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Determinants for the activation and autoinhibition of the diguanylate cyclase response regulator WspR

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Abstract

The bacterial second messenger c-di-GMP controls secretion, cell adhesion and motility leading to biofilm formation and increased cytotoxicity. Diguanylate cyclases containing GGDEF and phosphodiesterases containing EAL or HD-GYP domains have been identified as the enzymes controlling cellular c-di-GMP levels, yet less is known regarding the molecular mechanisms governing regulation and signaling specificity. We recently determined a product-inhibition pathway for the diguanylate cyclase response regulator WspR from *Pseudomonas*, a potent molecular switch that controls biofilm formation. In WspR, catalytic activity is modulated by a helical stalk motif that connects its phospho-receiver (REC) and GGDEF domains. The stalks facilitate the formation of distinct oligomeric states that contribute to both activation and autoinhibition. Here, we provide novel insights into the regulation of diguanylate cyclase activity in WspR based on the crystal structures of full-length WspR, the isolated GGDEF domain, and an artificially dimerized catalytic domain. The structures highlight that inhibition is achieved by restricting the mobility of rigid GGDEF domains, mediated by c-di-GMP binding to an inhibitory site at the GGDEF domain. Kinetic measurements and biochemical characterization corroborate a model in which the activation of WspR requires the formation of a tetrameric species. Tetramerization occurs spontaneously at high protein concentration or upon addition of the phosphomimetic compound beryllium fluoride. Our analyses elucidate common and WspR-specific mechanisms for the fine-tuning of diguanylate cyclase activity.

Keywords

signaling; biofilm formation; bacterial response regulator; diguanylate cyclase; cyclic nucleotide

Introduction

Two-component signaling systems relay external information to internal signaling events controlling gene transcription and cellular behavior ¹. In *Pseudomonas*, the Wsp operon encodes a signaling system that receives signals from a membrane-spanning methyl-accepting chemotaxis protein, ultimately controlling biofilm formation, cytotoxicity and other adaptive

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phenotypes^{2; 3; 4}. Receptor activation triggers phosphorylation of a response regulator diguanylate cyclase, WspR, via a soluble histidine kinase, triggering the synthesis of the unique bacterial second messenger bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP)³.

WspR is conserved in *Pseudomonas* and related species, and contains an N-terminal phosphoreceiver (REC) domain and a C-terminal diguanylate cyclase (GGDEF) domain with the characteristic GGDEF motif at the active site (Figure 1A). A long helical stalk, an extension of the C-terminal helix of the REC domain, connects both domains⁵. The stalks are key regulatory elements for the oligomerization, activation and autoinhibition of WspR (Figure 1B). WspR crystallized as a tetramer with c-di-GMP bound at a conserved inhibitory site (I-site) on the GGDEF domains. In a previous study, we could show that the tetramer can assemble from a dimeric enzyme. As part of a negative feedback loop, c-di-GMP binding to the I-site would coordinate the tetramer in a conformation observed in the crystal lattice. The nucleotide-bound tetramer subsequently dissociates into an inactive dimer species with elongated shape. The two distinct dimer-tetramer transitions occur in the absence of post-translational modification but are sensitive to protein concentration. The structural and biochemical consequences of phosphorylation leading to its activation remain elusive³.

WspR and PleD, a diguanylate cyclase from *Caulobacter crescentus* with similar but not identical domain organization, utilize homologous regulatory features to assume feedback inhibition but the exact mechanisms appear to be different regarding the implementation of some of these features^{5; 6; 7; 8; 9}. Both enzymes become activated upon phosphorylation^{3; 8}. Both enzymes display an I-site with a characteristic RxxD motif as primary c-di-GMP binding site that is located distal to the GGDEF motif-containing active site loop^{5; 6; 9}. In the structures of WspR and the dimeric state of PleD, c-di-GMP bound to the I-site bridges two adjacent GGDEF domains, involving a second arginine residue on the neighboring domain, and stabilizes an inactive conformation^{5; 9}. Monomeric PleD adopts an alternative conformation in which the secondary I-site is located at its second REC domain within the same molecule⁶.

For PleD, two modes for c-di-GMP-mediated autoinhibition have been proposed, an allosteric mode in which c-di-GMP binding to the I-site stabilizes an inactive conformation of the active site, and a mode that relies on the sequestration of the active sites away from each other^{6; 7; 9}. In contrast, in nucleotide-bound WspR tetramers, the stalk motifs of adjacent molecules physically block the active sites providing another mechanism for inhibition. Cyclic di-GMP-mediated cross-linking of the GGDEF domains in such a tetrameric assembly resembles a conformation observed for dimeric PleD but stabilizes a distinct oligomeric structure.

Here we elucidate a functional role for the WspR tetramer and identify regulatory motifs and post-translational modifications, in particular the stalk region and phosphorylation, that facilitate the formation of distinct oligomeric states and modulate diguanylate cyclase activity. We provide a refined model for the molecular mechanism underlying WspR function based on enzyme kinetics and structures of an inactive monomeric and a constitutively dimeric, hyperactive cyclase domain. The results show that the stalk motifs bring rigid and intrinsically inactive GGDEF domains into close proximity establishing an active conformation. Yet, the native coiled-coil motif appears to have evolved to restrict the enzymatic activity of WspR and, in conjunction with the REC-homology domain, allow for regulation via phosphorylation. In contrast to PleD, the establishment of catalytically active GGDEF domain dimers in the context of full-length WspR relies on the tetramerization of WspR. Cyclic di-GMP binding to the I-site exerts its inhibitory function by stabilizing a catalytically incompetent conformation in which the active sites are separated from each other, consistent with a model proposed for

PleD⁹. Taken together, we describe both conserved and enzyme-specific features that contribute to the regulation of diacylglycerol cyclases and c-di-GMP signaling.

Results and discussion

Crystal structure of full-length WspR from *P. syringae*

We recently described an autoinhibition mechanism for WspR from *Pseudomonas aeruginosa*, and could show its conservation in other *Pseudomonas* species on a biochemical level⁵. Here, we determined the crystal structure of full-length WspR from *P. syringae* (76% identical to the protein sequence of WspR from *P. aeruginosa*) at 2.8 Å resolution (Supplemental Table 1). Crystals were obtained under similar conditions reported previously, and the structure was solved by molecular replacement using the coordinates of *P. aeruginosa* WspR as the search model (PDB code: 3BRE; chain B)⁵. While crystals of WspR from *P. aeruginosa* belonged to space group C2 and contained two molecules in the asymmetric unit with slightly different orientations of the GGDEF domains relative to the regulatory domains⁵, crystals grown with the protein from *P. syringae* had higher symmetry (space group C222) with only one molecule in the asymmetric unit (Figure 1C). The conformations of WspR protomers from both species were very similar showing only minor differences in the angle of the stalk-like protrusion relative to the REC domain (Supplemental Figure 1A). Subtle changes in the quaternary structure compared to that of *P. aeruginosa* WspR might provide insight into the transition from a tetrameric assembly to an elongated dimer state.

