

1 **Activation mechanism of a small prototypic Rec-GGDEF**

2 **diguanylate cyclase**

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7

8 **Abstract**

9 Diguanylate cyclases (DGCs) synthesising the bacterial second messenger c-di-GMP are found to be
10 regulated by a variety of sensory input domains that control the activity of their catalytical GGDEF
11 domain. As part of two-component systems, they are activated by cognate histidine kinases that
12 phosphorylate their Rec input domains. DgcR from *Leptospira biflexa* is a constitutively dimeric
13 prototype of this class of DGCs. Full-length crystal structures revealed that BeF₃- pseudo-
14 phosphorylation induces a relative rotation of two rigid halves in the Rec domain. This is coupled to a
15 reorganisation of the dimeric structure with concomitant switching of the coiled-coil linker to an
16 alternative heptad register. Finally, the activated register allows the two substrate-loaded GGDEF
17 domains, which are linked to the end of the coiled-coil *via* a localised hinge, to move into a
18 catalytically competent dimeric arrangement. Bioinformatic analyses suggest that the binary register
19 switch mechanism is utilised by many DGCs with N-terminal coiled-coil linkers.

20 **Introduction**

21 C-di-GMP is a near-ubiquitous bacterial second messenger responsible for the coordination of a
22 variety of cellular processes and behaviour, including motility, biofilm formation, virulence and cell
23 cycle progression (Jenal et al., 2017). Intracellular levels of c-di-GMP are regulated by the opposing
24 actions of diguanylate cyclases (DGCs), which contains a GGDEF domain and synthesize c-di-GMP,
25 and phosphodiesterases (PDEs), responsible for the degradation of this second messenger via an EAL
26 or HD-GYP domain (Simm et al., 2004). Not unfrequently, dozens of these enzymes can be encoded
27 by one single genome with each of the proteins containing distinct sensory input domains that can
28 sense/receive diverse signals like O₂, light and metals (Tarnawski et al., 2015)(Glantz et al., 2016)
29 (Zähringer et al., 2013). This allows bacteria to detect intracellular and environmental cues and
30 respond promptly by adjusting c-di-GMP levels which will then be detected by specific receptors.
31 Common input domains are GAF and PAS, which can recognise a variety of molecules, and response
32 regulator receiver domains (Rec), which as part of two component systems are phosphorylated by
33 cognate histidine kinases (HKs) (Zoraghi et al., 2004) (Henry and Crosson, 2011) (Gao and Stock,
34 2010).

35 DGCs catalyse the condensation of two molecules of GTP to yield the 2-fold symmetric c-di-GMP
36 product. This requires the juxtaposition of two GTP loaded DGC domains in appropriate 2-fold
37 related arrangement to form a catalytically competent GGDEF dimer that enables nucleophilic attack
38 of the deprotonated O3' hydroxyl onto the phosphorous of the other GTP molecule (Schirmer, 2016).
39 The first characterized full-length DGCs were PleD and WspR of Rec – Rec - GGDEF and Rec -
40 GGDEF domain organization, respectively, which were also studied in the beryllofluoride (BeF₃-)
41 modified form known to mimic phosphorylation (Wemmer and Kern, 2005). It was shown that, upon
42 this modification, PleD shifts from a monomer to a catalytically active dimer (Wassmann et al., 2007),
43 whereas the behaviour of WspR was more complex in that it enhanced tetramer formation (De et al.,
44 2009). Later, structural and biochemical analyses on DGCs with other input domains revealed that
45 these enzymes can exist also as constitutive dimers. Zinc binds to the CZB domain of DgcZ and

46 prevents productive encounter of the GGDEF domains by restraining domain mobility (Zähringer et
47 al., 2013). DosC has a globin domain with bound heme to sense oxygen (Tarnawski et al., 2015), and
48 lastly, the bacteriophytochrome PadC senses red light through its PHY domain to activate the GGDEF
49 domain (Gourinchas et al., 2017).

50 Almost invariably, input and catalytic domains of DGCs are connected by a dimeric coiled-coil that
51 can vary in length. We proposed earlier that the constituting helices could change their crossing angle
52 and/or azimuthal orientation to allow or prevent productive encounter of the two GGDEF domains
53 (chopstick model, (Schirmer, 2016)). This mechanism would be a generalization of the scissors with
54 fixed pivot blades model ascribed to histidine kinases signalling (Lowe et al., 2012). To test for the
55 mechanism, we selected a prototypical minimal DGC with known input signal. LEPBI_RS18680
56 from *Leptospira biflexa*, hereafter called DgcR (**d**iguanylate-**c**yclase controlled by **R**ec), is a Rec -
57 GGDEF protein with a short domain linker (Fig. 1a), thus making this enzyme attractive for studying
58 its conformational states by crystallography.

59 *Leptospira* is a bacterial genus composed of more than 30 species, among them some pathogenic
60 representatives responsible for causing leptospirosis, a worldwide zoonosis that affects more than one
61 million people and accounts for 60,000 deaths per year (Karpagam and Ganesh, 2020). *Leptospira*
62 *biflexa* is a saprophytic species used as a model to study *Leptospira* biology (Pětrošová and Picardeau,
63 2014). It contains an additional extra-chromosomal element of 74 kb (p74) that codes for 56 proteins
64 including DgcR. DgcR was also chosen, because it shares the same domain organization and linker
65 length as Rrp1 from *Borrelia burgdorferi*, the pathogen responsible for causing the Lyme disease.
66 Rrp1 is the only DGC encoded by *B. burgdorferi* genome and is essential for bacterial survival in the
67 tick host (He et al., 2011).

68 Here we show by biophysical and crystallographic analyses that DgcR is a constitutive dimer that
69 changes coiled-coil geometry and domain arrangement upon pseudo-phosphorylation. The chopstick
70 model is generally confirmed, but, upon activation, DgcR shows an unexpected translational register

71 shift. Bioinformatic analyses suggest that the observed activation mechanism is most likely
72 operational in most diguanylate cyclases of Rec – GGDEF organisation, but also in some other DGCs.

73 **Results and discussion**

74 **DgcR is a constitutive dimer that gets activated by domain rearrangements**

75 To reveal the structural changes accompanying the activation of Rec - GGDEF DGCs we determined
76 the full-length crystal structures of DgcR in native and pseudo-phosphorylated (BeF₃- modified) state.
77 A DgcR variant (R206A/D209A, abbreviated DgcR_AxxA) that had the putative allosteric inhibition
78 site (Fig. 1a) mutated was used to avoid locking the enzyme in a product inhibited conformation.
79 Crystallization was performed in presence of 3'-deoxy-GTP (3'dGTP), which is a non-competent
80 substrate analogue due to the absence of the 3'-hydroxyl group.

81 The structure of native DgcR_AxxA (called DgcR_nat) was solved by molecular replacement to 2.2 Å
82 resolution. There is one dimer in the asymmetric unit with the protomers held together by extensive
83 isologous contacts between the Rec domains involving their $\alpha 4$ - $\beta 5$ - $\alpha 5$ face (Fig. 1b). The Rec
84 domain shows the canonical ($\beta\alpha$)₅ fold (rmsd of 1.5 Å for 116 C α atoms with respect to PhoP, 2PKX),
85 but with the C-terminal $\alpha 5$ helix considerably extended and forming together with its symmetry mate
86 a coiled-coil leading to the GGDEF domains. A Mg²⁺ ion is bound to the acidic pocket formed by
87 E12, D13, and the phosphorylatable D56.

88 The structure of the GGDEF domain is very similar to others in the PDB database (rmsd of 1.4 Å for
89 157 C α atoms with respect to PleD, 2V0N) and shows the canonical ($\beta 1$ - $\alpha 1$ - $\alpha 2$ - $\beta 2$ - $\beta 3$ - $\alpha 3$ - $\beta 4$ - $\alpha 4$ - $\beta 5$)
90 topology of nucleotidyl cyclases of group III (Sinha and Sprang, 2006) with an N-terminal extension
91 that starts with a characteristic wide turn showing a DxLT motif followed by helix $\alpha 0$ that leads to $\beta 1$
92 (Fig. 1b, see also (Schirmer, 2016)). The GG(D/E)EF motif is located at the turn of the $\beta 2$ - $\beta 3$ hairpin.
93 Again as observed in other structures (Wassmann et al., 2007), the guanine base of the substrate
94 analogue is bound to a pocket between $\alpha 1$ and $\alpha 2$ and forms H-bonds with N182 and D191, whereas
95 the two terminal phosphates are H-bonded to main chain amides of the short loop between $\beta 1$ and $\alpha 1$.

96 Additionally, the γ -phosphate forms ionic interactions with K289 and R293. Two magnesium ions are
97 bound to the usual positions being complexed to the β - and γ -phosphates and the side-chain
98 carboxylates of D174, E217, and E218.

99 The GGDEF domains do not obey the 2-fold symmetry of the Rec domains, but form a relative angle
100 of about 90° . Thus, the two active sites with the bound GTP analogues do not face each other
101 rendering this constellation clearly non-productive. Though the constellation may be determined to
102 some extent by crystal packing, it demonstrates considerable inter-domain flexibility. Comparison of
103 the main-chain torsion angles reveals that the relative rotation can be traced back to a 169° change in a
104 single torsion, namely around the $C\alpha - C$ bond of residue 136 (ψ_{136} , Fig 2a). Thus, the hinge locates
105 to the C-terminal end of Rec $\alpha 5$, with the following residue I137 being packed against the Y149 from
106 the end of the GGDEF $\alpha 0'$ helix in both chains (Figs. 2b and c). As noted before (Schirmer, 2016), the
107 conserved residue N146 (see sequence logo in Fig. 2 - figure supplement 1) is capping both $\alpha 5$ and
108 $\alpha 0'$, but only in the A-chain.

109 The structure of activated DgcR_AxxA (called DgcR_act) obtained by BeF_3^- modification was solved
110 by molecular replacement to 2.8 Å resolution (Fig. 1c). There are two dimers in the asymmetric unit
111 that show virtually the same Rec dimer structure, but slightly different $\alpha 5$ -helix bending and GGDEF
112 orientations (Figure 1—figure supplement 1). As in DgcR_nat, the dimer is formed by isologous
113 contacts between the $\alpha 4$ - $\beta 5$ - $\alpha 5$ Rec faces and the extension of $\alpha 5$ forms a coiled-coil, but with an
114 altered relative disposition, which will be described in detail further below. D56 is found fully
115 modified by BeF_3^- and its immediate environment is different compared to DgcR_nat as will be
116 discussed in detail hereafter. The GGDEF domains are arranged symmetrical with the two bound
117 3'dGTP ligands facing each other, but too distant for catalysis (Fig. 1c, bottom). The GGDEF
118 orientation relative to the Rec domain is similar as in the A-chain of DgcR_nat.

119 Consistent with the crystal structures and the presence of the coiled-coil in both states, in solution,
120 DgcR is a constitutive dimer as measured by MALS both in the native and the activated form (Figure

121 1—figure supplement 2). Addition of substrate analogue or product was not changing this quaternary
122 state.

123 **Aspartate modification induces a relative rigid-body rotation within the Rec domain**

124 Comparison of DgcR_act with DgcR_nat (Fig. 3a) shows that, on activation, the hydroxyl group of
125 T85 is moved towards the BeF₃⁻ moiety to form an H-bond. The void left by this movement is claimed
126 by Y105 that undergoes a small side-chain rotation, but does not change its rotamer. Furthermore,
127 K108 forms ionic interactions with BeF₃⁻ and E12 in the activated structure (Fig. 3a). In the native
128 state, a magnesium ion is bound loosely to E13 and D56, whereas, in the active state, it is additionally
129 coordinated by the BeF₃⁻ moiety.

