Characterization of a dual-active enzyme, DcpA, involved in cyclic diguanosine monophosphate turnover in *Mycobacterium smegmatis*

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We have reported previously that the long-term survival of *Mycobacterium smegmatis* is facilitated by a dual-active enzyme MSDGC-1 (renamed DcpA), which controls the cellular turnover of cyclic diguanosine monophosphate (c-di-GMP). Most mycobacterial species possess at least a single copy of a DcpA orthologue that is highly conserved in terms of sequence similarity and domain architecture. Here, we show that DcpA exists in monomeric and dimeric forms. The dimerization of DcpA is due to non-covalent interactions between two protomers that are arranged in a parallel orientation. The dimer shows both synthesis and hydrolysis activities, whereas the monomer shows only hydrolysis activity. In addition, we have shown that DcpA is associated with the cytoplasmic membrane and exhibits heterogeneous cellular localization with a predominance at the cell poles. Finally, we have also shown that DcpA is involved in the change in cell length and colony morphology of *M. smegmatis*. Taken together, our study provides additional evidence about the role of the bifunctional protein involved in c-di-GMP signalling in *M. smegmatis*.

### INTRODUCTION

Cyclic diguanosine monophosphate (c-di-GMP) is a ubiquitous second messenger in the bacterial kingdom (Hengge, 2009; Romling et al., 2013) and has been found recently in a eukaryote *Dictyostelium discoideum* (Chen & Schaap, 2012). c-di-GMP is synthesized by diguanylate cyclase (DGC), having a conserved GGDEF motif, and hydrolysed by phosphodiesterases (PDEs), having conserved EAL or HD-GYP motifs. The state of balance in the two opposing activities determines a healthy cellular concentration of c-di-GMP. Based on its concentration, c-di-GMP participates in the regulation of various aspects of cellular physiology, including motility, biofilm formation, cell differentiation, virulence and quorum sensing, amongst many others (Hengge, 2009; Romling et al., 2013). We have reported previously that c-di-GMP facilitates the long-term survival of mycobacteria under carbon-limiting conditions (Gupta et al., 2010b; Bharati et al., 2012, 2013). The genes associated with such a sustained capacity to resist carbon-limiting conditions were identified as Rv1354c in *Mycobacterium tuberculosis* (Gupta et al., 2010b) and MSMEG_2196 in *Mycobacterium smegmatis* (Bharati et al., 2012). These genes encode the bifunctional enzymes MtbDGC and MSDGC-1 (renamed DcpA for ‘diguanylate cyclase and phosphodiesterase A’), respectively. These proteins exhibit both c-di-GMP synthesis as well as hydrolysis activity (Gupta et al., 2010b; Bharati et al., 2012). Our bioinformatics analysis also revealed that many mycobacterial species harbour at least one copy of a homologous protein that may be involved in c-di-GMP turnover (Bharati et al., 2012).

DcpA is a multidomain bifunctional protein in which the GAF, GGDEF and EAL domains are arranged in tandem. Its close orthologue MtbdGC (Rv1354c) in *M. tuberculosis* shares high sequence similarity as well as conserved signature motifs and domain architecture. Most of the c-di-GMP-metabolizing proteins studied so far have either DGC or PDE activities (Hengge, 2009; Romling et al., 2013). The presence of both the activities in tandem GGDEF–EAL domain proteins is intriguing and has been referred to as an ‘enzymatic conundrum’ (Seshasayee et al., 2010; Romling et al., 2013). The investigations of the regulatory switches that may result in the catalytic dominance of one activity over the other in such cases are sparse.

However, there are a few reports that deal with the mechanistic details of the dual-active enzymes. For example,
Rhodobacter spheroides encodes a bacteriophytochrome BphG1 (RSP _4191) that has PAS–GAF–PHY–GGDEF–EAL domains in tandem. The full-length protein shows PDE activity, but a site-specific hydrolysis results in a PAS–GAF–PHY–GGDEF domain protein that exhibits DGC activity (Tarutina et al., 2006). In Vibrio parahaemolyticus, a bifunctional GGDEF–EAL protein, ScrC (VPA1511), shows DGC activity in isolation; however, it switches to PDE activity upon interaction with ScrA (VPA1513) and ScrB (VPA1512). This interaction is induced by a novel autoinducer that binds to ScrB (Ferreira et al., 2008). In Legionella pneumophila, a REC–PAS–GGDEF–EAL domain protein, Lp0329, has both DGC and PDE activities. A site-specific phosphorylation of the REC domain results in a lowering of the DGC activity, whereas PDE activity remains unaltered (Levet-Paulo et al., 2011). In Shewanella woodyi, a GGDEF–EAL protein shows predominant DGC activity when it interacts with the H-NOX domain. However, the binding of NO with the H-NOX domain results in the dominance of PDE activity (Liu et al., 2012). Therefore, DcpA, having GAF–GGDEF–EAL domains, and both synthesis and hydrolysis activities, presents itself as an attractive model to study such bifunctional behaviour.

Additionally, it has been reported previously that persistor mycobacterial cells show characteristic cellular structures, colony morphology and staining patterns (Ojha et al., 2000; Dahl et al., 2005). As DcpA is involved in regulating the long-term survival in M. smegmatis, we wanted to investigate if it is also involved in regulating the phenotypes of the persistor cells. It is generally believed that a better understanding of cellular physiology of persistent mycobacteria may lead to the development of effective inhibitors (Parrish et al., 1998). Therefore, in this work we focused on the mechanism of activity of the protein involved in regulating such phenotypes in M. smegmatis. We report here that DcpA exists in monomeric and dimeric forms, with different activities. The activities of these forms have been determined using various biochemical approaches. Furthermore, we have also observed that DcpA appears to be associated with the membrane mostly at the cell poles, and affects cell length and colony morphology of M. smegmatis.

**METHODS**

**Strains, media and culture conditions.** Table 1 contains details of the various strains and plasmids used in this study. M. smegmatis mc² 155 (WT), M. smegmatis mc² 155 harbouring pIJAM-DcpA (DOE) and DcpA knockout M. smegmatis cells (DKO) were grown in Middlebrook 7H9 (Difco) broth supplemented with 2 % glucose and 0.05 % Tween 20 with or without agar, wherever necessary. Acetamide and IPTG were used for the induction of genes in pJAM2 and pET21b vectors, respectively. Escherichia coli BL21(DE3) cells were used for the expression of various proteins. Protein purification protocols were adapted from our previous work (Sharma et al., 2012). When required, antibiotics were added at the following concentrations: 100 μg ampicillin ml⁻¹ and 25 μg kanamycin ml⁻¹. Most of the chemicals/reagents and restriction enzymes/polymerases were purchased from Sigma and New England Biolabs, respectively.

