Substrate-induced domain movement in a bifunctional protein, DcpA, regulates cyclic di-GMP turnover: Functional implications of a highly conserved motif

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In eubacteria, cyclic di-GMP (c-di-GMP) signaling is involved in virulence, persistence, motility and generally orchestrates multicellular behavior in bacterial biofilms. Intracellular c-di-GMP levels are maintained by the opposing activities of diguanylate cyclase (DGCs) and cognate phosphodiesterases (PDEs). The c-di-GMP homeostasis in Mycobacterium smegmatis is supported by DcpA, a conserved, bifunctional protein with both DGC and PDE activities. DcpA is a multidomain protein whose GAF-GGDEF-EAL domains are arranged in tandem and are required for these two activities. To gain insight into how interactions among these three domains affect DcpA activity, here we studied its domain dynamics using real-time FRET. We demonstrate that substrate binding in DcpA results in domain movement that prompts a switch from an “open” to a “closed” conformation and alters its catalytic activity. We found that a single point mutation in the conserved EAL motif (E384A) results in complete loss of the PDE activity of the EAL domain and in a significant decrease in the DGC activity of the GGDEF domain. Structural analyses revealed multiple hydrophobic and aromatic residues around Cys579 that are necessary for proper DcpA folding and maintenance of the active conformation. On the basis of these observations and taking into account additional bioinformatics analysis of EAL domain-containing proteins, we identified a critical putatively conserved motif, GCXXQGFG, that plays an important role in c-di-GMP turnover. We conclude that a substrate-induced conformational switch involving movement of a loop containing a conserved motif in the bifunctional diguanylate cyclase-phosphodiesterase DcpA controls c-di-GMP turnover in M. smegmatis.

Cyclic di-GMP is a global bacterial second messenger that controls a wide range of cellular processes that contribute to intracellular communication, surface adaptation, biofilm formation, cell cycle progression, virulence, and long-term survival. The c-di-GMP4 homeostasis is maintained by synthesis and hydrolysis activities of diguanylate cyclases (DGCs; consisting of a GGDEF domain) and cognate phosphodiesterases (PDEs; consisting of an EAL domain), respectively (1–6). The c-di-GMP is synthesized from two GTP molecules and is hydrolyzed via the linear intermediate pGpG to GMP (7–13). Extensive studies demonstrating the role of c-di-GMP in different physiological functions have been performed in Gram-negative bacteria (3, 6, 14, 15). Recent evidence supports the importance of c-di-GMP–mediated signaling in Bacillus subtilis wherein motility and biofilm formation were observed to be regulated by modulation of c-di-GMP concentration (16). Similar observations were made in the eukaryote Dictyostelium discoideum (17). The c-di-GMP–mediated signaling network and its regulation are complex due to its abundance. Multiple copies of proteins containing GGDEF and EAL/HD-GYP domains are present in bacterial genome, rendering it difficult to ascribe any function to a particular gene (15). The HD-GYP domain proteins are less abundant than EAL domain proteins and are able to hydrolyze c-di-GMP into two molecules of GMP. The c-di-GMP production can be controlled at transcriptional, post-transcriptional, and post-translational levels. In 2008, Breaker and co-workers (18) reported the discovery of a riboswitch that binds c-di-GMP and modulates gene expression in response to this second messenger.

A comparative genomics study involving 11,248 GGDEF and EAL domain–containing proteins across prokaryotic genomes revealed a very high degree of sequence conservation among the domains (19). In Gram-positive mycobacterial species, ~99 sequences containing either one copy or multiple copies of GAF, GGDEF, EAL, and HD-GYP domains were found. However, at least one protein with GAF-GGDEF-EAL domain organization is conserved in most mycobacterial species (20, 21). GAF domains in proteins in general may regulate the catalytic activities by nucleotide or small-molecule binding (22, 23).

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This article contains Figs. S1–S12, Tables S1–S6, and supporting Appendix.

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4 The abbreviations used are: c-di-GMP, bis(3′–5′)-cyclic dimeric guanosine monophosphate; DGC, diguanylate cyclase; PDE, phosphodiesterase; IAEDANS, 5-[2-(iodoacetamido)ethylamino]napththalene-1-sulfonic acid; IAF, 5-(iodoacetamido)fluorescein; ppGpp, guanosine 3′,5′-bispyrophosphate; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; pGpG, 5′-phosphoguananyl(3′-5′)-guanosine; ESI, electrospray ionization; Tm, melting temperature; DLS, dynamic light scattering; A, anisotropy; τr, rotational correlation time; Rf, Förster distance; IAM, iodoacetamide; CID, collision-induced dissociation; CCD, charge-coupled device; Q, quantum yield; τf, fluorescence lifetime; I, spectral overlap integral; E, energy transfer efficiency.
c-di-GMP turnover in *M. smegmati*
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Figure 1. Mutation in the EAL domain (hydrolysis domain) affects the synthesis activity of GGDEF domain. 

**a**, purified DcpA-E384A protein (lane 1, molecular weight marker; lane 2, DcpA-E384A); **b**, DGC and PDE-A activities of DcpA-E384A (lane 1, control (GTP); lane 2, PleD; lane 3, DcpA; lane 4, DcpA-E384A); and **c**, relative DGC and PDE-A activities of DcpA and DcpA-E384A. E384A substitution in the conserved motif (EAL) completely abolished the c-di-GMP degradation with a significant decrease in the DGC activity of GGDEF domain. The data represent the mean, and error bars represent the S.D. obtained from three different experiments.

Recently, we identified a single copy of the gene *dcpA* that is essential for long-term survival under carbon starvation, and the *dcpA* promoter activity increases several fold during starvation (24). DcpA is a multidomain protein with GAF-GGDEF-EAL domains arranged in tandem, and it was observed to exist as a dimer with a plausible implication in regulating diguanylate cyclase activity (25). In addition to phosphorylation-mediated oligomerization, c-di-GMP syntheses were reported to be controlled by several coupled signaling systems that respond to signal ligands, including nitric oxide and heme in mono- and bifunctional enzymes (15, 21, 26). In *Mycobacterium smegmati*, DcpA shows both DGC and PDE activities in vitro as a full-length protein (with GAF-GGDEF-EAL domains). The individual domains are inactive, indicating a possible interplay between the different domains of DcpA during synthesis or degradation of c-di-GMP (20).

In this work, we show that a point mutation in the EAL domain of DcpA can affect both the DGC and PDE-A activities significantly. This was further supported by fluorescence resonance energy transfer (FRET) experiments, which showed relative domain movements in DcpA as a function of substrate addition. Notably, in the presence of a substrate, we did not observe any change in the distance between the FRET reporters (5-[2-(iodoacetamido)ethylamino]naphthalene-1-sulfonic acid (IAEDANS) and 5-(iodoacetamido)fluorescein (IAF)) in a mutant enzyme bearing a single point substitution that abrogates its catalytic activity. Based on these observations, we were inclined to believe that a plausible domain–domain interaction is involved in regulating the DGC and PDE-A activities in DcpA.