Previously, we proposed a model for the inhibition of WspR in which compact, parallel WspR dimers can assemble into tetramers (Figure 1B and D). Cyclic di-GMP would bind to the I-sites and the complex would dissociate into an elongated dimer state, with both nucleotide-bound tetramers and elongated dimers being inactive. The structure of *P. syringae* WspR supports such a mechanism. Similar to *P. aeruginosa* WspR, WspR from *P. syringae* formed a tetramer in the crystal lattice, in this case a 222-symmetric structure, mediated by parallel and anti-parallel interactions of the helical stalks and by c-di-GMP, which was bound to the I-site at the GGDEF domains (Figure 1D, middle panel). Based on the tetrameric structure, calculation of buried surface area and functional considerations, we can propose models for the compact (parallel) and elongated (anti-parallel) dimer conformations (Figure 1D, left and right panels, respectively)⁵. For WspR from *P. aeruginosa* and *P. syringae*, both states bury roughly equivalent surface area at the dimer interfaces (Supplemental Table 2) with only a noticeable difference in the parallel dimer state. In the parallel dimer of *P. syringae* WspR the interfacial area is about ~580 Å² smaller (~330 Å² within the REC dimer interface; Supplemental Figure 1B) compared to the homologous state of *P. aeruginosa* WspR, suggesting that the interface might be weaker in the first case.

The extent of buried surface area in the antiparallel dimer is almost identical but their estimated stability appears to be different. Free energy (Δ^iG) calculations based on the structure of *P. aeruginosa* WspR provided a positive value for the solvation free energy gain suggesting that such an anti-parallel packing would not be favorable (Supplemental Table 2)¹⁰. In contrast, similar calculation for the elongated dimer model from *P. syringae* resulted in negative values for the free energy gain upon dimer formation (Figure 1D; Supplemental Table 2). While these calculations only provide rough estimates for the relative stability of complexes, they indicate that such conformations may exist in solution.

Although burying significantly less surface area, an alternative model for the elongated, anti-parallel WspR dimer, in which two GGDEF domains were bridged by c-di-GMP molecules bound to the I-sites, was also estimated to be a stable interface, even when c-di-GMP-mediated interactions were not considered (Supplementary Figure 1C). At this point, the model is ambiguous with regard to the exact dimer conformations, and structural changes may be

involved that cannot be predicted based on the available crystallographic data. It is also feasible that a fast association of the dimers into tetramers has a positive effect on dimer stability.

Taken together, we hypothesize that the structure of *P. syringae* WspR represents an early intermediate in the transition from a tetrameric to an elongated dimer state, which has been observed *in vitro* ⁵. Such a structural rearrangement would involve the dissociation of the REC domain dimers while other interactions within the anti-parallel dimer may form, a proposal that is consistent with our data. Such interactions may involve the GGEEF motif and would block the active site for substrate binding. The mixture of c-di-GMP-bound tetramers and elongated dimers was catalytically inactive, corroborating such an autoinhibition mechanism ⁵. Alternatively, differences in the REC dimer interface of WspR from *P. syringae* and *P. aeruginosa* may reflect slightly different interactions in orthologous proteins from different species.

Activation of WspR

We can follow the transitions between oligomeric states of WspR in gel filtration chromatography that has been standardized by using static multi-angle light scattering and analytical ultracentrifugation (Figure 2A, Table 1, and Supplementary Figure 2) ⁵. As shown previously, WspR purifies in a state of equilibrium between c-di-GMP-bound tetramers and elongated dimers ⁵. Removal of c-di-GMP by incubation with a phosphodiesterase (PDE) such as SadR from *P. aeruginosa* yielded a dimeric species that appeared to be more compact than the original state based on their elution times (Figure 2A). We refer to this state as PDE-treated WspR. Based on homology to adenylate cyclases and structural arguments it was assumed that the compact dimer would be the active state ⁵. Upon chromatographic removal of PDE and the PDE-reaction product pGpG from the nucleotide-free species involving concentration steps, a tetrameric WspR fraction assembled spontaneously in addition to the compact dimers. We refer to this preparation as nucleotide-free WspR.

To our surprise, when we assayed diguanylate cyclase activity of *P. aeruginosa* WspR directly after PDE treatment in a coupled assay that monitors pyrophosphate release, the compact dimer of WspR was as inactive as the initial, nucleotide-bound (NB) protein (Figure 2B). In contrast, nucleotide-free WspR was highly active. The main differences between the two samples are the presence of a tetrameric species in the nucleotide-free WspR preparation and the fact that the PDE had been purified away in this sample. Addition of PDE to nucleotide-free WspR had no effect on diguanylate activity, ruling out an adverse effect mediated by the PDE. Next, we separated the dimeric and tetrameric fractions of the nucleotide-free protein that were resolved by gel filtration, and assayed their activities independently. Robust diguanylate cyclase activity could only be detected in fractions of the peak that corresponded to tetrameric WspR (Figure 2B) ⁵. Fractions of the peak containing dimeric nucleotide-free WspR showed low activity consistent with results obtained for PDE-treated WspR.

We next studied the effect of phosphorylation on the oligomerization and activity of WspR using beryllium fluoride (BeF_3^-), a chemical compound that can bind to the REC domains non-covalently mimicking aspartate phosphorylation (Figure 3 and 4) ^{9; 11; 12}. Upon treatment of full-length WspR with BeF_3^- for 5 minutes, proteins were analyzed by size exclusion chromatography. Longer incubations of nucleotide-free, wild-type WspR with BeF_3^- compromised protein solubility and promoted aggregation (data not shown).

Nucleotide-bound, inactive WspR eluted in a single peak at 12.4 ml (Figure 3A), corresponding to an equilibrium state consisting of a tetramer and an elongated dimer based on static light scattering experiments ⁵. BeF_3^- incubation had no effect on the elution peak volume of this conformational ensemble. In contrast, nucleotide-free, active WspR was sensitive to BeF_3^- addition. At low concentration of approximately 10 M (considering the dilution factor

associated with gel filtration), the majority of the protein was dimeric with an elution peak volume of 13.5 ml, and only a smaller fraction contained tetramers eluting at 11.8 ml (Figure 3B; Supplementary Figure 2). Both peaks are distinct from that of the nucleotide-bound, inhibited sample. Increasing the protein concentration shifted the equilibrium towards the tetrameric species⁵. A similar effect could be achieved by addition of BeF_3^- at low protein concentration, suggesting that phosphorylation facilitates the dimerization of dimers (Figure 3B). BeF_3^- -induced tetramer formation was also observed in a WspR mutant with disrupted I-site (WspR^{R242A}; data not shown). WspR^{D70N}, a protein with a mutation at the predicted phosphorylation site in the REC domain of WspR, was used to establish specificity regarding the response to the phospho-mimetic compound. In contrast to the wild-type protein, nucleotide-free WspR^{D70N} showed no change in elution profile upon incubation with BeF_3^- (Figure 3C).

In order to elucidate the functional role of tetramer formation, we correlated the oligomerization data with kinetic measurements of cyclase activity. We analyzed both WspR^{D70N} and wild-type (wt) WspR proteins in the following states: 1. Nucleotide-free (compact dimer-tetramer equilibrium); 2. c-di-GMP-bound, inactive (tetramer-elongated dimer equilibrium); and 3. PDE-treated (compact dimer) (Figure 4). Both WspR^{D70N} and WspR^{wt} purified in a c-di-GMP-bound, inhibited state. Both proteins were active when PDE-treatment was included in the purification protocol yielding partially tetrameric, nucleotide-free protein. No enhanced activity over that of the initially nucleotide-bound sample was observed upon incubation with PDE or BeF_3^- , respectively. However, PDE treatment followed by BeF_3^- addition significantly increased the activity of wild-type WspR, consistent with previously reported phosphorylation-dependent activation of WspR in cells and by acetyl-phosphate³. WspR^{D70N} was insensitive to such a treatment establishing specificity (Figure 4B). In summary, we observed an increase in cyclase activity for samples that underwent tetramerization upon BeF_3^- binding, whereas dimeric WspR was only poorly active.