**Cloning experiments.** For overexpression of DcpA (His₆-tagged) in M. smegmatis, a PCR amplicon of the full-length MSMEG_2196 gene predigested with BamHI and XhoI was cloned in pIAM2, resulting in pIAM-DcpA. The expression of DcpA was checked in M. smegmatis by acetamide (0.5 %) induction and validated by probing the cell lysate with anti-His, antibodies. For construction of DcpA fused with the leucine zipper, the DNA sequence of the leucine zipper was amplified from pKT25-zip (Euromedex), and Ndel and Nhel restriction sites were incorporated. Both pET-DcpA and PCR products were digested with the same sets of enzymes and ligated. For construction of pIAM-Rfp-DcpA, the red fluorescent protein (RFP) sequence was amplified from pCherry3 (Addgene) that possesses a BamHI site and inserted at the N terminus of DcpA in pIAM-DcpA. Next, RFP was further amplified from the same source possessing BamHI and XhoI sites, and cloned in pIAM2, resulting in pIAM-Rfp. The clones constructed in this study were confirmed by sequencing at Eurofins Genomics.

**Native PAGE analysis.** To determine the oligomeric status of DcpA, 10 % native polyacrylamide gel was used. The gel loading dye did not contain any reducing agents and the samples were not boiled prior to loading. The calculated pl of DcpA is 5.2. Hence, BSA (66 kDa, pl 4.8) and ferritin (440 kDa, pl 4.5) were used as molecular mass standards, when required. The gel was visualized by silver staining.

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**Table 1. Bacterial strains and plasmids used**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>description</th>
<th>Source</th>
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<tr>
<td><strong>M. smegmatis</strong></td>
<td><strong>WT strain</strong></td>
<td>Snapper et al. (1990)</td>
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<tr>
<td>DOE</td>
<td>M. smegmatis WT strain harbouring pIAM-DcpA (Kan’)</td>
<td>This study</td>
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<tr>
<td>DKO</td>
<td>M. smegmatis strain in which MSMEG_2196 was deleted (Kan’)</td>
<td>Bharati et al. (2012)</td>
</tr>
<tr>
<td>pET-PleD</td>
<td>PleD gene was cloned in pET (Ampr)</td>
<td>Gift from Professor Urs Jenal (Biozentrum, Basel, Switzerland)</td>
</tr>
<tr>
<td>pET-DcpA</td>
<td>ORF of MSMEG_2196 was cloned in pET21b (Ampr)</td>
<td>Bharati et al. (2012)</td>
</tr>
<tr>
<td>pET-Dcp-N-zip</td>
<td>Leucine zipper was fused at the N terminus of DcpA in pET-DcpA (Ampr)</td>
<td>This study</td>
</tr>
<tr>
<td>plAM2</td>
<td>Acetamide-inducible vector for mycobacterial protein expression (Kan’)</td>
<td>Triccas et al. (1998)</td>
</tr>
<tr>
<td>plAM-DcpA</td>
<td>plAM2 with ORF of MSMEG_2196 (Kan’)</td>
<td>This study</td>
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<tr>
<td>plAM-Rfp-Dcp</td>
<td>RFP was fused at the N terminus of DcpA in pIAM-DcpA (Kan’)</td>
<td>This study</td>
</tr>
<tr>
<td>pIAM-Rfp</td>
<td>ORF of RFP in plAM2 (Kan’)</td>
<td>Euromedex (BACTH system kit)</td>
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<tr>
<td>pKT25-zip</td>
<td>Source of leucine zipper</td>
<td>Carroll et al. (2010)</td>
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<tr>
<td>pCherry3</td>
<td>Source of RFP (Hygr)</td>
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Gel filtration. Size-exclusion chromatography was also performed to determine the oligomeric status of DcpA. A Superdex 200 10/300 column (Amersham Pharmacia) connected to an AKTA FPLC instrument (GE healthcare) was pre-equilibrated with the equilibration buffer (Tris/glycine buffer, pH 8.3). The purified protein (−0.5 mg mL⁻¹) was then injected into the column and allowed to pass from it at a flow rate of 0.6 mL min⁻¹ at 25 °C. The protein was detected automatically by measuring A₂₈₀.

Cross-linking experiments. The ability of the DcpA to form dimers was further assessed by chemical cross-linking using glutaraldehyde (Mercck) as described previously with modifications (Paul et al., 2007). The purified DcpA was dialysed against cross-linking buffer [5 mM NaPO₄, pH 7.8, 10 mM MgCl₂, 100 mM NaCl, 5 mM β-mercaptoethanol (β-ME) and 5 % glycerol]. DcpA (8 μM) was incubated with glutaraldehyde (0.01 %) at 37 °C for 0, 1 and 2 min. The reactions were quenched by the addition of 100 mM Tris/HCl, pH 7.8. The cross-linked products were then separated on a SDS-10% polyacrylamide gel and stained with silver stain to visualize the oligomeric forms.

Induced disulfide bond formation. The protocol was adapted and modified from the work of others (Indu et al., 2010). O-Phenanthroline and CuSO₄ . 5H₂O (Sigma)-mediated cross-linking was performed to determine the alignment of the two protomers in dimeric DcpA. For this purpose, DcpA was first dialysed in a buffer containing 10 mM NaPO₄, pH 7, 150 mM NaCl and 1 % glycerol. The protein (200 μg) was incubated with 5 μM o-phenanthroline and 1.5 μM CuSO₄ at 37 °C for 1 min in the dark. The cross-linked samples were then separated on a non-reducing SDS-10% polyacrylamide gel and stained with Coomassie brilliant blue dye. The cross-linked dimeric DcpA and non-cross-linked monomer were excised out from the gel, processed for trypsin digestion under reducing and native conditions, and MS was performed as described previously (Gupta et al., 2010a). In brief, for validity of the protein and identification of disulfide-bonded peptides, tryptic digests were mixed with a matrix solution containing 2-cyano-4-hydroxycinnamic acid saturated in 50 % acetonitrile/water containing 0.1 % (v/v) trifluoroacetic acid. It was then spotted onto a MALDI-TOF MS plate, air-dried and analysed with a ultraFlxExtreme MALDI-TOF spectrometer (Bruker Daltonics) using a smart beam laser in positive-ion mode.