**Construction and characterization of cysteine variants of DcpA**

With an aim to follow the intradomain interactions in DcpA, we decided to carry out FRET measurements with thiol-specific fluorescent probes. DcpA harbors four cysteine residues at positions 124, 424, 474, and 579 (Fig. 2a), and multiple alignments of GAF-GGDEF-EAL domains from various mycobacterial species revealed that Cys424, Cys474, and Cys579 are conserved, unlike Cys124 (Fig. 2b). It is possible that these conserved cysteines are involved in intramolecular disulfide linkages and thus regulate the DGC and PDE-A activities. Therefore, before creating point mutations at cysteine residues and labeling the remaining cysteines with thiol-specific fluorophores, it was prudent to identify their oxidation state. ESI-MS of N-ethylmaleimide–labeled DcpA (Fig. S1) suggested that all

**Results**

**Mutation in EAL domain of DcpA modulates synthesis and hydrolysis of c-di-GMP**

We have shown earlier that the domains of DcpA are inactive when separated (20). In another study, it was reported that a mutation in the conserved EAL motif abolished the PDE-A activity in the phosphodiesterase family of proteins (27). The goal of this study was to delineate the relationship between the EAL domain (hydrolysis) and the GGDEF domain (synthesis) activities of the DcpA protein. Here, we created a single point mutation (E384A) in the EAL domain (EAL motif) of DcpA (Fig. 1a) and purified the mutant protein (DcpA-E384A) that was used to measure the individual domain’s activity. The purity of the protein was estimated using 10% SDS-PAGE (~90% pure), which showed the presence of a few higher oligomers, which were confirmed by MALDI-MS analysis (not shown). The c-di-GMP synthesis and degradation activity of DcpA-E384A was monitored and compared with that of the WT DcpA. As expected, the E384A substitution in the EAL domain of DcpA resulted in complete abrogation of the c-di-GMP hydrolysis (PDE-A) activity (Fig. 1, b and c). However, the c-di-GMP synthesis activity was found to be reduced significantly, by up to ~60%. It is intriguing to note that a mutation in the EAL domain directly affects the DGC activity of the GGDEF domain in DcpA, and this suggests that the activity of the GGDEF domain is regulated by the PDE-A activity of the EAL domain. Based on these observations, we were inclined to believe that a plausible domain–domain interaction is involved in regulating the DGC and PDE-A activities in DcpA.
four cysteines are freely accessible for chemical modification, and they are present in their reduced form in solution. Furthermore, to support this observation, mass spectrometric analysis was performed on iodoacetamide-labeled (Figs. S2 and S3) and native DcpA (Fig. S4), and none of the cysteines were found to be involved in disulfide linkage (Table S2).
The conserved cysteines of the EAL domain (Cys^{424}, Cys^{474}, and Cys^{579}) were mutated to serine without altering the Cys^{124} at the N-terminal GAF domain, and proteins were purified using nickel-affinity chromatography (Fig. S5). The DGC and PDE-A activities of the mutants were estimated and compared with that of the WT DcpA (Fig. 2, c and d). It can be seen that serine substitutions at positions 424 and 474 were tolerated (DcpA-C424S and DcpA-C424S,C474S), whereas both the synthesis and hydrolysis activities were reduced considerably when Cys^{579} was mutated to serine (DcpA-C579S).

Substituting two cysteines, including the one at position 579 (DcpA-C474S,C579S and DcpA-C424S,C579S), also resulted in the loss of both the c-di-GMP synthesis and hydrolysis activities. With an aim to determine the secondary structure of DcpA and its variants, particularly in response to substrate binding, circular dichroism (CD) studies were performed. Secondary structure estimation showed that DcpA is predominantly α-helical (~54% by K2D2 server) in nature with no significant change upon GTP binding (Fig. 3a). We also recorded the CD spectra of all the mutant proteins, and it can be seen from Fig. 3b and Table S3 that a single mutation (C424S) resulted in a substantial increase in α-helical content without any alteration in its enzyme activity. The double mutant DcpA-C424S,C474S retained the DGC and PDE-A activities as well as the secondary structures. Surprisingly, a single mutation at position 579 (DcpA-C579S) retained the secondary structures but showed a complete loss of the DGC and PDE-A activities. This is an interesting observation and will be discussed in a later section. In addition, the double mutants DcpA-C424S,C579S and DcpA-C474S,C579S showed collapse of the characteristic structure of DcpA with complete abrogation of the DGC and PDE-A activities. From the mutational and CD analyses in corroboration with bioinformatics analyses of the mutant proteins it can be surmised that these conserved cysteine residues are important from structural and functional points of view.

Furthermore, thermal melting was carried out to monitor the stability of these proteins. The stability of a protein decreases with increasing temperature and at the midpoint of transition.

![Image](https://example.com/image.png)
where the concentrations of folded and unfolded proteins are equal, the temperature is considered as the melting temperature ($T_m$). The thermal melting profiles of DcpA and DcpA-C579S were largely similar (41.4 ± 0.2 and 39.9 ± 0.2 °C, respectively); however, the intensities of fluorescence emission varied (Fig. 3c and Fig. S6). This suggests that the Cys-to-Ser substitution at position 579 does not affect the stability of the mutant protein DcpA-C579S appreciably, although the activity is drastically compromised. We did not observe a significant change in the apparent $T_m$ values of the other mutants.

It was necessary to determine the steady-state kinetic parameters of the mutant DcpA-C424S,C474S that was used for fluorescence measurements. The substrate GTP is converted to c-di-GMP by the DGC activity and subsequently hydrolyzed to pGpG by the PDE-A activity. Therefore, both of these activities in DcpA-C424S,C474S can be monitored by adding a single substrate, GTP (Fig. 3f). Notably, the turnover number (apparent $k_{cat}$) of DcpA-C424S,C474S was found to be double that of the WT DcpA (Table 1); however, the catalytic efficiency (apparent $k_{cat}/K_m$) was similar in both cases. It is worth mentioning here that, for the measurement of the total DGC activity of the enzyme, both the synthesis and hydrolysis of c-di-GMP were taken into consideration as we cannot measure individual domain activities.

Furthermore, because our earlier studies found DcpA to exist and show activity in its monomeric and dimeric forms, we monitored the oligomeric nature of DcpA-C424S,C474S by dynamic light scattering (DLS). DLS studies revealed that the major population of the DcpA and DcpA-C424S,C474S proteins are monomeric, and upon incubation with GTP, they attain a dimeric state. In contrast, in the presence of c-di-GMP, they remain in the monomeric form (Fig. 3d, f, S8, and Table S4).

**Fluorescently labeled DcpA-C424S,C474S is active**

The DcpA-C424S,C474S protein was labeled with thiol-specific fluorophores IAEDANS (donor) and IAF (acceptor). The DcpA-C424S,C474S protein was labeled with IAEDANS first, and the molar ratio of protein:probe measured by spectroscopy was estimated to be 1:1.1. To ensure energy transfer between the donor and the acceptor fluorophores, the fluorescence emission spectra of IAEDANS-labeled DcpA-C424S,C474S protein (monolabeled) were recorded with increasing concentrations of free IAF. Here, the samples were excited at 336 nm, and emission was monitored at 470 nm. Fig. 4a shows the quenching of emission maxima of the donor in the presence of the acceptor, which tends to saturate at a 1:1 molar ratio of donor to acceptor. When the IAEDANS-labeled (monolabeled) protein was subjected to IAF labeling, the corresponding molar ratio between the donor and the acceptor was estimated and found to be substoichiometric (1:0.7), indicating that complete labeling was not achieved. Furthermore, IAEDANS and IAF labeling at Cys124 and Cys579 positions was confirmed by MALDI-TOF MS analyses (Fig. S9, a–e). The spectrophotometric measurements of the IAEDANS- and IAEDANS-IAF–labeled proteins indicated that the majority of the proteins were labeled (~90%). Evaluation of the DGC and PDE-A activities of the IAEDANS- and IAEDANS-IAF–labeled proteins suggested that the labeled protein was as equally active as the unlabeled protein (Fig. 4b, lanes 5 and 6). It should be mentioned here that the labeling of Cys579 with a bulky fluorescent group (IAEDANS and/or IAF) did not alter the catalytic activity. However, a serine substitution at the same position was not tolerated (DcpA-C579S; Fig. 2, c and d). On the contrary, both DGC and PDE-A activities were retained when the cysteines at positions 124 and 579 were labeled with fluorophores. With this background, we planned to proceed further with Förster’s distance estimation between N-terminal GAF and C-terminal EAL domains as a function of binding to substrates GTP and c-di-GMP. First, it is important to mention that any unlabeled or monolabeled protein that may be present as contaminants with the labeled protein will not interfere with the energy transfer studies. Second, two probes may be heterogeneously distributed between two specific sites; however, this distribution will presumably not interfere with the results because two sites on the protein are fixed, and the Förster’s distance will not vary according to the location of the donor or acceptor.

