eco_orn**

Temperature-induced conformational changes confer that Ms_orn is capable of tolerating higher temperatures than the C-terminal deletion mutant. To further investigate the conformational stability and denaturant-dependent effect on stability calculations, equilibrium Gdm-HCl–induced denaturation measurements were done by far-UV CD and intrinsic fluorescence measurements and thermodynamic parameters were calculated.

To monitor Gdm-HCl–induced denaturation of Ms_orn, Ms_ornΔC, and Eco_orn, intrinsic fluorescence spectra were measured at 25 °C in the presence of increasing concentrations of Gdm-HCl. The \( \lambda_{\text{max}} \) of fluorescence in the unfolded state reaches 356 nm for Ms_orn, 354 nm for Ms_ornΔC and 360 nm for Eco_orn. The denaturation curve of Eco_orn shows a monophasic manner of transition from folded to unfolded state, whereas both mycobacterial proteins exhibit a biphasic transition. Ms_orn denaturation profile shows a pre-transition state from 0 to 0.75 M Gdm-HCl concentration and intermediate transition shows stable conformation at 1.1 to 1.3 M Gdm-HCl followed by a completely unfolded form at 3.0 M Gdm-HCl (Fig. 7A). A size-exclusion profile of Ms_orn with increasing concentrations of Gdm-HCl over a Superdex-75 column revealed the presence of a dimer up to 1.0 M concentration, suggesting that the first transition is more likely to be local conformational changes (rather than a monomer–dimer transition) followed by unfolding (Fig. S5). A similar kind of nearly identical biphasic transition curve is observed for Ms_ornΔC with its pre-transition state from 0 to 0.6 M Gdm-HCl, intermediate transition from 1.1 to 1.3 M Gdm-HCl followed by a completely unfolded form at 3.0 M Gdm-HCl.

Gdm-HCl–induced denaturation was also monitored through far-UV CD, by measuring the change in \([\theta]_{222}\) nm as a function of increasing molar concentrations of Gdm-HCl (Fig. 7B). Ms_orn and Ms_ornΔC once again show a biphasic denaturation curve, as seen in their respective fluorescence spectra. The change in secondary structure of Eco_orn also follows a biphasic transition when monitored by \([\theta]_{222}\) nm although not by tryptophan fluorescence. The biphasic transitions as monitored by change in \(\lambda_{\text{max}}\) or \([\theta]_{222}\) are subtle yet highly reproducible in several experimental runs with varying...
concentrations of GdmCl between 0 to 3 and 4 M. The unfolding curve with indicated set of Gdm-HCl concentrations (i.e., 0–6 M) was finally plotted as shown in Figure 7, A and B for estimation of thermodynamic parameters. The thermodynamic parameters for unfolding of Ms_orn, Ms_ornΔC, and Eco_orn, as monitored by [θ]_{222} nm, were estimated for both transitions, that is, transition I (N to X) and transition II (X to U), where N represents native state, X is thermodynamically stable intermediate and U is unfolded state (Table 2 and Fig. S6). There is a major difference in [θ]_{222} nm of Ms_ornΔC in the 0 to 0.6 M Gdm-HCl range, which corresponds to transition I, that is, N ↔ X with much lower values of C_{ml} and ΔG_{I}. In addition, the overall ΔG value of denaturation (ΔG_{I0} + ΔG_{II}) for Ms_ornΔC is also lower than that observed for Ms_orn (or Eco_orn) (Table 2), implying much lower stability of the C-terminal deletion mutant.

Figure 7. Gdm-HCl–induced denaturation. Gdm-HCl–induced denaturation of Ms_orn (black), Ms_ornΔC (red), and Eco_orn (green) monitored by following changes in (A) λ_{max} and (B) [θ]_{222} nm. Inset shows enlarged view for Ms_orn and Ms_ornΔC in the range of 0 to 2.0 M Gdm-HCl. C: ANS binding measurements for Ms_orn, Ms_ornΔC, and Eco_orn with increasing concentrations of Gdm-HCl, as indicated. The biphasic transition was highly reproducible. The unfolding curve with indicated set of of Gdm-HCl concentrations (i.e., 0–6 M), used for estimation of parameters, was repeated twice before plotting. ANS, 8-anilino-1-naphthalene sulfonic acid; Eco_orn, Orn of Escherichia coli; Gdm-HCl, guanidium hydrochloride; Ms_orn, Orn of Mycobacterium smegmatis.
Table 2
Thermodynamic parameters associated with Gdm-HCl-induced denaturation of Ms_orn, Ms_ornΔC, and Eco_orn

| Transition | Thermodynamic parameter | Ms_orn | Ms_ornΔC | Eco_orn |
|------------|-------------------------|--------|----------|---------|
| N ↔ X     | ΔG^Gd-HCl^0             | 3.9 ± 0.03 | 1.94 ± 0.06 | 3.7 ± 0.17 |
|           | C_mol                   | 0.86 ± 0.0 | 0.24 ± 0.01 | 0.58 ± 0.03 |
| X ↔ U     | ΔG^Gd-HCl^C            | 1.6 ± 0.06 | 1.5 ± 0.14 | 1.3 ± 0.02 |
|           | C_mol                   | 2.1 ± 0.02 | 1.98 ± 0.01 | 1.75 ± 0.00 |

ΔG^Gd-HCl^0, Gibbs free energy change associated with transition II where X state exists.
Units of ΔG^Gd-HCl^C is kilocalorie mole^-1, Cmol < CmII or CmII < Cmol is M.
“z” represents the mean error from duplicate measurements.

To monitor conformational state of the intermediate in the first transition, 8-anilino-1-naphthalene sulfonic acid (ANS)–
binding measurements of Ms_orn, Ms_ornΔC and Eco_orn were carried out with increasing Gdm-HCl concentrations.

Effect of deletion of C-terminal tail of Ms_orn in vivo

To establish the role of C-terminal tail in the activity of Ms_orn in vivo, a knockout of orn in M. smegmatis mc^2155
(Δorn) (P.B. and B.T. [unpublished observations]) was complemented with clones expressing proteins corresponding to
full length (orn), 1–185 residues (ornΔC) or 1–180 residues (ornΔC'). Growth of knockout and complemented strains was monitored by measuring optical density at 600 nm and compared with wild-type (WT) strain. Deletion of orn (Δorn strain) exhibited slightly slower growth as compared to WT, which was partially restored in the complemented Δorn/orn and Δorn/ornΔC strains but not in Δorn/ornΔC', which lacks the complete C-terminal tail of Ms_orn (Fig. 9A). Expression of orn (or its mutant forms) in the soluble fractions was confirmed by monitoring transcript levels by quantitative RT–
PCR (qRT–PCR) (Fig. 9B) and Western blotting (Fig. 9C), before comparing growth of WT, knockout and the complemented strains.

Partial deletion of the C-terminal tail in the Ms_ornΔC mutant indicated subtle changes in stability of the protein, as estimated by thermal unfolding and Gdm-HCl–induced denaturation experiments. In order to assess the effect of deletion of the complete tail, we carried out an all-atom molecular dynamics (MD) simulations of Ms_orn, Ms_ornΔC, Ms_ornΔC' and Eco_orn for 500 ns and analyzed C^β-rmsd with respect to time. Our analysis indicated that with the deletion of the entire C-terminal tail of Ms_orn, the average rmsd (rmsd_avg) values are higher in Ms_ornΔC' (0.44 nm) than that of native Ms_orn (0.29 nm) or Ms_ornΔC (0.26 nm), suggesting larger structural deviations in Ms_ornΔC' during the MD run, than the other two mycobacterial proteins (Fig. 8A). The rmsd plot of Eco_orn also shows no major fluctuations during the timescale of MD simulations up to 500 ns (Fig. S7).