For validation of oligomeric forms of DcpA, the peptide spectra for both monomeric and dimeric forms were acquired in the MALDI-TOF instrument. Each spectrum generated a set of m/z values that corresponded to the individual peptides. These sets of m/z values for both monomeric and dimeric DcpA were searched separately against the Matrix science MASCOT Peptide Mass Fingerprint search tool (http://www.matrixscience.com). The following parameters were set for these analyses: database, Msme; taxonomy, Mycobacterium tuberculosis complex; enzyme, trypsin; fixed modification, carbamidomethyl (C); variable modification, oxidation (M); missed cleavages, 2; protein mass, 67 kDa; peptide tolerance, ± 50 p.p.m.; mass values, MH⁺.

For the sequencing of disulfide-bonded peptide, tryptic digests generated under non-reducing conditions were passed through a reverse-phase column (Agilent Poroshell 120 EC-C18, 3 × 30 mm, 2.7 μm) attached to an Agilent 1260 HPLC coupled to a maxis impact electrospray ionization (ESI)-quadrupole-time-of-flight spectrometer (Bruker Daltonics) with a flow rate of 0.2 ml min⁻¹. The peptides eluted were then characterized by ESI-MS and ESI-MS/MS as described previously (Gupta et al., 2010a; Banerjee et al., 2011). The collision-induced dissociation (CID) experiments were performed through collision with argon gas and data were acquired in auto MS/MS mode.

Radiometric activity assays. For the in-gel activity assay, purified DcpA was loaded onto a 10% native polyacrylamide gel in 10 successive wells. After electrophoresis, three lanes of the gel were cut and stained separately, leaving the remaining seven lanes unstained. The stained gel part was then aligned with the unstained gel part, and the gel portions corresponding to the monomer, dimer and aggregate bands in the unstained gel were excised separately. The monomer, dimer and aggregate bands were then sliced separately in small pieces; care was taken not to crush them completely. These gel bands were then incubated with c-di-GMP synthesis buffer and c-di-GMP hydrolysis buffer separately. The synthesis buffer contained 50 mM Tris/HCl, pH 7.8, 500 mM NaCl, 10 mM MgCl₂, 5 mM β-ME, 0.1 mM GTP and [α-³²P]-GTP (0.01 μCi μl⁻¹, 250 Bq μl⁻¹; BRIT). A DGC from Caulobacter crescentus, PleD, which could synthesize c-di-GMP but could not hydrolyse it, was used to synthesize radioactive [³²P]-di-GMP for the hydrolysis assay. For this, PleD was incubated with the synthesis buffer (mentioned above) for 60 min and subsequently removed by boiling the reaction mixture followed by centrifugation. The supernatant was used as the substrate for the subsequent hydrolysis assays. After 3 h of incubation of gel pieces in synthesis or hydrolysis buffer, the reaction mixtures were subjected to centrifugation, and the reaction products in the supernatants were concentrated using a SpeedVac and spotted onto polyethylenamine cellulose-TLC plates in the separation buffer [saturated 1:1.5 (v/v) (NH₄)₂SO₄ and 1.5 M KH₂PO₄, pH 3.6]. These sheets were then visualized using a phosphorimager.

In the case of the activity assay in solution, all components were the same as for the in-gel activity assay, except that the protein was in solution and not in the gel, as described previously (Bharati et al., 2012; Sharma et al., 2012).

Bioinformatics analysis. The amino acid sequences of MSMEG_2196 and its orthologues in other mycobacterial species were obtained from the KEGG database (http://www.genome.jp). These sequences were analysed for domain architecture using the Pfam database (http://pfam.sanger.ac.uk/search/sequence). The membrane association of the protein was analysed using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and PSORTb (http://www.psort.org/psortb/) tools. The data generated by Seshasayee et al. (2010) were used to determine the distribution of proteins similar to DcpA across the bacterial kingdom.

Preparation of subcellular fractions. To prepare subcellular fractions, a DOE (M. smegmatis WT:pJAM-DcpA) culture pellet was resuspended in PBS (pH 7.5) containing 1 mM PMSF and then subjected to cell lysis using a French press. All the steps were performed at 4 °C. The lysate was subsequently subjected to differential ultracentrifugation as described previously (Delogu et al., 2004; Gibbons et al., 2007; Rezwan et al., 2007). Whole-cell lysate, cytosol and cytoplasmic membrane fractions were obtained at 27 000 (supernatant), 100 000 (supernatant) and 100 000 g (pellet), respectively. The protein concentration in each fraction was estimated by measuring A₂₈₀ using a NanoDrop ND-1000 spectrophotometer.

Western blot experiments. The protein or cellular fractions were subjected to PAGE under denaturing or non-denaturing, reducing or non-reducing conditions, as and when required. They were then transferred onto PVDF membranes and probed with polyclonal anti-His6 antibodies. The bound antibodies were detected by using a chemiluminescence kit (GE Healthcare), when required.

In-cell DGC activity assay. This experiment was performed following the protocol reported by De et al. (2008, 2009). E. coli BL21(DE3) cells were transformed with vectors pET21b, pET-PleD, pET-DcpA and pET-DcpN-zip to produce BL21(DE3):pET21b...
aliquot of 2 Luria–Bertani (LB) media at 37 °C with the leucine zipper), respectively. These cells were grown in WT DcpA) and BL21(DE3) : pET-Dcp-N-zip (expressing DcpA fused with the leucine zipper), respectively. These cells were grown in Luria–Bertani (LB) media at 37 °C and with shaking at 180 r.p.m. An aliquot of 2 μl from each culture was used as inoculum and spotted onto LB agar plates containing 50 μg Congo red ml⁻¹, 100 μg ampicillin ml⁻¹ and various concentrations of IPTG in triplicate. These plates were incubated at 30 °C overnight and then photographed. The cell lysates of these strains grown under similar conditions were used to check the induction level of the respective proteins.

**Colony morphology experiments.** *M. smegmatis* WT (harbouring pJAM2 vector), DOE and DKO cells were grown in liquid culture under the conditions described above. The growth rate was monitored for 32 h with shaking at 180 r.p.m. The cultures (of equal optical density) were spotted in the middle of 90 mm Petri dishes containing 1.5 % MB7H9 agar (40 ml), 25 μg kanamycin ml⁻¹, 2 % glucose and 0.5 % acetamide. After inoculation, plates were incubated at 37 °C in a humid chamber for 24 days and photographed. Scale bars were incorporated using the ImageJ tool (http://imagej.nih.gov/ij/).

**Microscopy and image analysis.** The *M. smegmatis* strains were grown and harvested after 40 h, and the preparation of cells for imaging was done as described elsewhere with modification (Thanky *et al.*, 2007). In brief, cells were washed three times using PBS and fixed using a fresh solution of paraformaldehyde (4 %). Additional PBS washes were used to remove the fixer. The cells were then mounted onto glass slides, covered with a coverslip and air-tightened. A Zeiss microscope with a x100/1.40 oil objective was used to take differential interference contrast and fluorescent images. For RFP visualization, excitation and emission wavelengths were fixed at 587 and 610 nm, respectively. All the images were processed with AxioVision Rel (Zeiss).