**Measurement of steady-state anisotropy (A) and rotational correlation time ($\tau_r$)**

Next, we conducted steady-state $A$ measurements of the fluorescent moiety when attached to DcpA-C424S,C474S. This experiment was performed to monitor the environment of the probe when attached to a protein and in the presence of the substrate. Anisotropy values for IAEDANS-labeled protein showed an increment upon GTP binding and a minor decrease upon c-di-GMP binding (Table S5). Dimerization of the protein upon GTP binding as seen in DLS studies was also reflected in the change of anisotropy although to a lesser extent. $\tau_r$ was measured as a function of GTP and c-di-GMP binding. We observed that the $\tau_r$ values are very high (>200 ns) and were not altered much due to dimerization in the presence of GTP (Table S5). This is not unexpected as the $\tau_r$ values were higher at the start and appeared to have reached saturation.

**Estimation of Förster’s distance between N and C termini of DcpA as a function of substrate binding**

Fluorescence lifetimes ($\tau$) of the donor as estimated for IAEDANS- and IAEDANS-IAF–labeled DcpA-C424S,C474S...
**c-di-GMP turnover in M. smegmatis**

![Image](a.png)

**Figure 4.** The DGC and PDE-A activities of IAEDANS- and IAEDANS-IAF–labeled DcpA-C424S,C474S. a, quenching of fluorescence emission of IAEDANS (donor)-labeled DcpA-C424S,C474S (0.9 μM) with varying concentration of IAF (acceptor). The samples were excited at 336 nm, and emission spectra were recorded from 350 to 480 nm. The quenching of the emission maxima of the donor (IAEDANS) observed at 470 nm was plotted against the increasing concentration of acceptor (IAF), which tends to saturate at a 1:1 molar ratio of the donor to acceptor (spectra not shown). The data represent the mean, and error bars represent the S.D. obtained from three different experiments. b, DGC and PDE-A activities of DcpA-C424S,C474S are not impaired upon labeling with thiol-specific fluorescence probes. Lane 1, negative control (assay buffer); lane 2, positive control (PleD); lane 3, DcpA; lane 4, DcpA-C424S,C474S; lane 5, IAEDANS-labeled DcpA-C424S,C474S; lane 6, IAEDANS-IAF–labeled DcpA-C424S,C474S. The experiments were performed in triplicates. a.u., arbitrary units.

**Table 2**

The lifetime of the donor fluorophore (IAEDANS) in the presence and absence of the acceptor (IAF) and the estimated Förster’s distance between the donor–acceptor pair in DcpA-C424S,C474S

| Sample                        | Monolabeled (IAEDANS) | Double-labeled (IAEDANS-IAF) | $R (\kappa^2 = 0.48)$ |
|-------------------------------|-----------------------|------------------------------|-----------------------|
|                               | $\alpha_1$ | $\tau_{\alpha_1}$ | $\alpha_2$ | $\tau_{\alpha_2}$ | $\chi^2$ | $\alpha_1$ | $\tau_1$ | $\alpha_2$ | $\tau_2$ | $\chi^2$ | $\kappa$ |
| Protein                       | 0.76      | 142 ± 0.4 ns         | 0.24     | 2.9              | 1.07    | 0.54      | 6.6 ± 0.5 ns | 0.46     | 1.5 ± 0.3 ns | 0.65 | 41.7 ± 0.9 |
| Protein with GTP (100 μM)     | 0.81      | 137 ± 0.5 ns         | 0.19     | 2.3              | 0.64    | 0.55      | 1.6 ± 0.5 ns | 0.45     | 7.4 ± 0.5 ns | 1.01 | 32.8 ± 0.6 |
| Protein with c-di-GMP (25 μM) | 0.80      | 140 ± 0.3 ns         | 0.20     | 2.0              | 0.34    | 0.57      | 2.0 ± 0.5 ns | 0.43     | 7.8 ± 0.7 ns | 1.19 | 33.8 ± 0.5 |

are shown in Table 2. We measured the lifetime using a phase modulation technique where the minimum $\chi^2$ fit is taken as the best fit for any estimation. We observed that, in the case of IAEDANS-labeled protein, both a one- and two-component fit can be performed; however, a two-component fit does not give a better $\chi^2$ value. In addition, the total contribution from the second component ($\alpha_2$, $\tau_2$) was calculated using the equation ($\alpha_2 \times \tau_2$)/($\alpha_1 \times \tau_1 + \alpha_2 \times \tau_2$), and it was within 3–6% of the total decay process and hence may be ignored. Conversely, in the case of IAEDANS- and IAF-labeled protein, the lifetime of the donor can be described by a two-component fit ($\alpha_1$, $\tau_1$ and $\alpha_2$, $\tau_2$) where the amplitude of the second component is appreciable. However, in this case too, the contribution from the second component is around 16%, and hence we ignored the second lifetime, which was calculated using the above expression. Upon considering the lifetime of the donor in the absence of the acceptor as 14.2 ± 0.4 ns and in the presence of the acceptor as 6.6 ± 0.5 ns (Table 2), the distance between the pair turned out to be 41.7 Å with a $\kappa^2$ value of 0.67. In the presence of the substrate GTP or c-di-GMP, the lifetime of the donor ($\tau_1$) was reduced considerably, indicating the shortening of the distance between the pair of fluorescent probes used here. However, Table 2 shows that, in the presence of the substrates, the second component of the lifetime contributes significantly. Therefore, we calculated the distances using two approaches. First, we considered only the $\alpha_1$, $\tau_1$ values like the previous case. In addition, we also considered the average $\alpha$ and $\tau$ values from the two-component fit. It can be seen from Table 2 that the distance decreased appreciably by 7–9 Å when $\alpha_1$, $\tau_1$ values were only considered. We also calculated the distances using the weighted average of lifetimes; the distance did not change in that case. When the averages of $\alpha_1$, $\tau_1$ and of $\alpha_2$, $\tau_2$ values were considered, the distance decreased by 4.0 Å. However, our DLS and anisotropy studies suggest that protein attained a compact conformation upon GTP binding. Thus, we reported the distances based on major components ($\alpha_1$, $\tau_1$) where the estimated distances decreased as a result of GTP binding. The distance between the N-terminal GAF (Cys124) and C-terminal EAL (Cys579) domains decreased from 41.7 to 32.8 Å in the presence of GTP (100 μM) and to 33.8 Å (by ~20%) upon c-di-GMP (25 μM) binding to DcpA. Our measurements indicate that the protein acquired a more compact conformation upon GTP and/or c-di-GMP binding.
Several reviews have been published about difficulties in assigning a certain value for $\kappa^2$ (47, 49, 50). However, if both the donor and the acceptor are rotating freely or are isotropic in nature, the $\kappa^2$ value stands at 0.67. In addition, sixth root dependence (Equation 3 under “Experimental procedures”) minimizes the error in the Förster distance ($R_0$) estimation even if the $\kappa^2$ value has some inherent error. Therefore, it is necessary to check the isotropic character of the donor, particularly when a large protein is labeled. One can test such an assumption by measuring the time-dependent anisotropy, and if $\tau_c$ is less than the lifetime of the excited state, then it can safely be assumed that the fluorophore is rotating fast during the excited-state lifetime and hence is isotropic in nature. On the contrary, in our case, time-dependent anisotropy measurements showed that $\tau_c$ is abnormally high, and therefore the rotation of the fluorophore is restricted. In such cases, the $\kappa^2$ value is usually taken as 0.48 (28). We assumed two different distances with two different orientation factors, $\kappa^2$ values of 0.48 and 0.67, and interpreted our results accordingly. Taking these into consideration, the energy transfer efficiency between IAEDANS and IAF in the labeled protein was found to be 54 ± 3%. The $R_0$ value at 50% transfer efficiency was 42.5 Å for $\kappa^2 = 0.48$ and 45.0 Å for $\kappa^2 = 0.67$, within 1% error of the value reported in the literature (45.5 Å for $\kappa^2 = 0.67$) (29, 30). This can happen due to variation in the buffer condition and the nature of the protein, etc. The quantum yield, spectral overlap integral, and $R_0$ values are shown in Table 3, and the spectral overlap is shown in Fig. S10.

