 Activation of the Diguanylate Cyclase PleD by Phosphorylation-mediated Dimerization*

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Ralf Paul, Sören Abel, Paul Wassmann, Andreas Beck, Heiko Heerklotz1, and Urs Jenal2

From Biozentrum, University of Basel, Klingelbergstrasse 70, Basel CH-4056, Switzerland

Diguanylate cyclases (DGCs) are key enzymes of second messenger signaling in bacteria. Their activity is responsible for the condensation of two GTP molecules into the signaling compound cyclic di-GMP. Despite their importance and abundance in bacteria, catalytic and regulatory mechanisms of this class of enzymes are poorly understood. In particular, it is not clear if oligomerization is required for catalysis and if it represents a level for activity control. To address this question we perform in vitro and in vivo analysis of the Caulobacter crescentus diguanylate cyclase PleD. PleD is a member of the response regulator family with two N-terminal receiver domains and a C-terminal diguanylate cyclase output domain. PleD is activated by phosphorylation but the structural changes inflicted upon activation of PleD are unknown. We show that PleD can be specifically activated by beryllium fluoride in vitro, resulting in dimerization and c-di-GMP synthesis. Cross-linking and fractionation experiments demonstrated that the DGC activity of PleD is contained entirely within the dimer fraction, confirming that the dimer represents the enzymatically active state of PleD. In contrast to the catalytic activity, allosteric feedback regulation of PleD is not affected by the activation status of the protein, indicating that activation by dimerization and product inhibition represent independent layers of DGC control. Finally, we present evidence that dimerization also serves to sequester activated PleD to the differentiating Caulobacter cell pole, implicating protein oligomerization in spatial control and providing a molecular explanation for the coupling of PleD activation and subcellular localization.

Cyclic 3',5'-guanylyl and adenylyl nucleotides function as second messengers in signal transduction pathways of eukaryotes and prokaryotes. The synthesis of these molecules is catalyzed by a wide variety of nucleotidyl cyclases, which are active as homo- or heterodimers (1). Monocyclic nucleotidyl cyclases that catalyze the formation of cAMP or cGMP are regulated by small molecules, endogenous domains, or exogenous protein partners, many of which alter the interface of the catalytic domains and therefore the integrity of the catalytic site. Much less is known about catalysis and regulation mechanisms of the recently discovered family of diguanylate cyclases (DGCs). DGCs are responsible for the synthesis of cyclic di-GMP, a ubiquitous second messenger involved in bacterial biofilm formation and persistence (2). Cellular levels of c-di-GMP are controlled through the opposing activities of DGCs and phosphodiesterases, which form two large families of output domains found in bacterial one- and two-component systems (3). The DGC activity is contained within the highly conserved GGDEF domain, whose three-dimensional fold is similar to the catalytic core of adenylate cyclase and the “palm” domain of DNA polymerases (4, 5). Because GGDEF domains are often associated with sensory input domains, it was proposed that these regulatory proteins serve to directly couple environmental or internal stimuli to a specific cellular response through the synthesis of the second messenger c-di-GMP. Similar to monocyclic nucleotidyl cyclases, the controlled formation of catalytically competent GGDEF domain dimers may be a key mode of DGC regulation (2, 5, 6). A simple model proposes that dimerization mediates an antiparallel arrangement of two DGC domains, each of which is loaded with one GTP substrate molecule. Such an arrangement would allow deprotonation of the GTP 3’OH groups and subsequent intermolecular nucleophilic attacks onto the α-phosphate to occur (5).

The diguanylate cyclase PleD controls pole morphogenesis during the Caulobacter crescentus cell cycle (4, 7–10). PleD is an unorthodox member of the response regulator family of two-component signal transduction systems with two receiver domains arranged in tandem fused to a GGDEF output domain (5). Phosphorylation by two cognate kinases, PleC and DivJ, is required for the activation and dynamic sequestration of PleD to the differentiating Caulobacter cell pole (4, 9). Although the first receiver domain (Rec1) serves as phosphoryl acceptor (at the conserved Asp-53 residue), the second receiver domain (Rec2) was proposed to function as an adaptor for dimerization of activated PleD (5, 9). A simple mechanistic model for the activation of PleD proposes that phosphorylation at the conserved Asp-53 of Rec1 induces repacking of the Rec1/Rec2 interface. This in turn would mediate dimer formation by isologous Rec1-Rec2 contacts across the interface and thereby facilitate reorientation and assembly of two C-terminal DGC domains (5). Here we demonstrate that PleD activity can be greatly stimulated in vitro...
by the phosphoryl mimic BeF$_3$ and that activation of PleD results in dimer formation. Cross-linking experiments revealed that the DGC activity resides entirely in the dimer fraction of activated PleD. Furthermore, controlled dimerization not only modulates DGC activity but is also employed to couple PleD activity to its subcellular sequestration. This is the first demonstration that GGDEF protein dimers represent the active conformation of diguanylate cyclases and confirms that oligomerization can be used to regulate the activity of this abundant class of signaling proteins.