Oligomerization requirement for cyclase activity

To further elucidate the effect of oligomerization and role of the stalks for WspR function, we compared the following constructs based on *P. aeruginosa* WspR with respect to their cyclase activity and regulation: The isolated GGDEF domain (WspR^{GGDEF}), the stalk-GGDEF domain unit (WspR^{Stalk-GGDEF}), and a fusion protein in which we replaced the natural stalk motif with the leucine zipper motif of GCN4 from yeast (WspR^{GCN4-GGDEF}) (Figure 5A). The leucine zipper of GCN4 has been shown to form parallel coiled-coils and hence serves as a strong dimerization module^{13; 14}. The GCN4 motif was fused to the GGDEF domain of WspR that included the natural linker between the GGDEF and helical stalk to allow for inter-domain flexibility comparable to wild-type WspR.

First, we analyzed the activity of these constructs in cells using a phenotypic assay (Figure 5B). The expression of active diguanylate cyclases and c-di-GMP production in *E. coli* correlates with an increased Congo Red (CR) staining characteristic of cellulose production^{15; 16}. *E. coli* BL21 were transformed with an empty expression vector (pET21), WspR^{full-length} or one of the constructs introduced above, and were plated onto media containing CR and either no or 0.1 mM IPTG for low and elevated expression levels, respectively⁵. While colonies of the vector control and colonies expressing WspR^{GGDEF} remained colorless in this assay, leaky expression of WspR^{full-length}, WspR^{Stalk-GGDEF} or WspR^{GCN4-GGDEF} in the absence of IPTG was sufficient to cause a red colony phenotype (Figure 5B). Increased, IPTG-induced protein expression attenuated the red phenotype for WspR^{full-length} and WspR^{Stalk-GGDEF}. In contrast, WspR^{GCN4-GGDEF} colonies remained red even in the presence of IPTG. These results indicate that both WspR^{full-length} and WspR^{Stalk-GGDEF} were subject to autoinhibition upon overexpression, whereas

WspR^{GCN4-GGDEF} was constitutively active independent of its cellular concentration. Higher IPTG concentrations or mutation of the I-site rendered WspR^{GCN4-GGDEF} cytotoxic suggesting that this construct was hyperactive with respect to WspR^{full-length} and partially inhibited by c-di-GMP in cells (data not shown).

Next, we expressed and purified WspR^{GGDEF}, WspR^{Stalk-GGDEF} and WspR^{GCN4-GGDEF}, enabling us to characterize these proteins *in vitro*. To analyze the nucleotide-bound state, purified proteins were heat-denatured, and pelleted by centrifugation. Supernatants were filtered and analyzed by reverse-phase HPLC (Figure 5C)⁵. This indicated that the two proteins containing stalk motifs, WspR^{Stalk-GGDEF} and WspR^{GCN4-GGDEF}, co-purified with c-di-GMP (bound to the I-site; see below) whereas WspR^{GGDEF} purified nucleotide-free, in agreement with the cell-based assays. WspR^{GGDEF} was able to bind purified c-di-GMP (Supplemental Figure 3A), but low activity levels of the isolated domain in *E. coli* may have prevented effective complex formation.

WspR^{GGDEF}, WspR^{Stalk-GGDEF} and WspR^{GCN4-GGDEF} showed distinct elution characteristics in size exclusion chromatography (Figure 6A). While both WspR^{GGDEF} and WspR^{GCN4-GGDEF} eluted in a single peak corresponding to a monomeric and dimeric species, respectively, based on their molecular weights measured by light scattering, WspR^{Stalk-GGDEF} showed a bimodal distribution (Figure 6). According to the light scattering experiments, both peaks contained dimeric states of WspR^{Stalk-GGDEF} with some higher oligomeric species detected in the earlier eluting peak (Figure 6B, bottom panel). The conformational heterogeneity could be attributed to the specific properties and oligomerization propensities of the native stalk region of WspR since the isolated GGDEF domain and the obligate parallel dimer, WspR^{GCN4-GGDEF}, did not show such behavior. Addition of c-di-GMP to the isolated GGDEF domain resulted in partial dimerization of the protein (Supplemental Figure 3B). In contrast, enzymatic removal of c-di-GMP from the I-site of WspR^{GCN4-GGDEF} by incubation with PDE had no significant effect on its oligomerization behavior indicating that the GCN4 motif is sufficient for the formation of a stable dimer (Supplemental Figures 3C and 3D). WspR^{Stalk-GGDEF} was significantly less soluble upon PDE treatment suggesting that the oligomeric states were stabilized by bound nucleotide (data not shown).

The formation of dimers and higher-order oligomers has been shown to be involved in the regulation of WspR⁵, and the experiments described here corroborate these findings and identify the stalk motif as a major determinant for the underlying mechanism. The native stalk motif in WspR has oligomerization properties that are distinct from a canonical, parallel coiled-coil forming sequence such as the leucine zipper from GCN4. In addition, since the native stalk motifs are sufficient for dimerization of the GGDEF domains, they are likely to contribute significantly to the overall oligomerization of full-length WspR, which oligomerizes even in the absence of REC domain phosphorylation.

Nucleotide-bound WspR^{Stalk-GGDEF} was inactive *in vitro* although initial activity in cells was detected and was required for obtaining c-di-GMP-bound protein (Figure 7A, bottom panel; Figure 5). Consistent with the cell-based assays, WspR^{GGDEF} had no activity *in vitro*, while GCN4-mediated dimerization yielded a hyperactive enzyme compared to nucleotide-free, full-length WspR (Figure 7A). At equal cyclase concentration (0.5 M), WspR^{GCN4-GGDEF} activity exceeded that of full-length, nucleotide-free WspR by more than 2-fold. Under these conditions, the enzymes of the detection system became rate limiting in reactions containing the GCN4-fusion protein, and lower concentrations were used in subsequent experiments. While apparent substrate affinities were comparable for full-length WspR and WspR^{GCN4-GGDEF}, their catalytic rates were significantly different, with the latter being almost 10-fold faster than full-length WspR. This difference in activity suggests that the native stalk

motif contributes to a reduction in cyclase activity possibly by restricting GGDEF dimerization and/or by preventing the active oligomer to form.

In contrast to the linear kinetics observed with nucleotide-free, full-length WspR (Figure 7A)⁵, we noticed a non-linear activity with at least two phases for WspR^{GCN4-GGDEF} consistent with a mode of product inhibition (Figure 7B). Since WspR^{GCN4-GGDEF} was hyperactive yet bound to c-di-GMP, we studied the effect of c-di-GMP binding on WspR^{GCN4-GGDEF} activity by repeating the experiments with PDE-treated, nucleotide-free protein. As for preparations of nucleotide-free, full-length WspR, PDE and pGpG was removed from WspR prior to the activity assays. Removal of c-di-GMP increased the catalytic activity of WspR^{GCN4-GGDEF} slightly (Figure 7B and Table 2). This may suggest that purified WspR^{GCN4-GGDEF} was not quantitatively bound to c-di-GMP, and that c-di-GMP production exerted an inhibitory effect. Although no inhibition was observed for the GCN4-fusion protein in cell-based assay (Figure 5B), I-site mutants of WspR^{GCN4-GGDEF} generated by site-directed mutagenesis were toxic to *E. coli* (data not shown), supporting the notion of an inhibitory role of c-di-GMP-binding to the I-site of the GCN4-fusion protein.

