Activation mechanism of a small prototypic Rec-GGDEF diguanylate cyclase

Raphael D. Teixeira1, Fabian Holzschuh1 & Tilman Schirmer1

Diguanylate cyclases synthesising the bacterial second messenger c-di-GMP are found to be regulated by a variety of sensory input domains that control the activity of their catalytical GGDEF domain, but how activation proceeds mechanistically is, apart from a few examples, still largely unknown. As part of two-component systems, they are activated by cognate histidine kinases that phosphorylate their Rec input domains. DgcR from Leptospira biflexa is a constitutively dimeric prototype of this class of diguanylate cyclases. Full-length crystal structures reveal that BeF₃⁻ pseudo-phosphorylation induces a relative rotation of two rigid halves in the Rec domain. This is coupled to a reorganisation of the dimeric structure with concomitant switching of the coiled-coil linker to an alternative heptad register. Finally, the activated register allows the two substrate-loaded GGDEF domains, which are linked to the end of the coiled-coil via a localised hinge, to move into a catalytically competent dimeric arrangement. Bioinformatic analyses suggest that the binary register switch mechanism is utilised by many diguanylate cyclases with N-terminal coiled-coil linkers.
DgcR is a constitutive dimer that is activated by domain rearrangements. To reveal the structural changes accompanying the activation of Rec-GGDEF DGCs we determined the full-length crystal structures of DgcR in native and pseudo-phosphorylated (Be⁵⁺) modified state. A DgcR variant (R206A/D209A, abbreviated DgcR_AxxA) that had the putative allosteric inhibition site (Fig. 1a) mutated was used to avoid locking the enzyme in a product-inhibited conformation. Crystallisation was performed in presence of 3'-deoxy-GTP (3'GTP), which is a non-competent substrate analogue due to the absence of the 3'-hydroxyl group. The structure of native DgcR_AxxA (called DgcR), as shown in Table 1, was solved by molecular replacement to 2.2 Å resolution (Supplementary Table 1). There is one dimer in the asymmetric unit with the protomers held together by extensive isologous contacts between the Rec domains involving their α4-β5-α5 face (Fig. 1b). Notably, the electron density of the Rec domains is considerably weaker than that of the GGDEF domains, indicating a larger mobility. The Rec domain shows the canonical (β1αα) fold (rmsd of 1.5 Å for 116 Ca atoms with respect to PhoP, 2PKX), but with the C-terminal α5-helix considerably extended and forming together with its symmetry mate a coiled-coil, leading to the GGDEF domains. A Mg²⁺ ion is bound to the acidic pocket formed by E12, D13, and the phosphorylatable D56.

The structure of the GGDEF domain is very similar to others in the PDB database (rmsd of 1.4 Å for 157 Ca atoms with respect to PleD, 2V0N) and shows the canonical (β1-α1-α2-β2-β3-α3-β4-α4-β5) topology of nucleotidyl cyclases of group III¹⁹ with an N-terminal extension that starts with a characteristic wide turn showing a DxLT motif followed by helix a0 that leads to β1 (Fig. 1b, see also ref. 10). The GG(D/E)EF motif is located at the turn of the β2-β3 hairpin. Again as observed in other structures¹², the guanine base of the substrate analogue is bound to a pocket between α1 and α2 and forms H-bonds with N182 and D191, whereas the two terminal phosphates are H-bonded to mainchain amides of the short loop between β1 and α1. Additionally, the γ-phosphate forms ionic interactions with K289 and R293. Two magnesium ions are bound to the usual positions being complexed to the β- and γ-phosphates and the side-chain carboxylates of D174, E217, and E218.

The GGDEF domains do not obey the twofold symmetry of the Rec domains, but form a relative angle of about 90°. Thus, the two active sites with the bound GTP analogues do not face each other. Rendering this constellation non-productive. Though the constellation may be determined to some extent by crystal packing, it demonstrates considerable inter-domain flexibility. Comparison of the main-chain torsion angles reveals that the relative rotation can be traced back to a 169° change in a single torsion, namely around the Cα–C bond of residue 136 (ψ136, Fig. 2a). Thus, the hinge locates to the C-terminal end of Rec α5, with the following residue I137 being packed against the Y149 from the beginning of the GGDEF α0’ helix in both chains (Fig. 2b, c). As noted before¹⁰, the conserved residue N146 (Supplementary Fig. 6) is capping both α5 and α0’, but only in the A-chain.

The structure of activated DgcR_AxxA (called DgcR*) obtained by BeF⁵⁻ modification was solved by molecular replacement to 2.8 Å resolution (Fig. 1c) (Supplementary Table 1). There are two dimers in the asymmetric unit that show virtually...
the same Rec dimer structure, but slightly different α5-helix bending and GGDEF orientations (Supplementary Fig. 1). As in DgcR', the dimer is formed by isologous contacts between the α4-β5-α5 Rec faces and the extension of α5 forms a coiled-coil, but with an altered relative disposition, which will be described in detail further below. D56 is found fully modified by BeF3- and its immediate environment is different compared to DgcR' as will be discussed in detail hereafter. The GGDEF domains are arranged symmetrical with the two bound 3'dGTP ligands facing each other, but too distant for catalysis (Fig. 1c, bottom). The GGDEF orientation relative to the Rec domain is similar as in the A-chain of DgcR'. As in the DgcR' structure, there are no direct contacts between the domains of a protomer.