Compactness and structural changes during the course of MD simulations were also calculated through estimation of R_g as a function of time. Again, Eco_orn shows no major changes in R_g during the timescale of MD simulations up to 500 ns (Fig. S7). The mycobacterial proteins, Ms_orn and Ms_ornΔC, also show only a small deviation in R_g values at the end of the MD run, when compared with the initial values. Ms_ornΔC', in contrast, exhibits the highest average R_g (R_g-avg) = 2.26 nm and largest deviation of ΔR_g = 0.11 nm among the three mycobacterial Orn forms, despite estimated smaller size upon deletion of the C-terminal tail (Fig. 8, B and C).

A closer analysis of the simulated structures shows that there is a slight opening of the Orn dimer during the course of the MD run. While a marginal opening between 4.5º and 5.3º is observed in Ms_orn (or Eco_orn) dimers, Ms_ornΔC' shows largest deviation of nearly 15º, corresponding with larger observed changes in R_g in this mutant (Fig. 8, C and D). A larger deviation in Ms_ornΔC when compared with Ms_ornΔC' is possibly because of loss of all possible interactions at the C-terminal dimer interface in the former, whereas interacting residues, 181, 183 and 184, are retained in Ms_ornΔC.
Figure 8. MD simulation studies. A, rmsd and (B) Rg plot of Ms_orn (black), Ms_ornΔC (red), and Ms_ornΔC' (blue). C, measurement of changes in rmsd, Rg, and opening of dimers (measured as angle deviation between helices α9 of the A and B subunits, shown as cylinders) indicate Ms_ornΔC' has largest deviations. D, conformational analysis of Ms_orn, Ms_ornΔC, Ms_ornΔC', and Eco_orn reveals opening up of dimers in all proteins after the MD simulations, with largest deviations in Ms_ornΔC'. Eco_orn, Eco_orn, Orn of Escherichia coli; MD, molecular dynamics; Ms_orn, Orn of Mycobacterium smegmatis.
Figure 9. In vivo effect of deletion of C-terminal tail of Ms_orn. A, growth curve of WT (Mycobacterium smegmatis mc²155, shown in black), Δorn (red), Δorn/orn (green), Δorn/ornΔC (yellow), and Δorn/ornΔC’ (blue) in 7H9 media. B, relative expression of orn with respect to WT in Δorn, Δorn/orn, Δorn/ornΔC, and Δorn/ornΔC’. Average values of three independent biological replicates are plotted after normalization with the β’ subunit of RNA polymerase, rpoC; error bars indicate ± standard deviation. The p values were calculated by one-way ANOVA Tukey’s post hoc test. Asterisks in figure represent significant difference between two groups (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001). C, expression of C-terminal deletion mutants in the soluble fractions was confirmed by Western blotting with a mouse anti-His antibody against full-length complement (orn/Δorn) as a control. A prestained molecular weight marker is indicated (M). D, growth of indicated serial dilutions of WT, Δorn, Δorn/orn, Δorn/ornΔC, and Δorn/ornΔC’ on 7H9 agar plates after exposure to UV for 15 min. E, upper panels, growth of WT, Δorn, Δorn/orn, Δorn/ornΔC, and Δorn/ornΔC’ in 7H9 media with constant shaking. Growth of serial dilutions of WT, Δorn, Δorn/orn, Δorn/ornΔC, and Δorn/ornΔC’ strains on 7H9 agar plate after incubation for 3 h with indicated concentrations of H2O2 (milimolar). All growth experiments were performed with two biological replicates. F, in vitro activity of Ms_orn. Phosphatase (gray bars) and phosphodiesterase (slant lines) activity of Ms_orn, Ms_ornΔC, and Eco_orn on pNPP and bNPP substrates, respectively. The
dilutions on 7H9 plates. Δorn and Δorn/ornΔC’ showed no growth up to 48 h after exposure, suggesting that the presence of complete extended C-terminal tail is required for proper functioning of Ms_orn and to enable optimal growth of M. smegmatis under both stress and stress-free growth conditions.

The PDE activity of Ms_orn and Ms_ornΔC was also tested in vitro with bis-(p-nitrophenol) phosphate (bNPP) as a substrate that mimics the two rings of its in vivo pGpG substrate. Specific activity of Ms_orn on bNPP was found to be 74.36 μmol/min/mg. Deletion of the C-terminal tail in Ms_ornΔC leads to a slight decrease in PDE activity to 61.11 μmol/min/mg. The absence of the C-terminal helix, however, leads to more than nine-fold higher phosphatase activity in Ms_ornΔC than Ms_orn (Fig. 9F), suggesting that the C-terminal tail may play a role in substrate access and selection.

Discussion

Homeostasis of c-di-GMP is key for normal physiology and survival of M. smegmatis as well as M. tuberculosis. C-di-GMP regulates lipid transport and metabolism (23), survival under hypoxic and reductive stress (21, 22, 49, 50), modulation of pathogenicity and antibiotic response in both mycobacterial species (20, 23, 24). The intracellular levels of c-di-GMP in bacteria are maintained by two antagonistic enzyme families: DGCs for its biosynthesis and PDEs containing either HD-GYP or EAL domain for its degradation (1, 3, 4) that hydrolyze c-di-GMP into GMP or pGpG, respectively. In mycobacteria, no homolog for HD-GYP domain containing PDE is present and c-di-GMP is hence cleaved to pGpG by EAL domain containing PDEs; the bifunctional DGC (MSMEG_2196) in M. smegmatis (21) and DGC (Rv1354c) or MtbPDE (Rv1357c) in M. tuberculosis (20, 51). Removal of pGpG is important to maintain the pool of free nucleotides inside the cell and to prevent mispriming of transcripts (41) and is brought about by Orns (33, 34). Any excess uncleaved pGpG can cause feedback inhibition on EAL domain containing PDEs and extend the half-life of c-di-GMP in the cell (4, 33, 34). Despite the importance of complete metabolism of c-di-GMP to GMP, when not required for its regulatory roles, the mycobacterial Orn remains uncharacterized so far. In addition, mycobacterial Orns have an extended C-terminal tail in contrast to other bacterial homologs. In order to obtain better insights into this enzyme, we have determined the crystal structure of MSMEG_4724, the Ms_orn, to investigate the role of its unique structural features and to examine the molecular interactions of the protein with a potential substrate (Fig. 5B). In order to aid the structural basis of substrate binding in Ms_orn, pGpG was modeled in the active site using Vc_orn as a reference structure. Interestingly, the sulphate ion is present at the same site as the 5’ phosphate of the modeled pGpG and makes similar interactions with two serines of one subunit (Ser106 and Ser133) and a tyrosine and arginine of the

Ms_orn constrains a 2-mer substrate in its binding pocket though P-cap and 3’OH cap

We identified two bound ligands (a sulphate ion and a glycerol molecule) in the active site of Ms_orn that helped map molecular interactions of the protein with a potential substrate (Fig. 5B). In order to aid the structural basis of substrate binding in Ms_orn, pGpG was modeled in the active site using Vc_orn as a reference structure. Interestingly, the sulphate ion is present at the same site as the 5’ phosphate of the modeled pGpG and makes similar interactions with two serines of one subunit (Ser106 and Ser133) and a tyrosine and arginine of the

p values of relative specific activity on pNPP were calculated by two-way ANOVA multiple comparison test (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001). The phosphatase (100.75 μmol/min/mg) and phosphodiesterase activity of Eco_orn (52.67 μmol/min/mg) on the same substrates is also shown for comparison. The error bars represent standard deviation of two independent experiments. bNPP, bis-(p-nitrophenol) phosphate; Eco_orn, Orn of Escherichia coli; Ms_orn, Orn of Mycobacterium smegmatis; pNPP, p-nitrophenol phosphate.
other (Tyr127 and Arg128), as seen in Vc_orn. These residues have been defined as a 5′ P-cap that constricts the active site of Orns and prevent binding of substrates longer than diribonucleotides (46).