Crystal structure of WspR^{GCN4-GGDEF}

In order to obtain further structural insight into the molecular mechanism controlling WspR activity, we determined the crystal structure of WspR^{GCN4-GGDEF} at 1.94 Å resolution (Figure 8A; Supplemental Table 1). WspR^{GCN4-GGDEF} crystallized with two molecules in the asymmetric unit that were slightly different in the orientation of the GGDEF domain relative to the GCN4 helix, and distinct from the orientations observed in the full-length structures (Figure 8B). The two molecules form a dimer via a canonical coiled-coil motif mediated by the GCN4 sequence^{13; 14} and via c-di-GMP-mediated contacts within the GGDEF domains (Figure 8A). The structure resembled an inactive state reminiscent of the one observed for BeF₃⁻-bound PleD (Figure 8C)⁹. In this state, c-di-GMP is bound to the primary and secondary I-sites on the GGDEF domains, stabilizing a catalytically incompetent conformation in which the active sites are pointing away from each other.

The structural analysis demonstrates that the GGDEF domains of WspR can in principle adopt a PleD-like inactive conformation if the GGDEF domains were brought into close proximity (Figure 8)⁹. WspR dimers in which the stalk motifs may form parallel coiled-coil structures bringing the GGDEF domains into close proximity may be conceivable, yet such a conformation has not been observed crystallographically. While a parallel dimer can be extracted from the available crystal structures, the tips of the stalks are splayed, holding the active sites at a distance (Figure 8D)⁵. It is interesting to note, though, that a mode of GGDEF domain dimerization similar to that obtained in structures of WspR^{GCN4-GGDEF} and PleD has been observed in the tetrameric state of WspR, mediated by an anti-parallel stacking of coiled-coils. Consequently, the respective stalk motifs point in opposite directions relative to the GGDEF domain in the structures of full-length WspR and WspR^{GCN4-GGDEF} (Figure 8B and C).

In order to achieve an active conformation in which the GGDEF motifs face each other in an anti-parallel configuration, the GGDEF domains of WspR^{GCN4-GGDEF} (and full-length WspR) would have to rotate by about 45°. Flexible linker regions that connect the stalk and GGDEF domain might facilitate such a conformational degree of freedom. As seen in structures of PleD and WspR^{5; 9}, the protein-protein interface between adjacent GGDEF domains is not very extensive and the domains are predominantly cross-linked via c-di-GMP. Given the nature of the interface, a rotational motion unlocking the domains may be feasible, resulting in a toggling between active and inactive conformations even when c-di-GMP is bound to the I-site. In this scenario, c-di-GMP binding may alter the kinetics of interconversion. This model provides an alternative explanation for the hyperactivity of WspR^{GCN4-GGDEF} (see above; Figure 7).

In summary, it has been established that diguanylate cyclase activity relies on an oligomerization event that brings two GGDEF domains into close proximity. The native stalks of WspR appear to prevent the formation of such a complex in the context of a dimeric protein, which may explain the requirement of higher-order WspR oligomers for establishing an active state that can be inhibited subsequently by c-di-GMP (Figure 8)⁵. More structural information regarding the active, inactive and intermediate states of WspR and other GGDEF domain-containing proteins, including their dynamics, will be required to describe the molecular mechanisms and pathways that control c-di-GMP synthesis at higher resolution.

Structural comparison of c-di-GMP-bound and unbound GGDEF domains of WspR

We also determined the crystal structure of WspR^{GGDEF} at a resolution of 2.03 Å (Figure 9A and Supplemental Table 1). The protein used for crystallization purified free of nucleotide (see above; Figure 5). Superposition of WspR^{GGDEF} with GGDEF domains from WspR^{GCN4-GGDEF} and full-length WspR using all C atoms as reference revealed that the structures of the GGDEF domain in the different crystals and nucleotide-bound states were virtually identical (Figure 9A). The active site loop conformation is very similar and independent of the nucleotide-bound state of the GGDEF domain (Figure 9B). Only in one protomer of full-length WspR a subtle conformational change was observed around Gly251 of the GGDEF motif (Figure 9B and C). A minor systematic difference between the apo-structure and the nucleotide-bound states was observed at position 254 in the GGDEF motif with Glu254 adopting a different rotamer conformation in the structure of WspR^{GGDEF} that lacked c-di-GMP at the I-site (Figure 9C). Such rigidity in the overall structure and the conformation of the active site loop and I-site is consistent with an inhibition mechanism that relies on the separation of active sites as has been proposed before for PleD, and less so with an allosteric mechanism that would involve coupling of the I- and active sites (Figure 9)^{7; 9}.

Conclusions

Bacterial biofilm formation and other virulence pathways are under the control of signaling networks that utilize c-di-GMP as a second messenger^{4; 17; 18}. GGDEF domains responsible for catalyzing c-di-GMP production occur in a multitude of proteins, often as domains in transmembrane receptors or as fusions with regulatory modules in soluble proteins¹⁹.

Considering the large number of these enzymes encoded in many genomes and the diversity with regard to domain composition, it is probable that these enzymes are activated by distinct inputs and may create specific responses^{4; 19}. Given the modular nature of signaling proteins, some regulatory principles will apply to many enzymes with similar catalytic and/or regulatory domains but variations in configuration may also suggest that their activity is controlled by distinct mechanisms specific for a particular protein. PleD and WspR, two diguanylate cyclases with similar but not identical domain architecture, appear to be regulated by similar global mechanisms, e.g. the activation via phosphorylation, product inhibition via separation of their GGDEF domains, and most likely the enzymatic mechanism of c-di-GMP production^{3; 5; 6; 7; 8; 9; 20}. Yet, WspR and PleD appear to employ these mechanisms somewhat differently to realize the same task. The differences might be important for the fine-tuning of catalytic activity that could determine signal strength, duration or transmission.

Until now, crystal structures described inhibited states of diguanylate cyclases suggesting that other states might be more flexible or dynamic. In particular, assemblies in which the GGDEF domains of adjacent molecules are bridged by I-site-bound c-di-GMP have been observed for PleD and WspR. Even the artificial and hyperactive WspR^{GCN4-GGDEF} crystallized in such a conformation, which is intriguing. It is likely that such a structure represents a general mode for the inhibition of diguanylate cyclases that contain primary and secondary I-sites within their GGDEF domains. Such conformation may also allow fine-tuning of diguanylate cyclase

activity. While the apparent catalytic rate of purified WspR^{GCN4-GGDEF} was less affected by c-di-GMP binding to the I-site than that of PleD, cell-based assays were less conclusive since a dampened activity was crucial to minimize cytotoxicity but inhibition was less strict than in the case of full-length WspR and PleD^{5; 9}. Other inhibited states appear to be enzyme-specific and dependent on domains adjacent to the GGDEF domain⁶. Distinct inhibitory states in PleD and WspR might be non-redundant taking effect at different stages of the signaling reaction. They would collectively contribute to a constant negative feedback on the system, potentially restricting its signaling potential or radius.