130 Activation of DgcR is accompanied with a change in the backbone structure as identified by a
131 DynDom analysis (Girdlestone and Hayward, 2015). The Rec domain can be divided into two parts
132 that undergo a relative 16° rotation as shown in Fig. 3b. Thereby secondary structure elements α3 to
133 β5 (residues 54 to 108) behave as one rigid body (rmsd = 0.83 Å /49) that rotates relative to the rest
134 (8-53, 109-135) that superimposes with an rmsd of 1.19 Å for 67 Cα positions. Figure 3b shows that
135 the rotation axis passes roughly perpendicular to the β-sheet through the centre of β4 (L83). Note that
136 the phosphorylatable D56 is close to the junction between the two rigid bodies and that its Cα position
137 only changes slightly during the transition. T86, however, with its distance of 7.5 Å from the rotation
138 axis moves by 2.0 (Cα) to 3.3 Å (Oγ) and the motion is most pronounced (5.2 Å) for the N-terminus
139 of α3 (P91) with its distance of about 15 Å from rotation axis. Thus, the rigid body motion changes
140 significantly the arrangement of α4 with respect to α5, which has a profound effect on the packing of
141 the Rec domains in the dimer.

142 For many Rec domains, a Y-T coupling mechanism has been described, where, upon (pseudo-)
143 phosphorylation a threonine/serine (T86 in DgcR) is dragged towards the phosphate and the
144 conserved tyrosine/phenylalanine (Y105 in DgcR) follows suite with a rotameric change from
145 *gauche*₊ to *trans* (Birck et al., 1999), (Bachhawat et al., 2005), (Wassmann et al., 2007). In DgcR, the

146 conserved tyrosine is already in *trans* conformation before activation and the T and Y move
147 concertedly towards the beryllofluoride moiety as part of a rigid-body ($\alpha 3$ to $\beta 5$) movement (Fig. 3).

148 **Rigid body rotation induces repacking of Rec domains within the dimer**

149 In both states, the Rec domains form 2-fold symmetric dimers with the contacts mediated by
150 isologous interactions between the $\alpha 4$ - $\beta 5$ - $\alpha 5$ surfaces (Fig. 4). However, due to the rigid-body
151 motion within the protomer and the concomitant relative displacement of $\alpha 4$ and $\alpha 5$ (Fig. 3), the
152 association of the $\alpha 4$ - $\beta 5$ - $\alpha 5$ faces is different in the native and the activated state. Therefore, the
153 two dimers superimpose rather poorly (rmsd = 3.1 Å/119 C α positions) with the β -sheets of the
154 protomers showing a difference in orientation of about 15° (Fig. 4a).

155 The native Rec dimer (Fig. 4b) with a buried surface area of 980 Å² is held together by an extended
156 apolar contact of $\alpha 5$ (A117, F120) with $\alpha 4$ (F94, I98), an ionic interaction of D104 with R118, an H-
157 bond between main-chain carbonyl 102 and R124 (both $\beta 5$ - $\alpha 5$ contacts). All aforementioned
158 residues are well defined with the exception of the R118 side-chain, which probably has several
159 alternative conformations, but all placing the guanidinium group close to D104 and to its symmetry
160 mate. Finally, and most relevant for the allosteric regulation of the C-terminal GGDEF effector
161 domains, there are regular coiled-coil interactions across the symmetry axis between the C-terminal
162 halves of the $\alpha 5$ helices starting with S121. These will be discussed in the next chapter.

163 The activated Rec dimer (Fig. 4c) with a buried surface area of 850 Å² shows the same apolar $\alpha 5$ - $\alpha 4$
164 cluster as the native dimer, but with the residues repacked in-line with the aforementioned relative
165 displacement of $\alpha 4$ and $\alpha 5$ within the protomer. At the centre of the interface, D104 shows a well-
166 defined, intermolecular salt-bridge with R118, but also with R118 from the same chain. As in the
167 native dimer, the R124 and S121 side-chains form intermolecular H-bonds, but with other partners
168 compared to the native interactions (main-chain carbonyls of 98 and 103, respectively).

169 A BLAST search revealed that, apart from Rec - GGDEF orthologs, the sequence of the DgcR Rec
170 domain is most similar to that of OmpR-like transcription factors (Fig. 4d). These have a Rec - DNA-

171 binding domain architecture and have been shown to dimerize *via* the Rec $\alpha 4$ - $\beta 5$ - $\alpha 5$ face upon
172 activation to allow binding of their effector domains to DNA (Draughn et al., 2018). Indeed, a
173 structure search of the DgcR_act dimer against the PDB retrieved as top hit (rmsd = 1.5 Å/228 Ca
174 positions) the BeF₃- activated Rec domain of PhoB (1ZES) (Bachhawat et al., 2005). Most of the
175 intermolecular interactions are thereby conserved, in particular the central salt-bridge D109 - R118
176 (DgcR numbering), or conservatively replaced (Fig. 4d). To our knowledge, no response regulator
177 with a DNA binding effector domain has yet been observed as a constitutive $\alpha 4$ - $\beta 5$ - $\alpha 5$ dimer (for a
178 review, see (Gao et al., 2019), which is probably due to their small or absent coiled-coil linkers.

179 Summarizing, beryllofluoride- modification of D56 induces a relative rigid body motion in the Rec
180 domain that changes the relative disposition of $\alpha 4$ and $\alpha 5$. Consequently, since both helices are part of
181 the Rec - Rec interface, the relative arrangement of the protomers and, thus, of the two $\alpha 5$ helices of
182 the dimer is changed (compare top panels of Figs. 4b and c). This change is supposed to be crucial for
183 the allosteric regulation of the C-terminal GGDEF domains as will be discussed in the following.

184 **Relative translation of C-terminal Rec helices changes coiled-coil register**

185 The DgcR Rec $\alpha 5$ helix is longer by about 3 turns (10 residues) compared to that of canonical Rec
186 domains. In the dimer, these protrusions form a 2-fold symmetric coiled-coil both in the native and
187 the activated state (Figs. 1b-c), though with distinct relative arrangement. Both constellations are
188 stabilized by isologous contacts between predominantly hydrophobic residues that obey a heptad-
189 repeat pattern (Figs. 5a and b). Thereby, I125 and L132 contribute to the contact in both structures
190 (position a; persistent contacts), but with the side-chains interacting with their symmetry mates from
191 opposite sides depending on the state (see e.g. the 132 - 132 contact in Fig. 5a). In contrast, other
192 residues contribute either only to the native (L128, T135) or the activated (H129, A136) constellation
193 (positions *d*, *e*; conditional contacts).

194 The two contact modes represent alternative knobs-into-holes packing as best seen in the helical net
195 diagram of Fig. 5c suggesting a relative translation of the interacting helices. Indeed, superposition of
196 one of the helices as in Figs. 5d and e reveals a large relative translation of about 9 Å. In other words,

197 upon activation, the two helices do not roll over each other (which would be accompanied by a
198 change in their azimuthal angles), but are translated with respect to each other to realise an alternative
199 knobs-into-holes packing. Note, that for steric reasons this shift would require dissociation and
200 reassemble of the constituting helices. Thus, the coiled-coil behaves like a binary switch that can
201 assume two clearly defined states, i.e. two distinct registers.

202 Recently, an analogous transition in the coiled-coil linker of a diguanylate cyclase has been proposed
203 for phytochrome-regulated PadC (Gourinchas et al., 2017). Indeed, the C-terminal end of the coiled-
204 coil of the dark-state enzyme is in the same register as native DgcR with, amongst others, N518 and
205 L525 forming (conditional) contacts (see PadC in Fig. 5 - figure supplement 1). Inspection of the
206 linker sequence and dynamic considerations prompted the authors to propose an alternative register
207 involving the neighbouring residues N519 and A526 for the illuminated state. Indeed, mutations
208 designed to stabilize this second register were constitutively active and the coiled-coil was found in
209 the active register (see PadC_mut in Fig. 5 - figure supplement 1), (Gourinchas et al., 2018). Though
210 the structure of light-activated wild-type PadC is not known, it is very likely that PadC and DgcR use
211 the same binary coiled-coil switch mechanism for DGC regulation, despite unrelated input domains.

212 WspR, another well-studied Rec - GGDEF diguanylate cyclase, also exhibits a “slippery” *axxdexx*
213 heptad-repeat with *e* of the last repeat in position -3 with respect to the DxLT motif (Fig. 5 - figure
214 supplement 1). Unfortunately, only product bound structures are available that reveal a non-
215 productive, c-di-GMP cross-linked tetramer in which the coiled-coils emanating from the two Rec
216 dimers are splayed apart at their ends (De et al., 2008). Most revealing, however, a GCN4 – GGDEF
217 WspR with GCN4 interface residues in the active register (Fig. 5-figure supplement 1) was reported
218 to be highly active and a corresponding structure (compact dimer) was predicted for active WspR (De
219 et al., 2009).

220 Summarizing, the change in coiled-coil registration upon DgcR activation is accompanied by a
221 substantial shift between the constituting helices that lead directly to the catalytic domains. Structural
222 data on other DGCs are consistent with this finding.

223 **Small rotation around inter-domain hinge allows formation of competent GGDEF dimer**

224 In the activated structure, the two GGDEF domains show no mutual interactions and their precise
225 orientation appears to be determined by crystal contacts. However, the two bound 3'dGTP ligands
226 face each other, though their distance ($> 10 \text{ \AA}$) is clearly too large for catalysis (see Fig. 1c). Having
227 identified the CA - C main-chain bond of A136 as an inter-domain hinge (Fig. 2a), we tried, by small
228 changes in ψ_{136} and adjoining main-chain dihedrals, to symmetrically move the GGDEF domains as
229 rigid bodies into a catalytically competent arrangement (Michaelis-Menten complex). Indeed, only
230 small torsional changes (Fig. 6a) were necessary to bring the (reconstructed) 3'-hydroxyl groups of
231 each bound substrate in line with the scissile PA - O3A bond of the other substrate as required for
232 catalysis (Figs. 6b and c). It should be considered, however, that the optimal arrangement of the
233 catalytic domains depends obviously on the conformation of the bound substrates, as discussed in
234 (Zähringer et al., 2013). In fact, comparison of the bound 3'dGTP ligands in DgcR_nat and DcrR_act
235 shows variability in the ribose and α -phosphate orientation (Fig. 6 – figure supplement 1), probably
236 due to the lack of strong interactions with the binding site. Here, we used the conformation as seen in
237 DgcR_act. Scenarios with other substrate conformations were not explored, but the relative GGDEF
238 arrangement would probably be similar considering the fixed hinges at the end of the activated Rec
239 dimer.

240 The details of the Michaelis-Menten complex shown in Fig. 6c are consistent with the model
241 proposed in (Schirmer, 2016) with metal M2 coordinating the 2'-hydroxyl group and K179 hovering
242 over the α -phosphate of the incoming substrate. There is no titratable residue close to the O3'-group.
243 Most likely, deprotonation of the hydroxyl-group proceeds via a water molecule that could be
244 activated by the close-by metal(s) as e.g. in adenylate cyclases (Steegborn, 2014).

245 In the competent dimer, there are no clashes between the catalytic domains. Molecular dynamics
246 simulations would be required to refine the model, but it appears that D183 and D282 may interact
247 with Y286 and H187, respectively. All these residues are conserved in diguanylate cyclases (Fig. 2 –
248 figure supplement 1). Indeed, in the apo-structure of the constitutively active mutant of PadC (6ET7,

249 (Gourinchas et al., 2018)) the proposed interactions seem well possible, albeit only in one half of the
250 asymmetric structure.

251 There is one more conserved residue that projects to the other subunit, namely R147 (Fig. 2 – figure
252 supplement 1). Judged by the model, it appears possible that this arginine may interact with the
253 guanyl Hoogsteen-edge of the opposing substrate. This would be supported by a recent study on the
254 promiscuous (accepting GTP and ATP) DGC GacA, wherein the reason for the relaxed substrate
255 specificity was attributed to an aspartate-serine replacement of a base-binding residue (Hallberg et
256 al., 2019). In the sub-group of promiscuous DGCs, the position homologous to R147 of DgcR is not
257 conserved (sequence logo in Fig. 6 – figure supplement 1D of (Hallberg et al., 2019)) suggesting that
258 the arginine is no longer important, since it cannot interact with a adenyl Hoogsteen-edge. In the same
259 paper, the fourth residue of the GGDEF motif (equivalent to E218 in DgcR) was proposed to
260 deprotonate the 3'-hydroxyl group of the substrate bound to the other subunit. In our model (Fig. 6c),
261 this residue is coordinating metal M2 and clearly not close to this substrate hydroxyl group.