Modeling of a 3-mer oligo with an additional nucleotide at the 5′ end of modeled pGpG showed steric clashes with Tyr127 and Arg128 of the P-cap (Fig. 5C). On the 3′ OH side of the 2-mer, the binding pocket is lined by the catalytic Glu11 along with His63, Val59 and Met12 of Ms_orn (Fig. 5D). These residues prevent the fitting of a 3-mer oligo with an additional nucleotide at the 3′ end of the 2-mer modeled pGpG. A similar observation was also made in REXO2 structure earlier (47). Indeed, examination of Orn sequences confirmed that residues corresponding to Glu11, Met12 and His63 of Ms_orn were conserved (Fig. 2) and we propose that they are likely to play a conserved “3′ OH capping” role. The presence of both a conserved P-cap at 5′ site and the proposed 3′ OH cap on the 3′ site of modeled pGpG thereby limits the space in binding pocket for a 2-mer substrate only. Interestingly, crystal structures of Orns with bound oligos, all show only a 2-mer oligo in the binding pocket and additional nucleotides were found to be disordered or with very high B-factors, despite being used in crystallization trials (46, 48, 52).

Although a 2-mer has been shown to be a preferred substrate in both bacterial (P. aeruginosa and V. cholerae) and mammalian mitochondrial orn (REXO2), recently (33, 46, 53), in vitro biochemical assays show that Orns bind and cleave longer ribonucleotides (typically 2–5 mer) as well (37, 38, 47, 54, 55). The active site face of Ms_orn is positively charged and additional nucleotides (longer than 2-mer) may be accommodated outside the binding pocket through nonspecific interactions on this side (Fig. 5C).

C-terminal helix, a10, renders higher stability features to Ms_orn

Proteins of intracellular pathogens like M. tuberculosis have often been shown to have higher stability to retain their function in vivo (56, 57). We examined whether the C-terminal tail of Ms_orn could play such a role to enable higher stability to the protein by thermal and Gdm-HCl–induced denaturation experiments. First, thermal stability of Ms_orn and Ms_ornΔC was examined through [θ]_222 nm. Both Ms_orn and Ms_ornΔC appear to be thermostable under our experimental conditions and no major change in [θ]_222 nm was observed as a function of temperature for either protein. Thermal denaturation was hence next carried out in the presence of small amount of a denaturant to enhance the unfolding of proteins and yielded an estimated T_m of 70 °C for Ms_orn, which was much higher than that obtained for Ms_ornΔC (estimated T_m of 50 °C). Interestingly, estimated T_m (50 °C) of Eco_orn (which naturally lacks the C-terminal extended region) was similar to that of Ms_ornΔC, suggesting a10 aids the higher observed thermostability of Ms_orn (under mild denaturing conditions).

The structural stability of Ms_orn and role of the C-terminal tail were also examined by Gdm-HCl–induced denaturation experiments for Ms_orn and Ms_ornΔC. The denaturation profile of both Ms_orn and Ms_ornΔC exhibited a biphasic transition when monitored by either changes in λ_max of intrinsic fluorescence or [θ]_222 nm (Fig. 7, A and B). The biphasic transition and the presence of the intermediate unfolding state for Ms_orn and Ms_ornΔC were confirmed by ANS binding experiments as well (Fig. 7C). SEC profile with low concentrations of Gdm-HCl (<1.0 M Gdm-HCl), (Fig. S5) shows the dimer is retained at these concentrations. The presence of hydrophobic regions (ANS binding) and apparent dimeric state (SEC with Gdm-HCl) suggests that Ms_orn unfolding is initiated by local perturbations first, followed by complete unfolding and loss of the dimeric state.

A lower C_m of Gdm-HCl was required for first transition of Ms_ornΔC by both intrinsic fluorescence and CD measurements, possibly because of loss of additional dimer packing (that would have been provided by α10) in the full-length protein. Once again, Eco_orn, naturally lacking the C-terminal extended region, appears to follow a different unfolding pattern from Ms_orn when monitored by either changes in λ_max of intrinsic fluorescence or [θ]_222 nm, suggesting that the unfolding pathways of Ms_orn with the extended C-terminal tail are different from other Orns.

The extended C-terminal tail of Ms_orn hence transmits several unique features to the protein. Examination of additional unique sequence features among RNase-H fold–containing proteins using DALI (58) indicated the presence of an additional C-terminal helix in one of the REXO2 (human mitochondrial Orn) crystal structures (PDB ID: 6N6J) (Fig. 10A). The C-terminal tail of REXO2, however, is involved in crystal contacts (Fig. 1B) and hence does not appear to be in its native conformation. Unfortunately, none of the other REXO2 structures exhibit this extended C-terminal region (and were presumed disordered), preventing further analysis of this region in REXO2. The C-terminal tail of Ms_orn is hence unique and likely to have organism-specific roles for maintaining the stability of this key enzyme for c-di-nucleotide homeostasis and its regulatory roles in growth and stress response in the harsh intracellular environments.

In conclusion, we present the structure of mycobacterial orn that helps in complete degradation and maintenance of c-di-GMP homeostasis in the cell. Ms_orn structure consists of the canonical RNase-H fold and contains an additional C-terminal helix that packs against the other protomer in a “handshake” conformation providing additional interactions at the dimer interface. We identify preference of a 2-mer substrate in Ms_orn through site constrictions in the substrate binding pocket by conserved residues constituting the P-cap or “3′ OH cap.” Deletion of the C-terminal tail leads to opening up of the dimer as seen by MD simulation experiments, highlighting its importance. C-terminal tail is required for proper functioning of the enzyme as deletion of C-terminal tail of Ms_orn.
Structure and stability features of Orn of M. smegmatis

in bacterial cell affects its growth in both normal and stress conditions. Finally, Gdm-HCl–mediated unfolding and thermal denaturation experiments suggest that the C-terminal region helps in higher stability of Ms_orn, highlighting the role of intrinsic sequence features in mycobacterial sequences that enable its stable state under harsh intracellular conditions. This study sheds insights on the structure–function relationship of Ms_orn and its unique C-terminal region, which will be key in evaluating the intracellular levels and homeostasis of c-di-GMP in regulation of key pathways by this signaling molecule.

Experimental procedures

Chemicals and reagents

Sodium chloride, trizma, glycine, SDS, EDTA, ANS, Gdm-HCl, PMSF, and imidazole were obtained from Sigma Chemical Co. Nickel–nitritotriacetate (Ni–NTA) agarose–based resin was brought from Qiagen. Mono-Q ion-exchange and superdex-75 (10/300 GL) gel filtration columns were purchased from GE Healthcare. LB agar, LB broth, glycerol, ammonium persulphate, IPTG, and kanamycin were purchased from Himedia Laboratories. Syringe filters (0.22 and 0.02 μm cutoff) were purchased from Millipore Corporation. All chemicals and reagents used were of analytical grade.