### MATERIALS AND METHODS

**Strains, Plasmids, and Media**—Bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* strains were grown in Luria Broth (LB) media supplemented with antibiotics for selection, when necessary. The exact procedure of strain and plasmid construction is available on request.

**Expression and Purification of PleD**—*E. coli* cells carrying the respective expression plasmid were grown in 200 ml of LB medium with ampicillin (100 μg/ml), and expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside to 0.4 mM final concentration. After harvesting by centrifugation, the cells were resuspended in TN buffer (50 mM Tris-HCl at pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol) and lysed by passage through a French press cell. The suspension was clarified by centrifugation, followed by a high spin centrifugation step (100,000 × g, 1 h). The supernatant was loaded onto Ni-NTA affinity resin (Qiagen), washed with TN buffer, and eluted with an imidazole gradient. Elution fractions were examined for purity by SDS-PAGE, and fractions containing pure protein were pooled. PleD was extensively dialyzed first against 500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 5 mM β-mercaptoethanol, and then against 250 mM NaCl, 25 mM Tris-HCl, pH 7.8, 5 mM β-mercaptoethanol. Prior to cross-linking experiments PleD was dialyzed against a buffer containing 250 mM NaCl, 5 mM PO$_4$$^-$, and 5 mM β-mercaptoethanol. Analytical size exclusion chromatography (SEC) was performed with a Superdex 200 column on a SMART system (Amersham Biosciences) at a flow rate of 50 μl/min. Preparative SEC to quantitatively strip nickel-nitrilotriacetic acid-purified PleD from bound c-di-GMP was performed on a preparative scale Superdex 200 column on an AKTA system (Amersham Biosciences).

**Enzymatic Assays**—Diguanylate cyclase assays were adapted from procedures described previously (Paul et al. 4). The standard reaction mixtures with purified PleD contained 50 mM Tris-HCl, pH 7.8, 250 mM NaCl, 10 mM MgCl$_2$, in a 50-μl volume and were started by adding 100 μM GTP/[α-32P]GTP (PerkinElmer Life Sciences, 0.01 μCi/μl). To calculate the initial velocity of product formation, aliquots were withdrawn at regular time intervals, and the reaction was stopped with an equal volume of 50 mM EDTA, pH 6.0. Reaction products (2 μl) were separated on polyethyleneimine-cellulose plates (Machery-Nagel) in 1.5 M K$_2$PO$_4$/5.5 M (NH$_4$)$_2$SO$_4$ (pH 3.5), mixed in a 2:1 ratio. Plates were exposed to a phosphorimaging screen, and the intensity of the various radioactive species was calculated by quantifying the intensities of the relevant spots using ImageQuaNT software (Amersham Biosciences). Measurements were always restricted to the linear range of product formation.

**Cross-linking Assays**—The purified protein (20 or 25 μM in 100 mM NaCl, 5 mM NaPO$_4$, pH 7.8, 10 mM MgCl$_2$, 5 mM β-mercaptoethanol, ± 1 mM BeCl$_2$/10 mM NaF) was incubated with 2 μM disuccinimidyl suberate (DSS, Pierce) for 0, 1, 5, and 10 min. The cross-linker was inactivated by adding Tris-HCl, pH 7.8, to 50 mM final concentration. After separation on 10% SDS-PAGE and transfer to a PVDF membrane, PleD monomeric and dimeric forms were detected by staining with an anti-PleD antibody (8).