262 **Structural coupling of Rec modification with competent dimer formation**

263 The knowledge of the structures of full-length DgcR both in its native and activated form and the
264 model of the Michaelis-Menten complex allows to discuss in detail how signal perception
265 (phosphorylation) is coupled to output activation in a prototypic response regulator with enzymatic
266 function. In figure 7, this process is dissected into 5 notional steps.

267 (1) Starting with a symmetrized version of DgcR_{nat} (Fig. 7a), aspartate pseudo-phosphorylation
268 induces a rigid-body motion within each Rec domain (Fig. 7b, tertiary change). With an unchanged
269 coiled-coil packing, the intermolecular $\alpha 4 - \alpha 5$ contacts would break up. (2) This is counteracted by a
270 repacking of the two Rec domains (Fig. 7c, quaternary change. (3) The clashing of the C-terminal
271 ends of the coiled-coil is relieved by slight outward bending of the helices (Fig. 7d). Obviously, these
272 first three steps, which describe the transition of the native to the activated Rec stalk, will be tightly
273 coupled.

274 The following steps invoke no direct Rec - GGDEF communication, but only an unrestricted rotation
275 of the GGDEF domains around the inter-domain hinges. With the Rec stalk in its activated
276 constellation, the hinges are positioned such that the GGDEF domains can attain (4) a constellation as
277 in DgcR_act (Fig.7e) and, finally, assemble to form (5) the catalytically competent constellation
278 Michaelis-Menten complex (Fig.7f). An animation of the entire structural transition from native to
279 competent DgcR is shown in Fig.7g.

280 The aspect of conformational sampling and its dependence on the coiled-coil register and the
281 dynamics of the entire enzyme has been discussed before for PadC (Gourinchas et al., 2018). Whether
282 the asymmetric GGDEF dimer obtained for mutated PadC is of functional importance needs further
283 studies. Although such a state would probably be compatible with our model, it is not mandatory for
284 the proposed mechanism in which the two phosphodiester bonds could be formed quasi-
285 simultaneously. Furthermore, we suggest that the competent GGDEF dimer would assemble
286 autonomously due to electrostatic and steric complementarity, in particular in presence of the
287 substrates that interact with K179 and M2 of the opposing domain (Fig. 6c), thus not requiring any
288 direct interaction between input and output domains.

289 **Allosteric inhibition by product mediated domain cross-linking**

290 Allosteric product inhibition by c-di-GMP is a well-known feature of many DGCs (Christen et al.,
291 2005)(Wassmann et al., 2007)(Schirmer, 2016)(Chou and Galperin, 2016). Hereby, dimeric c-di-GMP
292 mutually cross-links a RxxD motif (primary I-site, I_p) on one GGDEF domain with a secondary I-site
293 (I_s) on the other GGDEF domain and vice-versa. The crystal structure of DgcR obtained in presence
294 of c-di-GMP (DgcR_inh) was determined to 3.3 Å by molecular replacement and is shown in Fig. 8a.
295 There are three symmetric dimers in the asymmetric unit. Each dimer shows a Rec stalk in native
296 conformation and the two GGDEF domains have their active sites facing outwards. Dimeric c-di-
297 GMP is cross-linking the domains by interacting with the R206xxD209 motif of one subunit and
298 R163* from α0' of the other subunit (Fig. 8b). Due to symmetry, there are two isologous crosslinks
299 within the DGC dimer. Comparison with the PleD/c-di-GMP complex (Wassmann et al., 2007) (Fig.

300 8c) shows very similar binding, but with PleD providing an additional arginine (R390) to the I_p-site.
301 The DgcR equivalent (R237) is too distant to interact, but this may happen with the Rec stalk in the
302 activated conformation. Arginines 163* (DgcR) and R313* (PleD) fulfil the same role in c-di-GMP
303 binding, but are not homologous on the sequence level. Indeed, it has been noted earlier that among
304 GGDEF sequences (Paul et al. 2007) arginines are enriched at either position. The unique c-di-GMP
305 stabilized GGDEF arrangement that differs drastically from DgcR_{nat} (Fig. 1b) again demonstrates
306 the large flexibility provided by the inter-domain hinge.

307 **Kinetic analysis of DgcR activity reveals delay in non-competitive feed-back inhibition**

308 The effect of activation and I_p-site mutation on DgcR catalysed c-di-GMP production was studied by
309 a real-time nucleotide quantification assay (online ion-exchange chromatography, oIEC, Agustoni et
310 al., in preparation, see Methods). Figure 9a shows that product formation catalysed by native wild-
311 type DgcR (wt) gradually decreases early-on (when there is still a large excess of substrate),
312 indicative of non-competitive product inhibition. Indeed, the progress curve was found consistent with
313 the respective classical model with a low k_{cat} of about 0.01 s⁻¹ and a relatively large K_i of about 30 μM
314 (Tab. 2).

315 A very different behaviour was observed for activated DgcR (wt*) that produced very quickly (< 75 s)
316 a substantial amount of product yielding a lower boundary for k_{cat} of 0.1 s⁻¹ (Fig. 9a). This was
317 followed by a phase of very small, virtually constant velocity. Such phenotype was clearly
318 inconsistent with classical equilibrium models and seemed indicative of a slow transition to the
319 product-inhibited state. Mechanistically, this transition would comprise (fast) product binding and
320 (slow) re-organization of the two GGDEF domains to acquire the inactive product cross-linked
321 configuration (Fig. 8).

322 The progress curves were fitted with the kinetic model shown in Fig. 9b. Independent binding of two
323 substrate molecules (S) to the dimeric enzyme (EE) was parametrised with an equilibrium constant K_d
324 (assuming fast substrate binding), whereas the transition between active and inactive states was
325 modelled kinetically with an effective second-order rate constant k_{on} (dependent on product and

326 enzyme concentration) and a first-order rate constant k_{off} with the inhibitory constant given by $K_i =$
327 $k_{\text{off}}/k_{\text{on}}$. Note that for simplicity the model considers only one product binding site on the dimeric
328 enzyme, while there are actually four (two c-di-GMP dimers). This simplification will affect the
329 nominal value of K_i . Full kinetic modelling without this simplification and with explicit modelling of
330 the conformational enzyme transition has been postponed to a follow-up study.

331 The kinetic model fits the biphasic curve of wt* very well (Fig. 9a) yielding the parameters given in
332 Table 2. The k_{cat} of 0.2 s⁻¹ together with the slow kinetics of the active to inactive transition explains
333 the large build-up of product in the initial phase, which is followed by very low residual activity of the
334 (equilibrated) sample due to the low K_i of 62 nM.

335 To validate the involvement of the RxxD motif in feed-back product inhibition as suggested by the
336 crystal structure (Fig. 8) and shown for many other DGCs, but also to scrutinise the kinetic model, the
337 motif was mutagenised to AxxA. Indeed, the activated mutant (mut_{AxxA}*) was found to be drastically,
338 though not fully, inhibition relieved (Fig. 9a, inset). The curve is consistent with an unchanged k_{cat} ,
339 but a drastically (almost 200-fold) increased K_i as compared to wild-type (Tab. 2). Apparently, the
340 mutations did not completely abolish inhibition with the remaining residues of the primary and
341 secondary I-site possibly still enabling (weak) product binding (Fig. 8).

342 In contrast to the native wild-type enzyme (wt), the activity of the native mutant (mut_{AxxA}) was lower,
343 which may be due to a detrimental, long-range effect of the mutations on the active site geometry.
344 Notably, there was no indication of product inhibition. Thus, for both wild-type and mutant DgcR the
345 activated state is more susceptible to product inhibition. Whether this is due a sub-optimal product
346 mediated backside cross-linking in the native state as suggested by the crystal structure (Fig. 8) has to
347 await further structural investigations.

348 Summarizing, activated DgcR shows a pronounced initial burst of activity before entering the product
349 inhibited state with a rather slow kinetics probably reflecting domain reorganization. The kinetic
350 model (Fig. 9b) proved to reproduce all measured progress curves and the parameters (Tab. 2) reflect
351 the impact of activation and I_p-site mutation.

352 **Rec - GGDEF linker sequence profiles are consistent with register shift mechanism**

353 DgcR has been selected as a prototypic Rec - GGDEF enzyme of relatively small size (298 residues),
354 but bioinformatic analysis showed that the linker length can vary considerably in this class of DGCs.
355 This was surprising considering that the linker has a defined structure and seems crucial for signal
356 transduction. However, the linker length histogram (Fig. 10a) shows that the lengths are not
357 distributed uniformly, but exhibit discrete values separated by multiples of 7 (groups 1 to 6, with
358 DgcR and WspR belonging to groups 1 and 4, respectively). Thus, members of the groups would
359 merely differ in the number of double helical turns when forming parallel coiled-coils. Indeed, the
360 individual sequence logos can easily be aligned (Fig. 10b) to reveal the striking repeat of leucines
361 in every 7th position (heptad position *a*). Most interestingly, the last (and to a lesser degree the last but
362 one) heptad repeat at the C-terminal end (Fig. 10c) shows a conserved *axxdexx* pattern as in DgcrR
363 (Fig. 5). Thus, a common binary register shift mechanism seems likely for members of all the
364 groups. Group 0 (Figs. 10a, b) does not obey the linker length rule. Since it also has an (S/N)PLT
365 instead of a DxLT motif, it probably has a different linkage and, therefore, activation mechanism.

366 A similar pattern of discretized coiled-coil lengths has been reported for PAS - GGDEF and LOV –
367 GGDEF proteins (Möglich et al., 2009) (Glantz et al., 2016), which makes it tempting to speculate
368 that input to effector signal transduction might work similarly as in Rec - GGDEF enzymes. However,
369 further investigations into their sequence profiles are needed to see whether they also exhibit
370 ambiguous *axxdexx* heptad repeats.

371 **Conclusion**

372 The presence of coiled-coil linkers between N-terminal regulatory and catalytic GGDEF domains in
373 many diguanylate cyclases has been described and their role in signal transduction discussed (De et
374 al., 2009)(Glantz et al., 2016; Möglich et al., 2009; Schirmer, 2016). Changes in the crossing angle or
375 the azimuthal orientation of the helices upon activation were anticipated, but a repacking of the
376 interface was not discussed, which was then seen first in the comparison of inactive and a
377 constitutively activated mutant of light-regulated PadC (Gourinchas et al., 2018). The now presented

378 detailed structural analysis of DgcR in its native and pseudo-phosphorylated form allowed a
379 comprehensive dissection of the activation process for a full-length, wild-type Rec - GGDEF enzyme
380 (Fig. 7). Tertiary and quaternary changes in the Rec input domains lead to a register shift in the
381 coiled-coil linker repositioning the inter-domain hinge and, thus, the propensity of the GGDEF
382 domains to attain the catalytically competent dimer constellation.

383 A register shift in the coiled-coil linker may be operational also for other enzymes with predicted
384 coiled-coil linkers, e.g. DGCs with N-terminal GAF domains or trans-membrane helices. LOV sensor
385 domains that carry a flavin-nucleotide chromophore and have been studied very well as part of HKs
386 (Glantz et al., 2016)(Möglich, 2019) are different in that the coiled-coil forming $J\alpha$ helix is not part of
387 the core fold, but rather an extension of the C-terminal $I\beta$ -strand that projects outward in the same
388 direction. It has been shown that, upon light activation, the two $I\beta$ - $J\alpha$ junctions of the dimer increase
389 their distance considerably (Röllen et al., 2016) probably causing a change in the crossing angle
390 and/or the super-twist of the $J\alpha$ coiled-coil in the full-length protein to control activity as discussed in
391 the recent review by Möglich (Möglich, 2019). Most likely, GAF domain proteins control GGDEF
392 activity in a similar way, due to the structural similarity with LOV including the predicted C-terminal
393 coiled-coil (Möglich et al., 2009). HAMP domains have been shown to operate as rotary switches
394 (Hulko et al., 2006). How such a change will affect the geometry of the C-terminal coiled-coil in
395 respective DGCs has not been studied, but it will surely affect the relative disposition of the hinges
396 that lead to the catalytic domains and, thus, activity.