Cloning, expression, and purification

Rxv2511 is annotated as M. tuberculosis Orn (Mtb_orn). The open reading frame of MSMEG_4724 (GenBank accession ID: CP000480.1), corresponding to a gene product of 216 amino acids, was identified as the ortholog of Mtb_orn and amplified from genomic DNA of M. smegmatis mc²155 using gene-specific forward and reverse primers (Table S1) to obtain two different clones. Clone A encodes full-length protein of 216 amino acid residues, whereas the second clone (clone B) encodes a product starting from eighth amino acid of MSMEG_4724, corresponding to N terminus of Mtb_orn. Both forward primers introduce a BamHI restriction site at 5' end, and the reverse primer introduces a Xhol restriction site at 3' end after the stop codon. The PCR amplicon was digested with BamHI and Xhol restriction enzymes and purified using gel-extraction kit. The digested product was cloned in pET-28-His10-Smt3 expression vector at BamHI and XhoI sites. The vector hence encodes clone A or clone B of MSMEG_4724 fused to His₁₀–Smt3 (His₁₀–tagged yeast SUMO protein, Smt3) tag at the N terminus. The recombinant construct was transformed into E. coli BL21 (DE3) for expression, and the tagged proteins were purified as described previously (59). In brief, recombinant protein was purified using Ni–NTA agarose beads, and His₁₀–Smt3 tag was cleaved by Smt3-specific protease (Ulp1). Clone A of MSMEG_4724 yielded insoluble protein in inclusion bodies and could not be used further. The final purification steps to obtain purified protein from clone B required purification over an anion exchange column (Mono-Q; GE Healthcare) followed by SEC using Superdex-75 (10/300 GE) column (GE Healthcare) with 20 mM Tris–HCl, pH 8.5, and 100 mM NaCl as column buffer. Purity of the protein was checked on 12% SDS-PAGE. The purified MSMEG_4724 from clone B, thus obtained, was stored at −20 °C until further use and is referred to as Ms_orn in the rest of the article.

A C-terminal deletion mutant of Ms_orn, lacking residues 181 to 209 and annotated Ms_ornAC, was constructed using specific forward and reverse primers (Table S1) to yield the truncated protein. However, Ms_ornAC was found in inclusion bodies and could not be purified in soluble form. Ms_ornAC (lacking residues 186–209 at the C terminus of Ms_orn) was alternately expressed and purified following the same procedure as described previously for Ms_orn and used for all experiments reported for the C-terminal deletion mutant of Ms_orn.

The open reading frame corresponding to Orn (orn) of E. coli (National Center for Biotechnology Information accession ID: NC_000913.3) was amplified using PCR, from genomic DNA of E. coli K-12 strain, using gene-specific forward and reverse primers and cloned in pET-28-His₁₀–Smt3 expression vector at BamHI and HindIII restriction sites. The protein, referred to as Eco_orn, was purified as described for Ms_orn and finally resuspended in 20 mM Tris–HCl, pH 8.5, and 100 mM NaCl and stored at −20 °C in 100 μl aliquots until further use.

Figure 10. C-terminal tail of REXO2. A, superposition of two REXO2 dimer structures, 6N6J (brown) and 6STY (green) over Ms_orn dimer (cyan and blue) to show the orientation of extended C termini. Ms_orn shares 48.8% sequence identity with both REXO2 structures. 6N6J and 6STY superpose very well with Ms_orn with rmsd of 0.969 and 1.109 Å for 352 and 348 aligned residues of the dimer, respectively. However, extended C-terminal helix is seen only in 6N6J but not in 6STY. B, crystal packing of REXO2 (6N6J) shows that the extended helix of REXO2 (magenta) is involved in crystal contacts. Ms_orn, Orn of Mycobacterium smegmatis.
Structure and stability features of Orn of *M. smegmatis*

SEC–MALLS of purified protein

SEC with in-line MALLS (SEC–MALLS) was performed using AktaPure (GE Healthcare) FPLC connected in series to Dawn Heleos8+ multianalyte laser scattering detector and Optilab T-rEX, a refractive index detector (Wyatt Technologies) (60). SEC was performed using Superdex-75 (10/300 GE) column at room temperature in 20 mM Tris–HCl, pH 8.5, 100 mM NaCl, and 5% glycerol, operated at 0.5 ml/min flow rate. About 200 µl of 3 mg/ml of Ms_orn or 6 mg/ml of Eco_orn were injected in the column in separate runs. ASTRA 7.3.1.9 software (Wyatt Technologies) was used to collect data from UV, refractive index and light scattering detectors for further analysis. Absolute molecular weight of protein (M_m) was determined using dn/dc of 0.1850 ml/g for both Ms_orn and Eco_orn. To normalize light scattering signal across detectors, bovine serum albumin (Millipore Sigma) was used as a standard in the same buffer and at same flow rate. All measurements were carried out in duplicates.

Crystallization, data collection, and structure refinement

Ms_orn was crystallized by hanging drop diffusion method by mixing 2 µl of 0.3 mM protein in 20 mM Tris–HCl buffer, pH 8.5, 100 mM NaCl, and 2 mM MgCl2 and 2 µl of reservoir solution (0.1 M Tris–HCl buffer, pH 8.4, 0.2 M lithium sulphate, and 30% v/v PEG 4000) at 24 °C. Long and thick rod-shaped crystals were obtained after 8 to 10 days and flash frozen in liquid nitrogen in cryo-solution (reservoir solution containing 10% glycerol) before data collection.

Diffraction data were collected remotely at ID29 beamline at European Synchrotron Radiation Facility. Data were processed and integrated using autoPROC 1.1.7 (20171219) (61) and scaled using XSCALE (62). Statistics for data collection and refinement are summarized in Table 1.

Ms_orn crystals belong to P212121 space group and have four molecules in the crystallographic asymmetric unit. The structure of Ms_orn was solved by molecular replacement using polyalanine models of bacterial Orns (PDB IDs: 2IGI, 1YTA, 1J9A, 2GBZ, and 3TR8) as templates in Phaser of CCP4 suite (63). The best solution was obtained with the polyalanine model of *E. coli* Orn (PDB ID: 2IGI) as the starting model. The model was built and refined using iterative cycles of COOT (64) and Refmac5 (65). The final structure of Ms_orn was validated using MolProbity (66) and the refinement statistics are summarized in Table 1.

Crystallization of 0.3 mM Ms_orn was also set up with 10-fold molar excess of GMP. Crystals were obtained in P412121 space group and diffracted to 2.73 Å. The structure was solved by molecular replacement using coordinates of high-resolution data as template. However, no unambiguous density for ligand was observed, so it was not considered further, and higher resolution structure of apo form at 1.87 Å is used to describe structural features of Ms_orn in the article.