**Isothermal Titration Calorimetry**—The interaction of PleD with cyclic-di-GMP was measured with a VP-ITC isothermal titration calorimeter from MicroCal (Northampton, MA), with 3 μM PleD in the cell and 90 μM c-di-GMP in the syringe (buffer: 100 mM NaCl, 25 mM Tris-HCl, pH 7.8, 5 mM MgCl$_2$, and 1 mM β-mercaptoethanol). All solutions were thoroughly degassed and equilibrated to 25°C before filling into the calorimeter. The delay between the injections was set to 5–10 min to ensure complete re-equilibration between subsequent injections. The heat capacity of the interaction between the inhibitor and the protein was estimated through measurements between 5 and 25°C.

**Microscopy and Photography**—*C. crescentus* strains were grown in 5 ml of peptone-yeast extract media containing 5 μg/ml tetracycline (PYE/tet) for 18 h at 30°C on a roller incubator. The stationary phase cultures were diluted 1/50 and grown for another 8–10 h in 5 ml of PYE/tet. For fluorescence imaging 1 μl of bacterial culture was placed on a microscope slide layered with a pad of 2% agarose dissolved in water. An Olympus IX71 microscope equipped with an UPlanSapo 100×/1.40 oil objective (Olympus) and a coolSNAP HQ (Photometrics) charge-coupled device camera were used to take differential interference contrast and fluorescence photomicrographs. For GFP fluorescence fluorescein isoethiocyanate filter sets (Ex 490/20 nm, Em 528/38 nm) were used with exposure times of 0.15 and 1.0 s, respectively. Images were processed with softWoRx version 3.3.6 and Photoshop CS version 8.0.

## RESULTS

**Activation of the PleD Diguanylate Cyclase by Beryllium Fluoride**—To investigate the specific requirements for PleD DGC activity and activation in vitro, we first set out to define the optimal reaction conditions with respect to pH, the concentrations of monovalent and divalent cations, and protein concentration. PleD was enzymatically active between pH 6.5 and 10.0, with maximal activity between pH 7.5 and 8.5 (supple-

### Table 1

| Protein         | Without BeF$_3$ | With BeF$_3$ |
|-----------------|-----------------|--------------|
|                 | nanomoles c-di-GMP min$^{-1}$ mg$^{-1}$ | nanomoles c-di-GMP min$^{-1}$ mg$^{-1}$ |
| PleD            | 3.32 (±0.7)*     | 159.97 (±22.6) |
| PleDD53N        | 2.38 (±0.3)      | 1.10 (±0.2)   |
| PleDY26A        | ND*             | 0.036 (±0.017) |

* In parentheses: standard deviation.
* ND, not detectable.
Enzymatic activity was strictly dependent on the presence of either Mg\(^{2+}\) (supplemental Fig. S1) or Mn\(^{2+}\) (not shown), with Mn\(^{2+}\) resulting in a slightly higher activity compared with Mg\(^{2+}\). The strict requirement for bivalent cations is in agreement with the recent finding of coordinating metal ions in the catalytic center of the PleD DGC (11). Activity decreased with increasing NaCl concentration (supplemental Fig. S1). Also, the addition of KCl (25 mM) to reaction assays (4) slightly decreased the enzymatic activity and was omitted in subsequent experiments.

Activation of the PleD DGC \textit{in vitro} and \textit{in vivo} requires the transfer of a phosphoryl group onto the aspartic acid acceptor residue Asp-53 of the first receiver domain (Fig. 1) (4). However, \textit{in vitro} phosphorylation experiments with PleD resulted in an exiguous increase of DGC activity, possibly due to suboptimal assay conditions or to low stability of the phosphorylated form (4). For this reason we tested activation of PleD by beryllium fluoride (BeF\(_3\)), a molecular mimic of a phosphoryl group that has been widely used for biochemical and structural studies of bacterial response regulators (12–18). As shown in Fig. 2, BeF\(_3\) significantly stimulated the enzymatic activity of PleD,

**FIGURE 1.** Crystal structure of the non-activated PleD (5). A front view of a PleD dimer is shown in A with the first receiver domain (Rec1) in red, the second receiver domain (Rec2) in yellow, and the GGDEF domain in green. The active site (A-site) loop (blue), the phosphoryl acceptor Asp-53, and the position of Tyr-26 are indicated. The 2-fold symmetry axis is drawn in dark blue. B, bottom view along the axis of the Rec1-Rec2 dimerization stem. The domain coloring is equivalent to A.