Consistent with the crystal structures and the presence of the coiled-coil in both states, in solution, DgcR is a constitutive dimer as measured by Multi-Angle Light Scattering (MALS) both in the native and the activated form (Supplementary Fig. 2). Addition of substrate analogue, product or lowering salt concentration did not change the quaternary state.

Aspartate modification induces a relative rigid-body rotation within the Rec domain. Comparison of native and activated DgcR' (Fig. 3a) shows that, on activation, the hydroxyl group of T86 is moved towards the BeF3- moiety to form an H-bond. The void left by this movement is claimed by Y105 that undergoes a small side-chain rotation, but does not change its rotamer.
Furthermore, K108 forms ionic interactions with BeF$_3^-$ and E12 in the activated structure (Fig. 3a). In the native state, a magnesium ion is bound loosely to E13 and D56, whereas, in the active state, it is additionally coordinated by the BeF$_3^-$ moiety.

Activation of DgcR is accompanied with a change in the backbone structure as identified by a DynDom analysis$^{20}$. The Rec domain can be divided into two parts that undergo a relative 16° rotation as shown in Fig. 3b. Thereby secondary structure elements a3 to b5 (residues 54 to 108) behave as one rigid body (rmsd = 0.83 Å/49) that rotates relative to the rest (8–53, 109–135) that superimposes with an rmsd of 1.19 Å for 67 Ca positions. Figure 3b shows that the rotation axis passes roughly perpendicular to the β-sheet through the centre of β4 (L83). Note that the phosphorylatable D56 is close to the junction between the two rigid bodies and that its Ca position only changes slightly during the transition. T86, however, with its distance of 7.5 Å
from the rotation axis moves by 2.0 (Ca) to 3.3 Å (Oy) and the motion is most pronounced (5.2 Å) for the N-terminus of α3 (P91) with its distance of about 15 Å from rotation axis. Thus, the rigid-body motion changes significantly the arrangement of α4 with respect to α5, which has a profound effect on the packing of the Rec domains in the dimer.

For many Rec domains, a Y-T coupling mechanism has been described, where, upon (pseudo-) phosphorylation a threonine/serine (T86 in DgcR) is dragged towards the phosphate and the conserved tyrosine/phenylalanine (Y105 in DgcR) follows suite with a rotameric change from gauche\(^+\) to trans\(^12,21,22\). In DgcR, the conserved tyrosine is already in trans conformation before activation and the T and Y move concertedly towards the beryllofluoride moiety as part of a rigid-body (α3 to β5) movement (Fig. 3).

**Rigid-body rotation induces repacking of Rec domains within the dimer.** In both states, the Rec domains form twofold symmetric dimers with the contacts mediated by isologous interactions between the α4 - β5 - α5 surfaces (Fig. 4). However, due to the rigid-body motion within the protomer and the concomitant relative displacement of α4 and α5 (Fig. 3), the association of the α4-β5-α5 faces is different in the native and the activated state. Therefore, the two dimers superimpose rather poorly (rmsd = 3.1 Å/119 Ca positions) with the β-sheets of the protomers showing a difference in orientation of about 15° (Fig. 4a).

The native Rec dimer (Fig. 4b) with a buried surface area of 980 Å\(^2\) is held together by an extended apolar contact of α5 (A117, F120) with α4 (F94, I98), an ionic interaction of D104 with R118, an H-bond between main-chain carbonyl 102 and R124 (both β5-α5 contacts). All aforementioned residues are well-defined with the exception of the R118 side-chain, which probably has several alternative conformations, but all placing the guanidinium group close to D104 and to its symmetry mate.

Finally, and most relevant for the allosteric regulation of the C-terminal GGDEF effector domains, there are regular coiled-coil interactions across the symmetry axis between the C-terminal
halves of the α5 helices starting with S121. These will be discussed in the next chapter. The activated Rec dimer (Fig. 4c) with a buried surface area of 850 Å² shows the same apolar α5-α4 cluster as the native dimer, but with the residues repacked in-line with the aforementioned relative displacement of α4 and α5 within the protomer. At the centre of the interface, D104 shows a well-defined, intermolecular salt-bridge with R118, but also with R118 from the same chain. As in the native dimer, the R124 and S121 side-chains form intermolecular H-bonds, but with other partners compared to the native interactions (main-chain carbonyls of 96 and 103, respectively). A BLAST search revealed that, apart from Rec-GGDEF orthologs, the sequence of the DgcR Rec domain is most similar to that of OmpR-like transcription factors (Fig. 4d). These have a Rec-DNA-binding domain architecture and have been shown to dimerise via the Rec α4-β5-α5 face upon activation to allow binding of their effector domains to DNA. Indeed, a structure search of the DgcR* dimer against the PDB retrieved as top hit (rmsd = 1.5 Å/228 Ca positions) the BeF₃-activated Rec domain of PhoB (1ZES). Most of the intermolecular interactions are thereby conserved, in particular the central salt-bridge D109 - R118 (DgcR numbering), or conservatively replaced (Fig. 4d). To our knowledge, no response regulator with a DNA binding effector domain has yet been observed as a constitutive α4-β5-α5 dimer (for a review, see ref. 8), which is probably due to their small or absent coiled-coil linkers. A special case is the Rec-Rec'-GGDEF protein PleD, where, in the activated state, the two Rec domains of each chain form a quasi twofold intra-molecular dimer involving their α4-β5-α5 faces and exhibiting OmpR-like inter-domain salt-bridges. Such assembled Rec-Rec' domains are then instrumental for the subsequent dimerisation of two protomers and, thus, enzyme activation. Summarising, beryllium-fluoride modification of D56 induces a relative rigid-body motion in the Rec domain that changes the relative disposition of α4 and α5. Consequently, since both helices are part of the Rec–Rec interface, the relative arrangement of the protomers and, thus, of the two α5 helices of the dimer is changed (compare top panels of Fig. 4b, c). This change is supposed to be crucial for the allosteric regulation of the C-terminal GGDEF domains as will be discussed in the following.