**Statistical analysis.** The Kruskal–Wallis test for ANOVA was performed in which cell lengths of DOE and DKO strains were compared with WT *M. smegmatis* as suggested previously (Schoonmaker *et al.*, 2014). P values <0.0001 were considered significant. The number of cells in each case was 100. This analysis was performed using GraphPad Prism 5.

**RESULTS**

**Oligomeric status of DcpA**

The role of dimerization in regulating the DGC activity of a protein with an active GGDEF domain is accepted widely (Hengge, 2009; Schirmer & Jenal, 2009). However, the information about the oligomeric requirement for the activity of the EAL domain is not completely understood (Hengge, 2009; Schirmer & Jenal, 2009). The studies showing a relationship between oligomerization and its role in c-di-GMP turnover have been done mostly with proteins possessing either the active GGDEF or EAL domains (Paul *et al.*, 2007; Rao *et al.*, 2009). We have reported previously that *M. smegmatis* harbours a protein called DcpA that is a multidomain dual-active enzyme (Bharati *et al.*, 2012). It has GAF–GGDEF–EAL domains arranged in tandem and is able to synthesize as well as hydrolyse c-di-GMP. DcpA orthologues are present in many mycobacterial species that share high degree of similarities with respect to sequence and domain organization (Table S1, available in the online Supplementary Material). Proteins with similar domain architecture and sequence homology have also been found in several other bacterial species. According to Seshasayee *et al.* (2010), ~15 % of the total bacterial genomes that encode c-di-GMP-metabolizing proteins are comprised of the GAF–GGDEF–EAL domain architecture. Therefore, DcpA can be considered as a suitable model to investigate the mechanistic details of dual-active proteins involved in c-di-GMP turnover. We have attempted to perform extensive biochemical and related phenotypic characterizations of DcpA. The following results were obtained during our study of DcpA oligomerization.

**DcpA exists in monomeric and dimeric forms.** DcpA (His₆-tagged) was purified to homogeneity (Fig. 1a), and subjected to native PAGE and analytical gel-filtration analyses. These experiments showed that DcpA existed in monomeric and dimeric forms (Fig. 1b, c). On native polyacrylamide gel, we observed that the migration of monomeric and dimeric forms of DcpA was similar to the oligomers of BSA (both had a similar mass to charge ratio, on the basis of which proteins are separated on a native polyacrylamide gel; therefore, BSA acts as a suitable marker for the determination of oligomers of DcpA). These forms were then validated by performing native Western blotting experiments using polyclonal anti-His₆ antibodies (Fig. S1). Further, chemical cross-linking experiments with glutaraldehyde were also performed that showed that DcpA had the ability to form dimers (Fig. 1d). It was reasoned that the amount of cross-linked product would be proportional to the amount of dimers present in the solution. Moreover, it has been shown that the extent of dimerization could be increased in the presence of certain factors or modifications, such as phosphorylation (Hengge, 2009; Paul *et al.*, 2007).

**Dimerization of DcpA is due to non-covalent interactions between protomers.** DcpA has four cysteine residues in its polypeptide chain; therefore, we wanted to know if the nature of the interaction between the two protomers in the dimer was covalent. The purified protein was run on a SDS-10 % polyacrylamide gel in the absence and presence of a reducing agent. Here, we observed the presence of only monomeric form and not the dimeric form (Fig. 2a, lanes 2 and 3). On a 10 % native polyacrylamide, the presence of a reducing agent did not change the oligomeric forms (Fig. 2b, lane 3). These experiments indicated clearly that dimerization was not due to covalent (disulfide) bonding, but was due to non-covalent interactions. We also performed cysteine labelling and MS analysis of DcpA under native conditions that indicated all four cysteines were in the reduced state (unpublished data), further supporting our conclusion.

**Interconversion exists between the monomeric and dimeric forms.** Next, we investigated whether the monomeric and dimeric forms were individually stable or
interconvertible. To answer this, the separated monomer and dimer eluates of the gel-filtration experiments were analysed individually on a 10% native polyacrylamide gel. We were able to see the presence of monomers in the dimer pool and dimers in the monomer pool (Fig. 2c, lanes 2 and 3). In another experiment, we separated DcpA into monomeric and dimeric forms on a 10% native polyacrylamide gel. These forms were then excised from the gel and extracted separately, and subsequently reloaded on another 10% native polyacrylamide in separate wells. Here, also, we observed similar results (Fig. 2d). These experiments suggested the interconvertible nature of the two forms. However, how fast such interconversion occurred was unclear.

Two protomers align in parallel orientation in dimeric DcpA. Subsequently, we attempted to characterize the mode of alignment of the two protomers in the dimer to assume the quaternary structure of DcpA. To accomplish this, we performed induced cross-linking of DcpA using cysteine-oxidizing agents o-phenanthroline and CuSO₄ (see Methods), followed by MS characterization. Before performing the cross-linking experiments, the suitability of the cysteine residues for cross-linking in DcpA was checked. DcpA has four sulfydryl groups distributed along the polypeptide chain with the GAF and EAL domains possessing one (C124) and three (C424, C474 and C579) cysteines, respectively. Iodoacetamide and N-ethylmaleimide labelling and subsequent MS analysis revealed that all cysteines were accessible (unpublished data).

After cross-linking, the protein was loaded in triplicate onto a non-reducing SDS-10% polyacrylamide gel in which we were able to see the presence of dimeric bands along with the prominent monomeric bands (Fig. 3a, lanes 2–4). The addition of DTT resulted in the disappearance of the dimeric form (lane 1), indicating that there had been disulfide bond formation. Although the amount of cross-linked product was less, we confirmed the identity of both monomeric and dimeric bands to be DcpA using trypsin digestion and MALDI-TOF analysis (Fig. S2). Similarly, these bands were excised and subjected to trypsin digestion under native conditions. The tryptic digests were then analysed using MALDI-TOF and ESI-MS/MS MS. In the MALDI-TOF spectra, we were able to see the presence of a peak at m/z 3556 corresponding to a disulfide-bonded dipeptide (TAC⁵²⁴AEFSRWRANGVGR)₂ that was absent in
The monomer (Fig. 3b). This experiment suggested that only C424 was able to form disulfide bonds between the protomers under the experimental conditions tested.