**FIGURE 2.** Beryllium fluoride-mediated activation of the PleD diacylguanylate cyclase. A, PleD (5 \(\mu\)M) activity (nanomoles of c-di-GMP min\(^{-1}\) mg\(^{-1}\)) as a function of BeCl\(_2\) concentration in the presence of 5 mM NaF. B, PleD (2.5 \(\mu\)M) activity (nanomoles of c-di-GMP min\(^{-1}\) mg\(^{-1}\)) as a function of NaF concentration in the presence of 1 mM BeCl\(_2\). C, PleD\(_{53N}\) (5 \(\mu\)M) activity (nanomoles of c-di-GMP min\(^{-1}\) mg\(^{-1}\)) as a function of BeCl\(_2\) concentration in the presence of 5 mM NaF. D, specific activities (nanomoles of c-di-GMP min\(^{-1}\) mg\(^{-1}\)) for PleD (open circles), PleD activated with BeF\(_3\) (filled circles), and the constitutively active PleD\(^*\) mutant protein (triangles) as a function of the protein concentration.
with optimal concentrations of 1 mM BeCl₂ (Fig. 2A) and 10 mM NaF (Fig. 2B), respectively. DGC activation was reversible and was immediately abolished upon removal of BeF₃ (data not shown). A PleD mutant protein lacking the phosphoacceptor site Asp-53 (PleD<sub>D53N</sub>) could not be activated, suggesting that BeF₃ activates the protein by specifically interacting with this residue in the first receiver domain (Fig. 2C). A constitutively active mutant of PleD, PleD<sup>*</sup> (4) was also stimulated by BeF₃, but only by a factor of 2 (data not shown). This is consistent with the view that PleD<sup>*</sup> is locked in an active state (see below). Concentrations of BeCl₂ or NaF above 1 mM and 10 mM, respectively, had a negative effect on DGC activity (Fig. 2, A–C). At these concentrations BeF₃ probably interacts nonspecifically with surface residues that are required for diguanylate cyclase activity.

**BeF₃ Activation Results in PleD Dimerization**—The specific activities of non-activated and BeF₃-activated PleD wild-type protein and of the constitutively active PleD<sup>*</sup> mutant increased with increasing protein concentration (Fig. 2D). This suggested that PleD might be active in a dimeric (or oligomeric) form, with dimer formation being concentration-dependent. The observation that PleD<sup>*</sup> and BeF₃ activated PleD reach an activity plateau at much lower protein concentrations than non-activated PleD further suggested that the PleD dimerization constant is affected either by genetic changes or by chemical activation of the protein. To test this hypothesis, cross-linking studies were performed with PleD using the chemical cross-linker DSS (see "Materials and Methods"). We reasoned that the amount of covalently cross-linked dimers was proportional to the amount of dimers in solution. When the DSS cross-linker was incubated with non-activated wild-type PleD or PleD<sub>D53N</sub> at a protein concentration of 20 μM (well below the estimated dissociation constant <i>K<sub>d</sub></i> of dimerization of 10<sup>-4</sup> μM (11)), only a minor fraction of the protein was captured as covalently cross-linked dimers (Fig. 3A). This is consistent with the low basal level of enzymatic activity observed for non-activated PleD (Table 1). Activation of PleD by BeF₃ not only increased DGC activity (Table 1) but also the amount of cross-linked dimer species (Fig. 3A). In contrast, the non-activated PleD<sub>D53N</sub> (Fig. 2 and Table 1) showed no increase in cross-linked dimers (Fig. 3A).

The crystal structure of non-activated PleD predicted a specific dimerization interface in the Rec1-Rec2 receiver domain stem with a small contact patch around the surface exposed Tyr residue at position 26 of the first receiver domain (Fig. 1). Tyr-26 is strictly conserved in PleD homologs that share a specific dimerization interface in the Rec1-Rec2 receiver domain. To test if this residue plays a role in PleD dimerization, DGC activity and dimerization behavior of the PleD<sub>Y26A</sub> mutant protein were analyzed. Indeed, PleD<sub>Y26A</sub> was completely inactive in the absence and only marginally active in the presence of BeF₃ (Table 1). Consistent with this, only a minor fraction of the protein could be cross-linked in the dimer form, irrespective of the presence of BeF₃ (Fig. 3A). In agreement with these <i>in vitro</i> data, the pleD<sub>Y26A</sub> allele failed to complement the pleiotropic developmental defects of a <i>C. crescentus</i> pleD null mutant. Together these results strongly support the view that Tyr-26 residue forms part of the interaction surface of PleD dimers. This is consistent with the finding that, although additional inter-chain contacts are formed in the crystal structure of BeF₃ activated PleD, the specific contact around Tyr-26 is maintained (11).