We also investigated the role of the stalk motifs and phosphorylation during the activation of WspR. To our surprise, diguanylate cyclase activity appeared to be dependent on the presence of WspR tetramers, and tetramerization was facilitated by beryllium fluoride. This requirement could be alleviated by the substitution of the regulatory REC-stalk module of WspR with the coiled-coil motif of yeast GCN4 that is known to form parallel dimers. Although the biochemical studies reported here might suggest a canonical monomer-dimer equilibrium for WspR activation, as observed for other response regulator proteins^{8; 9; 21; 22; 23}, all efforts to determine the quaternary structure by standard size exclusion chromatography, multi-angle light scattering, analytical ultracentrifugation, and electron spin resonance spectroscopy implicate that WspR exists as higher-order oligomers (Supplementary Figure 3; data not shown). The data is most consistent with a dimer-tetramer equilibrium that is regulated by phosphorylation. Conceptually (and in some regard) structurally, such a phosphorylation-dependent oligomerization switch may be similar to the ATPase regulation of the transcriptional activators DctD/NtrC^{24, 25, 26}. In these response regulators, the dimeric, unphosphorylated REC domains and their helical extensions hold the ATPase domains in an inactive conformation. Phosphorylation introduces changes in the REC dimerization interface triggering an higher-order oligomerization and activation of the ATPase domains. Further experiments will be required to characterize the exact states and conformations of WspR during catalysis and autoregulation.

Material and Methods

Protein expression and purification

The coding regions corresponding to full-length WspR from *P. syringae* (PSPTP1499) was amplified by standard PCR using genomic DNA isolated from *Pseudomonas syringae* pv. tomato DC3000 as template. Coding regions corresponding to WspR^{GGDEF} (residues 172–347) and WspR^{stalk-GGDEF} (residues 140–347) from *P. aeruginosa* were PCR-amplified from an expression plasmid described previously⁵. The DNA fragment encoding the fusion protein WspR^{GCN4-GGDEF} was generated by overlap-extension PCR using a fragment corresponding to the coiled-coil segment of GCN4 (residues 249–278)¹³ amplified from genomic DNA of *S. cerevisiae* and the plasmid encoding WspR^{GGDEF} as templates. DNA inserts were cloned into the pET21 (Novagen) expression plasmid yielding C-terminally hexahistidine-tagged proteins. For the crystallization of WspR^{GGDEF}, the insert was cloned into a modified pProExHT expression vector (Invitrogen) yielding an N-terminally hexahistidine-tag that could be removed using Precision Protease.

Transformed *Escherichia coli* cells BL21 (DE3) (Novagen) were grown in TB medium supplemented with 100 mg/l Ampicillin at 37°C. At a cell density corresponding to an absorbance of 1.0 at 600 nm the temperature was reduced to 18°C, and protein production was induced with 1 mM IPTG, except in the case of WspR^{GCN4-GGDEF} where no IPTG was added. Protein was expressed for 12–16 hr. Cells were collected by centrifugation, resuspended in NiNTA buffer A (25 mM Tris-Cl pH 8.2, 500 mM NaCl, 20 mM imidazole, and 5 mM 2-mercaptoethanol). After cell lysis by sonication, cell debris was removed by centrifugation at 40,000 × g for 1 hr at 4°C. Clear lysates were loaded onto HisTrap NiNTA columns (GE

Healthcare) equilibrated in NiNTA buffer A. The resin was washed with 20 column volumes of NiNTA buffer A, and proteins were eluted on a single step with NiNTA buffer A supplemented with 500 mM imidazole. For crystallization trials, WspR^{GGDEF} was incubated with Precision Protease for removal of the hexahistidine tag, and the cleaved protein was collected in the flow-through during NiNTA affinity chromatography. All proteins were further subjected to size exclusion chromatography on a Superdex200 column (GE Healthcare) equilibrated in gel filtration buffer (25 mM Tris-Cl pH 7.5, 100 mM NaCl, and 1 mM DTT). Fractions containing protein were pooled and concentrated on a Centricon ultrafiltration device (10 kDa cutoff; Millipore) to a final concentration of approximately 50 mg/ml. Protein aliquots were frozen in liquid nitrogen and stored at -80°C . Full-length SadR/RocR/PA3947 and nucleotide-free, full-length WspR from *Pseudomonas aeruginosa* PAO1 was produced as described previously^{5; 27; 28}.

Point mutations were introduced into the coding region of expression plasmids using the QuikChange XL Mutagenesis Kit (Stratagene) following the manufacturer's instructions. Expression and purification of mutant proteins was identical to the procedure for wild-type proteins. All mutant proteins described above expressed to comparable levels.

Crystallization, X-ray data collection, and structure solution

All crystals were obtained by hanging drop vapor diffusion by mixing equal volumes of protein (5–30 mg/ml) and reservoir solution followed by incubation at 20°C . For *P. syringae* WspR the reservoir solution comprised 0.1 M Tris-Cl pH 8.0–8.5, 2.0 M NaCl, 0.1 M SrCl_2 , and 20% xylitol. Crystals appeared within 4 days with typical dimensions of $0.15\text{ mm} \times 0.1\text{ mm} \times 0.1\text{ mm}$. Crystals of the GGDEF domain of WspR were obtained by incubation over a reservoir solution comprising 0.1 M imidazole pH 7.0, 0.2M NaCl and 1 M Na-K-tartrate. Clusters of plate-like crystals appeared within 2–4 days with typical dimensions of $0.04\text{ mm} \times 0.1\text{ mm} \times 0.1\text{ mm}$. For cryoprotection, single crystals were separated from the clusters and soaked in reservoir solution supplemented with 15% xylitol. For crystals of WspR^{GCN4-GGDEF} the reservoir solution contained 0.1 M Hepes-NaOH pH 7.5 and 0.5 M Magnesium formate dihydrate. Diamond-shaped crystals of typical dimensions of $0.15\text{ mm} \times 0.1\text{ mm} \times 0.1\text{ mm}$ were obtained after 4–5 days. Reservoir solution supplemented with 15% xylitol was used as cryoprotectant. All crystals were flash-frozen in liquid nitrogen and kept at 100K during data collection.

Crystallographic statistics for data collections and refinements are shown in Supplemental Table 1. Data sets were collected using synchrotron radiation at the Cornell High Energy Synchrotron Source (CHESS, Ithaca, beamline A1). Data reduction was carried out with the software package HKL2000²⁹. Structures were solved by molecular-replacement using the software package Phenix³⁰ and coordinates for *P. aeruginosa* WspR (PDB code 3BRE)⁵ as search models. The models were adjusted manually starting from the molecular replacement solution and refined using Coot³¹ and Phenix³⁰. Illustrations were made in Pymol (DeLano Scientific). For WspR^{GCN4-GGDEF}, the structure of the GCN4 leucine zipper from *Saccharomyces cerevisiae* (PDB code 2ZTA)¹³ was utilized for model building.

Structure were analyzed using the program package CNS³² and the Protein interfaces, surfaces and assemblies service PISA at European Bioinformatics Institute (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html)¹⁰.

Diguanylate cyclase assays

Two experimental systems for assaying diguanylate cyclase activity *in vitro* were used and have been described in detail previously⁵. Briefly, a coupled spectrophotometric assay quantifies the amount of inorganic pyrophosphate, a product of the cyclization reaction, in

solution (EnzChek Pyrophosphate Assay, Invitrogen)³³. Pyrophosphate production was monitored by a shift in the absorption peak of the phosphorylated substrate from 330 nm to 360 nm. The second assay uses reverse-phase HPLC to separate nucleotides for the determination of nucleotide loading states of purified proteins and for the analysis of reaction products after incubation of proteins in the presence of 0.5 mM GTP and 2 mM MgCl₂. Nucleotides were separated on a reverse phase column (Phenomenex Gemini C18) using a methanol-phosphate gradient (buffer A: 100 mM potassium phosphate, pH 6.0; buffer B: 30% methanol/70% buffer A). Reaction products were collected and identified by comparison to standard nucleotides or by mass spectroscopy. Synthetic c-di-GMP was purchased from Biolog (Bremen, Germany).