To further substantiate the identity of the disulfide-bonded dipeptide, the tryptic digest of the dimer was analysed by liquid chromatography LC-ESI-MS. In this analysis, the peak corresponding to m/z 3556 was identified as the triply charged species with m/z 1186.2 (Fig. 3c, inset). Further, this peak with m/z [1186.2]⁺ was subjected to tandem MS/MS analysis, in which the bonded peptide was sequenced. First, the fragmented species of disulfide-bonded peptide were identified in the ESI-MS/MS spectrum (Fig. 3c). This analysis was further verified using an established tool, Disconnect (Bhattacharyya et al., 2013). Second, we also tried to sequence the peptide by identifying the b and y ions. We were able to identify many of these ions, thereby validating the sequence of the cross-linked peptide (Fig. S3).

Enzymatic activity of the monomeric and dimeric forms

As we observed that DcpA existed in the interconvertible monomeric and dimeric forms, we embarked upon the investigation of the activity present in each form. We performed several in vitro and in-cell activity assays to address this issue.

In vitro activity assays

In-solution activity. It was suggested previously by several groups that the concentration of a protein may play a crucial role in determining its local and global structure, and its inherent activity. A well-known approach was adapted here to shift the monomer–dimer interconversion in favour of the dimer by increasing the protein concentration and then the activity was measured as suggested previously (Paul et al., 2007; Sundriyal et al., 2014). As expected, we observed an increase in the synthesis activity as a function of the protein concentration (Fig. 4a, i). However, as the WT DcpA has both synthesis and hydrolysis activity, it was difficult to interpret the activity assay conclusively. Previously in our laboratory we observed that the hydrolysis activity of DcpA can be blocked by labelling it with iodoacetamide (unpublished data). When such an iodoacetamide-labelled protein was assayed for its activity, we noticed an increase in c-di-GMP formation as a function of protein concentration (Fig. 4a, ii), suggesting that the synthesis activity was perhaps dimerization dependent. The hydrolysis activity (appearance of the product pGpG) also appeared to be affected by a change in the protein concentration, suggesting that the dimer perhaps had hydrolysis activity as well (Fig. 4a, iii).

In-gel activity assay. As the monomeric and dimeric forms are interconvertible, it was extremely difficult to achieve homogeneous populations of each species. Therefore, we adapted the strategy of an in-gel activity assay. The two forms of DcpA were separated on a 10% native polyacrylamide gel and excised individually from the gel such that they were still entrapped in the small gel pieces. These gel pieces with monomeric and dimeric DcpA in their native conditions were subjected to an in-gel activity assay. Fig. 4(b, i) shows the in-gel synthesis assay in which we observed that only the dimer (lane 3) was able to show the synthesis activity, whereas the aggregates (lane 2) and the monomer (lane 4) did not show any synthesis activity. Calf intestinal alkaline phosphatase (CIAP, which removes linear phosphate moieties)-treated PleD reaction product was loaded in lane 1 to show the authentic position of c-di-GMP. A similar in-gel activity assay was performed to monitor hydrolysis activity (Fig. 4b, ii). In this experiment, the PleD reaction product (not treated with CIAP) was loaded in lane 2 to show the migration of c-di-GMP and it also acted as a negative control because it could not hydrolyse c-di-GMP into pGpG. Lanes 4 and 5 show that both the dimer and monomer are able to hydrolyse c-di-GMP as indicated by the appearance of the pGpG spot in these lanes. Lane 3 shows that the aggregated DcpA could not hydrolyse c-di-GMP under the tested conditions.


I. M. Sharma and others

(a)  

DTT  
DcpA  
α-Phenanthroline  
CuSO₄

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<tr>
<td>Dimer</td>
<td>+</td>
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<td>Monomer</td>
<td>+</td>
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(b)  

0_D9/1: +MS. Baseline subtracted (0.80)

(c)  

TACAEFSRWRANGVGR

TACAEFSRWRANGVGR

TACAEFSRWRANGVGR

TACAEFSRWRANGVGR

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Activity in the leucine zipper fusion protein. We engineered a fusion protein, Dcp-N-zip (Fig. 5a), in which a leucine zipper (amino acid sequence given in Fig. S4a) was fused in-frame at the N terminus of DcpA. The clone was verified by nucleotide sequencing, and the molecular mass of both Dcp-N-zip and DcpA were determined by LC-ESI-MS (Fig. S4b, c). The masses of Dcp-N-zip and DcpA were found to be m/z 71 442 and 67 009, respectively. It is known that the leucine zipper that has a parallel coiled-coiled structure and acts as a strong dimerization module. Hence, purified Dcp-N-zip (Fig. 5b) was examined for its oligomeric status using native gel electrophoresis (Fig. 5c, i) followed by native Western blotting using anti-His 

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\text{antibody (Fig. 5c, ii). As expected, we observed the presence of the dimeric form of Dcp-N-zip alone and no monomeric form. We then performed a radiometric activity assay in which we observed that the dimeric Dcp-N-zip showed both c-di-GMP synthesis and hydrolysis activities (Fig. 5d, lane 3) supporting the in-gel activity assay. A similar assay for the dimerization and activity (using a different method) of a DGC fused with the leucine zipper has also been reported by others (De et al., 2009). As the leucine zipper was fused at the N terminus of DcpA and this fusion protein showed biochemical activity, it indirectly supported our previous data that the two protomers are aligned in a parallel orientation.

In-solution and in-gel activity assays. The synthesis assay involved the conversion of two molecules of GTP into one molecule of c-di-GMP. The assay mix contained the test protein, 50 mM Tris/HCl, pH 7.8, 500 mM NaCl, 10 mM MgCl2, 5 mM β-ME, 0.1 mM GTP (cold) and [\text{\textsuperscript{32}}P]GTP. The hydrolysis assay involved the conversion of c-di-GMP into pGpG where the assay mix contained the test protein, 50 mM Tris/HCl, pH 7.8, 500 mM NaCl, 10 mM MgCl2, 5 mM β-ME and [\text{\textsuperscript{32}}P]c-di-GMP. 

Concentration-dependent (0–6.4 \text{ mg} \text{ protein}) in-solution activity assay where (i) and (ii) represent the synthesis activity of native DcpA and DcpA labelled with iodoacetamide (IAM), respectively, for 30 min, and (iii) shows the hydrolysis activity of DcpA for 30 min. (b) In-gel activity assay. (i) Synthesis assay: lanes 1, PleD (a bona fide c-di-GMP synthase) treated with CIAP (that removes linear phosphate moieties) to confirm the position of c-di-GMP; 2, aggregates; 3, dimer; 4, monomer. (ii) Hydrolysis assay: lanes 1, GTP negative control; 2, PleD reaction product showing the presence of c-di-GMP; 3, aggregates; 4, dimer; 5, monomer.