**Lateral shift of C-terminal Rec helices changes their coiled-coil register.** The DgcR Rec α5-helix is longer by about three turns (10 residues) compared to that of canonical Rec domains. In the dimer, these protrusions form a twofold symmetric coiled-coil both in the native and the activated state (Fig. 1b, c), though with distinct relative arrangement. Both constellations are stabilised by isologous contacts between predominantly hydrophobic residues that obey a heptad repeat pattern (Fig. 5a, b). Thereby, I125 and L132 contribute to the contact in both structures (position a; persistent contacts), but with the side-chains interacting with their symmetry mates from opposite sides depending on the state (see, e.g., the 132–132 contact in Fig. 5a). In contrast, other residues contribute either only to the native (L128, T135) or the activated (H129, A136) constellation (positions b, c; conditional contacts).

The two contact modes represent alternative knobs-into-holes packing as best seen in the helical net diagram of Fig. 5c, suggesting a relative lateral translation of the interacting helices. Indeed, superposition of one of the helices as in Fig. 5d, e reveals a large lateral shift of about 9 Å. In other words, upon activation, the two helices do not roll over each other (which would be accompanied by a change in their azimuthal angles), but are translated with respect to each other to realise an alternative knobs-into-holes packing. Note, that for steric reasons this shift would require dissociation and reassembly of the constituting helices. Thus, the coiled-coil behaves like a binary switch that can assume two clearly defined states, i.e., two distinct registers.

Recently, an analogous transition in the coiled-coil linker of a diguanylate cyclase has been proposed for phytochrome-regulated PadC. Indeed, the C-terminal end of the coiled-coil of the dark-state enzyme is in the same register as native DgcR with, amongst others, N518 and L525 forming (conditional) contacts (see PadC in Supplementary Fig. 4). Inspection of the linker sequence and dynamic considerations prompted the authors to propose an alternative register involving the neighbouring residues N519 and A526 for the illuminated state. Indeed, mutations designed to stabilise this second register were constitutively active and the coiled-coil was found in the active register (Supplementary Fig. 4). Although the structure of light-activated wild-type PadC is not known, it is very likely that PadC and DgcR use the same binary coiled-coil switch mechanism for DGC regulation, despite unrelated input domains.

WspR, another well-studied Rec-GGDEF diguanylate cyclase, also exhibits a “slippery” ααxdexx hexad repeat with e of the last repeat in position −3 with respect to the DxLT motif (Supplementary Fig. 4). Unfortunately, only product bound structures are available that reveal a non-productive, c-di-GMP cross-linked tetramer in which the coiled-coils emanating from the two Rec dimers are splayed apart at their ends. Most revealing, however, a GCN4-GGDEF WspR with GCN4 interface residues in the active register (Supplementary Fig. 4) was reported to be highly active and a corresponding structure (compact dimer) was predicted for active WspR. Summarising, the change in coiled-coil registration upon DgcR activation is accompanied by a substantial lateral shift of the constituting helices, which lead directly to the catalytic domains. Structural data on other DGCs are consistent with this finding.

Small rotation around inter-domain hinge allows formation of competent GGDEF dimer. In the activated structure, the two GGDEF domains show no mutual interactions and their precise orientation appears to be determined by crystal contacts. However, the two bound 3′dGTP ligands face each other, though their distance (>10 Å) is clearly too large for catalysis (see Fig. 1c). Having identified the CA-C main-chain bond of A136 as an inter-domain hinge (Fig. 2a), we tried, by small changes in the linker sequence and dynamic considerations prompted the authors to propose an alternative register involving the neighbouring residues N519 and A526 for the illuminated state. Indeed, mutations designed to stabilise this second register were constitutively active and the coiled-coil was found in the active register (Supplementary Fig. 4). Although the structure of light-activated wild-type PadC is not known, it is very likely that PadC and DgcR use the same binary coiled-coil switch mechanism for DGC regulation, despite unrelated input domains.

WspR, another well-studied Rec-GGDEF diguanylate cyclase, also exhibits a “slippery” ααxdexx hexad repeat with e of the last repeat in position −3 with respect to the DxLT motif (Supplementary Fig. 4). Unfortunately, only product bound structures are available that reveal a non-productive, c-di-GMP cross-linked tetramer in which the coiled-coils emanating from the two Rec dimers are splayed apart at their ends. Most revealing, however, a GCN4-GGDEF WspR with GCN4 interface residues in the active register (Supplementary Fig. 4) was reported to be highly active and a corresponding structure (compact dimer) was predicted for active WspR. Summarising, the change in coiled-coil registration upon DgcR activation is accompanied by a substantial lateral shift of the constituting helices, which lead directly to the catalytic domains. Structural data on other DGCs are consistent with this finding.