397 Apparently, the coiled-coil linker is a versatile and effective means of transmitting a signal between
398 domains without requiring direct interactions between them, which, obviously, is of paramount
399 advantage for their modular combination in evolution. The same principle seems to apply also for
400 HKs, many of them both are controlled by the same kind of input domains as DGCs and exhibit a
401 coiled-coil preceding the DHp $\alpha 1$ bundle (Diensthuber et al., 2013) (Wang et al., 2013).

402 Bioinformatic analyses (Anantharaman et al., 2006)(Glantz et al., 2016) may now be extended to test
403 for the occurrence of “slippery” heptad repeats in coiled-coil proteins in general to reveal proteins
404 potentially signaling *via* a coiled-coil register shift.

405 **Material and methods**

406 **Protein expression and purification**

407 *E. coli* BL21 (DE3) cells transformed with pET-28a vector containing DgcR full-length construct
408 purchased from Genescript Inc. were incubated at 37 °C with agitation until they reached the optical
409 density of 0.8 - 0.9. Expression was then induced by the addition of IPTG (Isopropyl β -D-1-
410 thiogalactopyranoside) at a final concentration of 400 μ M for 4 hours at 30 °C. The cells were
411 harvested after centrifugation and resuspended in a buffer composed by 20 mM Tris pH 8.0, 500 mM
412 NaCl, 5 mM MgCl₂, 5 mM 2-Mercaptoethanol and protease inhibitor (Roche). The lysis proceeded by
413 3 passages in a French press cell at a pressure of 1500 psi. After a centrifugation at 30000 x g for 50
414 minutes, the soluble fraction was loaded onto a His Trap HP 5 mL column (GE Healthcare) in 20 mM
415 Tris pH 8.0, 500 mM NaCl, 5mM MgCl₂, 5mM 2-Mercaptoethanol and 20 imidazole. DgcR was
416 eluted using imidazole gradient of 20 mM to 500 mM in 15 column volumes. The fractions containing
417 DgcR were further purified by size exclusion chromatography using a Superdex 200 26/600 column
418 (GE Healthcare) in 20 mM Tris pH 8.0, 20 mM NaCl, 5 mM MgCl₂ and 1 mM DTT. Protein was
419 quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

420 **BeF₃⁻ modification of DgcR**

421 In order to produce BeF₃⁻ modified DgcR, approximately 300 μ M of DgcR in 20 mM Tris pH 8.0, 20
422 mM NaCl and 5 mM MgCl₂ were incubated with a mixture of NaF at 10 mM and BeCl₂ at 1 mM,
423 final concentration. After gentle mixing to achieve a homogeneous solution, the sample was left at
424 room temperature for at least 15 minutes. DgcR BeF₃⁻ mix was then centrifuged at 4°C at 18.000 x g
425 to remove a light precipitation formed during the process. Protein concentration was measured after
426 the activation process and was found virtually unaltered.

427 **SEC-MALS analysis**

428 Light scattering intensity and protein concentration were measured at elution from the column using
429 an in-line multi-angle light-scattering and differential refractive index detectors (Wyatt Heleos 8+ and
430 Optilab rEX). These data were used to calculate molar mass for proteins by standard methods in Astra
431 6 (Wyatt). Corrections for band-broadening, inter-detector delays and light-scattering detector
432 normalisation were performed using a sample of bovine serum albumin in the experimental buffer,
433 according to the manufacturer's protocol. Samples were loaded (100 μ L) at concentrations ranging
434 from 0.4 to 10 mg/mL in presence of various ligands at a constant flow of 0.5 mL/min in 20 mM Tris
435 pH 8.0, 500 mM NaCl, 5 mM MgCl₂, 1 mM DTT.

436 **Crystallization**

437 Crystallization attempts were performed using vapour diffusion method prepared in 3-drop MRC
438 plates by Gryphon robot (Art Robbins Instruments) with DgcR (wild-type or I-site mutant AxxA) at a
439 concentration of 10 mg/mL (280 μ M) in 20 mM Tris pH 8.0, 20 mM NaCl, 5 mM MgCl₂ and 1 mM
440 DTT at 18 °C. For DgcR_{nat} crystallisation, 3'dGTP was added at a final concentration of 2 mM.
441 After 3 days, crystals could be observed in 0.2 M Magnesium sulfate, 20 % PEG 3350 from condition
442 C8 of PEG/Ion HT crystallization kit (Hampton Research). DgcR_{act} was crystallised by the same
443 protocol, but with BeF₃⁻ treatment prior to the crystallisation set-up. After 7 days crystals were
444 observed in a condition composed by 0.3 M Magnesium chloride hexahydrate, 0.3 M calcium chloride
445 dehydrate, 1.0 M imidazole, MES monohydrate (acid), pH 6.5, EDO_P8K, 40% v/v ethylene glycol,
446 20 % w/v PEG 8000 present in condition A2 from Morpheus I crystallization kit (Molecular
447 Dimensions). Crystallization of DgcR in the inhibited conformation (DgcR_{inh}) was achieved by the
448 presence of 2.0 mM c-di-GTP. Crystals appeared after 5 days in 0.2 M potassium thiocyanate, 0.1 M
449 Tris pH 7.5, 25% PEG 2000 MME, condition optimized from H11 of Index HT crystallization kit
450 (Hampton Research). Crystals were frozen in liquid nitrogen and stored in a transport Dewar prior to
451 data collection.

452 **Crystal data collection and structure determination**

453 Data was collected at the Swiss Light Source (SLS), Villigen, Switzerland at 100 K and was
454 processed using XDS (DgcR_nat), iMosflm (DgcR_act data and DgcR_inh) and CCP4i suite (Kabsch,
455 2010)(Potterton et al., 2018) (Battye et al., 2011). DgcR_nat structure was solved by molecular
456 replacement using homologous structures generated from the Auto-Rickshaw pipeline web server
457 (Panjikar et al., 2009). Subsequently, the DgcR_act and DgcR_inh structures were solved by
458 molecular replacement using the Rec and GGDEF domains of DgcR_nat separately using Phaser
459 (McCoy et al., 2007). The model was built using COOT and refinement was carried using Refmac5
460 (Emsley and Cowtan, 2004) (Murshudov et al., 2011). Structure figures were prepared using Dino
461 (<http://dino3d.org>). Morphing was calculated using UCSF Chimera (Pettersen et al., 2004).

462 **Enzymatic analysis**

463 DgcR wild type and DgcR_AxxA activity assays were performed at 5 μ M in the presence of
464 500 μ M of GTP in a reaction buffer composed of 100 mM Tris pH 8.0, 100 mM NaCl and 5 mM
465 MgCl₂. The reaction was started by substrate and product progress curves were acquired by a novel
466 automatic chromatographic method, named online ion exchange chromatography (oIEC) (Agustoni,
467 manuscript in preparation), in which aliquots (68 μ L) are automatically withdrawn from the large
468 reaction vessel (650 μ L) and loaded into a Resource Q column (GE Healthcare) without the need for
469 prior quenching of the reaction. This was followed by ammonium-sulfate (0 to 1 M, 20 mM tris, pH
470 8.0) gradient elution of the bound substances (enzyme, substrate, product). Peak areas corresponding
471 to the c-di-GMP product were integrated and converted to concentrations using a scale factor obtained
472 from calibration. Data was plotted and fitted using proFit (QuantumSoft).

473 To calculate theoretical progress curves, the partial differential equations corresponding to the kinetic
474 scheme in Fig. 9b were set-up in ProFit and solved by numerical integration. Global fitting of this
475 function using the Levenberg algorithm implemented in ProFit to the measured time courses of
476 product and substrate concentration yielded the parameters listed in Table 2.

477 **Bioinformatic analysis**

478 Rec and GGDEF domain HMM profiles were taken from Pfam (Finn et al., 2010) and used as input to
479 an hmmsearch on the HMMER web server against the reference proteome database rp55 (E-values
480 0.01; hit 0.03) (Potter et al., 2018). 8016 sequences were found and filtered by size (< 360 residues) to
481 exclude Rec - GGDEF sequences with additional domains. This procedure reduced the data size to
482 1991 sequences. A redundancy filter (< 80% pairwise identity) finally reduced the number of
483 sequences to 1408. Global alignment was performed using Muscle (Madeira et al., 2019). From this
484 alignment, the linker sequences (as defined ranging from the KP-motif in the Rec $\beta 5$ - $\alpha 5$ loop to the
485 DxLT motif at the beginning of the GGDEF domain) were extracted and clustered according to length
486 by a custom-made Python script. For the major clusters, corresponding logos were generated using
487 Geneious Prime 2020.1.2 (www.geneious.com) and manually aligned to account for the distinct linker
488 lengths.

489 **Accession codes**

490 Coordinates and structure factors of DgcR_nat, DgcR_act, and DgcR_inh have been deposited in the
491 Protein Data Bank under the accession codes 6ZXB, 6ZXC, and 6ZXM.

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640 **Tables**

641 **Table 1. Data collection and refinement statistics.**

642

	DgcR_nat	DgcR_act	DgcR_inh
Data collection			
Synchrotron source	SLS, PXIII	SLS, PXI	SLS, PXIII
Resolution	41 - 2.2 (2.3 - 2.2)	41 - 2.8 (2.9 - 2.8)	48 - 3.3 (3.4 - 3.3)
Space group	C 2	P 2 ₁ 2 ₁ 2	P 2 ₁
a, b, c (Å)	137, 39, 146	133, 247, 41	123, 73, 126
α, β, γ (°)	90, 109, 90	90, 90, 90	90, 118, 90
Total reflections	191690 (18770)	205975 (19551)	111858 (9780)
Unique reflections	38087 (3727)	34912 (3320)	29577 (2768)
Multiplicity	5.0 (5.0)	5.9 (5.7)	3.8 (3.5)
Completeness (%)	98 (98)	94 (97)	99 (94)
Mean I/sigma(I)	10.7 (1.2)	8.6 (1.5)	6.7 (1.6)
R-merge	0.09 (1.20)	0.29 (1.13)	0.17 (0.75)
R-pim	0.05 (0.60)	0.13 (0.51)	0.10 (0.46)
CC1/2	0.99 (0.70)	0.90 (0.60)	0.99 (0.70)
Refinement			
R-work/R-free (%)	23.1/25.7	24.8/30.1	25.7/31.4
Number of molecules/a.u	2	4	6
Number of atoms	4804	9545	14641
protein	4672	9312	14070
ligands	101	184	552
water	31	49	19
RMS(bonds)	0.008	0.009	0.023
RMS(angles)	1.45	1.52	3.96
Ramachandran favored (%)	97.08	95.14	95.19
Ramachandran allowed (%)	2.23	4.86	3.89
Ramachandran outliers (%)	0.69	0	0.92
Average B-factor (Å ²)	65.91	59.80	81.37
protein	66.24	59.87	82.06
ligands	56.65	57.08	64.83
water	46.87	55.74	55.87
PDB code	6ZXB	6ZXC	6ZXM

643

644 Statistics for the highest-resolution shell are shown in parentheses.

645 **Table 2. Kinetic parameters of DgcR diguanylate cyclase activity**

646

	K_d (M) +	k_{cat} (s⁻¹)	inhibition type	k_{on} (10³/M⁻¹·s⁻¹)	K_i (M)
DgcR	1.0·10 ⁻⁵	0.0089	non-competitive, equilibrium	n/a	2.8·10 ⁻⁵
DgcR*	1.0·10 ⁻⁵	0.2 ++	non-competitive, slow	5.2	6.2·10 ⁻⁸
mut_AxxA	1.0·10 ⁻⁵	0.0015	none	n/a	-
mut_AxxA*	1.0·10 ⁻⁵	0.2 ++	non-competitive, slow	7.9	1.1·10 ⁻⁵