Size-exclusion chromatography (SEC) and multi-angle light scattering

Purified protein (~5 mg/ml) was subjected to SEC using a Superdex 200 10/300 column (GE Healthcare) equilibrated in gel filtration buffer (25 mM Tris-Cl pH 7.5, 100 mM NaCl, and 1 mM DTT). For SEC-coupled multi-angle light scattering³⁴, purified protein (4 mg/ml) was subjected to SEC using a Shodex KW-803 column (JM Science, Inc.) equilibrated in gel filtration buffer. The chromatography system was coupled to an 18-angle light scattering detector (DAWN EOS) and refractive index detector (Optilab DSP) (Wyatt Technology). Data were collected every 0.5 s at a flow rate of 0.4 ml/min. Data analysis was carried out using the program ASTRA, yielding the molar mass and mass distribution (polydispersity) of the sample. For normalization of the light scattering detectors and data quality control, monomeric bovine serum albumin (BSA; Sigma) was used.

Analytical ultracentrifugation—Sedimentation velocity experiments were carried out using an XL-I analytical ultracentrifuge (Beckman Coulter) equipped with an AN-60 Ti rotor. Proteins (0.5–2 mg/ml) were diluted in assay buffer and were analyzed at a centrifugation speed of 130,000 × g. Data collection was carried out at 280 nm, followed by data analysis using the program SedFit (version 11.0).

Cell-based diguanylate cyclase assay—*E. coli* BL21(DE3) transformed with expression plasmids were grown in LB media supplemented with 100 mg/l ampicillin to a cell density corresponding to an absorbance of 0.5 at 600 nm. From each culture, 2.5 l was spotted onto LB plates supplemented with 100 mg/l ampicillin, 50 mg/l Congo Red (CR; Sigma-Aldrich) and IPTG at the indicated concentrations. Plates were incubated at 30°C over night.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

c-di-GMP

	bis-(3'-5')-cyclic dimeric guanosine monophosphate
GTP	guanosine triphosphate
PDE	phosphodiesterase
REC	receiver domain
SEC	size-exclusion chromatography

References

1. Stock AM, Robinson VL, Goudreau PN. Two-component signal transduction. *Annu Rev Biochem* 2000;69:183–215. [PubMed: 10966457]
2. Goymer P, Kahn SG, Malone JG, Gehrig SM, Spiers AJ, Rainey PB. Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. II. Role of the GGDEF regulator WspR in evolution and development of the wrinkly spreader phenotype. *Genetics* 2006;173:515–26. [PubMed: 16624907]
3. Hickman JW, Tifrea DF, Harwood CS. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc Natl Acad Sci U S A* 2005;102:14422–7. [PubMed: 16186483]
4. Kulasakara H, Lee V, Brencic A, Liberati N, Urbach J, Miyata S, Lee DG, Neely AN, Hyodo M, Hayakawa Y, Ausubel FM, Lory S. Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. *Proc Natl Acad Sci U S A* 2006;103:2839–44. [PubMed: 16477007]
5. De N, Pirruccello M, Krasteva PV, Bae N, Raghavan RV, Sondermann H. Phosphorylation-independent regulation of the diguanylate cyclase WspR. *PLoS Biol* 2008;6:e67. [PubMed: 18366254]
6. Chan C, Paul R, Samoray D, Amiot NC, Giese B, Jenal U, Schirmer T. Structural basis of activity and allosteric control of diguanylate cyclase. *Proc Natl Acad Sci U S A* 2004;101:17084–9. [PubMed: 15569936]
7. Christen B, Christen M, Paul R, Schmid F, Folcher M, Jenoe P, Meuwly M, Jenal U. Allosteric control of cyclic di-GMP signaling. *J Biol Chem* 2006;281:32015–24. [PubMed: 16923812]
8. Paul R, Abel S, Wassmann P, Beck A, Heerklotz H, Jenal U. Activation of the diguanylate cyclase PleD by phosphorylation-mediated dimerization. *J Biol Chem* 2007;282:29170–7. [PubMed: 17640875]
9. Wassmann P, Chan C, Paul R, Beck A, Heerklotz H, Jenal U, Schirmer T. Structure of BeF3⁻-modified response regulator PleD: implications for diguanylate cyclase activation, catalysis, and feedback inhibition. *Structure* 2007;15:915–27. [PubMed: 17697997]
10. Krissinel E, Henrick K. Inference of macromolecular assemblies from crystalline state. *J Mol Biol* 2007;372:774–97. [PubMed: 17681537]
11. Wemmer DE, Kern D. Beryll fluoride binding mimics phosphorylation of aspartate in response regulators. *J Bacteriol* 2005;187:8229–30. [PubMed: 16321925]
12. Yan D, Cho HS, Hastings CA, Igo MM, Lee SY, Pelton JG, Stewart V, Wemmer DE, Kustu S. Beryll fluoride mimics phosphorylation of NtrC and other bacterial response regulators. *Proc Natl Acad Sci U S A* 1999;96:14789–94. [PubMed: 10611291]
13. O'Shea EK, Klemm JD, Kim PS, Alber T. X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science* 1991;254:539–44. [PubMed: 1948029]
14. Rasmussen R, Benvegna D, O'Shea EK, Kim PS, Alber T. X-ray scattering indicates that the leucine zipper is a coiled coil. *Proc Natl Acad Sci U S A* 1991;88:561–4. [PubMed: 1988953]