Fig. 3. Alignment of protomers in dimeric DcpA. (a) A SDS-10 % polyacrylamide gel showing dimer formation due to induced disulfide bond formation in the presence of α-phenanthroline and CuSO\textsubscript{4} (lanes 2–4). It was converted to the monomeric form in the presence of DTT (lane 1). (b) MALDI-TOF analysis of the tryptic digests showing a peak with m/z 5556 (disulfide-bonded dipeptide) present in the dimer that was absent in the monomer. (c) Cid-MS/MS spectrum to identify various fragmented species of the disulfide-bonded dipeptide.

Fig. 4. In-solution and in-gel activity assays. The synthesis assay involved the conversion of two molecules of GTP into one molecule of c-di-GMP. The assay mix contained the test protein, 50 mM Tris/HCl, pH 7.8, 500 mM NaCl, 10 mM MgCl2, 5 mM β-ME, 0.1 mM GTP (cold) and [\text{\textsuperscript{32}}P]GTP. The hydrolysis assay involved the conversion of c-di-GMP into pGpG where the assay mix contained the test protein, 50 mM Tris/HCl, pH 7.8, 500 mM NaCl, 10 mM MgCl2, 5 mM β-ME and [\text{\textsuperscript{32}}P]c-di-GMP. 

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In-cell activity assay. As it is the dimer that has synthesis activity, an increase in the dimer population should augment the c-di-GMP-dependent cellular phenotype. To test this hypothesis and to further substantiate the activity of the two forms of DcpA, we performed an in-cell activity assay. In order to do so, we adapted the well-established
Congo red dye-binding assay (De et al., 2008, 2009). This assay has been established by several researchers in E. coli, wherein the pathway emerging from c-di-GMP synthesis to extra-polysaccharide production and subsequent Congo red binding is well known. Although M. smegmatis has been shown to exhibit Congo red binding, the involvement of c-di-GMP in this case has not been studied. Therefore, we preferred E. coli rather than M. smegmatis for demonstrating in-cell activity of the dimer. We chose four strains of E. coli: BL21(DE3) : pET21b (negative control), BL21(DE3) : pET-PleD (positive control expressing the DGC PleD), BL21(DE3) : pET-DcpA (WT DcpA that exhibits both the monomeric and dimeric forms) and BL21(DE3) : pET-Dcp-N-zip (DcpA fused with the leucine zipper that exhibits only the dimeric form). These strains expressed the respective protein upon induction with IPTG (data not shown). As BL21(DE3) : pET-Dcp-N-zip cells possess only dimeric DcpA, they are expected to produce more intracellular c-di-GMP when compared with BL21(DE3) : pET-DcpA that possesses both the monomer and dimer. The increased intracellular levels of c-di-GMP in turn should upregulate extra-polysaccharide production, which can then bind to Congo red dye and result in enhancement of the red colour of the colony. From Fig. 7, it can be seen that BL21(DE3) : pET-Dcp-N-zip colonies appeared darker than those of BL21(DE3) : pET-DcpA as a function of increasing IPTG concentrations (panels i–iii). However, at higher IPTG concentration, the colonies of both the strains appeared similar (panel iv). The BL21(DE3) : pET21b strain (negative control) did not show any increase in dye binding, whereas in the BL21(DE3) : pET-PleD strain (positive control), the leaky expression of the protein in the absence of IPTG appeared to be enough to show reasonable dye binding (panel i). This observation was similar to that reported by De et al. (2009) in the case of WspR. However, at higher IPTG concentrations, the overexpression of PleD appeared to be lethal, probably due to excessive production of c-di-GMP. A similar case of toxicity has been observed as a result of overproduction of a GGDEF protein, RSP3513, in BL21 cells (Ryjenkov et al., 2005). Hence, the BL21(DE3) : pET-PleD strain did not show significant dye binding at higher IPTG concentrations (panels iii and iv). These experiments demonstrated that, inside the cell, the dimer possesses predominantly c-di-GMP synthesis activity.

**DcpA is associated with the cytoplasmic membrane in M. smegmatis**

Several enzymes responsible for c-di-GMP turnover are known to be spatially and temporally distributed to fine tune the signalling cascade in the bacteria (Hengge, 2009; Romling et al., 2013). Their spatial segregation results in an increased local concentration of c-di-GMP. M. smegmatis has only one copy of the gene encoding the enzyme involved in c-di-GMP turnover. We had noticed previously that the level of c-di-GMP appeared to be low in M. smegmatis (Bharati et al., 2012, 2013). The reported cellular concentration in other bacteria is ~1–10 μM (Hengge, 2009). Therefore, the distribution of DcpA appeared to be

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**Fig. 5.** Leucine-zipper-induced dimerization and activity. (a) Schematic of the fusion protein [in which the leucine zipper (LZ) motif is fused at the N terminus of DcpA]. (b) A SDS-10 % polyacrylamide gel showing the purification profile of Dcp-N-zip. (c) (i) A 10 % native polyacrylamide gel showing the presence of the dimeric form of Dcp-N-zip*, which is validated by a native Western blot using anti-His6 antibodies in (ii). (d) Radiometric activity assay in which GTP (cold) and [α-32P]GTP were used as substrates of DcpA (10 μM) for 1 h.
restricted in *M. smegmatis*. Using membrane proteome analysis, Mawuenyega *et al.* (2005) reported that Rv1354c (an orthologue of DcpA in *M. tuberculosis*) is present in the membrane fraction. Subsequently, Cui *et al.* (2009) showed that Rv1354c interacts with the ATP-binding cassette (ABC) transporter in the membrane. To examine the cellular distribution of DcpA, the protein sequence was first analysed using the TMHMM tool and then the PSORTb tool. These analyses did not show the presence of any transmembrane helix in DcpA, but the protein was predicted to be associated with the cytosolic membrane. To substantiate this analysis, subcellular fractions of *M. smegmatis* cells from early stationary phase were prepared using a differential ultracentrifugation method. We failed to substantiate these methods.

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**Fig. 6.** pH-dependent change in oligomerization and activity. (a) A decrease in pH results in monomer formation of DcpA. (b) Radiometric activity assay. (i) Synthesis and (ii) hydrolysis activity assays for 10 min. (iii) Synthesis and (iv) hydrolysis activity assays for 3 h.