Co-crystallization of Eco_orn was also attempted by incubating 1.0 µl of pre-mixed protein–ligand solution (7 mM GMP and 0.75 mM purified protein) in 20 mM Tris–HCl buffer, pH 8.5, 100 mM NaCl, and 5% glycerol with 1.0 µl of reservoir solution (0.1 M Hepes buffer, pH 7.5, 0.25 M sodium acetate, and 25% v/v PEG-3350) at 24 °C. Crystals were obtained after 1 week and cryo-protected in a solution containing 10% glycerol in addition to the crystal reservoir solution and flash frozen in liquid nitrogen before collecting diffraction data to 2.3 Å resolution. The structure of Eco_orn was solved by molecular replacement using coordinates of 2IGI but could not identify a bound ligand. The final structure was validated using MolProbity (66) and the refinement statistics are summarized in Table 1. The final refined structure of apo form of Eco_orn was hence used for comparative structural analysis with Ms_orn.

The atomic coordinates and structure factors of Ms_orn and Eco_orn have been deposited with the PDB with accession codes 7WIK and 7VH4, respectively.

MD simulations

All atom MD simulations were carried out for Ms_orn using Gromacs, version 5.1.4 software package (67, 68). The coordinates of Ms_orn were first stripped of all crystallographic waters and ions, topologies were generated using CHARMM27 force field (69), and then solvated using SPC/E water model (70). The solvated system was minimized using 50,000 steps of steepest descent algorithm. The models were then separately subjected to position restrained canonical defined NVT (N: constant number of atoms, V: volume, and T: temperature) and NPT (N: constant number of atoms, P: pressure, and T: temperature) ensemble for 5 ns. The temperature of the system during NVT equilibrium was maintained at 300 K using Berendsen weak coupling method, and pressure in NPT equilibrium was maintained at 1 bar by using Parrinello–Rahman barostat. LINCS algorithm was used for constraining bonds (67). The long-range electrostatic interactions were calculated by using particle mesh Ewald (71). The van der Waals interactions were calculated through Lennard–Jones potential with cutoff of 0.1 nm. The production run of 500 ns was performed with integration time of 2 fs.

An all-atom MD simulation on Eco_orn using Gromacs, version 5.1.4 for 500 ns before further analysis.

To monitor the effect of C-terminal tail of Ms_orn, in *silico* deletion of the tail from residues 181 to 198 or 186 to 198 was carried out to yield coordinates corresponding to Ms_ornΔC or Ms_ornΔC. Further MD simulation of Ms_ornΔC or Ms_ornΔC was carried out as described for full-length aforementioned Ms_orn. The generated MD trajectories were analyzed by gmx rms and gmx gyrate utilities of Gromacs to calculate rmsdavg and R_g values across the timescale of MD simulations of 500 ns. Graphical analysis of MD trajectories was done using Xmrgace.

CD measurements

Far-UV CD measurements for Ms_orn, Ms_ornΔC, or Eco_orn were carried out with Jasco J-815 spectrophotometer.
Structure and stability features of Orn of M. smegmatis

equipped with a temperature controller (PTC-517). The CD instrument was continuously purged with nitrogen gas at a flow rate of 5 to 8 l/min and routinely calibrated with D-10 camphor sulfonic acid. Far-UV CD spectra were measured in the wavelength range of 250 to 195 nm with 0.2 mg/ml of respective protein in 20 mM Tris–HCl, pH 8.5, 100 mM NaCl, and 5% glycerol. CD measurements were carried out in a 1 mm path length cuvette at a scan rate of 100 nm/s, 1 nm bandwidth, and 1 s response time. An average of three consecutive scans, corrected by subtraction of buffer (used as blank), was used for each spectrum. Obtained raw CD data were converted into mean residue ellipticity at a wavelength \([\theta]_\lambda\) (deg cm² dmol⁻¹) by using the equation, \([\theta]_\lambda = M_o \theta_i / 10lc\) (where, \(M_o\) is mean residue weight of a protein, \(\theta_i\) is observed ellipticity in mdeg at \(\lambda\) wavelength, \(c\) is concentration of protein (mg/ml), and \(l\) is cuvette path length in cm). All CD spectra measurements were carried out in triplicates.

**Thermal denaturation measurements by CD**

Heat-induced denaturation of Ms_orn, Ms_ornΔC, or Eco_orn were carried out with 0.2 mg/ml of respective protein on a Jasco J-815 spectrophotometer. Denaturation was measured in the temperature range of 20 to 85 °C at a heating rate of 1 °C/min. Changes in CD signal were recorded at 222 nm, as a function of temperature. Obtained raw data were converted to mean residue ellipticity at \([\theta]_{222}\). Reversibility after heat denaturation was checked by cooling the denatured protein to 20 °C and then matching the spectrum with the spectrum taken before heating the protein. All thermal denaturation measurements were carried out in triplicates.

**Intrinsic fluorescence measurements**

Fluorescence spectra of Ms_orn, Ms_ornΔC, or Eco_orn were measured with 0.2 mg/ml protein in 20 mM Tris–HCl, pH 8.5, 100 mM NaCl, and 5% glycerol using Jasco FP-6200 spectrofluorometer, equipped with an external thermostated water circulator to maintain constant sample temperature. The excitation was carried out at 295 nm followed by measurement of the emission spectra in the 300 to 400 nm wavelength range. All measurements were carried out in triplicates.

**Gdm-HCl-induced denaturation measurements by CD and fluorescence**

A stock solution of 7 M Gdm-HCl was prepared in 20 mM Tris–HCl, pH 8.5, 100 mM NaCl, and 5% glycerol, and its concentration was determined by refractive index measurements. Equilibrium unfolding of Ms_orn, Ms_ornΔC, or Eco_orn induced by Gdm-HCl was monitored by far-UV CD as well as by intrinsic fluorescence. About 0.2 mg/ml of respective protein was incubated with different concentrations of Gdm-HCl between 0 to 3-4 M in several experimental runs for 2 to 3 h before spectral measurements. The denaturation curves with Gdm-HCl were finally carried out between 0 and 6 M of the denaturant in two independent runs, and the transition curves of Ms_orn, Ms_ornΔC, and Eco_orn were analyzed to estimate the thermodynamic parameters \(\Delta G_1\) (\(\Delta G\) associated with transition I) and \(\Delta G_{II}\) (\(\Delta G\) associated with transition II) using the following equations,

\[
\Delta G_1 = -RT \ln \frac{y_{\text{U}} - y_{\text{N}}}{y_{\text{X}} - y}
\]

\[
\Delta G_{II} = -RT \ln \frac{y_{\text{U}} - y_{\text{N}}}{y_{\text{Y}} - y}
\]

where \(T\) is temperature in Kelvin, \(R\) is universal gas constant, and \(y_{\text{U}}, y_{\text{X}},\) and \(y_{\text{Y}}\), respectively, represent the properties of the protein molecules in the native, intermediate, or unfolded state at the same [Gdm-HCl] in which \(y\) (observed property of protein) was measured. A linear plot of \(\Delta G_1\) or \(\Delta G_{II}\) versus [Gdm-HCl] was obtained, enabling estimation of \(\Delta G^0_1\) (value of \(\Delta G_1\) at 0 M Gdm-HCl) associated with transition I and \(\Delta G^X_{II}\) (value of \(\Delta G_{II}\) in the presence of Gdm-HCl, where X state existed) associated with transition II, using least square analysis according to the equations,

\[
\Delta G_1 = \Delta G^0_1 - m_1 [\text{Gdm-HCl}]
\]

\[
\Delta G_{II} = \Delta G^X_{II} - m_{II} [\text{Gdm-HCl}]
\]

**ANS measurements**

The stock solution of ANS was prepared in water, and its concentration was determined using value of 5000 M⁻¹ cm⁻¹ for molar absorption coefficient at 350 nm (72). For ANS fluorescence measurements, the protein sample was incubated with 25-fold molar excess of ANS, for 2 to 3 h in the dark. Emission spectra were collected in the wavelength range of 400 to 600 nm after excitation at 360 nm. For all fluorescence measurements, quartz cuvette of 10 mm path length was used with excitation and emission slits, both set at 5 nm bandwidth. All experiments were repeated twice before plotting.