15. Romling U, Sierralta WD, Eriksson K, Normark S. Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the *agfD* promoter. *Mol Microbiol* 1998;28:249–64. [PubMed: 9622351]
16. Zogaj X, Nimtz M, Rohde M, Bokranz W, Romling U. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Mol Microbiol* 2001;39:1452–63. [PubMed: 11260463]
17. D'Argenio DA, Miller SI. Cyclic di-GMP as a bacterial second messenger. *Microbiology* 2004;150:2497–502. [PubMed: 15289546]
18. Romling U, Gomelsky M, Galperin MY. C-di-GMP: the dawning of a novel bacterial signalling system. *Mol Microbiol* 2005;57:629–39. [PubMed: 16045609]
19. Galperin MY, Nikolskaya AN, Koonin EV. Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol Lett* 2001;203:11–21. [PubMed: 11557134]
20. Sinha SC, Sprang SR. Structures, mechanism, regulation and evolution of class III nucleotidyl cyclases. *Rev Physiol Biochem Pharmacol* 2006;157:105–40. [PubMed: 17236651]
21. Gao R, Tao Y, Stock AM. System-level mapping of *Escherichia coli* response regulator dimerization with FRET hybrids. *Mol Microbiol* 2008;69:1358–72. [PubMed: 18631241]
22. Maris AE, Sawaya MR, Kaczor-Grzeskowiak M, Jarvis MR, Bearson SM, Kopka ML, Schroder I, Gunsalus RP, Dickerson RE. Dimerization allows DNA target site recognition by the NarL response regulator. *Nat Struct Biol* 2002;9:771–8. [PubMed: 12352954]
23. Toro-Roman A, Wu T, Stock AM. A common dimerization interface in bacterial response regulators KdpE and TorR. *Protein Sci* 2005;14:3077–88. [PubMed: 16322582]
24. Meyer MG, Park S, Zeringue L, Staley M, McKinsty M, Kaufman RI, Zhang H, Yan D, Yennawar N, Yennawar H, Farber GK, Nixon BT. A dimeric two-component receiver domain inhibits the sigma54-dependent ATPase in DctD. *FASEB J* 2001;15:1326–8. [PubMed: 11344129]
25. Lee SY, De La Torre A, Yan D, Kustu S, Nixon BT, Wemmer DE. Regulation of the transcriptional activator NtrC1: structural studies of the regulatory and AAA+ ATPase domains. *Genes Dev* 2003;17:2552–63. [PubMed: 14561776]
26. Doucleff M, Chen B, Maris AE, Wemmer DE, Kondrashkina E, Nixon BT. Negative regulation of AAA + ATPase assembly by two component receiver domains: a transcription activation mechanism that is conserved in mesophilic and extremely hyperthermophilic bacteria. *J Mol Biol* 2005;353:242–55. [PubMed: 16169010]
27. Kuchma SL, Connolly JP, O'Toole GA. A three-component regulatory system regulates biofilm maturation and type III secretion in *Pseudomonas aeruginosa*. *J Bacteriol* 2005;187:1441–54. [PubMed: 15687209]
28. Kulasekara HD, Ventre I, Kulasekara BR, Lazdunski A, Filloux A, Lory S. A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial cup genes. *Mol Microbiol* 2005;55:368–80. [PubMed: 15659157]
29. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. *Macromolecular Crystallography, Pt A* 1997;276:307–326.
30. Adams PD, Grosse-Kunstleve RW, Hung LW, Ioerger TR, McCoy AJ, Moriarty NW, Read RJ, Sacchettini JC, Sauter NK, Terwilliger TC. PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr D Biol Crystallogr* 2002;58:1948–54. [PubMed: 12393927]
31. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 2004;60:2126–32. [PubMed: 15572765]
32. Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* 1998;54:905–21. [PubMed: 9757107]
33. Webb MR. A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. *Proc Natl Acad Sci U S A* 1992;89:4884–7. [PubMed: 1534409]
34. Wyatt PJ. Multiangle light scattering: The basic tool for macromolecular characterization. *Instrumentation Science & Technology* 1997;25:1–18.

Figure 1. Structure of WspR from *P. syringae*

(A) Domain organization of WspR. The N-terminal phospho-receiver (REC) domain is connected via a helical stalk to the GGDEF domain with diguanylate cyclase activity. In WspR, the active site loop contains the GGEEF motif (residues 249–253 in *P. syringae* WspR).

(B) Model for the regulation of WspR. Based on structural and functional studies, the model outlines the activation and a c-di-GMP-dependent inhibition mechanism for WspR. Crystal structures of WspR were obtained for the product-inhibited tetrameric state⁵.

(C) Crystal structure of *P. syringae* WspR. The asymmetric unit of the crystals contain a single molecule of WspR with two intercalated c-di-GMP molecules bound to the I-site and a

strontium ion from the crystallization condition bound to the REC domain (left panel). WspR is colored as shown in (A).

(D) Crystallographic oligomers of *P. syringae* WspR. In the crystal, two WspR molecules from *P. syringae* pack in a parallel fashion via their REC domains and a portion of the stalk motif (left panel). Two crystal symmetry-related dimers are intertwined in the crystal lattice forming a 4-fold symmetric tetramer (middle panel). Anti-parallel interactions of the forked stalks and c-di-GMP-mediated contacts form the main interfacial contacts. A similar state has been observed in crystals for *P. aeruginosa* WspR⁵. A putative model of WspR in an elongated dimer state is shown in the right panel.

Figure 2. Oligomerization-dependent activation of WspR

(A) Size exclusion chromatography (SEC) profiles of distinct WspR states. Purified WspR was analyzed by SEC on a Superdex200 10/300 column (GE Healthcare) equilibrated in gel filtration buffer (25 mM Tris-Cl pH 7.5, 100 mM NaCl, and 1 mM DTT). WspR purifies c-di-GMP-bound from *E. coli* (red trace) and exists in a tetramer-dimer equilibrium. Upon incubation in the presence of a c-di-GMP-specific phosphodiesterase (PDE), the protein adopts a dimeric conformation that elutes in a single peak (black trace). During prolonged incubation and purification involving concentration steps, a tetrameric species forms in addition to the dimeric species (green trace). See Table 1 and De et al., 2008 for details ⁵.

(B) Correlating quaternary structure and diguanylate cyclase activity. The diguanylate cyclase activity of WspR (1 M; in gel filtration buffer supplemented with 2 mM MgCl₂) in distinct states was measured by using a coupled enzyme assay that quantifies the amount of pyrophosphate, the second product of the cyclase reaction, in solution. Cyclic di-GMP-bound WspR, PDE-treated and nucleotide-free WspR were analyzed. In addition, nucleotide-free WspR eluting in peaks corresponding to tetrameric and dimeric WspR were analyzed separately. The nucleotide loading state was confirmed by using the HPLC-based assays (data not shown) with “NF” and “NB” denoting nucleotide-free and c-di-GMP-bound WspR, respectively.

Figure 3. Effect of BeF_3^- on the oligomeric state of WspR

(A) Inhibited, c-di-GMP-bound WspR. Purified WspR (150 M) that is bound to c-di-GMP at its I-sites and is inhibited was incubated for 5 min in the presence of 10 mM NaF 1 mM $BeCl_3$ and 2 mM $MgCl_2$ (dark dashed trace) or in gel filtration buffer (red solid trace), and subjected to SEC.

(B) Nucleotide-free WspR. Cyclic di-GMP was degraded at the I-site of WspR by PDE-treatment, and free pGpG and PDE was removed in subsequent purification steps. Nucleotide-free WspR was treated as described in (A).

(C) Putative phosphorylation-deficient WspR mutant. A mutant WspR protein was generated with an aspartate-to-asparagine mutation at the putative phosphorylation site in the REC domain. The protein was made c-di-GMP-free following the protocol described for wild-type WspR⁵, and treated as described in (A).

Figure 4. Effect of BeF₃⁻ on the diguanylate cyclase activity of WspR

(A) Wild-type WspR. WspR (1 M) in distinct states was analyzed as described in Figure 2B. The nucleotide loading state of the input protein was confirmed by using the HPLC-based assays (data not shown) with “NF” and “NB” denoting nucleotide-free and c-di-GMP-bound WspR, respectively. For BeF₃⁻-treatment, purified WspR (150 M) was incubated for 5 min in the presence of 10 mM NaF 1 mM BeCl₃ and 2 mM MgCl₂, and subjected to SEC in gel filtration buffer. Activity assays were performed immediately after SEC.

(B) WpsR^{D70N}. The WpsR mutant with a mutation at the putative phosphorylation site was analyzed as described in Figure 2B. Samples were prepared as described in (A) and Figure 2B.

Figure 5. Functional characterization of the stalk motifs in WspR

(A) Overview of truncated and fusion proteins. Based in WspR of *P. aeruginosa*, constructs were generated for the expression of the isolated GGDEF domain, the stalk-GGDEF module of WspR, and an artificial fusion protein in which the native stalk motif was replaced by the coiled-coil forming leucine zipper of GCN4 from *S. cerevisiae*.

(B) Catalytic activity in cells. *E. coli* BL21 were transformed with expression plasmids introduced in (A). Cells were grown to mid-log phase at 37°C and 2 µl of the culture was spotted onto a CR-containing LB plate with or without IPTG (0.1 mM) and incubated for 24 hours at 30°C. Expression of an active diguanylate cyclase correlated with a red colony phenotype (rdar morphotype).