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**Fig. 7.** In-cell activity assay. BL21(DE3) cells, transformed with various plasmids, were grown in liquid culture and then spotted onto LB agar plates with ampicillin and Congo red dye with increasing concentrations of IPTG. Plates were incubated at 30 °C and the enhanced DGC activity of Dcp-N-zip was correlated with the appearance of a darker red colony phenotype. Leaky expression of PleD in the absence of IPTG was sufficient to result in a red colony phenotype, whereas higher concentrations of IPTG appeared to induce high and lethal expression of PleD or its product.
to detect DcpA in WT cells using its antibody, either due to a poor antibody or due to a low cellular concentration of the protein. Therefore, it was thought that the overexpression of the His6-tagged DcpA would help its detection by Western blot analysis. When the subcellular fractions from the overexpression strain (M. smegmatis: pJAM-DcpA) were probed with anti-His6 antibodies in the Western blotting experiments, the results were encouraging. We were able to see the presence of DcpA in the membrane fractions (Fig. 8a). However, due to the overexpression of DcpA, some amounts of protein were also detected in the other fraction. Considering the reports of Mawuenyega et al. (2005) and Cui et al. (2009), the presence of the protein in the other cellular fraction (cytosol) can be detected when the membrane sites are saturated. Also, it is known that a protein can be detected in the membrane fraction after differential centrifugation only if it is associated with it (Delogu et al., 2004; Gibbons et al., 2007; Rezwan et al., 2007). Dps1 (MSMEG_6467), a cytosolic protein (Chowdhury et al., 2007), was used as a marker and loading control to exclude any possibility of cross-contamination of the fractions. We reported previously that DcpA has at least one copy of its orthologues in many mycobacterial species (Bharati et al., 2012) and shares more than ~60% sequence similarity. Thus, it appears that the restricted distribution of such proteins could be conserved in the other mycobacterial species as well to regulate a common function. The TMHMM and PSORTb analyses were performed for all these orthologous proteins in mycobacteria, which resulted in similar predictions (Table S1).

**Localization of DcpA in M. smegmatis**

Subsequently, we engineered a construct for the expression of RFP-DcpA in pJAM2 vector, in which RFP was fused to the N terminus of DcpA. This construct (pJAM-Rfp-Dcp) was electroporated into WT M. smegmatis cells. The vector alone (pJAM-Rfp) was also electroporated separately as a negative control. The strains WT:pJAM-Rfp-Dcp and WT:pJAM-Rfp were grown to early stationary phase, fixed and imaged (see Methods). The localization analysis revealed that the WT:pJAM-Rfp-Dcp cells showed a strikingly different localization of DcpA than that of the control WT:pJAM-Rfp cells (Fig. 8b). The WT:pJAM-Rfp cells showed a uniform distribution of RFP across the entire cell, whereas the localization of RFP-DcpA appeared to be somewhat heterogeneous with a predominance at the cell poles. Such localization has been previously reported for other proteins with DGC activity (Paul et al., 2004; Huangyutitham et al., 2013). However, reports on the exact localization of proteins with c-di-GMP-specific PDE activity are sparse, with no information available in the case of bifunctional proteins.

**DcpA affects cell length and colony morphology in M. smegmatis**

It has been reported that c-di-GMP regulates several surface-associated properties in a variety of bacteria (Hengge, 2009; Romling et al., 2013). As DcpA appeared to be associated with the membrane, we speculated its involvement in regulating the surface-associated properties of M. smegmatis. In fact, a recent study by Li & He (2012) showed that c-di-GMP played a role in lipid metabolism in M. smegmatis and it is well known that lipids are major components of the mycobacterial cell wall. In our earlier attempt to delineate the role of DcpA in M. smegmatis, we reported that this protein was required for the long-term survival of the bacteria (Bharati et al., 2012). In the current study, we wanted to investigate the effect of DcpA on the cell structure and colony morphology of M. smegmatis by comparing WT (M. smegmatis mc² 155 harbouring pJAM2 vector), DOE (M. smegmatis mc² 155 harbouring pJAM-DcpA) and DKO (dcpA knockout M. smegmatis mc² 155) strains. The cells were grown to early stationary phase, harvested, fixed and imaged as described in Methods. We observed that the cell lengths were significantly different in all the three strains. As compared with the WT strain (2–3 μm), the DOE strains were shorter (1–2 μm) and the DKO strains were longer (3–4 μm) (Fig. 9a). When grown in planktonic culture, bacteria are known to exhibit differential nutrient uptake (Aldridge et al., 2012). Therefore, as a control, the length of at least 100 cells of each strain was measured and a size frequency graph was plotted (Fig. 9b). It appeared that the heterogeneity in the cell length increases in the order DOE<WT<DKO. It seems that DcpA is required to maintain the homogeneous growth of M. smegmatis in terms of the cell length. We also observed a clear change in colony morphology after 24 days of incubation at 37 °C. Generally, M. smegmatis

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**Fig. 8.** Localization of DcpA. (a) Western blot analysis of subcellular fractions of M. smegmatis using anti-His6 antibody showing the presence of DcpA in the cytoplasmic membrane fraction. WCL, whole-cell lysate; CM, cytoplasmic membrane; CT, cytosol. MsDps1 is a cytosolic protein, used here as a marker and loading control. (b) Imaging analysis to examine localization of DcpA in M. smegmatis. DIC, Differential interference contrast; FL, fluorescence.
grows in 3–5 days; however, our previous attempts to determine colony morphology within this period did not yield very obvious changes. This is why the cells were incubated for a longer period of time to achieve the maximum possible differences on 7H9 agar plates. The DOE strain formed colonies with larger diameters and differences in appearance from the WT and DKO strains (Fig. 9c).

DISCUSSION

The presence of proteins involved in c-di-GMP metabolism in both pathogenic and non-pathogenic species of mycobacteria (Fig. S6) suggests their important role in the bacterial survival strategy. Previously, we identified the \textit{M. smegmatis} gene MSMEG\_2196 (Bharati \textit{et al.}, 2012) and its orthologue in \textit{M. tuberculosis}, Rv1354c (Gupta \textit{et al.}, 2010b), to be involved in c-di-GMP metabolism and survival under carbon-limiting conditions. Here, we have presented additional evidence about the functional characterization of the product of MSMEG\_2196 (DcpA) in \textit{M. smegmatis}. In subsequent sections, we will discuss the importance of this study in the light of information available about other related proteins involved in c-di-GMP metabolism and/or mycobacterial survival capacity to resist carbon-limiting conditions.