**The Diguanylate Cyclase Activity of PleD Resides in the Dimer Fraction**—As indicated by cross-linking, PleD forms dimers in the presence of BeF₃. To investigate if the enzymatic activity coincided with the cross-linked PleD fractions, reactions were diluted 10-fold immediately after DSS treatment and quenching. At this BeF₃ concentration PleD showed only residual DGC activity (Fig. 2). As shown in Fig. 3B, cross-linking of BeF₃-activated PleD resulted in a 6-fold increase of DGC activity as...
compared with non-cross-linked samples. In contrast, cross-linking of PleD in the absence of BeF$_3$ and cross-linking of PleD$_{D53N}$ either in the presence or absence of BeF$_3$ did not result in increased DGC activity. Taken together, these results suggested that, in the presence of BeF$_3$, a fraction of the PleD protein is trapped by DSS cross-link in a dimerized form and that the dimer represents the active conformation of PleD. To further substantiate this idea we attempted to separate active dimers from inactive monomers by SEC (see “Materials and Methods”). Only PleD, but not PleD$_{D53N}$, changed its apparent molecular size in the presence of BeF$_3$ (Fig. 4, D). Enzymatic activity (nanomoles of c-di-GMP min$^{-1}$ mg$^{-1}$) of the PleD SEC fractions 11–15 from D with (open bars) or without (black bars) BeF$_3$. F, immunoblot analysis of PleD SEC fractions 11–15 from D with anti-PleD antibody. Arrows mark the monomeric and dimeric forms of PleD.

FIGURE 4. The enzymatic activity of PleD is contained within the dimer fraction. SEC of PleD (50 μg) in the presence (stippled line) or absence (solid line) of BeF$_3$ (A); PleD$_{D53N}$ (50 μg) in the presence (stippled line) or absence (solid line) of BeF$_3$ (B); PleD (solid line) and PleD* (stippled line) (C); and PleD cross-linked with 2 mM DSS in the absence (solid line) or presence (stippled line) of BeF$_3$ (D). E, enzymatic activity (nanomoles of c-di-GMP min$^{-1}$ mg$^{-1}$) of the PleD SEC fractions 11–15 from D with (open bars) or without (black bars) BeF$_3$. F, immunoblot analysis of PleD SEC fractions 11–15 from D with antipeleD antibody. Arrows mark the monomeric and dimeric forms of PleD.

that isolated dimers exhibited high DGC activity in support of an “activation by dimerization” mechanism.

DGC Activity Is Not Required for PleD Dimerization—To demonstrate that dimerization is required, but not sufficient for diguanylate cyclase activity, we analyzed if the PleD$_{E370Q}$ mutant formed dimers. The conserved Glu-370 residue is part of the A-site signature sequence GG(E/D)EF of the PleD diguanylate cyclase domain and was proposed to coordinate a Mg$^{2+}$ ion in the active site required for deprotonation of the 3’-OH group of one GTP substrate molecule and its subsequent nucleophilic attack onto the $\alpha$-phosphate of the other GTP molecule. As predicted, the PleD$_{E370Q}$ mutant lacked detectable enzymatic activity both in the presence and absence of BeF$_3$ (data not shown). We even failed to detect any enzymatic activity when the protein concentration was increased to >50 μM and with prolonged incubation times. However, in contrast to PleD$_{Y326A}$, the lack of activity was not due to a failure to dimerize. When BeF$_3$-activated PleD$_{E370Q}$ was used in cross-link experiments, the behavior of the mutant form was indistinguishable from PleD wild type (supplemental Fig. S3). Thus, dimerization is clearly a prerequisite for, rather than a consequence of diguanylate cyclase activity.