DcpA and DcpA-C424S,C474S showed dimerization upon GTP binding in DLS studies. One may argue that the distances we measured here reflect an intramolecular energy transfer process. Therefore, DcpA-C424S,C474S protein was labeled with either IAEDANS or IAF fluorophores separately and mixed together in equal proportions. Subsequently, intermolecular distances were measured in the presence and absence of GTP and/or c-di-GMP. The estimated distances between the Förster’s pair in this case was ~55.2 ± 0.8 Å and did not show any significant change due to GTP or c-di-GMP binding (data not shown). Thus, any contribution from intramolecular energy transfer in a dimer was ruled out.

Lastly, when two inactive proteins (DcpA-C474S,C579S and DcpA-C424S,C579S) were labeled with the donor–acceptor pair at two other free cysteine pairs (Cys$^{124}$–Cys$^{424}$ and Cys$^{124}$–Cys$^{474}$, respectively), the measured distances between the cysteine pairs (Cys$^{124}$–Cys$^{424}$ and Cys$^{124}$–Cys$^{474}$) were not altered in the presence of substrates, thus under scoring the correlation between biological activity and domain movement (Table 4). However, it is difficult to ascertain whether the complete abrogation of the DGC and PDE-A activities in the double mutant proteins is due to the inhibition of the domain movement or the loss of secondary structures.

### Table 3

| Sample                  | $Q_{\text{IAEDANS}}$ | $J$ | $R_0$ (Å) | $\kappa^2 = 0.48$ | $\kappa^2 = 0.67$ | $E$ (in the presence of IAF) % |
|------------------------|-----------------------|-----|-----------|-------------------|-------------------|-----------------------------|
| IAEDANS-labeled protein | 0.24                  | 2.32×10$^{-11}$ | 42.5 | 45               | 0.54 ± 0.03        |

### Table 4

| Distances               | Without GTP or c-di-GMP | With GTP | With c-di-GMP |
|-------------------------|-------------------------|----------|---------------|
| Distances measurement between cysteine pairs |                      |          |               |
| DcpA-C424S,C474S (Cys$^{124}$–Cys$^{474}$) | 41.7 ± 0.9 | 32.8 ± 0.6 | 33.8 ± 0.5 |
| DcpA-C424S,C579S (Cys$^{124}$–Cys$^{242}$) | 39.9 ± 0.6 | 39.6 ± 0.5 | 39.5 ± 0.8 |
| DcpA-C424S,C579S (Cys$^{124}$–Cys$^{474}$) | 38.7 ± 0.6 | 38.6 ± 0.5 | 38.5 ± 1  |

### Insights into the dual activity of DcpA and role of conserved cysteines (Cys$^{424}$, Cys$^{574}$, and Cys$^{579}$) in c-di-GMP turnover

Mass spectrometric analyses of DcpA protein ruled out the possibility of any intradisulfide linkages among the four cysteines, whereas mutational analyses suggest that C579S (DcpA-C579S) substitution is detrimental for both the DGC and PDE-A activities. It is not clear why Cys-to-Ser substitution at position 579 renders the protein inactive. All cysteines in native DcpA and the mutant (DcpA-C424S,C474S) were completely labeled with IAEDANS or iodoacetamide in the presence or absence of a reducing agent (tris(2-carboxyethyl)phosphine hydrochloride (TCEP)) and assayed for DGC and PDE-A activities. We noticed that the iodoacetamide-labeled DcpA protein showed only c-di-GMP synthesis activity without any hydrolysis of c-di-GMP, irrespective of the presence of TCEP (Fig. 5a, lanes 4 and 5). This was also true for DcpA treated with an excess of IAEDANS (Fig. 5a, lanes 6 and 7). However, when cysteines were substituted with serine at two central positions, DcpA-C424S,C474S, the protein was found to be catalytically active like the native protein, and labeling of this protein with IAEDANS/iodoacetamide at positions 124 and 579 did not alter the activity significantly (Fig. 5a, lanes 8–11).

To delineate the role of cysteine-mediated regulation of DGC and PDE-A activities, the DcpA protein was modeled using the I-TASSER server (58). The GGDEF and EAL motifs and distribution of all four cysteines are shown in the generated model of DcpA protein (Fig. 5b). The three-dimensional modeled structure of DcpA consists of predominantly α-helical regions, which was also evident from the CD studies. The absence of PDE-A activity in the EAL domain of DcpA, when labeled with an excess of bulky groups, can be explained from this model. It can be seen in Fig. 5b and Table S6 that all four cysteines are located far from the GGDEF motif (~25 Å), and Cys$^{124}$ and Cys$^{579}$ are also placed far from the EAL motif (~34.8 and 21.5 Å, respectively). However, Cys$^{424}$ and Cys$^{574}$, which are in close proximity of the EAL motif (~11.3 and 10.4 Å, respectively) when labeled with bulky molecules like IAEDANS and iodoacetamide, may create steric hindrance for the hydrolysis of c-di-GMP in DcpA but not for c-di-GMP synthesis.
Cys\textsuperscript{579} is part of a conserved motif, GCXXXQGF, influencing the c-di-GMP turnover

To investigate the structural and functional role of Cys\textsuperscript{579} in the regulation of the DGC and PDE-A activities, the sequence of DcpA was analyzed using the PSI-BLAST (Position-Specific Iterated BLAST) program against the nonredundant (nr) protein sequences. The result was used for multiple sequence alignments, which show that Cys\textsuperscript{579} is conserved in DcpA from all mycobacterial species as well as in other EAL domain–containing proteins (not shown). A WebLogo was generated to identify the conservation of cysteines in DcpA among the 100 proteins possessing GGDEF-EAL domains with 75% sequence coverage (31). It can be seen in Fig. 5c that Cys\textsuperscript{124} is the least conserved among DcpA homologs, whereas Cys\textsuperscript{579} is remarkably conserved among all the EAL-domain–containing proteins. WebLogo analyses and multiple sequence alignment show a conserved region comprising Cys\textsuperscript{579}, which may be part of a novel motif, GCXXXQGF, required to facilitate the hydrolysis of c-di-GMP (Fig. 5c, shown in a box). The DcpA protein sequence was also analyzed for evolutionary conservation of amino acids using the ConSurf server (32). Results indicate that Cys\textsuperscript{579} is a structurally conserved residue located in the conserved motif GCXXXQGF (Fig. S12). This signature motif is in the loop region of DcpA protein and might have a critical role in regulation of c-di-GMP turnover. Any mutation in this motif will destabilize the active conformation and result in loss of activity, and we have observed the same in the case of single and double mutants of Cys\textsuperscript{579}.