The details of the Michaelis–Menten complex shown in Fig. 6c are consistent with the model proposed in ref. 7 with metal M2 coordinating the 2′-hydroxyl group and K179 hovering over the α-phosphate of the incoming substrate. There is no titratable residue close to the O3′-group. Most likely,
deprotonation of the hydroxyl group proceeds via a water molecule that could be activated by the close-by metal(s) as, e.g., in adenylate cyclases26.

In the competent dimer, there are no clashes between the catalytic domains. Molecular dynamics simulations would be required to refine the model, but it appears that D183 and D282 may interact with Y286 and H187, respectively. All these residues are conserved in diguanylate cyclases (Supplementary Fig. 6). Indeed, in the apo-structure of the constitutively active variant of PadC (6ET724) the proposed interactions seem well possible, albeit only in one half of the asymmetric structure.

There is one more conserved residue that projects to the other subunit, namely R147 (Supplementary Fig. 6). Judged by the model, it appears possible that this arginine may interact with the guanyl Hoogsteen-edge of the opposing substrate. This would be supported by a recent study on the promiscuous (accepting GTP and ATP) DGC GacA, wherein the reason for the relaxed substrate specificity was attributed to an aspartate-serine replacement of a base-binding residue27. In the sub-group of promiscuous DGCs, the position homologous to R147 of DgcR is not conserved (sequence logo in Fig. 6—figure supplement 1D of ref. 27), suggesting that the arginine is no longer important, since it cannot interact with a adenyl Hoogsteen-edge. In the same paper, the fourth residue of the GGDEF motif (equivalent to E218 in DgcR) was proposed to deprotonate the 3'-hydroxyl group of the substrate bound to the other subunit. In our model (Fig. 6c), this residue is coordinating metal M2 and clearly not close to this substrate hydroxyl group.

Structural coupling of Rec modification with competent dimer formation. The knowledge of the structures of full-length DgcR both in its native and activated form and the model of the Michaelis–Menten complex allows to discuss in detail how signal perception (phosphorylation) is coupled to output activation in a prototypic response regulator with enzymatic function. In Fig. 7, this process is dissected into 5 notional steps.

1. Starting with a symmetrized version of DgcR (Fig. 7a), aspartate pseudo-phosphorylation induces a rigid-body motion within each Rec domain (Fig. 7b, tertiary change). With an unchanged coiled-coil packing, the intermolecular α4–α5 contacts would break up. 2. This is counteracted by a repacking of the two Rec domains (Fig. 7c, quaternary change. 3) The clashing of the C-terminal ends of the coiled-coil is relieved by slight outward bending of the helices (Fig. 7d). Obviously, these first three steps, which describe the transition of the native to the activated Rec stalk, will be tightly coupled.

The following steps invoke no direct Rec-GGDEF communication, but only an unrestricted rotation of the GGDEF domains around the inter-domain hinges. With the Rec stalk in its activated constellation, the hinges are positioned such that the GGDEF domains can attain (4) a constellation as in DgcR*...
and, finally, assemble to form (5) the catalytically competent constellation Michaelis–Menten complex (Fig. 7f). An animation of the entire structural transition from native to competent DgcR is shown in Supplementary Movies 1 and 2. Noteworthy, the two GGDEF domains most likely won’t be able to attain the productive arrangement when the Rec dimer is in the native form, since the distance between the inter-domain hinges is changed considerably upon activation/deactivation (Fig. 6b). The aspect of conformational sampling and its dependence on the coiled-coil register and the dynamics of the entire enzyme has been discussed before for PadC.

Allosteric inhibition by product mediated domain cross-linking. Allosteric product inhibition by c-di-GMP is a well-known feature of many DGCs. Hereby, dimeric c-di-GMP mutually cross-links a RxxD motif (primary I-site, I_p) on one GGDEF domain with a secondary I-site (I_s) on the other GGDEF domain and vice-versa. The crystal structure of DgcR obtained in presence of c-di-GMP (DgcR_inh) was determined to 3.3 Å by molecular replacement and is shown in Fig. 8a.
Supplementary Table 1). There are three symmetric dimers in the asymmetric unit. Each dimer shows a Rec stalk in native conformation and the two GGDEF domains have their active sites facing outwards. Dimeric c-di-GMP is cross-linking the domains by interacting with the R206xxD209 motif of one subunit and R163* from α0 of the other subunit (Fig. 8b). Owing to symmetry, there are two isologous cross-links within the DGC dimer. Comparison with the PleD/c-di-GMP complex12 (Fig. 8c) shows very similar binding, but with PleD providing an additional arginine (R390) to the Ip-site. The DgcR equivalent (R237) is too distant to interact, but this may happen with the Rec stalk in the activated conformation. Arginines 163* (DgcR) and R313* (PleD) fulfil the same role in c-di-GMP binding, but are not homologous on the sequence level. Indeed, it has been noted earlier that among GGDEF sequences (Paul et al. 2007) arginines are enriched at either position. The unique c-di-GMP stabilised GGDEF arrangement that differs drastically from DgcR (Fig. 1b) again demonstrates the large flexibility provided by the inter-domain hinge.