Oligomerization and activity

Oligomerization represents perhaps one of the most fundamental steps to control the activity of proteins. The activity of several monofunctional DGCs [PleD (Paul \textit{et al.}, 2007), WspR (De \textit{et al.}, 2009)] requires homodimerization of the protein, although the current literature lacks a very clear picture about the oligomeric requirement for the monofunctional cognate PDEs [RocR (Rao \textit{et al.}, 2009), BlrP1 (Barends \textit{et al.}, 2009)]. According to Romling \textit{et al.} (2013), the EAL domains retain activity as monomers, but most often exist as homodimers. This statement is also supported by recent observations (Sundriyal \textit{et al.}, 2014). Nevertheless, information about oligomerization of the hybrid bifunctional proteins is lacking (Tarutina \textit{et al.}, 2006; Ferreira \textit{et al.}, 2008; Levet-Paulo \textit{et al.}, 2011; Bharati \textit{et al.}, 2012; Liu \textit{et al.}, 2012).

Our study provides important evidence about the relationship between the oligomeric forms and the activities possessed in a multidomain dual-active enzyme, DcpA. Using native gel electrophoresis, chemical cross-linking and gel-filtration experiments, we have shown that DcpA exists in monomeric and dimeric forms. There are also other elegant methods to determine the oligomeric status of a protein conclusively: fluorescence correlation spectroscopy (Chakraborty \textit{et al.}, 2012), analytical centrifugation (Lebowitz \textit{et al.}, 2002), multi-angle light scattering (van Dieck \textit{et al.}, 2009), dynamic light scattering (Bitan \textit{et al.}, 2003), electron microscopy (Stahlberg \textit{et al.}, 2004), MS (van den Heuvel & Heck, 2004) and size-exclusion HPLC (Engelborghs, 2012). The dimer possesses both c-di-GMP synthesis and hydrolysis activities, whereas the monomer shows only hydrolysis activity. As DcpA is a multidomain protein that possesses a GAF domain in addition to the GGDEF and EAL domains in tandem, we hypothesize that the oligomerization and activity of DcpA requires additional layers of regulation. This is supported by the observation that the domain variants of DcpA are catalytically compromised (Bharati \textit{et al.}, 2012). Several other factors, such as phosphorylation (Paul \textit{et al.}, 2007), and the presence of oxygen species (Liu \textit{et al.}, 2012; Qi \textit{et al.}, 2009) and haem (Wan \textit{et al.}, 2009), have been shown to modulate activities of the monofunctional and bifunctional proteins involved in c-di-GMP turnover (Hengge, 2009; Schirmer & Jenal, 2009; Romling \textit{et al.}, 2013). As we purified DcpA from \textit{E. coli} and not from \textit{M. smegmatis} for biochemical analysis, we did not expect the proteins co-purified from \textit{E. coli} to have any effect on the activity of DcpA. Hence, we did not check such a possibility. As DcpA is required for the long-term survival of \textit{M. smegmatis} (Bharati \textit{et al.}, 2012), we also searched the literature for evidence regarding regulation by oligomerization of activities of other proteins involved in the mycobacterial capacity to
resist carbon-limiting conditions. The Rel/SpoT protein from *M. smegmatis* is one such example. It is a multidomain dual-active protein involved in the turnover of another second messenger, (p)ppGpp, and appears to require the monomeric form for both the activities (Jain et al., 2006), with interdomain interaction.

Additionally, we have shown that the two protomers are aligned in a parallel orientation in the dimeric DcpA. In PleD, a bona fide dimeric DGC, the GGDEF domain is the terminal domain and assumes an anti-parallel orientation in the dimeric form when performing the synthesis activity (Chan et al., 2004; Wassmann et al., 2007). Each domain binds with one GTP molecule to facilitate the synthesis of c-di-GMP. In DcpA, the GGDEF domain is present in the middle of the polypeptide chain and we observed parallel alignment of the protomers in the protein. Therefore, it appears that this interdomain distance may play a role in catalysis.

**Cellular localization**

Protein localization segregates its activity to avoid otherwise lethal cross-talk. Several DGCs [WspR (Huangyutitham et al., 2013), PleD (Paul et al., 2004), YfIN (Malone et al., 2010), MifAB (O'Shea et al., 2006)] and PDEs [DipA (Roy et al., 2012), BifA (Kuchma et al., 2007)] are spatially and temporally distributed to fine tune the signalling cascade. However, there is not much knowledge about such distribution in the case of multidomain dual-active proteins. The orthologue of DcpA, Rv1354c, has been shown to be associated with an ABC transporter in the membrane (Mawuenyega et al., 2005; Cui et al., 2009). In the present study, we observed the presence of DcpA in the cytoplasmic membrane fraction, especially at the cell poles. Therefore, in the light of the literature and our current analyses, one would assume that such proteins are associated with the membrane to possibly affect cell wall properties.

**Phenotype**

c-di-GMP is best known for regulating the transition between the motile and sessile form of bacteria (Hengge, 2009; Romling et al., 2013). It also affects several other cellular phenotypes, such as cell differentiation, virulence and biofilm formation, amongst many others (Hengge, 2009; Schirmer & Jenal, 2009; Romling et al., 2013). Persister mycobacteria exhibit phenotypes different from those of the WT strains. These phenotypes include changes in the cell length, colony morphology and staining pattern, amongst others (Ojha et al., 2000; Dahl et al., 2005). In this study, we have shown that DcpA appears to affect some of these phenotypes (cell length and colony morphology). In addition, Li & He (2012) have reported recently that increased expression of LtmA alters c-di-GMP signalling, which affects lipid metabolism (cell wall properties) and thereby the staining pattern of *M. smegmatis*. A similar case has been described for another multidomain bifunctional protein, Rel/SpoT (Ojha et al., 2000; Dahl et al., 2005). Therefore, our study not only provides evidence about the involvement of DcpA in regulating the typical phenotypes characterized for persister mycobacteria, but also hints at various stages in the pathway leading to the sustained capacity to resist carbon-limiting conditions.

Our study has raised several intriguing issues that require further research. (1) What are the factors that might affect the oligomerization and how does one activity dominate over the other in the dimer? (2) Which oligomeric form associates with the cytoplasmic membrane and influences their properties? (3) What controls the cellular localization of DcpA? (4) How does the change in the level of DcpA cause alterations in the cell wall components, cell length and colony morphology? A study performed by Seshasayee et al. (2010) showed that ~15 % of the bacterial genome encodes proteins with GAF–GGDEF–EAL domain architecture with high sequence conservation seen amongst them. Therefore, we believe that the findings in this study can be extrapolated to other similar cases. This would help in understanding the regulation of multidomain dual-active enzymes involved in c-di-GMP metabolism. In conclusion, this study provides evidence about the mechanism of action of the protein DcpA involved in c-di-GMP turnover leading to the long-term survival of *M. smegmatis*.

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