647

648 Parameters derived from Fig. 9a.

649 + represents lower limit as obtained from simulations

650 ++ represents upper limit as obtained from simulations

Figures

Fig 1

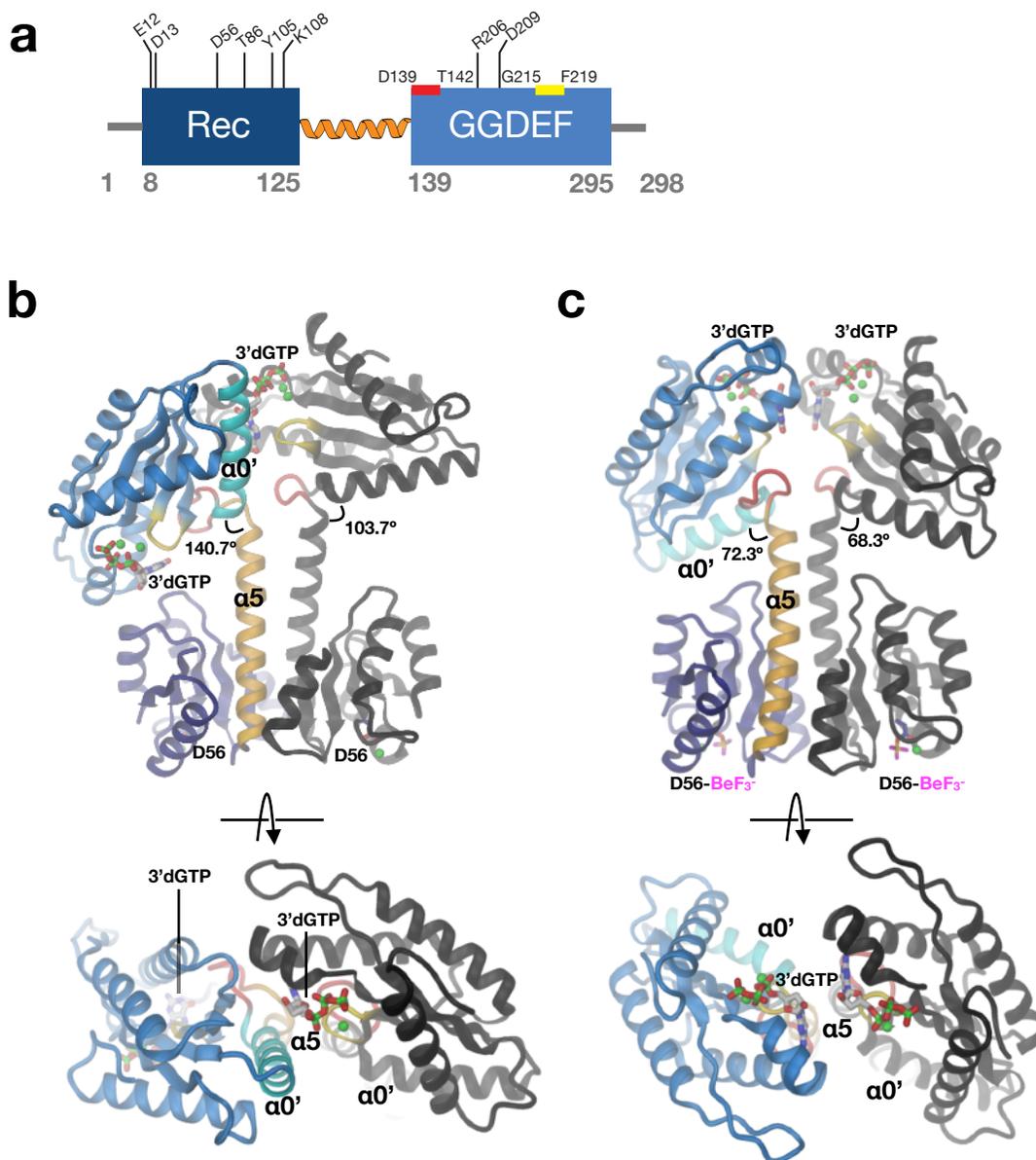


Figure 1. Native and activated DgcR dimers adopt distinct domain arrangements.

a) DgcR domain organisation with important features and residues highlighted. The Rec and the GGDEF domains are linked by the extension (orange) of the C-terminal Rec helix. Red and yellow bars indicate the DxLT and GGDEF motif, respectively. Note the presence of the RxxD inhibition site.

b-c) Side and top views of DgcR_{nat} (**c**) and DgcR_{act} (**d**) dimers. Within one protomer, domains and important elements are highlighted by color. The C-terminal Rec helix ($\alpha 5$) is colored in gold, the wide turn in red, the N-terminal GGDEF helix ($\alpha 0'$) in cyan and the characteristic β -hairpin (with GGEEF sequence) in yellow. The BeF_3^- modified aspartates of the Rec domains, the bound Mg^{++} ions (green), and the 3'dGTP substrate analogues bound to the GGDEF active sites are shown in full. In both cases, the Rec domains obey 2-fold symmetry. The GGDEF domains are related by 2-fold symmetry (with the two 3'dGTP ligands opposing each other) only in DgcR_{act}, while in DgcR_{nat} they are related by approximately 90°.

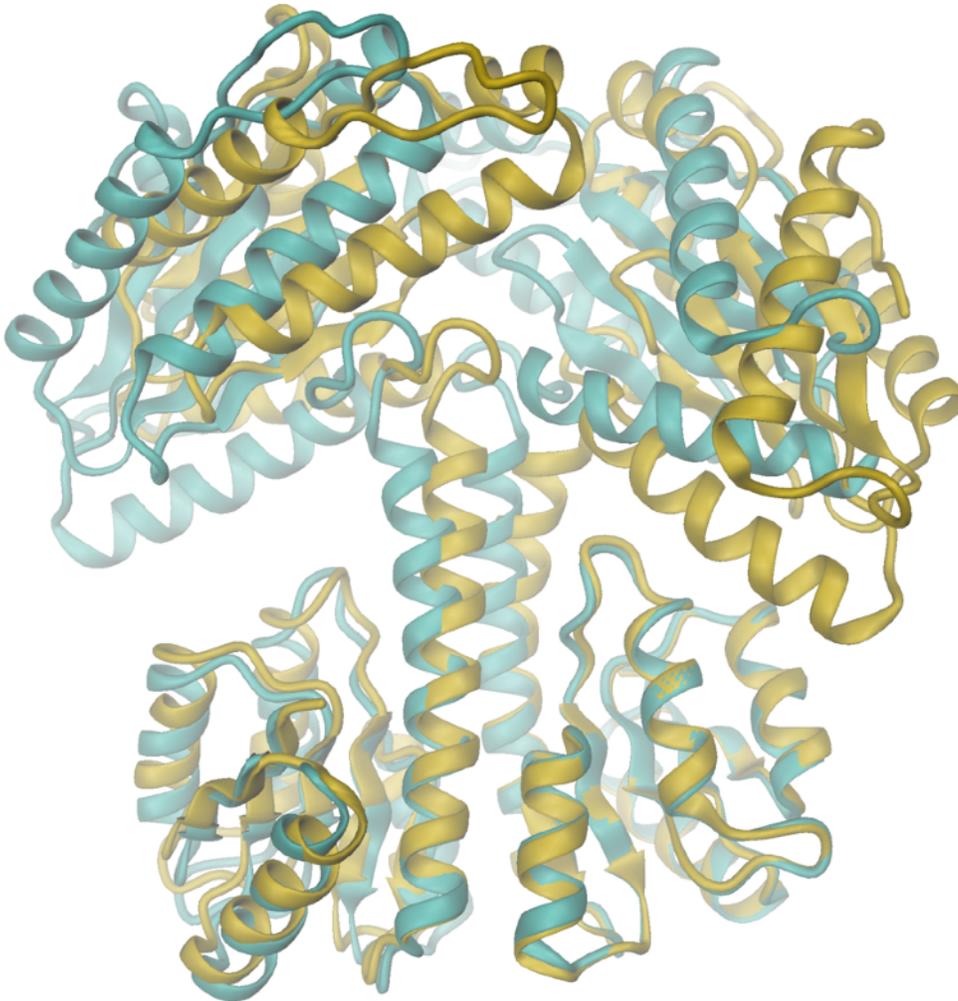
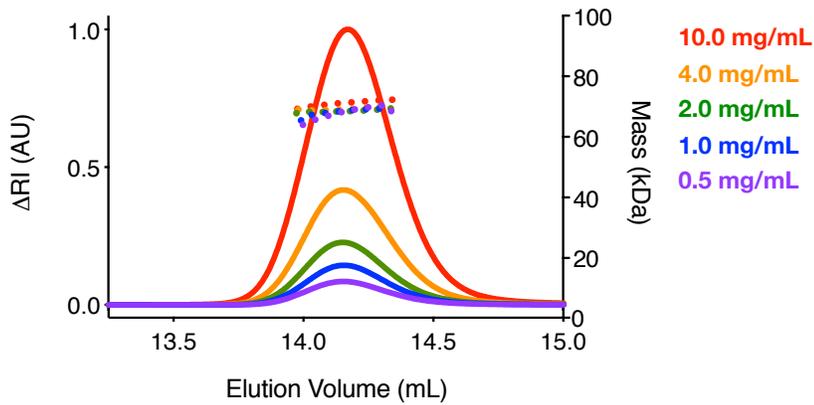


Figure 1—figure supplement 1. Superimposition of the two dimers of the asymmetric unit of the DgcR_act crystal structure. Dimers AB and CD were superimposed on their Rec part and are represented in turquoise and gold, respectively.

a



b

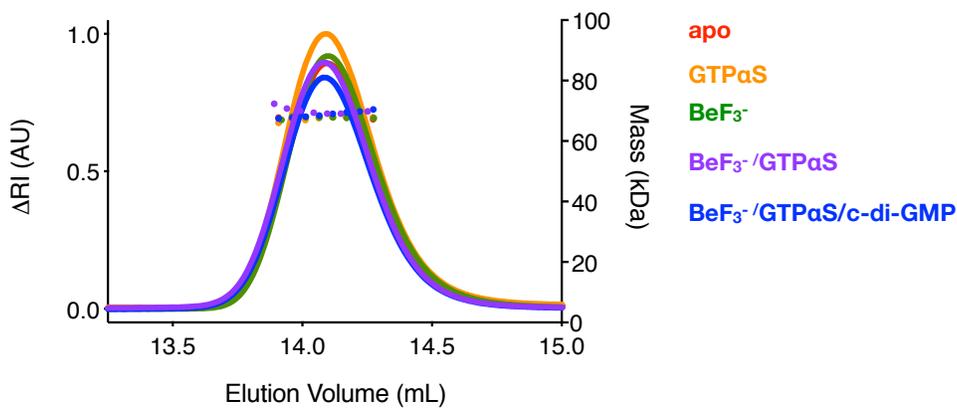


Figure 1 – figure supplement 2. DgcR is a constitutive dimer.

Oligomeric state of DgcR as analyzed by SEC-MALS as measured at the indicated **a**) loading concentrations (0.275 mM to 0.014 mM) and **b**) conditions (with GTPaS and c-di-GMP at a concentration of 2 mM and DgcR at a concentration of 0.14 mM). Molecular mass values (right axis) are shown by dotted lines and change in refractive index (left axis) by solid lines.