Kinetic analysis of DgcR activity reveals delay in non-competitive feed-back inhibition. The effect of activation and Ip-site mutation on DgcR catalysed c-di-GMP production was studied by a real-time nucleotide quantification assay (online ion-exchange chromatography, oIEC, Agustoni et al., in preparation, see Methods and example chromatograms in Suppl. Fig. 7). Figure 9b, c shows that product formation catalysed by native DgcR gradually decreases early-on (despite a large excess of substrate), indicative of non-competitive product inhibition. Indeed, the progress curve was found consistent with 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 (Supplementary Table 2).

A very different behaviour was observed for the activated enzyme (DgcR*) that produced very quickly (<75 s) a substantial amount of product (Fig. 9b, c). Figure 9a shows the initial burst as resolved by early time-point measurements using conventional IEC with EDTA to stop the reaction. The burst phase was followed by a phase of very small, virtually constant velocity. Such phenotype was clearly inconsistent with classical equilibrium models and seemed indicative of a slow transition to the product-inhibited state. Mechanistically, this transition would comprise (fast) product binding and (slow) reorganisation of the two GGDEF domains to acquire the inactive product cross-linked conformation (Fig. 8).

The progress curves were fitted with the kinetic model shown in Fig. 9d. Independent binding of two substrate molecules (S) to the dimeric enzyme (EE) was parametrised with both GGDEF domains in B-chain orientation (cf. with Fig. 1b). As in a, but with beryllium-induced tertiary change applied to Rec rigid_body 1 (see Fig. 3). 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). As in c, but with Rec dimer as found in symmetrized version of DgcR*. Symmetrized version of DgcR* (cf. with Fig. 1c). Model of catalytically competent DgcR as in Fig. 6b.

Fig. 7 Structural transitions in DgcR upon activation. Rec pseudo-phosphorylation induces steps 1 to 3, which are followed by GGDEF hinge motions of steps 4 and 5 to attain the catalytically competent state. See also Supplementary Movies 1 and 2. The structures are represented as in Fig. 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 beryllium moieties of the dimer are highlighted by magenta circles. a DgcR', symmetrized version with both GGDEF domains in B-chain orientation (cf. with Fig. 1b). b As in a, but with beryllium-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*. e Symmetrized version of DgcR* (cf. with Fig. 1c). f Model of catalytically competent DgcR as in Fig. 6b.
kinetically with an effective second-order rate constant $k_{\text{on}}$ (dependent on product and enzyme concentration) and a first-order rate constant $k_{\text{off}}$ with the inhibitory constant given by $K_i = k_{\text{off}}/k_{\text{on}}$. Note that for simplicity the model considers only one product binding site on the dimeric enzyme, while there are actually four (two c-di-GMP dimers). This simplification will affect the nominal value of $K_i$. Full kinetic modelling without this simplification and with explicit modelling of the conformational enzyme transition has been postponed to a follow-up study.

The kinetic model fits the biphasic curve of DgcR* very well (Fig. 9a, b) yielding the parameters given in Supplementary Table 2. The $k_{\text{cat}}$ of 0.33 s$^{-1}$ together with the slow kinetics of the active to inactive transition ($k_{\text{off}}$ about 10$^{-3}$ s$^{-1}$) explains the large build-up of product in the initial phase, which is followed by very low residual activity of the (equilibrated) sample due to the low $K_i$ of about 150 nM.

To validate the involvement of the RxxD motif in feed-back product inhibition as suggested by the crystal structure (Fig. 8) and shown for many other DGCs, but also to scrutinise the kinetic model, the AxxA variant (DgcR') was analysed. The activated variant (DgcR'*') is highly active (Fig. 9b, c) and the progress curve can be modelled with the same $k_{\text{cat}}$, but a drastically (almost 50-fold) increased $K_i$ as compared to wild-type (Supplementary Table 2). Thus, as intended, the mutations do not affect the catalytic efficiency of the activated enzyme, but render the enzyme largely insensitive to feed-back inhibition. Note that the mutations did not completely abolish inhibition, which may be explained by the remaining residues of the primary and secondary I-site (Fig. 8) still enabling (weak) product binding. The native variant showed a significantly lower activity than the native wild-type (Fig. 9b, c), but this difference in basal activity was not investigated further. Interestingly, for both wild-type and variant enzyme, the activated state was found to be more susceptible to product inhibition than the native states (Supplementary Table 2). A difference in the $K_i$ values is not surprising per se considering that formation of the back side cross-linked dimer should be influenced by the geometry of the dimeric Rec stem that is different in the two states.

Summarising, activated DgcR shows a pronounced initial burst of activity before entering the product-inhibited state with a rather slow kinetics probably reflecting domain reorganisation. The kinetic model (Fig. 9d) proved to reproduce all measured progress curves and the parameters (Supplementary Table 2) reflect the impact of activation and I_p-site mutation.