**Growth of M. smegmatis knockout and complemented strains**

A knockout of orn in M. smegmatis was generated by a suicidal vector strategy followed by homologous recombination method (PB and BT [unpublished observations]). M. smegmatis mc²155 (WT), knockout strain (orn-depleted strains, Δorn), and complemented strains (Δorn/orn, Δorn/ΔornΔC, and Δorn/ornΔC), complemented with clones in pTC0X1L, expressing proteins corresponding to full length (orn), 1 to 185 residues (ornΔC) or 1 to 180 residues (ornΔC'), were grown in Difco Middlebrook 7H9 broth supplemented with 0.25% glycerol, 0.05% Tween-80 and 0.4% glucose or on 7H9 medium containing 1.5% agar at 37 °C with constant shaking. Growth was monitored by measuring optical density at 600 nm using Bioscreen growth curve analyzer. Growth experiments were done for two independent biological

---

**ASBMBM**

J. Biol. Chem. (2022) 298(12) 102595 17
replicates. All growth experiments with WT and knockout strain (Δorn) were with an empty vector control (pTC0X1L).

**Growth under stress conditions**

For UV stress, serial dilutions of WT, Δorn, Δorn/orn, Δorn/ornΔC, and Δorn/ornΔC were spotted on 7H9 medium containing 1.5% agar plates and irradiated under UV light for 15 min. The plates were incubated at 37 °C in the dark for 2 to 3 days. For oxidative stress, WT, Δorn, Δorn/orn, Δorn/ornΔC, and Δorn/ornΔC were grown in 7H9 broth to early log phase to a cell density of 0.2. Strains were inoculated with different concentrations of H2O2 for 3 h. Cells were then serially diluted and spotted on 7H9 medium containing 1.5% agar and incubated at 37 °C for 2 to 3 days.

Growth under oxidative stress was also monitored by first growing WT, Δorn, Δorn/orn, Δorn/ornΔC, and Δorn/ornΔC’ in 7H9 medium at 37 °C until an absorbance of ~1.0 at 600 nm with constant shaking. The respective strains were then diluted 1:100 with fresh medium and 100 μl of each diluted culture was inoculated separately into honeycomb 100-well plates with varying concentrations of H2O2 (10, 5, 2.5, 1.25, and 0.625 mM H2O2). Growth was then monitored using Bioscreen growth curve analyzer every 3 h. The experiment was performed with two independent biological replicates.

**RNA isolation and qRT–PCR**

About 10 ml cultures of WT, Δorn, Δorn/orn, Δorn/ornΔC, and Δorn/ornΔC’ were grown to stationary phases (optical density of ~1.0 at 600 nm), and RNA was isolated using TRIzol method. RNA was quantified by measuring absorbance at 260 nm and quality was checked with absorbance at 260 nm/absorbance at 280 nm and absorbance at 260 nm/absorbance at 230 nm ratios and on a 2% agarose gel. To synthesize complementary DNA, 1.5 μg RNA was first treated with DNase at 37 °C, followed by complementary DNA synthesis using SuperScript IV first-strand synthesis system (Invitrogen) and random hexamers, according to the protocol provided by the manufacturer. SYBR Green (Applied Biosystems) was used as indicator dye for qRT–PCR. The RNA levels were normalized with respect to the rpoC gene of *M. smegmatis* (encoding β′ subunit of RNA polymerase). The experiments were performed for three biological replicates, and each experiment was performed in three technical replicates.

**Western blotting for detection of Ms_orn in complemented strains**

Complemented strains with clones expressing proteins corresponding to full-length Ms_orn (orn), 1 to 185 residues (ornΔC) or 1 to 180 residues (ornΔC’) with an N-terminal His6 tag were grown in 400 ml 7H9 medium for 24 h at 37 °C. Cells were harvested and respective protein from indicated strain was purified using Ni–NTA affinity chromatography as described for Ms_orn and concentrated to 100 μl. About 30 μl of protein aliquot was separated on 15% SDS-PAGE and electro-transferred to a nitrocellulose membrane. The membrane was blocked with 4% bovine serum albumin dissolved in 1× PBS for 2 h at room temperature and incubated overnight with 1:3000 dilution of mouse anti–His tag antibody at 4 °C. After washing the membrane two times with 1× PBS with Tween, followed by 1× PBS, the membrane was incubated with 1:1500 dilution of antimouse horseradish peroxidase–conjugated secondary antibody at room temperature for 3 h. The membrane was washed again with 1× PBS with Tween-20 and 1× PBS and incubated with chemiluminescent substrates. The developed membrane blot was imaged using ImageQuant LAS 500 (GE). Prestained protein ladder was used for molecular weight estimation.

**Enzyme activity assay**

Phosphatase or PDE activity of Ms_orn, Ms_ornΔC or Eco_orn was tested using pNPP or bNPP as respective substrates. The reactions were carried out in separate 100 μl reaction mix containing 4 μM of purified protein and 200 μM substrate in 20 mM Tris–HCl, pH 8.5, 100 mM NaCl, and 5 mM MgCl2. The reactions were incubated at 37 °C, and release of p-nitrophenol was monitored colorimetrically at λ405nm using Tecan infinite 200 Microplate reader. Activity assay was performed for two biological replicates and each replicate was performed in triplicate.

**Data availability**

All data described in the article are available from the authors on request. The atomic coordinates and structure factors of Ms_orn and Eco_orn have been deposited with the PDB with accession codes 7WIK and 7VH4, respectively.

**Supporting information**—This article contains supporting information (73).

**Acknowledgments**—We sincerely thank CSIR-Institute of Genomics and Integrative Biology for providing the infrastructure and central instrument lab facility. We thank “Open SESAME & Instruct-ERIC Workshop on Remote X-ray Data Collection from European Synchrotrons” at the Weizmann Institute of Science (May 14–18, 2018) for hands-on data collection at ID29, European Synchrotron Radiation Facility (ESRF). We also thank ESRF Access Program of Regional Centre for Biotechnology, Faridabad (supported by the grant no.: BT/INF/22/SP22660/2017 of the Department of Biotechnology, Ministry of Science and Technology to Regional Centre for Biotechnology) for data collection experiments at ESRF.

**Author contributions**—B. T. conceptualization; P. B., S. H. K., and B. T. methodology; P. B., S. H. K., and B. T. formal analysis; P. B. and B. T. investigation; P. B. data curation; P. B. and B. T. writing—original draft; P. B., S. H. K., and B. T. writing—review & editing; P. B. visualization; B. T. supervision.

**Funding and additional information**—P. B. and S. H. K. acknowledge financial assistance from CSIR as CSIR-Senior Research Fellow and CSIR-Research Associate, respectively.