Figure 5. Regulation of cysteine-mediated DGC and PDE-A activities and identification of conserved motif in DcpA. a, loss of phosphodiesterase activity upon iodoacetamide and/or IAEDANS labeling of the EAL domain cysteines in the WT DcpA. Lane 1, control (GTP); lane 2, PleD; lane 3, DcpA; lane 4, DcpA + iodoacetamide; lane 5, DcpA + TCEP + iodoacetamide; lane 6, DcpA + IAEDANS; lane 7, DcpA + TCEP + IAEDANS; lane 8, DcpA-C424S,C474S + iodoacetamide; lane 9, DcpA-C424S,C474S + TCEP + iodoacetamide; lane 10, DcpA-C424S,C474S + IAEDANS; lane 11, DcpA-C424S,C474S + TCEP + IAEDANS. b, cartoon representation of DcpA protein modeled using I-TASSER server (58) showing the distribution of all four cysteines in the GAF and EAL domains. c, graphical representation of conserved cysteines in DcpA among the phosphodiesterase family of proteins. The WebLogo was generated using 100 GGDEF-EAL domain proteins with 75% sequence coverage with DcpA. The putative motif, GCXXXQGF, that comprises Cys\textsuperscript{579} is shown in a box.

Structural analysis of the EAL domain of DcpA

As mentioned above, DcpA-C579S mutant is inactive when compared with the DGC and PDE-A activities of the WT enzyme. In contrast, IAEDANS, IAF, or iodoacetamide labeling of the same Cys\textsuperscript{579} does not affect either of the activities. One of
The close homologs of the EAL domain of DcpA with a known molecular structure is the phosphotransferase TBD1265 from *Thiobacillus denitrificans* (Protein Data Bank (PDB) code 2R6O) (33). The sequence alignment showed that it was 38% identical with the full-length DcpA (Fig. S12) with the presence of a conserved cysteine Cys719 (corresponding to Cys579 of DcpA), so it was used as a template for constructing a model for the EAL domain of DcpA. The proposed structure of DcpA showed that Cys579 is partially buried and surrounded overwhelmingly by hydrophobic amino acids except for a mainchain carbonyl oxygen. The hydrophobic amino acids form a cavity, keeping Cys579 at the center of the hydrophobic core (Fig. 6a). In the crystal structure of the EAL domain of TDB1265 from *T. denitrificans*, Cys719 is surrounded by a few aromatic residues, which are also conserved in the *M. smegmatis* variant (Fig. 6b). Thus, the sulfur–aromatic interaction could potentially tolerate the presence of a weakly polar thiol group in the midst of the aromatic residues of DcpA. It appears that cysteine at position 579 in DcpA protein cannot be replaced with any hydrophilic amino acid, and it helps to stabilize the highly hydrophobic and tightly packed environment.
In addition, the only polar group, at a distance of 3.4 Å from the thiol group of Cys579 (corresponding to Cys719 in T. denitrificans; Fig. 6c), is the main-chain carboxyl at position 581, and it may be viewed as a weak hydrogen bond acceptor. In the modeled structure, Gln383 (corresponding to Gln725 in T. denitrificans; Fig. 6c) is located proximal to Cys579 in the same loop as Gln583. It holds one of the carboxyl oxygens of Glu384 (corresponding to Glu723 in T. denitrificans; Fig. 6c), which interacts with magnesium ion through the other oxygen in the side-chain carboxyl. It seems possible that the interaction between Gln583 and Glu384 is important for Glu384 binding to Mg2+ liked to be critical for function.

In the mutant where Cys579 is replaced by serine, the side-chain hydroxyl oxygen is incompatible in the midst of apolar side chains. Given an extensive hydrophilic environment, the structure of the mutant is likely to be altered considerably to expose the side chain of serine. A profound alteration in the structure, suggested by CD studies of the mutant, could affect the spatial location of Gln383, thereby affecting its interaction with Glu384. In the protein labeled with probes, the aromatic groups favorably interact with surrounding hydrophilic amino acids and other nonpolar residues. In the crystal structure (PDB code 2R6O) of the template, the distance between Cys719 (equivalent to Cys579 in DcpA) and the carbonyl oxygen at position 721 is 3.41 Å, which corresponds to a weak hydrogen bond involving the hydroxyl group of Ser in the Cys-to-Ser mutants.

The crystal structure of another EAL domain protein from Caulobacter crescentus (PDB code 3U2E; UniProt ID Q9A310) in complex with 5′-pGpG (hydrolysis product of c-di-GMP) and Mg2+ is shown in Fig. 6d. Here, the guanosine base of pGpG is involved in the stacking interaction with Phe521 (corresponding to Phe585 in DcpA) of the predicted GCXXXQGF motif to stabilize c-di-GMP in the binding pocket for its hydrolysis. The conserved Gln383 in the predicted motif GCXXXQGF is in direct contact with Glu384 (E384AL motif) through hydrogen bonding. Therefore, our studies shows that GCXXXQGF is a novel motif for c-di-GMP degradation, and mutation of the cysteine in this motif, C579S, will destabilize the stacking interaction between the guanosine base of c-di-GMP and Phe585. In addition, the hydrogen bond between the EAL motif and Gln383 cannot be established in the DcpA-C579S mutant.

Discussion

In this study, we have highlighted domain–domain interaction in a bifunctional protein, DcpA, where the synthesis and degradation of c-di-GMP occur in tandem. Similar bifunctional activity is now reported for other bacteria as well. The protein Lp0329 from the Gram-negative Legionella pneumophila contains both GGDEF and EAL domains, and it has been found that phosphorylation of Lp0329 negatively controls the synthesis of c-di-GMP (34). Another bifunctional protein, Swoo_2750 (SwDGC), from Shewanella woodyi shows both c-di-GMP synthesis and hydrolysis activities (35, 36). The reason for control of the bifunctional activity by the same promoter is not immediately clear to us except for the fact that the level of this second messenger within a cell needs to be strictly maintained for its various biological roles.

The occurrence of synthesis and hydrolysis activities in a single protein and the possible interdependence of the dual functions have been observed earlier in Rel, required for the control of another second messenger, ppGpp, in M. smegmatis. Rel is a bifunctional, multidomain protein with HD (hydrolysis), RSD (synthesis), TGS, and ACT domains, which are arranged in tandem wherein the C-terminal TGS and ACT constitute the regulatory domains. A balance between the two opposing activities maintains the level of ppGpp according to the physiological requirement (29, 37, 38). It has been shown that intramolecular domain–domain interaction takes place during the stringent response to regulate Rel and SpoT activities (39, 40). Therefore, we found it intriguing to identify any specific domain movement in DcpA protein in response to the presence of substrates like GTP and c-di-GMP. In such a working model, it is obligatory to measure any conformational change in DcpA through the movement of interacting domains. We generated several cysteine variants of DcpA, and DcpA-C424S,C474S was found to be an active mutant with both DGC and PDE-A activities. The presence of biological activities in DcpA-C424S,C474S prompted us to label the two terminal cysteines with FRET pairs; the fluorescently labeled protein also showed enzymatic activities. We adopted the FRET method to measure the domain–domain interaction and observed that the distance between the N-terminal (GAF) and C-terminal (EAL) domain reduces 20% in the presence of the substrates compared with that of the substrate-free DcpA.