Activation of the PleD Diguanylate Cyclase Does Not Interfere with Feedback Inhibition—The diguanylate cyclase activity of PleD is subject to strong product inhibition through binding of c-di-GMP to an allosteric I-site widely conserved among GGDEF domain proteins (5, 19). To test if PleD activation with BeF$_3$ interferes with allosteric control, binding of c-di-GMP to the I-site was directly measured using ITC (Fig. 5). Integration
of the titration peaks of c-di-GMP injected from the syringe into the cell of the calorimeter containing PleD produced a sigmoidal enthalpy curve for the interaction between PleD and c-di-GMP. The slope of the binding curve implies a dissociation constant of 0.3 μM (±0.1 μM). This is in good agreement with the $K_I$ of 0.5 μM determined earlier (5, 19). In support of a c-di-GMP dimer bound at each I-site (5) the binding stoichiometry was measured as 2.1:1 (c-di-GMP:PleD) (Fig. 5).

When binding of c-di-GMP to PleD was compared in the non-activated and BeF$_3$-activated conformation, both binding affinity (0.4 ± 0.1 μM) and stoichiometry (2.1:1 ± 0.2) did not change significantly upon activation. In agreement with this, the $IC_{50}$ values for PleD inhibition measured at a protein concentration of 5 μM were very similar for the non-activated (5.1 ± 1.4 μM) and the BeF$_3$-activated (5.9 ± 1.3 μM) PleD. From these data we conclude that activation of PleD by dimerization does not interfere with allosteric control of the protein. It should be noted that the calorimetric data show a deviation at very low c-di-GMP concentrations that cannot be described in terms of the simple binding model used here. This may be related to the varying degree of dimerization of c-di-GMP in solution (20). Because this effect is limited to the first few injections and does not interfere with the sigmoidal part of the binding curve, we have eliminated the first three data points from the fit (Fig. 5B) rather than introducing a more complex model with additional parameters of questionable relevance. A surprising result was obtained when measuring the heat capacity for the interaction between c-di-GMP and PleD by plotting the binding enthalpy versus the temperature between 5 °C and 25 °C (dCp = dH/dT). The heat capacity was fitted as $-0.43 \text{kJ} / (\text{mol K})$ (data not shown). The negative value suggests a hydrophobic interaction between the inhibitor and the protein. However, in the crystal structure c-di-GMP interacts predominantly in a hydrophilic manner with charged amino acid residues (5). It is conceivable that the binding of c-di-GMP to PleD might cause a conformational change in the protein, with a corresponding change of the protein-solvent interactions.

A PleD Mutant Unable to Dimerize Fails to Sequester to the Cell Pole—During the C. crescentus cell cycle PleD dynamically sequesters to the pole in response to activation by phosphorylation (4). However, the molecular basis for pole discrimination between active and inactive PleD is unclear. To analyze if phosphorylation itself or rather phosphorylation-induced dimerization is required for polar sequestration, we fused PleDY26A to GFP and compared its subcellular localization to GFP fused versions of PleD$_{D53N}$ and PleD$_{D53N}^*$ as shown in Fig. 6. PleD$_{D53N}$ fails to localize to the pole, whereas PleD$_{D53N}^*$, the non-phosphorylatable but constitutive active form, sequesters to the cell pole. This indicates that the ability to form dimers is critical for polar localization of PleD (Fig. 6). When the Y26A mutation was introduced into the constitutive active form PleD*, the resulting PleD$_{Y26A}^*$-GFP fusion protein also failed to localize to the pole (Fig. 6). Levels of both PleD$_{Y26A}$ and PleD$_{Y26A}^*$ GFP fusion proteins were normal, arguing that the Y26A mutation did not affect the protein stability in vivo (data not shown). In summary, these data suggested that the oligomerization state of PleD provides the structural basis for cell cycle dependent dynamic recruitment of the protein to the cell pole.

**DISCUSSION**

Dimerization is a common property of proteins utilized to provide stability, transmit signals, or channel reagents across membranes (21). Dimer formation can also be a mechanism to control enzyme activity, as exemplified by the cell death protease caspase 9 (22) or the cytokine antagonist p40 (23). Here we present an example for dimerization-induced activation of an enzyme, diguanylate cyclase, which takes advantage of an regulatory domain that couples dimerization to phosphorylation input.