**Rec-GGDEF linker sequence profile are consistent with register shift mechanism.** DgcR has been selected as a prototypic
Fig. 9 Enzyme kinetics of DgcR. **a**–**c** Enzymatic progress curves of wild-type DgcR and inhibition relieved mutant DgcR* in the native and in the activated (indicated by asterisk) state. Experiments were performed with 5 μM enzyme and 500 μM GTP substrate concentrations. Symbols denote experimental values, continuous lines represent fit of the kinetic model shown in panel **d** to the data with parameters listed in Supplementary Table 2. **a** Progress curve of c-di-GMP production catalysed by DgcR* as measured by conventional IEC. **b** Progress curves as measured by oIEC catalysed with the indicated DgcR variants/states. **c** Zoom-in of **b**. **d** Kinetic model of diguanylate cyclase activity controlled by non-competitive product binding. Substrate (S) binding to the dimeric enzyme (EE) is modelled 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 modelled 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 catalyse the $S + S \rightarrow P$ condensation reaction (with turn-over number $k_{cat}$). Source data are provided as a Source Data file.
Rec-GGDEF enzyme of relatively small size (298 residues), but bioinformatic analysis showed that the linker length can vary considerably in this class of DGCs. This was surprising considering that the linker has a defined structure and seems crucial for signal transduction. However, the linker length histogram (Fig. 10a) shows that the lengths are not distributed uniformly, but exhibit discrete values separated by multiples of 7 (groups 1 to 6, with DgcR and WspR belonging to groups 1 and 4, respectively). Thus, members of the groups would merely differ in the number of double helical turns when forming parallel coiled-coils. Indeed, the individual sequence logos can easily be aligned (Fig. 10b) to reveal the striking repeat of leucines in every 7th position (heptad position a). Most interestingly, the last (and to a lesser degree the last but one) heptad repeat at the C-terminal end (Fig. 10c) shows a conserved axxdexx pattern as in DgcrR (Fig. 5). Thereby, the a positions are mainly occupied by Leu, while the d and e positions show more variation, but with most residue types (e.g., Ala, Ser, Asn) known to allow coiled-coil interaction. Probably, the variability in the conditional d and e positions reflects the requirement of weak interactions to allow conformational switching. Thus, a common binary register shift mechanism seems likely for members of all the groups. Group 0 (Fig. 10a, b) does not obey the linker length rule. Since it also has an (S/N)PLT instead of a DxLT motif, it probably has a different linkage and, therefore, activation mechanism.

A similar pattern of discretized coiled-coil lengths has been reported for PAS-GGDEF and LOV–GGDEF proteins4,30, which makes it tempting to speculate that input to effector signal transduction might work similarly as in Rec-GGDEF enzymes. However, further investigations into their sequence profiles are needed to see whether they also exhibit ambiguous axxdexx heptad repeats.

Conclusion

The presence of coiled-coil linkers between N-terminal regulatory and catalytic GGDEF domains in many diguanylate cyclases has been described and their role in signal transduction discussed4,9,13,30. Changes in the crossing angle or the azimuthal orientation of the helices upon activation were anticipated, but a repacking of the interface was not discussed, which was then seen first in the comparison of inactive and a constitutively activated variant of light-regulated PadC24. The now presented detailed structural analysis of DgcR in its native and pseudo-phosphorylated form allowed a comprehensive dissection of the
activation process for a full-length, wild-type Rec-GGDEF enzyme (Fig. 7). Tertiary and quaternary changes in the Rec input domains lead to a register shift in the coiled-coil linker repositioning the inter-domain hinge and, thus, the propensity of the GGDEF domains to attain the catalytically competent dimer constellation.

A register shift in the coiled-coil linker may be operational also for other enzymes with predicted coiled-coil linkers, e.g., DGCs with N-terminal GAF domains or trans-membrane helices. LOV sensor domains that carry a flavin-nucleotide chromophore and have been studied very well as part of HKs36,37 are different in that the coiled-coil forming α-helix is not part of the core fold, but rather an extension of the C-terminal β-strand that projects outward in the same direction. It has been shown that, upon light activation, the two β3-α1 junctions of the dimer increase their distance considerably38 probably causing a change in the crossing angle and/or the super-twist of the β-coiled-coil in the full-length protein to control activity as discussed in the recent review by Möglich.31 Most likely, GAF domain proteins control GGDEF activity in a different way, due to the structural similarity with LOV, including the predicted C-terminal coiled-coil39. HAMP domains have been shown to operate as rotary switches32. How such a change will affect the geometry of the C-terminal coiled-coil in respective DGCs has not been studied, but it will surely affect the relative disposition of the hinges that lead to the catalytic domains and, thus, activity.

Apparently, the coiled-coil linker is a versatile and effective means of transmitting a signal between domains without requiring direct interactions between them, which, obviously, is of paramount advantage for their modular combination in evolution.

The same principle seems to apply also for HKs, many of which are both controlled by the same kind of input domains as DGCs and exhibit a coiled-coil preceding the DHp α1 bundle34,35. Signalling, however, seems to proceed via helix rotation36,37 or depend on non-canonical coiled-coil geometry38 and does not invoke lateral helix translation as found here for DgcR. Bioinformatic analyses4,39 may now be extended to test for the occurrence of “slippery” heptad repeats in coiled-coil proteins in general to reveal proteins potentially signalling via coiled-coil register shifts.