Both the DGC and PDE-A activities of DcpA are not impaired by chemical modification of Cys579 because of its distance from the catalytic sites. We report here that the conserved cysteine at position 579 cannot accommodate a serine residue because the side chain of serine is strongly hydrophilic. The hydroxyl moiety tends to be oriented toward the surface for interaction with the solvent. Thus, cysteine-to-serine mutation will destabilize the hydrophilic interface. In addition, Cys579 is four residue positions away from glutamine (Gln583), which participates in the positioning of Mg2+ for the hydrolysis activity of the EAL domain (27, 41). It appears that cysteine (or its modified form) can facilitate hydrolysis activity through glutamic acid, whereas serine cannot. We also speculate that sulf-hydryl of Cys579 can participate in aromatic–sulfur interaction with the conserved phenylalanine rings (Phe585) that are adjacent but on the opposite side of cysteine. Modification of the sulfhydryl group with an aromatic moiety can add to the stability by way of a stacking interaction with the phenylalanine residue. Domain–domain interaction appears to modulate the DGC and PDE-A activities, and this could be the reason for the loss of DGC activity in DcpA with the C579S mutation in the EAL domain. It would be worthwhile to follow the interdependence of the two activities as a function of single mutation of each amino acid in the GGDEF and EAL domains. This long-range effect was observed in the case of a single E384A substitution at the EAL domain, which abrogated the c-di-GMP synthesis activity to some extent (Fig. 1). In addition, we have predicted a novel motif, GCXXXQGF, to be involved in c-di-GMP turnover in M. smegmatis.

\[^5\] E. V. Filippova, G. Minasov, L. Shuvalova, O. Kiryukhina, C. Massa, T. Schirmer, A. Joachimiak, W. F. Anderson, and Midwest Center for Structural Genomics (MCSG), unpublished data.
GMP homeostasis, and our hypothesis is supported by the crystal structure of the EAL domain of *C. crescentus*. It shows that Phe^{585} of the GCXXXQGF motif is involved in a stacking interaction with the guanosine base of pGpG or c-di-GMP. This is a strong interaction in the c-di-GMP–binding pocket of the EAL domain and is essential for phosphodiesterase activity. The functionally conserved active-site loop-6 (DFGTGYS), necessary for c-di-GMP degradation, is placed at the opposite side of the GCXXXQGF motif to facilitate c-di-GMP degradation (41). Cysteine-to-serine mutation at position 579 will destabilize the interaction between c-di-GMP and Phe^{585}, resulting in the loss of phosphodiesterase activity.

In summary, we have shown that a single point mutation in the EAL domain of DcpA results in complete loss of the PDE-A activity with a significant decrease in the DGC activity of the GGDEF domain, which possibly suggests that the EAL domain regulates the DGC activity of the GGDEF domain. We did not identify any intramolecular disulfide linkages that could be involved in such regulation; however, we did observe that the N and C termini of DcpA come in close proximity to regulate the DGC and PDE-A activities. Furthermore, our mutational studies and computational analyses suggest that the cysteine at the C-terminal EAL domain is structurally important and is a part of a novel motif, GCXXXQGF. In conclusion, this study provides evidence that a substrate-mediated conformational switch involving movement of a loop containing a novel conserved motif in DcpA controls c-di-GMP turnover in *M. smegmatis*. This domain movement, which leads to increased c-di-GMP synthesis, is probably linked with the substrate-induced enzyme dimerization that was found to predominantly display the c-di-GMP synthesis activity. Thus, it provides an additional layer of regulation in the activity of DcpA associated with persistent survival, colony morphology, and cell length.

Materials and methods

Reagents, bacterial strains, and culture conditions

All mutational studies were conducted in *Escherichia coli* DH5α strain, and *E. coli* BL21 (DE3) was used for protein expression and purification. *E. coli* was grown in LB broth and LB agar medium as and when required. Ampicillin (100 μg/ml) was added as needed. Restriction enzymes used in this study were obtained from New England Biolabs. Deep Vent polymerase and Phusion (Finnzymes) were used for site-directed mutagenesis following the manufacturer’s instructions. Unless otherwise stated, all the experiments were carried out at room temperature (−25°C).

Expression, purification, and characterization of WT DcpA and its mutants

The DcpA protein was expressed in *E. coli* and purified as described earlier (20). Briefly, DcpA-containing plasmid pETDGC-2196 was transformed into *E. coli* BL21 (DE3), and cells were grown in LB broth at 37°C to an *A*_{600} of ∼0.6. The cells were induced with 1 mM isopropyl-1-thio-β-galactopyranoside and further grown for an additional 3 h. The harvested cells were lysed by sonication in lysis buffer (50 mM Tris-HCl, pH 7.9, 500 mM NaCl, 1 mM phenylmethane sulfon fluoride, and 1 mg/ml lysozyme) at 4°C, and precleared lysate was loaded on nickel-nitrioltriacidic acid column pre-equilibrated with equilibration buffer (50 mM Tris-HCl, pH 7.9, and 500 mM NaCl). The column was washed with wash buffer (50 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 20 mM imidazole), and protein was eluted with elution buffer (50 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 500 mM imidazole). The protein was dialyzed against 50 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 5 mM β-mercaptoethanol and stored at 4°C for further use.

All the mutants were generated using pETDGC-2196 as a template. The conserved residue Glu^{384} (EAL motif) was mutated to Ala (E384A) using the megaprimer method, and for cysteine variants of DcpA, Cys^{424}, Cys^{474}, and Cys^{579} were mutated to Ser using two-step mutagenesis (42, 43). The mutant proteins were expressed and purified following the same procedure for the WT DcpA. Protein bands of high molecular weight in 10% SDS-polyacrylamide gels were identified as higher oligomers of the same proteins by MALDI-TOF MS analysis. We could not purify the protein further using a gel filtration column as these proteins had a greater tendency to bind to the column resin (Sephadex-200). Thus, the partially purified proteins were assayed for DGC and PDE-A activity using chromatographic analysis as described earlier (7, 20). The absorbance of the protein was recorded at *A*_{280 nm}, and protein concentration was calculated using the molar extinction coefficient of the WT protein (ε = 40, 910 M⁻¹ cm⁻¹) assuming all cysteine residues are reduced using ProtParam (44).

The DGC and PDE-A activities of the purified proteins were estimated and compared with those of the WT as described above (20). In short, the purified proteins (5 μM) were incubated with 50 mM Tris-HCl, pH 7.9, 500 mM NaCl, 10 mM MgCl₂, 5 mM β-mercaptoethanol, 0.1 mM GTP (cold), and [α-³²P]GTP (0.01 mCi μl⁻¹ (370 Bq μl⁻¹); Board of Radiation and Isotope Technology (BRIT), Hyderabad, India) at 37°C for 30 min in a 10-μl reaction volume. The reaction was stopped by placing them in boiling water for 5 min followed by centrifugation at 12,000 rpm (14,006 × g) for 20 min at 4°C. The supernatant (2 μl) was spotted on polyethyleneimine-cellulose plate (Merck); developed in 1:1.5 (ν/ν) saturated (NH₄)₂SO₄ and 1.5 M KH₂PO₄, pH 3.6; and exposed to phosphorimaging screens. The intensities of the spots were determined using Multi Gauge version 2.0 (Fujifilm) software from Science Laboratory.

Disulfide mapping in DcpA protein using MS

Approximately 100 μg of native DcpA protein was reduced with 10 mM DTT for 45 min at 37°C and further alkylated with 30 mM iodoacetamide (IAM; Sigma) for 1 h at 37°C in 50 mM Tris-HCl, pH 7.9. In the case of labeling without reduction, 100 μg of protein was buffer-exchanged into 50 mM Tris-HCl, pH 7.5, and alkylated with 30 mM IAM for 1 h at 37°C. In both cases, excess IAM was neutralized by further treating the alkylated protein with 20 and 30 mM DTT (Sigma) for 15 min at 37°C. The alkylated protein was digested with trypsin in a ratio of 25:1 (protein:enzyme) for 16 h at 37°C. To identify the disulfide-linked cysteines, the native protein was buffer-exchanged into 50 mM Tris–HCl, pH 7.5, and digested with trypsin in a ratio of 15:1 (protein:enzyme) for 16 h at 37°C. The reaction was stopped by adding 1 μl of formic acid (Sigma) in all
c-di-GMP turnover in M. smegmatis

cases, and the sample was analyzed by LC-MS for peptide mapping. The native protein was also separately labeled with 5 mM N-ethylmaleimide (Sigma) for 30 min at 37 °C in the presence of 2 M guanidinium chloride and 50 mM Tris-HCl, pH 6.5.