**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.
Structure and stability features of Orn of M. smegmatis

References

1. Romling, U., Galperin, M. Y., and Gomelsky, M. (2013) Cyclic di-GMP: the first 25 Years of a universal bacterial second messenger. Microbiol. Mol. Biol. Rev. 77, 1–52

2. Hengge, R. (2009) Principles of c-di-GMP signalling in bacteria. Nat. Rev. Microbiol. 7, 263–273

3. Schirmer, T., and Jenal, U. (2009) Structural and mechanistic determinants of c-di-GMP signalling. Nat. Rev. Structural Mol. Biol. 7, 724–735

4. Jenal, U., Reinders, A., and Lori, C. (2017) Cyclic di-GMP: second messenger extraordinaire. Nat. Rev. Microbiol. 15, 271–284

5. Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., et al. (2017) Cyclic di-GMP: second messenger and regulator of virulence in Pseudomonas aeruginosa. Mol. Microbiol. 107, 1283–1299

6. Banerjee, P., Sahoo, P. K., Sheenu, Adhikary, A., Ruhal, R., and Jain, D. (2021) Molecular and structural facets of c-di-GMP signalling associated with biofilm formation in Pseudomonas aeruginosa. Mol. Aspects Med. 81, 101001

7. Monds, R. D., Newell, P. D., Gross, R. H., and O’Toole, G. A. (2007) Phosphate-dependent modulation of c-di-GMP levels regulates Pseudomonas fluorescens PAO1 biofilm formation by controlling secretion of the adhesin LasA. Mol. Microbiol. 63, 656–679

8. Simm, R., Morr, M., Kader, A., Nimtz, M., and Römling, U. (2004) GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Mol. Microbiol. 53, 1123–1134

9. Chen, L.–H., Köseöglu, V. K., Güvener, Z. T., Myers-Morales, T., Reed, J. M., D’Orazio, S. E. F., et al. (2014) Cyclic di-GMP-dependent signaling pathways in the pathogenic Firmicute Listeria monocytogenes. PLoS Pathog. 10, e1004301

10. Duerig, A., Abel, S., Folcher, M., Nicollier, M., Schwede, T., Amiot, N., et al. (2009) Second messenger-mediated spatiotemporal control of protein degradation regulates bacterial cell cycle progression. Genes Dev. 23, 93–104

11. Kaczmarczyk, A., Hempel, A. M., von Arx, C., Böhm, R., Dubey, B. N., Nesper, J., et al. (2020) Precise timing of transcription by c-di-GMP coordinates cell cycle and morphogenesis in Caulobacter. Nat. Commun. 11, 816

12. Tschier, A. D., and Camilli, A. (2004) Cyclic guanylate (c-di-GMP) regulates Vibrio cholerae biofilm formation: c-di-GMP regulates V. cholerae biofilm. Mol. Microbiol. 53, 857–869

13. Tamayo, R., Schild, S., Pratt, J. T., and Camilli, A. (2008) Role of cyclic di-GMP during el tor biotype Vibrio cholerae infection: characterization of the in vivo-induced cyclic di-GMP phosphodiesterase CdpA. Infect. Immun. 76, 1617–1627

14. Ryan, R. P., Fouhy, Y., Lucey, J. F., Jiang, B.-L., He, Y.-Q., Feng, J.-X., et al. (2007) Cyclic di-GMP signalling in the virulence and environmental adaptation of Xanthomonas campestris. Mol. Microbiol. 63, 429–442

15. Ryan, R. P. (2013) Cyclic di-GMP signalling and the regulation of bacterial virulence. Microbiology 159, 1286–1297

16. Rosen, D. A., Twentymann, J., and Hunstad, D. A. (2018) High levels of cyclic di-GMP in Klebsiella pneumoniae attenuate virulence in the lung. Infect. Immun. 86, e00647–17

17. Hong, Y., Zhou, X., Fang, H., Yu, D., Li, C., and Sun, B. (2013) Cyclic di-GMP mediates Mycobacterium tuberculosis dormancy and pathogenicity. Tuberc. Edinb. Scotl. 93, 625–634

18. Bharati, B. K., Sharma, I. M., Kasetty, S., Kumar, M., Mukherjee, R., and Chatterjee, D. (2012) A full-length bifunctional protein involved in c-di-GMP turnover is required for long-term survival under nutrient starvation in Mycobacterium smegmatis. Microbiology 158, 1415–1427

19. Gupta, K. R., Kasetty, S., and Chatterjee, D. (2015) Novel functions of (p)pGpp and cyclic di-GMP in mycobacterial physiology revealed by phenotype microarray analysis of wild-type and isogenic strains of Mycobacterium smegmatis. Appl. Environ. Microbiol. 81, 2571–2578

20. Zhang, H.-N., Xu, Z.-W., Jiang, H.-W., Wu, F.-L., He, X., Liu, Y., et al. (2017) Cyclic di-GMP regulates Mycobacterium tuberculosis resistance to ethionamide. Sci. Rep. 7, 5860

21. Ryan, R. P., Lucey, J., O’Donovan, K., McCarthy, Y., Yang, L., Tolkner-Nielsen, T., et al. (2009) HD-GYP domain proteins regulate biofilm formation and virulence in Pseudomonas aeruginosa. Environ. Microbiol. 11, 1126–1136

22. Dow, J. M., Fouhy, Y., Lucey, J. F., and Ryan, R. P. (2006) The HD-GYP domain, cyclic di-GMP signaling, and bacterial virulence to plants. Mol. Plant-microbe Interactions 19, 1378–1384

23. Dow, J. M., Crossman, L., Findlay, K., He, Y.-Q., Feng, J.-X., and Tang, J.-L. (2003) Biofilm dispersal in Xanthomonas campestris is controlled by cell-cell signaling and is required for full virulence to plants. Proc. Natl. Acad. Sci. U. S. A. 100, 10995–11000

24. Schmidt, A. J., Ryjenkov, D. A., and Gomelsky, M. (2005) The ubiquitous protein domain EAL is a cyclic guanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. J. Bacteriol. 187, 4774–4781

25. Tao, F., Yang, Y., Qi, Y., and Liang, Z.-X. (2008) Catalytic mechanism of cyclic di-GMP-specific phosphodiesterase: a study of the EAL domain-containing RocR from Pseudomonas aeruginosa. J. Bacteriol. 190, 3622–3631

26. Christen, M., Christen, B., Folcher, M., Schauerte, A., and Jenal, U. (2005) Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. J. Biol. Chem. 280, 30829–30837

27. Yu, D., and Deutscher, M. P. (1995) Oligoribonuclease is distinct from the other known exoribonucleases of Escherichia coli. J. Bacteriol. 177, 4137–4139

28. Orr, M. W., Donaldson, G. P., Severin, G. B., Wang, J., Sintim, H. O., Waters, C. M., et al. (2015) Oligoribonuclease is the primary degradative enzyme for pGpG in Pseudomonas aeruginosa that is required for cyclic-di-GMP turnover. Proc. Natl. Acad. Sci. U. S. A. 112, E5048–E5057

29. Cohen, D., Mechold, U., Nevenzal, H., Yarmyshu, Y., Randall, T. E., Bay, D. C., et al. (2015) Oligoribonuclease is a central feature of cyclic guanylate signaling in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U. S. A. 112, 11359–11364