(C) Detection of protein-bound guanosine nucleotides by a reverse-phase HPLC. GTP, linear di-GMP (pGpG), and c-di-GMP were well separated in this assay (grey dashed line, inset). Purified proteins (WspR^{GGDEF}, green; WspR^{GCN4}-GGDEF, brown; WspRst-GGDEF, blue) were heat-denatured. Subsequent to centrifugation, clear supernatants were subjected to an

HPLC-based analysis. The left panel shows a close-up view of the region of the chromatograms that co-elute with the c-di-GMP standard. The inset shows the entire chromatogram.

Figure 6. Oligomerization states of WspR truncation mutants and GCN4-fusion protein
 (A) Size exclusion chromatography (SEC). Purified WspR^{GGDEF} (green), WspR^{Stalk-GGDEF} (blue), and WspR^{GCN4-GGDEF} (brown) were analyzed by size exclusion chromatography (SEC) on a Superdex200 10/300 column (GE Healthcare) equilibrated in gel filtration buffer (25 mM Tris-Cl pH 7.5, 100 mM NaCl, and 1 mM DTT).
 (B) SEC-coupled multi-angle light scattering. Proteins were analyzed on multi-angle light scattering detectors subsequent to SEC in gel filtration buffer. The primary signal (in volts, left axis) is plotted against the elution volume. The solid (colored) trace shows the signal of one of the light scattering detectors (at 90° to the incident beam). The signal from the refractive index detector is shown as dashed line and is used for protein concentration determination.

Molecular weight determinations (right axis) from each data slice across the protein-containing peaks (0.5 s increments) are plotted against the elution volume. The grey horizontal lines indicate the theoretical molecular weight for a monomeric ($W_{\text{spR}}^{\text{GGDEF}}$) or dimeric ($W_{\text{spR}}^{\text{Stalk-GGDEF}}$ or $W_{\text{spR}}^{\text{GCN4-GGDEF}}$) sample.

Figure 7. Activity of WspR truncation mutants and GCN4-fusion protein

(A) Comparison of diguanylate cyclase activity full-length WspR and WspR^{GCN4-GGDEF}. WspR (nucleotide-bound or -free) or WspR^{GCN4-GGDEF} (0.5 M) was incubated at 25°C in assay buffer (EnzChek Pyrophosphate Assay, Invitrogen) containing GTP/Mg²⁺ (0.5 mM/2 mM), and pyrophosphate production was measured continuously monitoring absorbance at 360 nm. The red curve corresponds to nucleotide-bound WspR, the green curve to nucleotide-free WspR, and the brown curve to WspR^{GCN4-GGDEF}. Error bars indicate standard deviations of three independent experiments. Incubation of inorganic pyrophosphate (PP_i; grey trace) (0.5 mM) in assay buffer determines the rate-limit of the assay system. In the buffer control (black

trace), GTP/Mg²⁺ is included in the reaction. Initial velocities (bottom panel) were determined by linear regression. Error bars indicate standard deviations of three independent experiments. (B) Concentration-dependence and effect of c-di-GMP binding. Diguanilate cyclase activity of W_{spR}^{G_{GCN4}-GGDEF} was monitored at the indicated enzyme concentrations using the assay described in (A). Activity of c-di-GMP-bound (solid lines) and nucleotide-free (dashed lines) protein was measured.

Figure 8. Structure of WspR^{GCN4-GGDEF}

(A) Crystal structure of WspR^{GCN4-GGDEF}. The crystals contain two molecules of WspR^{GCN4-GGDEF} in the asymmetric unit with two c-di-GMP dimers bound to the I-sites. The GGDEF domains of WspR are shown colored in green and grey, with their corresponding GCN4 stalks colored in brown and black, respectively. A close-up view of an I-site bound to c-di-GMP (shown in stick presentation) is shown in the right panel.

(B) Flexible linkage between the GGDEF domains and the stalk units. The two crystallographic chains of full-length WspR from *P. aeruginosa* and of WspR^{GCN4-GGDEF} were superimposed in their GGDEF domains. The REC domains of full-length WspR are not shown for clarity.

(C) Comparison of the structure of the WspR tetramer with activated PleD and a model of active, dimeric WspR. The crystal structure of dimeric PleD activated by beryllium fluoride is shown in a c-di-GMP-mediated inhibited conformation (PDB code: 2V0N)⁹ (left panel). The REC domains are shown colored in violet and blue (chain A) and light and dark grey (chain B). The GGDEF motif at the active site is shown colored in yellow. Beryllium fluoride and magnesium ions are shown as spheres, and c-di-GMP is shown as stick presentation. The right panel depicts

a WspR tetramer in a c-di-GMP-mediated inhibited conformation, highlighting the stalk-GGDEF domain modules that are coordinated by c-di-GMP binding to the I-sites of adjacent domains.

(D) Crystallographic, parallel WspR dimer. The ribbons presentation depicts a model for parallel dimer of WspR from *P. aeruginosa* (PDB code: 3BRE) ⁵ in colors according to Figure 1.

Figure 9. Crystal structure of nucleotide-free WspR^{GGDEF}

(A) Comparison of nucleotide-free and c-di-GMP-bound GGDEF domains of WspR. The crystal structures of the isolated GGDEF domain of WspR (WspR^{GGDEF}; two molecules/asymmetric unit), full-length WspR (PDB code: 3BRE), and WspR^{GCN4-GGDEF} were superimposed using their GGDEF domains as reference. The structures are shown as C traces. The position of the N- and C-termini, active and inhibitory site are indicated. A coloring key distinguishing the separate chains is provided (right panel).

(B) The GGEEF motif. A Close-up view of the GGDEF motif is shown.

(C) Comparison of rotamer conformations within the GGEEF motif. A similar view of the active site as in (B) is shown. Residues of the GGEEF motifs are shown in stick presentation and colored as listed in (A).

Table 1

Summary of oligomerization data for nucleotide-free WspR.

	AA-Seq ^a	SEC ^b	AUC	MALS ^c
Peak 1	156.8	296.0	154.0	159.6
Peak 2	78.4	143.0	68.8	82.1

^a Oligomeric weights were calculated assuming a tetramer-dimer equilibrium (De et al., 2008).

^b Apparent molecular weight estimates are based on calibrated SEC by using globular protein standards with known molecular weight. The calibration is only accurate for globular proteins since SEC is sensitive to shape of the protein. Molecular weights may be overestimated for proteins with extended conformations.

^c Polydispersity indices of 1.010 (peak 1) and 1.004 (peak 2) were comparable to the value obtained for monomeric protein BSA (polydispersity index=1.003; molecular weight^{MALS}=67.9 kDa; molecular weight^{AA-seq}=69.3 kDa). In addition, data and data quality was similar to results obtained previously (De et al., 2008).

Table 2

Kinetic data for WspRGCN4-GGDEF and full-length WspR.

	K_m (μM)	K_{cat} (sec^{-1})
WspR ^{full-length} (c-di-GMP-free)	5.97 $\pm$ 0.80	4.50 $\pm$ 0.12
WspR ^{GCN4-GGDEF} (c-di-GMP-bound)	13.07 $\pm$ 0.70	40.56 $\pm$ 0.49
WspR ^{GCN4-GGDEF} (c-di-GMP-free)	12.96 $\pm$ 1.00	59.05 $\pm$ 1.03