The native, reduced and the N-ethylmaleimide–labeled proteins were analyzed by LC-MS to obtain their molecular mass. Briefly, 20 µg of protein was injected into a reverse-phase column (Zorbax RV-C18, 5 µm, 4.6 × 150 mm, Agilent) and eluted with an increasing gradient of acetonitrile (Merck) in 0.1% formic acid (30–60% of acetonitrile in 30 min) at a flow rate of 200 µl min⁻¹ at 25 °C. The desalted protein was analyzed by ESI-MS (HCT-ultra with ETD-II, Bruker Daltonics) connected in tandem with reverse-phase HPLC. Tryptic digests were resolved through reverse-phase chromatography using a C₁₈ column (Poroshell-120, SB-C₁₈, 2.7 µm, 4.6 × 150 mm, Agilent) operating in an increasing gradient of acetonitrile in 0.1% formic acid (2–40% acetonitrile for 58 min followed by 40–95% acetonitrile in the next 10 min). The eluted peptides were monitored by ESI-MS and CID-MS/MS (HCT-ultra with ETD-II, Bruker Daltonics) connected online with HPLC. Nebulizer gas (N₂) was set at 35 p.s.i. with the drying gas at 7 liters min⁻¹ and a source temperature of 330 °C. Peptides were fragmented inside the ion trap using helium as the collision gas. An isolation width of 4 m/z and fragmentation amplitude of 1.5 V were set during collision-induced dissociation. The mass analyzer was operated in a range of 50–3000 m/z in positive ion, ultrascan mode (in the case of a protein sample) and extended scan mode (in the case of a peptide digest) with optimum ion charge control (ICC target, 200,000; maximum accumulation time, 100 ms). Data Analysis 3.4 (Bruker Daltonics) was utilized to deconvolute the charge-state distribution of intact mass spectra and for assigning the CID-MS/MS spectra of the tryptic peptides.

**CD and dynamic light scattering studies**

For all CD measurements, the purified DcpA protein and its variants were dialyzed against buffer (10 mM phosphate buffer, pH 8.0, and 150 mM KCl), and the spectra were recorded at 25 °C on a Jasco J-715 spectropolarimeter on the same day to minimize the formation of soluble aggregates in the absence of any reducing agents. The protein concentrations were determined carefully by measuring the absorbance at A₂₈₀ nm. Approximately, 2–3 µM DcpA and its variants were used in CD measurements. Scans were taken using a bandwidth of 2.0 nm, response time of 2 s, data pitch of 0.2 nm, and scanning speed of 50 nm/min. The spectra were recorded from 240 to 200 nm in a quartz cuvette of path length 2 mm. Spectra were averaged over five scans and buffer-subtracted. The GTP (5 molar ratio) was added to the DcpA protein, and spectra were recorded before and after incubation at 37 °C for 5 min. The CD data are represented as mean residue ellipticity, and the secondary structure estimations were carried out using the K2D2 program (45).

For DLS studies, the DcpA and DcpA-C424S,C474S proteins were dialyzed in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 5 mM β-mercaptoethanol. DLS experiments were performed on a Viscotek 802 DLS instrument in a 50-µl-volume quartz cuvette at 20 °C. Protein concentrations for the scattering experiments were in the range of about ~1 mg/ml, and data were analyzed using the software (Omnisize 3.0) associated with the Viscotek instrument.

**Thermal shift assay**

The thermal shift assay was conducted in an iCycle iQ5 Real-Time Detection System (Bio-Rad). The purified proteins were dialyzed against 10 mM phosphate buffer, pH 8.0, containing 150 mM KCl, and the final concentrations of the proteins used were ~2 µM. The SYPRO Orange solution (final 2.5×) (Sigma) was added to the wells of a 96-well thin-wall PCR plate (Bio-Rad), sealed with Optical-Quality Sealing Tape (Bio-Rad), and heated in the cycler from 10 to 95 °C in increments of 0.2 °C/30 s. Fluorescence changes in the wells of the plate were monitored simultaneously with a CCD camera. The wavelengths for excitation and emission were 490 and 575 nm, respectively. To obtain the temperature midpoint (Tₘ) for the protein unfolding transition, the fluorescence data from CCD detection were plotted as a function of temperature and fitted to a standard four-parameter sigmoidal equation.

\[
Y = LL + \frac{UL - LL}{1 + e^{(T_m - T)/a}} \quad \text{(Eq. 1)}
\]

where Y is the observed fluorescence signal and LL and UL are the minimum and maximum intensities during the transition, respectively. Tₘ is the melting temperature, T is the experimental temperature, and a is the slope of the transition (46).

**Substrate binding and steady-state kinetics of DcpA and mutant proteins**

Steady-state kinetic parameters Vₘₐₓ, Kₘ, k_cat, and k_cat/Kₘ for the DGC activity of DcpA-C424S,C474S were determined as described earlier (20) with GTP substrate concentration ranging from 0 to 180 µM with an equal amount of protein (5 µM). The reactions were stopped after 30 min and centrifuged at 12,000 rpm (14,006 × g) for 20 min, and supernatant (1 µl) was spotted on polyethyleneimine-cellulose plate. The plate was developed in 1:1.5 (v/v) saturated (NH₄)₂SO₄ and 1.5 M KH₂PO₄ (pH 3.6) and exposed to phosphorimaging screens. The intensities of the spots were determined using MultiGauge version 2.3 (Fujifilm) software from Science Laboratory. Kₘ and Vₘₐₓ were determined from a nonlinear Michaelis–Menten plot (v versus [S]) and a linear plot of initial velocity (1/v₀) as a function of substrate (1/[S]) concentration using GraphPad Prism (version 5.02). The experiments were carried out in triplicates, and the standard deviation was calculated and plotted in the graph using GraphPad Prism software.

**Labeling of cysteines with thiol-specific fluorophores**

The cysteines in DcpA-C424S,C474S, DcpA-C474S,C579S, and DcpA-C424S,C579S were labeled with thiol-specific fluorophores IAEDANS (donor) and IAF (acceptor) (Molecular Probes, Invitrogen), and labeling was carried out as described earlier with slight modification (43, 47). To achieve successful labeling of fluorescent probes, the protein was reduced with a 5-fold molar excess of TCEP for 30 min on ice, and 1,5-IAE-
DANS (1:1 molar ratio) was added to the protein solution and incubated for another 30 min on ice followed by a further incubation of 12 h at 4 °C under gentle agitation in the dark. To stop the reaction, a 10-fold molar excess of DTT was added and incubated for an additional 1 h. To remove the unbound probe, the labeled protein was rebuffered with nickel-nitriilotriacetic acid matrix for 3 h followed by washing with 20 column volumes of labeling buffer (40 mM HEPES, pH 7.9, 250 mM NaCl, 0.1 mM EDTA, and 5% glycerol) and eluted with labeling buffer containing 500 mM imidazole. The IAEDANS-labeled protein was further dialyzed extensively in labeling buffer containing 5 mM β-mercaptoethanol. The percentage of labeling was calculated by measuring the absorbance at 280 nm for protein (ε = 40,910 M⁻¹ cm⁻¹) and at 336 nm for IAEDANS (ε = 6,300 M⁻¹ cm⁻¹). The absorption of the IAEDANS-labeled protein was recorded at 336 nm, and the blank (absorbance of unlabeled protein at 336 nm) was subtracted for the correct estimation of the protein:dye ratio. For IAF labeling, the IAEDANS-labeled (monolabeled) protein was incubated with a 5-fold molar excess of TCEP for 30 min on ice, and a 20-fold molar excess of IAEDANS-labeled protein was incubated with a 5-fold molar excess of DTT for 30 min and further incubated for 12 h at 4 °C under gentle agitation in the dark. To stop the reaction, a 20-fold molar excess of DTT was added to the reaction mixture. The free IAF was removed as described above.