Abbreviations—The abbreviations used are: ANS, 8-anilino-1-naphthalene sulfonic acid; bNPP, bis-(p-nitrophenol) phosphate; c-di-GMP, cyclic-di-GMP; DGC, diguanylate cyclase; Eco_or, Orn of Escherichia coli; EAL, glutamate-alanine-leucine; ESRF, European Synchrotron Radiation Facility; Gdm-HCl, guanidium hydrochloride; HD-GYP, histidine-aspartate-glycine-tyrosine-proline; MALLS, multiangle laser light scattering; MD, molecular dynamics; Ms_or, Orn of Mycobacterium smegmatis; Mb_or, Orn of Mycobacterium tuberculosis; Ni–NTA, nickel–nitrilotriacetate; Orn, oligoribonuclease; P-cap, phosphate-cap; PDB, Protein Data Bank; PDE, phosphodiesterase; pNPP, p-nitrophenol phosphate; qRT–PCR, quantitative RT–PCR; SEC, size-exclusion chromatography; Vc_or, Orn of Vibrio cholerae.
Structure and stability features of Orn of M. smegmatis

35. Gupta, K. R., Baloni, P., Indi, S. S., and Chatterji, D. (2016) Regulation of growth, cell shape, cell division, and gene expression by second messengers (pppGpp and cyclic di-GMP in Mycobacterium smegmatis. *J. Bacteriol.* 188, 1414–1422

36. Liao, H., Liu, M., and Guo, X. (2018) The special existences: nanoRNA and nanoRNase. *Microbiol. Res.* 207, 134–139

37. Zhang, X., Zhu, L., and Deutscher, M. P. (1998) Oligoribonuclease is encoded by a highly conserved gene in the 3'-5' exonuclease superfamily. *J. Bacteriol.* 180, 2779–2781

38. Niyogi, S. K., and Datta, A. K. (1975) A novel oligoribonuclease of Eschericia coli. I. Isolation and properties. *J. Biol. Chem.* 250, 7307–7312

39. Zuo, Y., and Deutscher, M. P. (2001) Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucl. Acids Res.* 29, 1017–1026

40. Ghosh, S., and Deutscher, M. P. (1999) Oligoribonuclease is an essential component of the mRNA decay pathway. *Proc. Natl. Acad. Sci. U. S. A.* 96, 4372–4377

41. Goldman, S. R., Sharp, J. S., Vvedenskaya, I. O., Livny, J., Dove, S. L., and et al. (2011) Human REX02 controls short signaling molecules (p)ppGpp and cyclic di-GMP in Mycobacterium smegmatis. *J. Bacteriol.* 164, 3909–3921

42. Nickels, B. E. (2011) NanoRNAs prime transcription initiation in vivo. *Mol. Cell.* 42, 817–825

43. Chen, F., Chen, G., Liu, Y., Jin, Y., Cheng, Z., Liu, Y., et al. (2017) Pseudomonas aeruginosa oligoribonuclease contributes to tolerance to ciprofloxacin by regulating pyocin biosynthesis. *Antimicrob. Agents Chemother.* 61, e02256–16

44. Xia, B., Li, M., Tian, Z., Chen, G., Xia, Y., et al. (2019) Oligoribonuclease contributes to tolerance to aminoglycoside and β-lactam antibiotics by regulating KatA in Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* 63, e00212–19

45. Chen, G., Zhao, Q., Zhu, F., Chen, R., Jin, Y., Liu, C., et al. (2016) Oligoribonuclease is required for the type III secretion system and pathogenesis of Pseudomonas aeruginosa. *Microbiol. Res.* 188–189, 90–96

46. Krissinel, E., and Henrick, K. (2007) Inference of macromolecular assemblies from crystalline state. *J. Mol. Biol.* 372, 774–797

47. Kim, S.-K., Lormand, J. D., Weiss, C. A., Eger, K. A., Turdiev, H., Turdiev, A., et al. (2019) A dedicated diribonucleotidase resolves a key bottleneck for the terminal step of RNA degradation. *life* 8, e46313

48. Szewczyk, M., Malik, D., Borowski, L. S., Czarnomska, S. D., Kotrys, A. V., Kłosowska-Kosicka, K., et al. (2020) Human REXO2 controls short mitochondrial RNAs generated by mtRNA processing and decay machinery to prevent accumulation of double-stranded RNA. *Nucl. Acids Res.* 48, 5572–5590

49. Cho, L.-Y., Agrawal, S., Chen, Y.-P., Yang, W.-Z., and Yuan, H. S. (2019) A novel oligoribonuclease of Eschericia coli. II. Mechanism of action. *J. Biol. Chem.* 251, 7313–7319

50. Khan, S. H., Bijupuria, S., Mauya, A., and Taneja, B. (2020) Structural and thermodynamic characterization of a highly stable conformation of Rv2966c, a 16S rRNA methyltransferase, at low pH. *Int. J. Biol. Macromol.* 164, 3909–3921

51. Lack, N. A., Kawamura, A., Fullam, E., Laurieri, N., Beard, S., Russell, A. J., et al. (2009) Temperature stability of proteins essential for the intracellular survival of Mycobacterium tuberculosis. *Biochem. J.* 418, 369–378

52. Holm, L. (2020) Using dali for protein structure comparison. In: Gáspár, Z., ed., *29–42. 2112. Structural Bioinformatics*, Springer US, New York, NY: 29–42

53. Srivastav, R., Kumar, D., Grover, A., Singh, A., Manjasetty, B. A., Sharma, R., et al. (2014) Unique subunit packing in mycobacterial nanoRNase leads to alternate substrate recognitions in DHH phosphodiesterases. *Nucl. Acids Res.* 42, 7894–7910

54. Folta-Stogniew, E., and Williams, K. R. (1999) Determination of molecular mass of proteins in solution: implementation of an HPLC size exclusion chromatography and laser light scattering service in a core laboratory. *J. Biomol. Tech. JBT.* 10, 51–63

55. Vonrhein, C., Flensburg, C., Keller, P., Sharff, A., Smart, O., Piacorek, W., et al. (2011) Data processing and analysis with the autoPROC toolbox. *Acta Crystallogr. D Biol. Crystallogr.* 67, 293–302

56. Kabsch, W. (2010) XDS. *Acta Crystallogr. D Biol. Crystallogr.* 66, 125–132

57. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* 40, 658–674

58. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* 60, 2126–2132

59. Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., et al. (2011) REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr. D Biol. Crystallogr.* 67, 355–367

60. Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., et al. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr. D Biol. Crystallogr.* 66, 12–21

61. Hess, B., Bekker, H., Berendsen, H. J. C., and Fraaije, J. G. E. M. (1997) Lincs: a linear constraint solver for molecular simulations. *J. Comput. Chem.* 18, 1463–1472

62. Abraham, M. J., Murtola, T., Schulz, R., Páll, S., Smith, J. C., Hess, B., et al. (2015) Gromacs: high performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* 1–2, 19–25

63. Vanommeslaeghe, K., Hatcher, E., Acharya, C., Kundu, S., Zhong, S., Shim, J., et al. (2009) CHARMM general force field: a force field for drug-like molecules compatible with the CHARMM all-atom additive biological force fields. *J. Comput. Chem.* https://doi.org/10.1002/jcc.21367

64. Ziekiewicz, J. (2005) Structural properties of water: comparison of the SPC, SPCE, TIP4P, and TIP5P models of water. *J. Chem. Phys.* 123, 104501

65. Darden, T., York, D., and Pedersen, L. (1993) Particle mesh Ewald: an N log(N) method for Ewald sums in large systems. *J. Chem. Phys.* 98, 10089–10092

66. Weber, G., and Young, L. B. (1964) Fragmentation of bovine serum albumin by pepsin. I. The origin of the acid expansion of the albumin molecule. *J. Biol. Chem.* 239, 1415–1423

67. Wallace, A. C., Laskowski, R. A., and Thornton, J. M. (1995) LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Eng.* 8, 127–134