**Methods**

**Protein expression and purification.** E. coli BL21 (DE3) cells transformed with pET-28a vector containing DgcR full-length construct purchased from Genscript Inc. were incubated at 37 °C with agitation until they reached the optical density of 0.8–0.9. Expression was then induced by the addition of IPTG (Isopropyl β-D-1-thiogalactopyranoside) at a final concentration of 400 μM for 4 h at 30 °C. The cells were harvested after centrifugation and resuspended in a buffer composed of 20 mM Tris pH 8.0, 500 mM NaCl, 5 mM MgCl2, 5 mM 2-Mercaptoethanol and protease inhibitor (Roche). The lysate proceeded by 3 passages in a French press cell at a pressure of 1500 psi. After a centrifugation at 30,000 g for 50 min, the soluble fraction was loaded onto a His Trap HP 5 mL column (GE Healthcare) in 20 mM Tris pH 8.0, 500 mM NaCl, 5 mM MgCl2, 5 mM 2-Mercaptoethanol and 20 imidazole. DgcR was eluted using imidazole gradient of 20 mM to 500 mM in 15 column volumes. The fractions containing DgcR were further purified by size exclusion chromatography using a Superdex 200 26/60 column (GE Healthcare) in 20 mM Tris pH 8.0, 20 mM NaCl, 5 mM MgCl2 and 1 mM DTT. As a downstream purification step, the samples were dialysed against PBS with a concentration of 5 μM DgcR and stored in a freezer at −80 °C.

**Crystallisation.** Crystallisation attempts were performed using vapour diffusion method prepared in 3-drop MRC plates by Gryphon robot (Art Robbins Instruments) with DgcR wild-type or site variant AxxA at a concentration of 10 mg/ml in 20 mM Tris pH 8.0, 20 mM NaCl, 5 mM MgCl2 and 1 mM DTT at 18 °C. For DgcR crystallisation, 3 μl of the sample was added to a mother liquor with NaF at 10 mM and BeCl2 at 1 mM, 0.8 μM BeF3- modulation. The crystals were harvested after centrifugation and resuspended in a buffer composed by 0.8–1.0 M potassium chloride, 0.5 M calcium chloride dehydrate, 1.0 M imidazole, MES monohydrate (acid), pH 6.5, EDO_PK, 40% v/v ethylene glycol, 20% v/v PEG 8000 present in condition A2 from Merck, or DgcR_inh (Molecular Dimensions). Crystallisation of DgcR in the inhibited conformation (DgcR_inh) was achieved by the presence of 2.0 mM c-di-GMP. Crystals appeared after 5 days in 0.2 M potassium thiocyanate, 0.1 M Tris pH 7.5, 25% PEG 2000 MME, condition optimised from Hi1 of Index HT crystallisation kit (Hampton Research). Crystals were frozen in liquid nitrogen and stored in a transport Dewar prior to data collection.

**Enzymatic analysis.** DgcR wild type and DgcR_AxxA (DgcR_AxxA) activity assays were performed at 5 μM in the presence of 500 μM of GTP in a reaction buffer composed of 100 mM Tris pH 8.0, 100 mM NaCl and 5 mM MgCl2. The reaction was started by substrate and product progress curves were acquired by automatic chromatographic method, named online ion-exchange chromatography (oECC) (Agustoni, manuscript in preparation), in which aliquots (68 μL) are automatically withdrawn from the large reaction vessel (650 μL) and loaded into a Resource Q column (GE Healthcare) without the need for prior quenching of the reaction. This was followed by ammonium-sulfate (0 to 1 M, 20 mM tris, pH 8.0) gradient elution of the bound substances (enzyme, substrate, product). Peak areas corresponding to the c-di-GMP product were integrated and converted to concentrations using a scale factor obtained from calibration. To check for intermediate formation, 10 mM HCl was used as elution buffer with an NaCl gradient 0 – 400 mM. Data was plotted and fitted using proFit (QuantumSoft). To calculate the residual progress curves, the existing differential equations of the kinetic scheme in Fig. 9b were set-up in ProFit and solved by numerical integration. Global fitting of this function using the Levenberg algorithm implemented in ProFit to the measured time courses of product and substrate concentration yielded the parameters listed in Supplementary Table 2.

**Bioinformatic analysis.** Rec and GGDEF domain HHM profiles were taken from Pfam48 and used as input to an hmmsearch against the HMMER web server against the reference proteome database rp55 (E-values 0.01; hit 0.03)49. 8016 sequences were found and filtered by size (<360 residues) to exclude Rec-GGDEF sequences with additional domain(s). This procedure reduced the data size to 4941 sequences. A redundancy filter (<80% pairwise identity) finally reduced the number of sequences to 1408. Global alignment was performed using Muscle50. From this alignment, the linker sequences (as defined ranging from the KP-motif in the Rec β5-α5 loop to the DXLγ motif at the beginning of the GGDEF domain) were extracted and clustered according to length. The sample (length make-up Python script. For the major clusters, corresponding logos were generated using Geneious Prime 2020.1.2 (www.geneious.com) and manually aligned to account for the distinct linker lengths.
Data availability
Coordinates and structure factors of DgcR, DgcR2, and DgcR inh have been deposited in the Protein Data Bank (https://www.rcsb.org) under the respective, accession codes 6ZXB, 6ZXC and 6ZXM. Pfam database was accessed at https://pfam.xfam.org. Source data are provided with this paper.