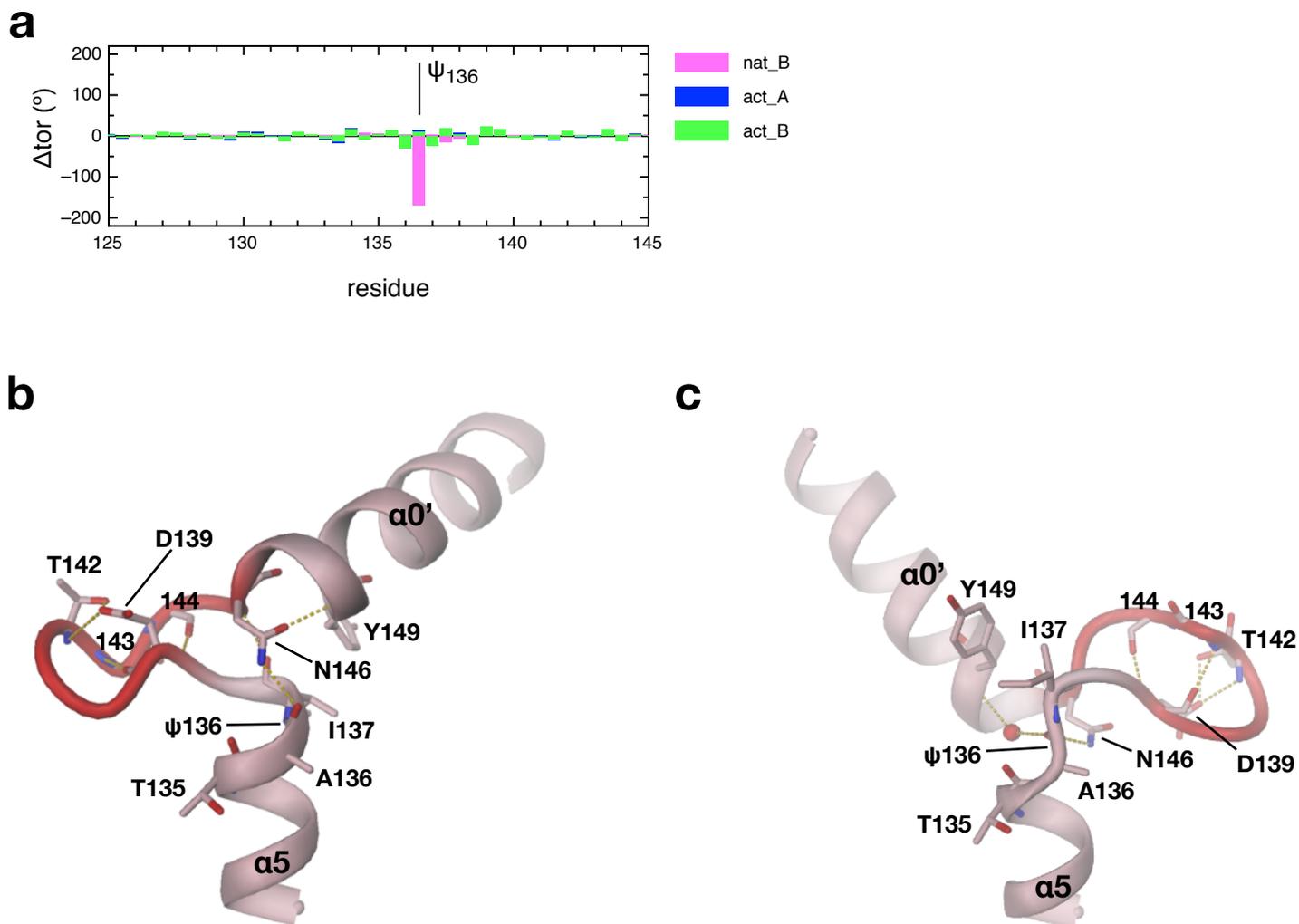


Figure 2. Inter-domain hinge revealed by comparison of the two DgcR_{nat} protomers.

a) Backbone torsion angle comparison of DgcR_{nat} and DgcR_{act} (nat_B, act_A and act_B) relative to nat_A. Note that the two DgcR_{nat} chains show a localized drastic difference in the main-chain torsion angle ψ_{136} .

b-c) Detailed view of the inter-domain hinge segment of chain A (panel **b**) and B (**c**) of DgcR_{nat}. The wide turn with the D139xLT142 motif is high-lighted in red. The 169° rotation of ψ_{136} drastically changes the relative angle between α_5 of the Rec and α_0' of the GGDEF domain.

a

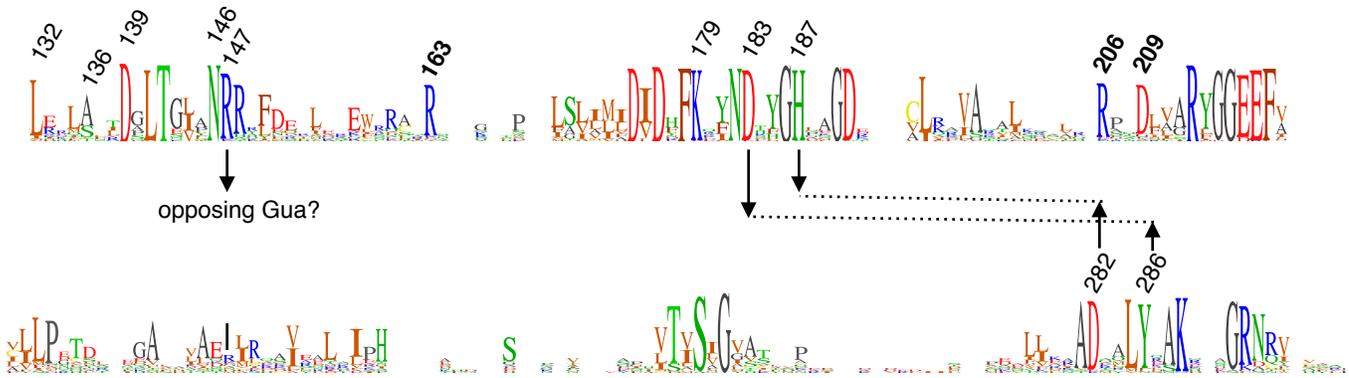


Figure 2—figure supplement 1. Sequence logo encompassing the GGDEF domain of Rec - GGDEF DGCs. The logo has been derived from the DgcR homologs of group 1 (see Fig. 10). Important residues are indicated by their number in DgcR (in bold for residues involved in c-di-GMP feed-back inhibition, see Fig. 8). Arrows indicate putative residues engaged in inter-domain contacts in the competent GGDEF dimer arrangement (Fig. 6b). R147 may interact with the Hogsteen-edge of the guanyl-base of the substrate bound to the opposing domain.

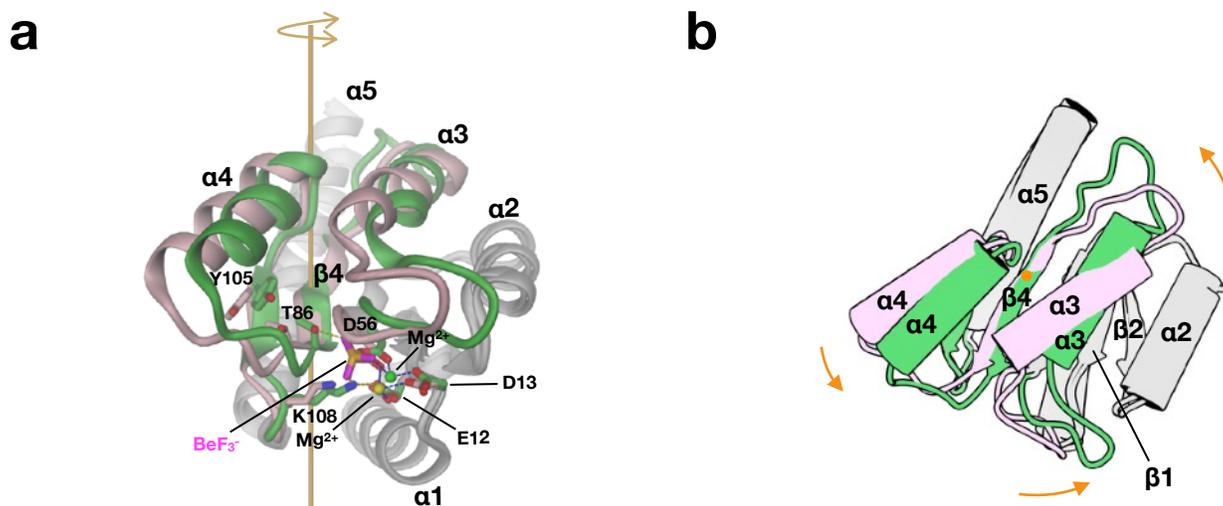


Figure 3. Beryllifluoride modification induces a relative 16° rotation of two Rec halves. Rigid-body 1 composed of $\alpha 3$, $\beta 4$, $\alpha 4$, $\beta 5$ (shown in pink and green for DgcR_nat and DgcR_act, respectively) is rotated with respect to the rest (rigid-body 2, grey) as seen after super-position of the two grey substructures.

a) Native and activated Rec domains with important residues shown in full view perpendicularly to the rotation axis of the relative rotation (orange). In DgcR_act, the beryllifluoride group forms an H-bond with T86 and an ionic interaction with K108 of DgcR_act.

b) Same as **a)**, but in cartoon representation and viewed along rotation axis.

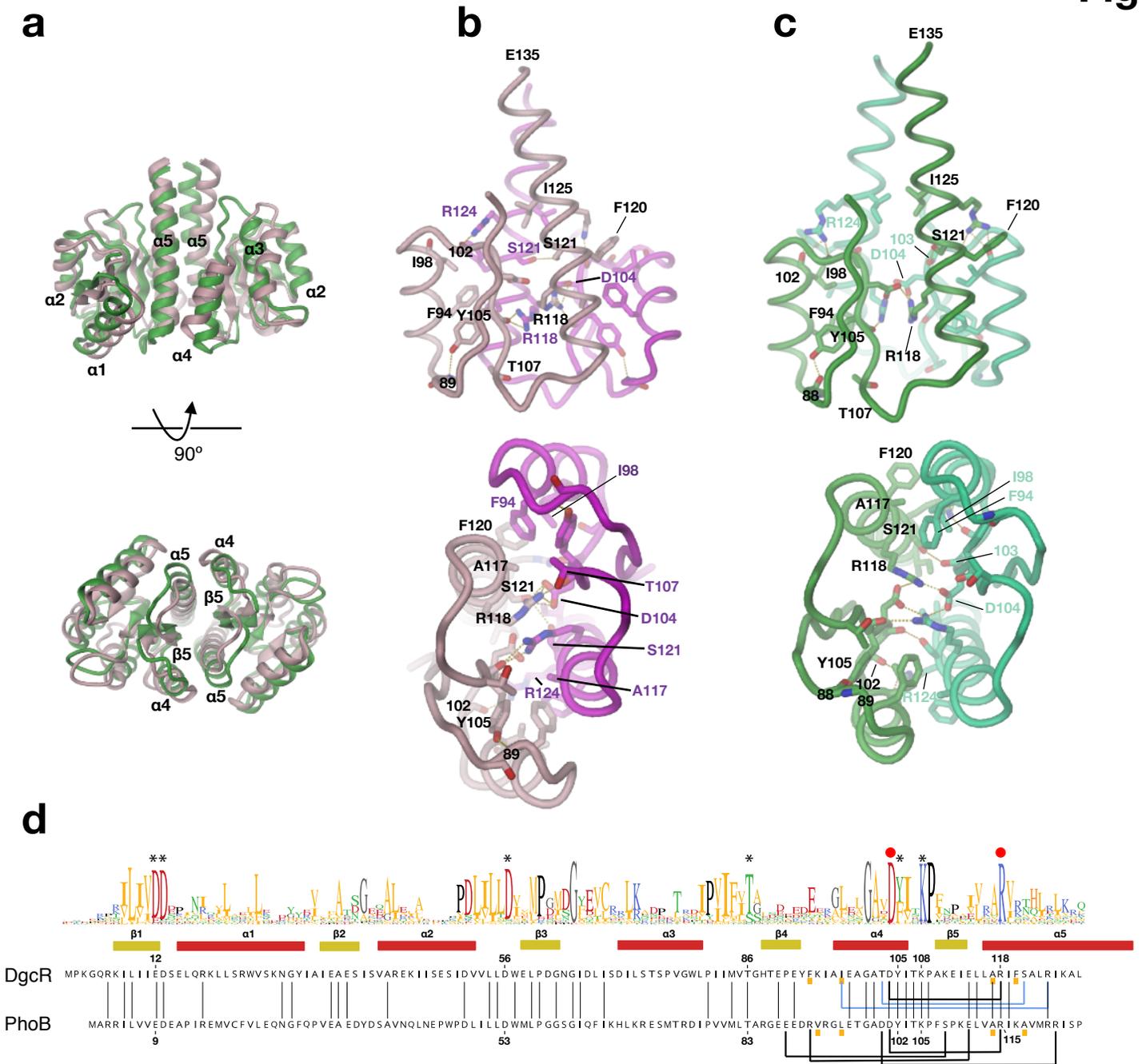


Figure 4. Distinct packing of Rec domains upon BeF₃⁻ modification.

a) Superimposed dimers with native structure in pink and activated structure in green.

b - c) Rec dimer of DgcR_{nat} (**b**) and DgcR_{act} (**c**) with contacts between the protomers <3.2 Å in yellow.

d) Sequence alignment of Rec domains of DgcR and PhoB with secondary structure elements of DgcR and sequence logo derived from DgcR group 1 homologs (see Fig. 10). Asterisks indicate conserved residues involved in Rec domain activation. Below the individual sequences, lines connect residues that participate in inter-domain contacts (side-chain - side-chain interactions in black, interactions involving the main-chain in blue). Red dots indicate D104 and R118 of the conserved inter-molecular salt-bridge.