The percentage of labeling was calculated by measuring the absorbance at 280 nm for protein (ε = 40,910 M⁻¹ cm⁻¹) and at 336 nm for IAEDANS (ε = 77,000 M⁻¹ cm⁻¹). The absorption of IAEDANS-labeled protein was also recorded at 492 nm and subtracted from values obtained with the IAEDANS-IAF-labeled protein at 492 nm for the correct estimation of the dye:protein ratio. The dye:protein ratio should be ~1:1. The IAEDANS-labeled DcpA-C424S,C474S protein was excited at 336 nm, and fluorescence emission intensity (arbitrary units) was monitored as a function of increasing concentration of free IAF at 470 nm. As shown in Fig. 4a, there was a saturation in fluorescence emission maxima when the IAEDANS-IAF molar ratio neared 1:1, suggesting that one of the cysteines was labeled with IAEDANS and the other cysteine is free for IAF labeling. However, we observed that the molar ratio of IAEDANS-labeled protein to IAF was 1:0.7. The purified DcpA, IAEDANS-labeled (monolabeled), and IAEDANS-IAF-labeled (doubly labeled) proteins were assayed for DGC and PDE-A activities as described above.

Furthermore, the IAEDANS- and IAEDANS-IAF-labeled proteins were digested with trypsin and analyzed using MALDI-TOF for the specific labeling of fluorophores at cysteine-containing peptides. The unlabeled DcpA-C424S,C474S protein was used as a control. The digested IAEDANS-labeled (monolabeled) and IAEDANS-IAF-labeled (doubly labeled) proteins were mixed with α-cyano-4-hydroxycinnamic acid and further analyzed using MALDI-TOF in positive ion mode.

**Measurement of quantum yield (Q), spectral overlap integral (J), and the distance between the donor–acceptor pair (r)**

To calculate the distance (r) between donor and acceptor fluorophore, energy transfer efficiency (E) was estimated, which is related to r according to Equation 2 (48).

\[ r = R_0 \left( \frac{1}{E} - 1 \right)^{1/6} \]  

(Eq. 2)

where \( R_0 \) is the Förster distance between the donor and acceptor pair when transfer efficiency is 50% (47, 49, 50). Thus, the value is constant for a specific pair and for an IAEDANS-IAF pair. We calculated \( R_0 \) according to Equation 3.

\[ R_0 = 9.79 \times 10^{-1} \left( \frac{J Q^n}{(\kappa^2)^{1/6}} \right) \]  

(Eq. 3)

\( J \) is the spectral overlap integral of the donor (IAEDANS) emission between 400 and 600 nm and the acceptor absorption between 420 and 535 nm. We calculated \( J (\text{m}^{-1} \text{cm}^3) \) according to Equation 4.

\[ J (\text{m}^{-1} \text{cm}^3) = \frac{\int F_d(\lambda) \varepsilon_d(\lambda) \lambda^4 d\lambda}{\int F_d(\lambda) d\lambda} \]  

(Eq. 4)

where \( F_d \) is the fluorescence emission intensities of the donor and \( \varepsilon_d \) is molar extinction coefficient of the acceptor. \( \lambda \) is the wavelength at a definite nanometer interval. The \( J \) value was calculated using a Fortran program as described previously (51) (see supporting Appendix). The overlap spectrum is shown in Fig. S11. The other variable factor for measuring \( R_0 \) is the \( \kappa^2 \), or orientation factor, for the donor and acceptor dipoles (described under “Results”). \( n \) is the refractive index of the medium and usually taken as 1.4. The quantum yield of the sample, \( Q_0 \), was calculated from the absorbance of IAF-labeled protein and the area enclosed by the corrected emission spectrum of IAEDANS-labeled protein according to Equation 5.

\[ Q_0 = \left( 1 - 10^{-A_d} \right) \frac{\text{Area}_{d}}{\text{Area}_{IAF} (1 - 10^{-A_A}) n_{IAF}^2} \]  

(Eq. 5)

where \( Q_0 \) is the quantum yield of the reference quinine sulfate in 0.1 M H₂SO₄ (52, 53). \( A_d \) and \( A_A \) are the absorbance of the reference and sample at 336 nm, respectively. \( \text{Area}_{IAF} \) and \( \text{Area}_{d} \) are the areas enclosed by the corrected emission spectra of the reference and donor fluorophores, respectively, when excited at 336 nm. The refractive indices of the sample, \( n_{IAF} \) and reference, \( n_{d} \), were taken as 1.36 and 1.4, respectively.

The energy transfer efficiency, \( E \), can be calculated according to Equation 6.

\[ E = 1 - \tau_0 / \tau \]  

(Eq. 6)

where \( \tau \) and \( \tau_0 \) are the lifetimes of the donor in the presence and absence of acceptor, respectively.

**Measurement of τ, A, and τ₀**

All fluorescence measurements were carried out in a Fluorolog Tau-3 (Jobin Yvon) with a source-corrected xenon lamp using a phase modulation technique, which is a frequency domain method. The theory of phase modulation has already been described elsewhere (53, 54). The samples were excited at 336 nm, and fluorescence emissions were monitored at 470 nm. Steady-state A gives information about the tumbling motion of biomolecules in the solution. We measured the steady-state...
anisotropy in the presence and absence of GTP and c-di-GMP. Time dependence of steady-state anisotropy yielded the $r_a$ value. All the experimental curves were finally fitted to the best-fit line with the least $\chi^2$ values. All the experiments were performed at 25 °C.

**Measurement of the intermolecular FRET in DcpA-C424S,C474S**

The DcpA-C424S,C474S protein was completely labeled with 1,5-IAEDANS and IAF separately in the presence of TCEP; the excess dyes were removed as described above, and the protein was dialyzed against the labeling buffer. The unlabeled DcpA-C424S,C474S protein was also dialyzed in the labeling buffer. The IAEDANS-labeled and unlabeled DcpA-C424S,C474S proteins were mixed (1:1 molar ratio), and the fluorescence efficiency of the only donor was recorded in the absence of acceptor. Similarly, the IAEDANS- and IAF-labeled proteins were mixed in a molar ratio of 1:1, and FRET efficiency and the distance between the IAEDANS-IAF was estimated in the presence and absence of GTP and c-di-GMP.

**Role of cysteines in the dual activity of the DcpA protein**

To investigate the biochemical role of cysteines in the DGC and PDE-A activities, the proteins DcpA and DcpA-C424S,C474S were purified as described earlier, and the undialyzed protein samples were labeled with a 50-fold molar excess of IAEDANS and iodoacetamide separately in the presence and absence of the reducing agent TCEP. For labeling in the presence of TCEP, the proteins were first reduced with a 10-fold molar excess of TCEP for 30 min in ice and further labeled with a 50-fold molar excess of IAEDANS or iodoacetamide. We used a higher molar excess of IAEDANS or iodoacetamide to achieve complete labeling of cysteines with either IAEDANS or iodoacetamide. All the samples were tested for DGC and PDE-A activities by densitometric TLC as assayed above for WT protein.

**Modeling of DcpA using I-TASSER and Modeller**

For the modeling studies, the DcpA protein sequence was submitted to the I-TASSER server (58), and the generated model was structurally aligned with the known crystal structures and compared using the CLICk server (data not shown) (55). The ConSurf server (http://consurf.tau.ac.il) was used to identify the structural and functional evolution of amino acids (32). The model for the EAL domain of DcpA was also generated using Modeller 9v7 (56). The DcpA protein was searched for homologs of known structure against the Protein Data Bank, and the EAL domain of *T. denitrificans* (PDB code 2R6O) was found to have 38% identity in sequence with DcpA in PSI-BLAST (33). PDB code 2R6O was used as a template for model building of the EAL domain of DcpA protein, and the generated model was superimposed with the crystal structure of diguanylate cyclase phosphodiesterase protein of *T. denitrificans*, and it was perfectly aligned. Fig. 6 was generated using SETOR software (57).

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6 Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party-hosted site.
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