References
1. Jenal, U., Reinders, A. & Lori, C. Cyclic di-GMP: second messenger extraordinaire. Nat. Rev. Microbiol. 10, 27–284 (2017).
2. Simm, R., Morr, M., Kader, A., Nimtz, M. & Römling, U. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Mol. Microbiol. 53, 1123–1134 (2004).
3. Tarnawski, M., Barends, T. R. M. & Schlichting, I. Structural analysis of an oxygen-regulated diguanylate cyclase. Acta Crystalogr. Sect. D., Biol. Crystallogr. 71, 2158–2177 (2015).
4. Glantz, S. T. et al. Functional and topological diversity of LOV domain photoreceptors. Proc. Natl Acad. Sci. USA 113, E1442–E1451 (2016).
5. Zähringer, F., Lacanna, E., Jenal, U., Schirmer, T. & Boehm, A. Structure and signaling mechanism of a zinc-sensory diguanylate cyclase. J. Mol. Biol. 29, 1128–1129 (2009).
6. Möglich, A., Ayers, R. A. & Moffat, K. Design and signaling mechanism of a cyclic di-GMP-binding protein and mechanisms. J. Bacteriol. 198, 56–56 (2016).
7. Möglich, A., Ayers, R. A. & Moffat, K. Structure and signaling mechanism of Per-ACTR-Sim domains. Structure (Lond., Engl.: 1993) 17, 1282–1294 (2009).
8. Möglich, A. Signal transduction in photoreceptor histidine kinases. Protein Sci. 28, 1923–1946 (2019).
9. Gao, R., Bouillet, S. & Stock, A. M. Structural basis of response regulator WspR. Structure (Lond., Engl.: 1993) 21, 1127–1136 (2013).
10. Diensthuber, R. P., Bonmer, M., Gleichmann, T. & Möglich, A. Full-length structure of a sensor histidine kinase pinpoint coaxial coiled coils as signal transducers and modulators. Structure (Lond., Engl.: 1993) 21, 1127–1136 (2013).
11. Wemmer, D. E. & Kern, D. Beryllo extraordinaire. J. Mol. Biol. 343, 3034–3039 (2014).
12. Costa, F. et al. Global moribidity and mortality of leptospirosis: a systematic review.PLoS Negl. Trop. Dis. 7, e2012133 (2011).
13. Crick, F. H. C. The packing of biological macromolecules. Proc. Natl Acad. Sci. USA 106, 16185–16190 (2009).
14. Schmidt, N. W., Grigoryan, G. & DeGrado, W. F. The accommodation index measures the perturbation associated with insertions and deletions in coiled-coils: Application to understand signaling in histidine kinases. Protein Sci. 36, 414–435 (2017).
15. Kabsch, W. XDS. Acta Crystalogr. Sect. D., Biol. Crystallogr. 66, 125–132 (2010).
16. Bottorff, L. et al. CCP4i2: the new graphical user interface to the CCP4 suite. Acta Crystalogr. Sect. D., Struct. Biol. 74, 68–84 (2018).
17. Battye, T. G. G., Kontogiannis, L., Johnson, O., Powell, H. R. & Leslie, A. G. W. iMOSFLM: a new graphical interface for diffraction-image processing with MOSELM. Acta Crystalogr. D, Biol. Crystallogr. 67, 271–281 (2011).
18. Panjikar, S., Parthasarathy, V., Lamzin, V. S., Weiss, M. S. & Tucker, P. A. On the combination of molecular replacement and single-wavelength anomalous diffraction phasing for automated structure determination. Acta Crystalogr. D, Biol. Crystallogr. 65, 1089–1097 (2009).
19. McCoy, A. J. et al. Phaser crystallographic software. J. Appl. Crystalogr. 40, 658–674 (2007).
20. Emxly, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystalogr. Sect. F., Struct. Biol. Crystallogr. Commun. 60, 2126–2132 (2004).
21. Emxly, P. C. & Powell, B. C. The P27 protein families database. Nucleic Acids Res. 38, D211–D222 (2010).
22. Potters, S. et al. HMMER web server: 2017 update. Nucleic Acids Res. 46, W200–W204 (2018).
23. Madeira, F., Madhusoodanan, N., Lee, J., Tivey, A. R. N. & Lopez, R. Using EMBL-EBI services via web interface and programmatically via web services.Curr. Protoc. Bioinform. 66, 674 (2019).
24. Crick, F. H. C. The packing of a-helices: simple coiled-coils. Acta Crystalogr. 6, 689–697 (1953).

Acknowledgements
We thank the beamline staff at the Swiss Light Source in Villigen and the Biophysics facility at the Biozentrum Basel for expert biophysical support. We thank T. Sharpes,
E. Agustoni, U. Jenal, and T. Maier for critical reading of the manuscript. This work was supported by Grant 31003A-166652 of the Swiss National Science Foundation.

Author contributions
R.D.T., F.H. and T.S. designed the experiments; R.D.T., F.H. performed the experiments; and R.D.T. and T.S. interpreted the results and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-22492-7.

Correspondence and requests for materials should be addressed to T.S.

Peer review information Nature Communications thanks Alejandro Buschiazzo and other, anonymous, reviewers for their contributions to the peer review of this work. Peer review reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021