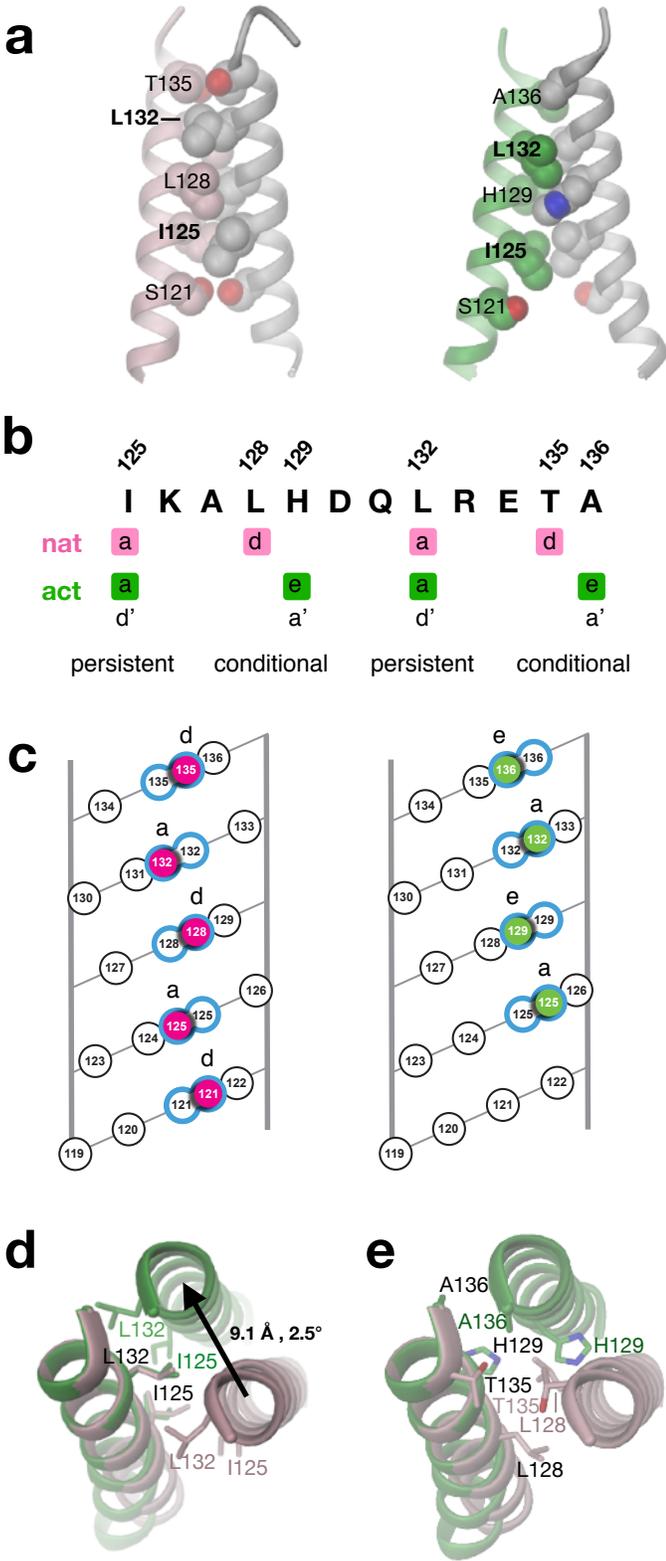


Figure 5. Coiled-coil linker adopts two alternative registers depending on activation state.

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Figure 5. Coiled-coil linker adopts two alternative registers depending on activation state.

a) Side view of parallel coiled-coil linker of DgcR_{nat} (left) and DgcR_{act} (right) with residues that form contacts with their symmetry equivalents in CPK representation. Only residues from the left helix are labeled. Bold labels indicate residues that are involved in both registers (persistent contacts).

b) Two alternative heptad repeat patterns (*a d a d* and *a e a e*), which are adopted by DgcR_{nat} and DgcR_{act}, respectively. Positions *a* are used in both registers (persistent contacts), whereas positions *d* or *e* are used only in the native or activated conformation (conditional contacts). Note, that the *a e a e* pattern can formally also be described by a *d' a' d' a'* pattern as indicated in the figure and used in (Gourinchas et al., 2017).

c) Helical net representation (Crick, 1953) of coiled-coil interactions in DgcR_{nat} (left) and DgcR_{act} (right). Of the front helix, only interacting residues are shown (high-lighted by color). Interacting residues pairs are outlined in blue.

d-e) Top view of coiled-coil after superposition of left helix. DgcR_{nat} and DgcR_{act} are shown in pink and green, respectively. For clarity, residues forming persistent and conditional contacts are shown in the separate panels **d** and **e**, respectively.

Figure 5—figure supplement 1



Figure 5—figure supplement 1. Alignment of selected DGCs comprising C-terminal end of Rec $\alpha 5$ and beginning of GGDEF domain.

DgcR and 3 other well studied DGCs were included in the alignment: Rrp1 from *Borrelia burgdorferi*, PadC from *Idiomarina sp. A28L* and WspR from *Pseudomonas aeruginosa* (wild type and GCN4 hybrid). Sequence numbers relative to DxLT motif are given on the top. Asterisks denote crystallographically observed coiled-coil contacts (black: persistent contacts; colored: conditional contacts, i.e. contacts formed only in the native (pink) or activated (green) register).

^{*}) DgcR_{nat} and DgcR_{act} of this study.

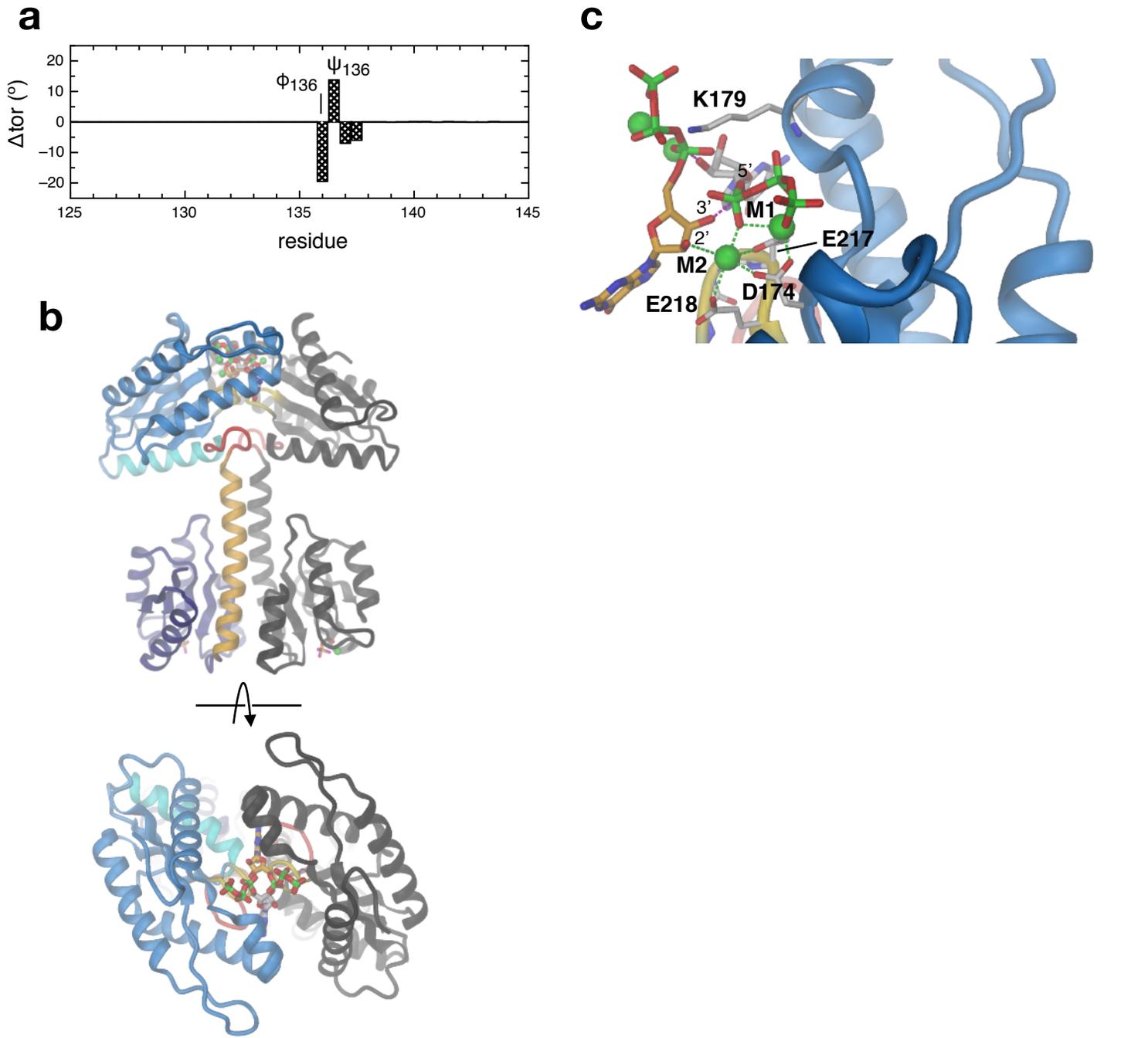


Figure 6. The activated Rec dimer allows formation of a catalytically competent GGDEF dimer.

a) Changes in main-chain dihedral angles applied manually to DgcR_act to move the two GGDEF domains into catalytically competent arrangement.

b) Model of competent DgcR (two orthogonal views) generated as described in **a**.

c) Detailed view of the competent juxtaposition of the two GGDEF bound GTP substrate molecules. The carbon atoms of the two GTP molecules are colored in orange and gray, respectively. The O3' hydroxyl of each ligand is poised for nucleophilic attack on the α -phosphorous (PA) of the other ligand being roughly inline with the scissile PA-O3A bond.

Figure 6—figure supplement 1

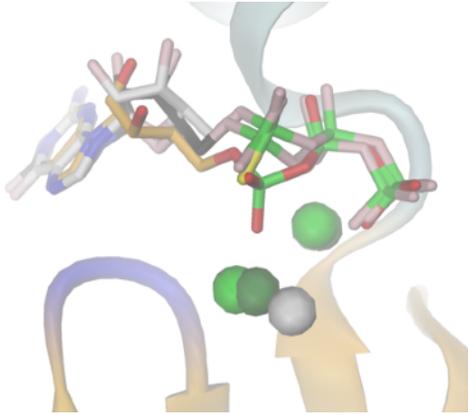


Figure 6—figure supplement 1. Superposition of GTP analog structures as bound to GGDEF domains.

3'dGTP/Mg⁺⁺ as found bound to DgcR_act (cartoon) is shown with yellow carbons and light green spheres. 3'dGTP/Mg⁺⁺ as found bound to DgcR_nat is shown with pink carbons and dark green spheres. The 3'-OH groups of 3'dGTP have been reconstructed. GTP- α S/Ca⁺⁺ as bound to DosC (4ZVF) is shown with pink carbons and a grey sphere.



Figure 7. Structural transitions in DgcR upon Rec pseudo-phosphorylation (a - d) followed by GGDEF hinge motions (e-f) to attain the catalytically competent state.

Continued on next page.

Figure 7. Structural transitions in DgcR upon Rec pseudo-phosphorylation (a - d) followed by GGDEF hinge motions (e-f) to attain the catalytically competent state.

The structures are represented as in Figs. 1b,c, but with the residues of the conditional coiled-coil contacts shown as CPK models (residues in *d* and *e* position are shown in pink and green). The beryllofluoride moieties of the dimer are highlighted by magenta circles.