The crystal structure of the non-activated form of the PleD response regulator suggested an “activation by dimerization” mechanism (5). However, because monomeric proteins can form non-physiological dimers or higher order oligomers in crystals, this hypothesis required experimental validation. In the crystal structure PleD forms a dimer with the two receiver domains mediating weak monomer-monomer interactions between a small contact patch around Tyr-26 of Rec1 and α3-α4 of Rec2 (Fig. 1). Thus, the apo structure not only provided the structural basis for activity control of PleD but also proposed the interaction surface for dimerization. Here we tested the hypothesis that, upon activation, PleD engages in
dimer formation, and we analyzed the specific role of Tyr-26 in this process. Because we failed to efficiently activate PleD in vitro by phosphorylation through one of its cognate kinases (4) we used the phosphoryl mimic BeF₃ to analyze the effect of activation on PleD oligomerization and activity (12, 15). Our results not only provide biochemical evidence for an "activation by dimerization" mechanism but also confirm an important role for Tyr-26 in dimerization. Both a genetically active form of PleD, PleD* (4), and BeF₃-activated PleD-stimulated DGC activity and oligomerization. Because stimulation of PleD DGC activity by BeF₃ specifically required the phosphoryl acceptor site Asp-53, the changes in structure and activity observed most likely reflect the activation mechanism normally evoked by phosphorylation. Because the constitutive active form PleD* still showed ~10-fold higher DGC activity and formed more stable dimers than BeF₃-modified PleD, it is possible that PleD is only partially activated by BeF₃ in the non-toxic concentration range used (Fig. 2). Alternatively, the PleD* mutant protein, which contains several amino acid changes contributing to the "locked-on" state (4, 9), might form particularly stable dimers. To demonstrate that the DGC activity specifically associates with PleD dimers, we chemically cross-linked BeF₃-activated protein and subsequently separated PleD dimers from non-cross-linked monomers by SEC. The finding that the cross-linked dimer fraction had a greatly increased enzymatic activity as compared with monomers strongly implied that PleD dimers represent the active form of the enzyme and argued that phosphorylation-mediated dimerization represents the main mechanism of PleD diguanylate cyclase activity control. A possible role for dimerization or oligomerization of diguanylate cyclases was suggested previously. Several full-length GGDEF domain proteins, and isolated GGDEF domains, that were expressed as fusions to maltose-binding protein, behaved as dimers or trimers when analyzed by SEC (6). However, although the significance of trimer formation is unclear, no correlation was reported between the oligomeric state and enzymatic activity of these proteins.

In contrast to PleD wild type, the PleD_Y26A mutant failed to efficiently form dimers upon activation with BeF₃. This, and the observation that, in the presence of BeF₃, PleD_Y26A showed an almost 10,000-fold lower DGC activity as compared with PleD wild type, is consistent with a specific requirement of residue Tyr-26 for PleD oligomerization. The weak monomer interactions observed in the PleD apo structure around Tyr-26 predicted that if this residue is part of the dimerization interface additional contacts would have to be formed upon activation to stabilize the complex. In agreement with the data presented here, the interaction surface around Tyr-26 is maintained in the crystal structure of the activated form of PleD (11) with Tyr-26 making specific contacts to Asp-209 and Arg-212 in the second receiver domain of the other chain. However, a series of additional inter-chain contacts are formed in the activated structure resulting in a tightening of the dimer interface (11). The Tyr residue at position 26 of the first receiver domain is strictly conserved in PleD homologs with an identical Rec1-Rec2-GGDEF domain structure (supplemental Fig. S2). In contrast, this residue is not conserved in response regulators with a different domain structure or composition (supplemental Fig. S2). Similarly, residues Asp-209 and Arg-212, the interaction partners of Tyr-26 in the crystal structure of activated PleD, show strict conservation only in proteins with a PleD-like domain structure (data not shown). Together with the experimental data presented here, this strongly suggested that Tyr-26 forms part of the dimerization surface of this protein family.