- a)** DgcR_{nat}, symmetrized version with both GGDEF domains in B-chain orientation (cf. with Fig. 1b).
- b)** As in **a**, but with beryllofluoride-induced tertiary change applied to Rec rigid_body 1 (see Fig. 3).
- c)** As in **b**, but with quaternary change applied to Rec domains. Note the clash between the C-terminal ends of the coiled-coil (red circle).
- d)** As in **c**, but with Rec dimer as found in symmetrized version of DgcR_{act}.
- e)** Symmetrized version of DgcR_{act} (cf. with Fig. 1c).
- f)** Model of catalytically competent DgcR as in Fig. 6b.
- g)** Animation (two views) of the structural transitions between the states shown in panels **a** to **f** obtained by morphing. The magenta broken lines (top view, right) connect the reacting atoms of the two substrates, i.e. O3' with Pa of the opposing substrate.

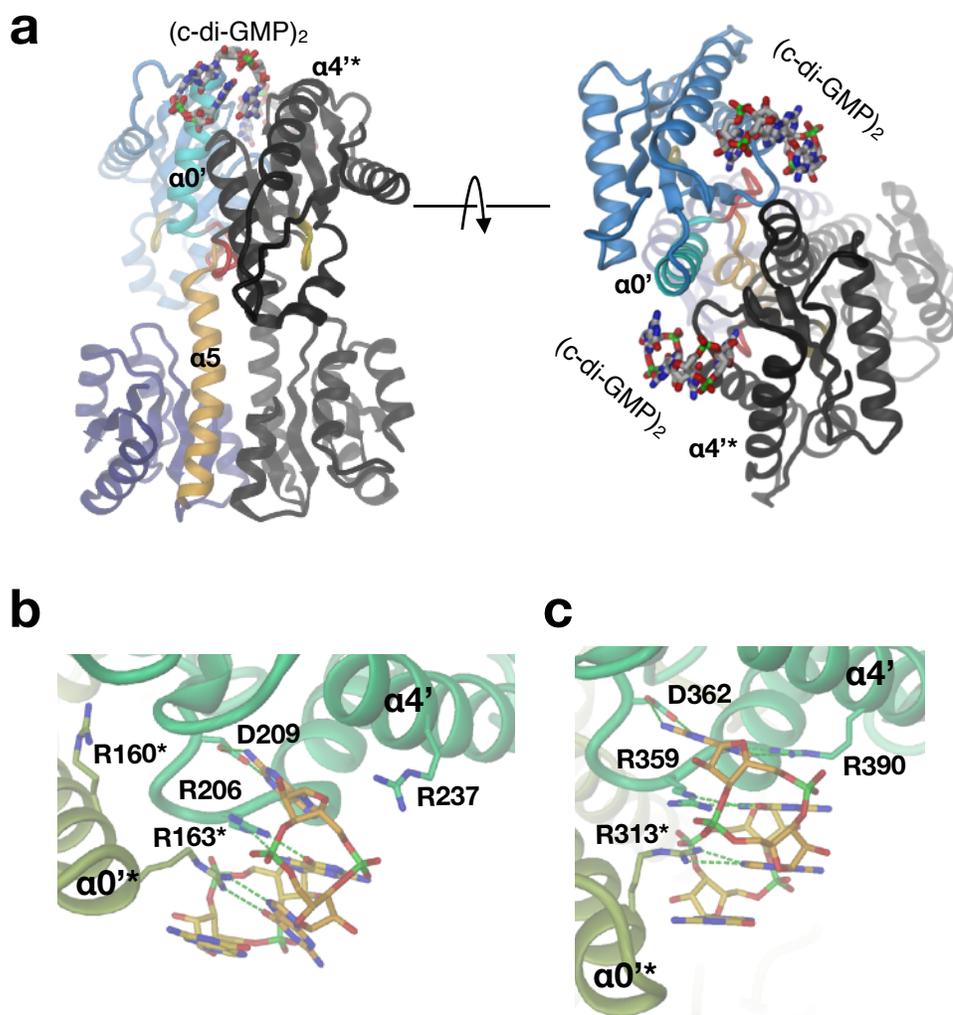


Figure 8. Dimeric c-di-GMP cross-links the GGDEF domain of the dimer.

a) Side and top views of the DgcR/c-di-GMP complex (DgcR_inh). Representation as in Fig. 1b.

b-c) Detailed comparison of the c-di-GMP binding mode in DgcR (**b**) and PleD (2V0N) (**c**). Protomers are distinguished by color hue. H-bonds are shown as green broken lines.

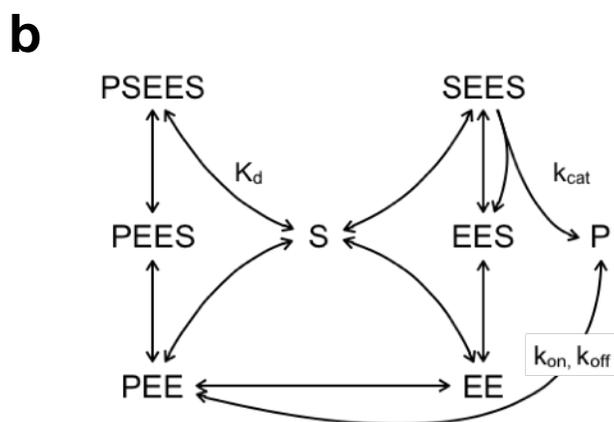
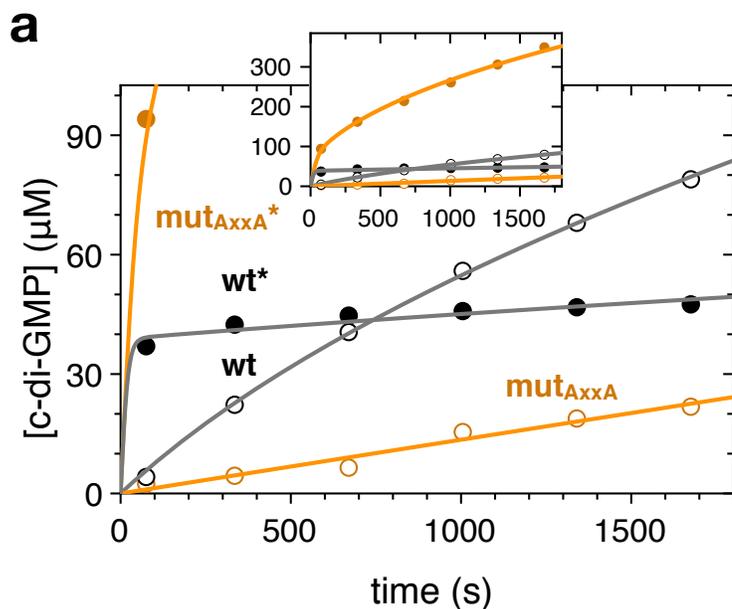
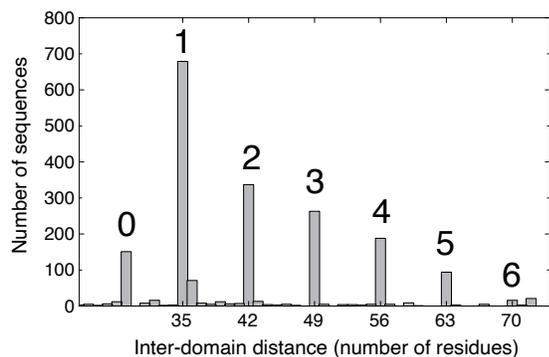


Figure 9. Enzyme kinetics of DgcR.

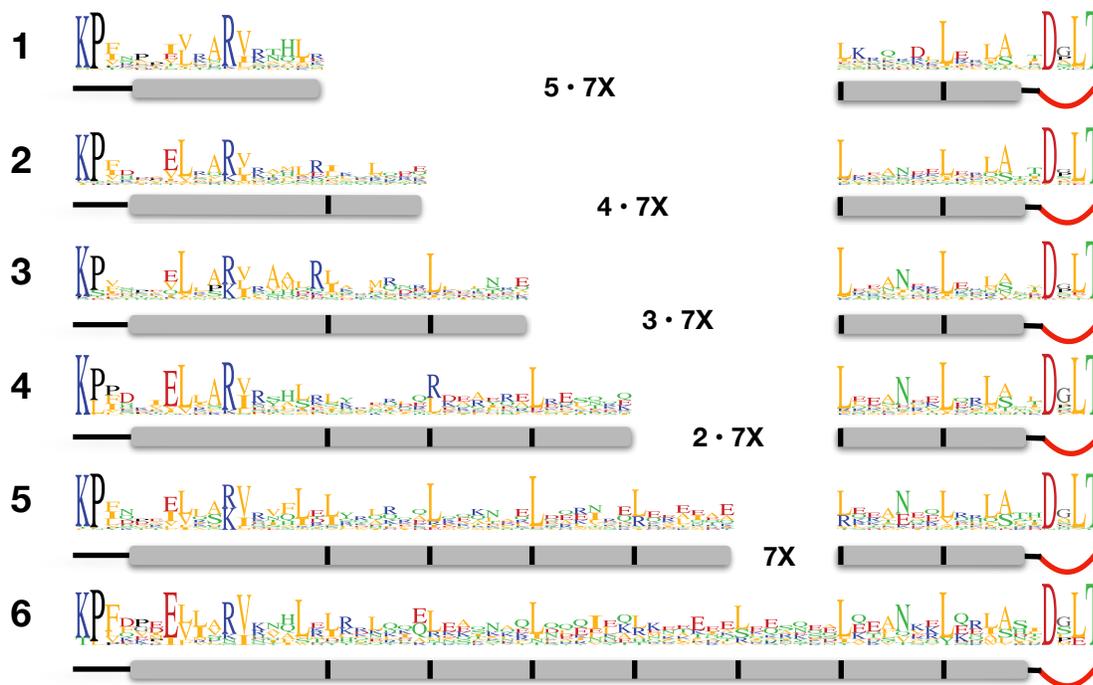
a) Enzymatic progress curves of DgcR and DgcR_{AxxA} in the native and in the activated (indicated by an asterisk) state. Experiments were performed at 5 μ M enzyme and 500 μ M GTP substrate concentration. Symbols denote oIEC measurements, continuous lines represent fit of the kinetic model shown in panel **b** to the data with parameters listed in Table 2.

b) Kinetic model of diguanylate cyclase activity controlled by non-competitive product binding. Substrate (S) binding to the dimeric enzyme (EE) is modeled with the equilibrium dissociation constant K_d and assumed to be unaffected by the presence of S in the second binding site or of product (P) in the allosteric site. Product binding is modeled kinetically with rate constants k_{on} and k_{off} . Note that the model considers simply one instead of four product binding sites on the enzyme. Only the Michaelis-Menten complex with two bound substrate molecules and no bound product (SEES) is competent to catalyze the $S + S \rightarrow P$ condensation reaction (with turn-over number k_{cat}).

a



b



c

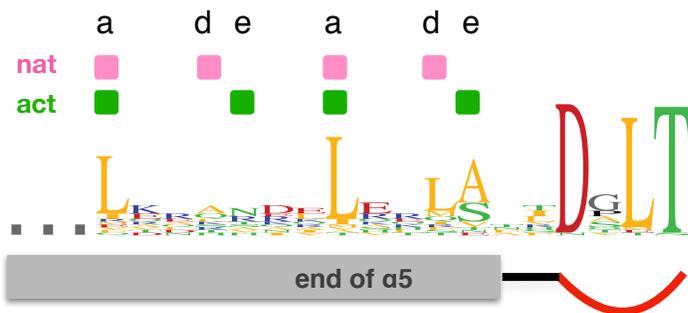


Figure 10. The linker helices of Rec-GGDEF proteins show heptad repeat patterns and discretized lengths.

a) Histogram of inter-domain distances as measured from Rec KP motif to GGDEF DxLT motif.

b) Sequence logos of inter-domain linkers of groups 1 to 6 of panel **a**. DgcR belongs to group 1. The grey rectangles symbolise the predicted $\alpha 5$ helices, black bars indicate recurring hydrophobic positions spaced with a distance of 7. Data were compiled from 1991 Rec-GGDEF sequences, see Methods.

c) Overall logo of C-terminal part of all sequences shown in panel **b**. Positions engaged in parallel coiled-coil interactions in DgcR_{nat} and DgcR_{act}, are indicated in pink and green, respectively (see also Fig. 5b).