Diguanylate cyclases catalyze the formation of a symmetric product by condensing two identical GTP substrate molecules. In contrast to monomeric nucleotidyl cyclases, which form non-symmetric products, dimerization is an apparent necessity for the catalytic mechanism of DGCs, because it creates a fully symmetrical active site at the interface of two subunits. In the simplest model two substrate-charged GGDEF domains would meet in a symmetric but antiparallel arrangement to properly position the 3'-OH groups for an intermolecular nucleophile attack onto the α-phosphate of the opposite substrate molecule. Moreover, because it is a prerequisite for catalysis, oligomerization of the DGC domains is obviously exploited to control PleD enzyme activity. Although phosphorylation-mediated dimerization of PleD represents the first example of controlled dimerization of a DGC, it is possible that promoting or inhibiting dimerization is a key mechanism of DGC activity control in general. The preponderance of potential DGCs present in many bacteria predicts complex signaling mechanisms and makes it obligatory for the cell to tightly control these enzymes (2). Although most DGCs have been postulated to be subject to strict product inhibition (19), little is known about how DGCs are activated in response to specific environmental or internal signals. Although it can be assumed that all GGDEF domains that are fused to receiver domains of two-component systems exhibit PleD-like phosphorylation-mediated control, GGDEF domains, which are associated with other signal input domains like GAF (24, 25), PAS (26), BLUF (27), or HAMP (28, 29), might function similarly. It is worth mentioning that most regulatory mechanisms used to control monomeric nucleotidyl cyclases involve the formation or dissolution of catalytically competent active sites, caused by rearrangement of the two catalytic domains of the dimer relative to each other (reviewed in Ref. 1). Future studies will show if this regulatory principle can be extended to the large family of bacterial DGCs.

Oligomerization of PleD is not only used to temporally control DGC activity but also contributes to its spatial distribution. PleD activity is required for the morphological changes that take place during the C. crescentus swarmer-to-stalked cell transition (7–9). During this cell differentiation step, PleD is activated by phosphorylation and as a result sequesters to the differentiating pole (4). The observation that non-phosphorylatable forms of PleD fail to localize to the cell pole, whereas the constitutively activated mutant form PleD* is predominantly found at this subcellular site, suggested that activation of PleD during development is directly coupled to its dynamic subcellular positioning (4). However, the molecular basis for this coupling event and for PleD recognition at the pole remained unclear. In principle, a polar interaction partner could recognize activated PleD by its phosphorylation status, by an altered monomer conformation, or by its oligomerization state. The observation, that PleD molecules lacking Tyr-26 not only fail to dimerize but also fail to sequester to the pole, suggested that the
oligomerization state dictates subcellular distribution of PleD during the *C. crescentus* cell cycle. Enzymatically active dimers of PleD would thus specifically sequester to the differentiating cell pole resulting in the predominant formation of c-di-GMP at this cellular localization. Because *Caulobacter* possesses markers that are laid down during or after cell division to tag the new poles (30–32), it is reasonable to assume that PleD interacts with one or several pre-existing proteins, which are able to discriminate between its monomeric and dimeric forms. Dimerization of PleD might increase the interaction diversity by enabling simultaneous binding of two interacting proteins or by creating new binding sites for additional proteins (21). Both of these possibilities could provide a molecular explanation for the discrimination of PleD oligomers at the differentiating pole.

Spatial discrimination based on receiver domain-mediated oligomerization could very well be a general cellular phenomenon in bacteria. Like PleD, the response regulator DivK dynamically localizes to the *C. crescentus* cell poles in a phosphorylation-dependent manner (33). It is not clear how the poles discriminate between activated and non-activated DivK, but it is attractive to speculate that oligomerization might also play a role in this behavior. CikA, a sensor histidine kinase and a key component of the circadian clock input pathway in the cyanobacterium *Synechococcus elongatus*, also localizes to the pole where it is believed to interact with a complex of clock-related proteins (34). Polar sequestration of CikA depends on a C-terminal pseudo-receiver domain that lacks the conserved phosphoryl acceptor side. It has been proposed that, through a docking/activation mechanism, pseudo-receiver domain couples the activity of CikA to its subcellular location (34). Because histidine kinases are active as dimers, the pseudo-receiver domain might serve as an adapter between the oligomeric state and polar positioning of CikA. Like the pseudo-receiver domain, the Rec2 receiver domain of PleD is not conserved and likely fulfills an adapter function. It is possible that the Rec1–Rec2-GGDEF domain structure that arose through duplication of the receiver domains in PleD homologs has evolved to provide for additional surface for the interaction with specific polar receptors. In such a scenario, Rec1 would be interacting with the histidine kinase and would provide dimerization surface. Rec2, in turn, would also be engaged in dimerization but in addition would mediate interaction with a polar receptor. If so, a distinct subcellular localization of enzymatically active DGCs might be common to all PleD homologs. Future studies are geared at identifying the polar receptor(s) for PleD, characterizing the molecular mechanisms required for the discrimination between PleD monomers and dimers, and analyzing the biological relevance of sequestering an enzymatically active form of PleD to this particular subcellular site.

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