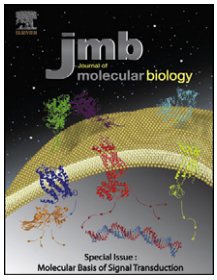
Review



C-di-GMP Synthesis: Structural Aspects of Evolution, Catalysis and Regulation

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Abstract

Cellular levels of the second messenger cyclic di-guanosine monophosphate (c-di-GMP) are determined by the antagonistic activities of diguanylate cyclases and specific phosphodiesterases. In a given bacterial organism, there are often multiple variants of the two enzymes, which are tightly regulated by a variety of external and internal cues due to the presence of specialized sensory or regulatory domains. Dependent on the second messenger level, specific c-di-GMP receptors then control fundamental cellular processes, such as bacterial life style, biofilm formation, and cell cycle control.

Here, I review the large body of data on structure–function relationships in diguanylate cyclases. Although the catalytic GGDEF domain is related to the respective domain of adenylate cyclases, the catalyzed intermolecular condensation reaction of two GTP molecules requires the formation of a competent GGDEF dimer with the two substrate molecules juxtaposed. This prerequisite appears to constitute the basis for GGDEF regulation with signal-induced changes within the homotypic dimer of the input domain (PAS, GAF, HAMP, etc.), which are structurally coupled with the arrangement of the GGDEF domains *via* a rigid coiled-coil linker. Alternatively, phosphorylation of a Rec input domain can drive GGDEF dimerization.

Both mechanisms allow modular combination of input and output function that appears advantageous for evolution and rationalizes the striking similarities in domain architecture found in diguanylate cyclases and histidine kinases.

Introduction

The second messenger c-di-GMP (cyclic di-guanosine monophosphate) mediates crucial cellular processes in bacteria. In particular, it is involved in the regulation of bacterial lifestyle in response to cell cycle phase and environment. Thereby, low and high c-di-GMP levels correlate with motile and sessile phenotype, respectively. This makes the second messenger a critical determinant for the state of a pathogenic infection, that is, virulence or persistence. Several reviews have been published recently covering the various biological and molecular aspects of c-di-GMP signaling [1–3], including its role in bacterial pathogens [4–7] and host immunity, for example, see Refs. [8,9]. Furthermore, a comprehensive account of the rather young history of c-di-GMP research has been published by Römling and coworkers few years ago [10].

Cellular c-di-GMP levels depend on the antagonistic activity of diguanylate cyclases and phosphodiesterases. Typically, in a given organism, there are several paralogous copies of both enzymes that carry distinct regulatory or sensory domain(s). This enables the bacterium to integrate various kinds of input signals to set the cellular c-di-GMP level, which, in turn, can affect the response of a multitude of specific c-di-GMP receptors to generate a coordinated cellular output.

General molecular mechanisms of c-di-GMP signaling have been reviewed [2,11,12]. Since then, a wealth of structures and functional data for c-di-GMP-related proteins has been acquired, which allows a reappraisal

and consolidation of current mechanistic models. Chou and Galperin have done this most recently for c-di-GMP-binding proteins [13], with particular emphasis on the role of reoccurring c-di-GMP-binding motifs. Whiteley and Lee have compiled the structures of selected diguanylate cyclases and c-di-GMP receptors that are of particular relevance for polysaccharide export [14].

Here, I focus on the rich data set of all known diguanylate cyclase structures (full-length and of separately determined domains) to study and compare their molecular organization. Hereby, the grand question is whether there are common principles used by the plethora of distinct input domains (Rec, PAS, GAF, HAMP, TMs, etc.) to control diguanylate cyclase activity. The analysis shows that, almost invariably, input domains are linked to the catalytic GGDEF output domain *via* a helical segment, which, in the dimeric protein, associates with its symmetry mate to form a coiled coil.

This organization appears very well suited to rigidly couple (and potentially amplify) signal-induced structural changes in the input domain with the relative distance and orientation of the two C-terminally linked GGDEF domains, which in turn would enable or impede their productive encounter.

The proposed mechanism has been inspired by recent structural insight into histidine kinase regulation [15–17] that seems to operate analogously, but with the regulatory coiled coil being merged with the N-terminal coiled coil of the histidine kinase output module.

C-di-GMP: Conformation and Oligomeric State

The structure of c-di-GMP molecules was first determined in isolation by small-molecule X-ray crystallography [18,19] where it crystallized as an intercalated dimer. The same dimer was observed later in protein complexes, for example, when bound to the I-site of diguanylate cyclases or to the PilZ receptor [20,21]. The c-di-GMP monomers are almost exactly twofold symmetric (Fig. 1a), with the conformation of the dinucleotide backbone similar to A-DNA, but with α/ζ in g+ instead of g– conformation [18,19] (Fig. 1a, inset). The low variation in the dihedral angles among the various high-resolution structures (standard deviation ~5°) and the closeness to standard backbone conformation indicate that the ring can be easily closed and that the structure is not easily perturbed [18]. The sugar pucker is invariably C3′-*endo*, and both glycosidic bonds are in *trans* conformation.

Interestingly, when bound as a monomer to the active site of EAL phosphodiesterases, the molecule shows an elongated shape due to a change in the ribose pucker and glycosidic χ angle of one of the

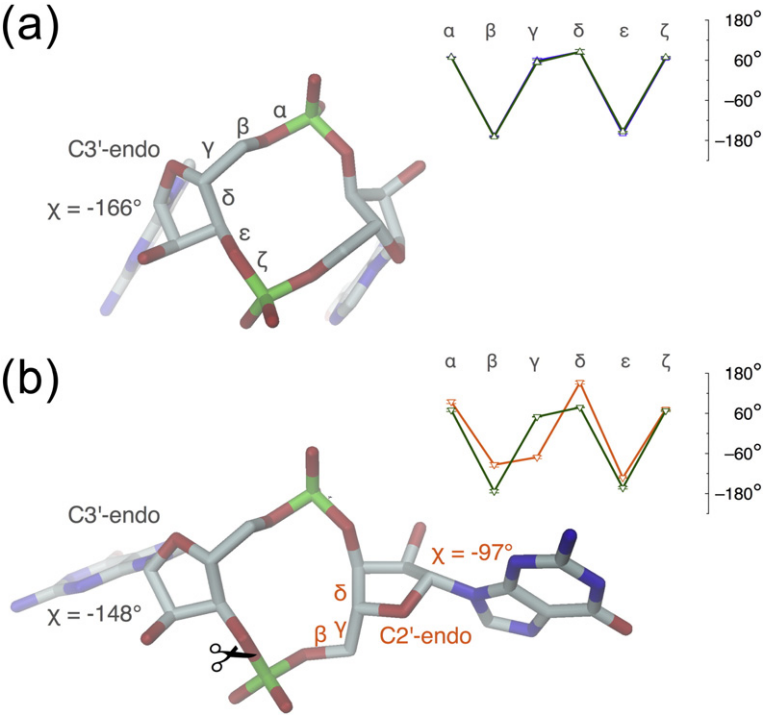

Fig. 1. Observed c-di-GMP conformations. The main part of the figure shows the c-di-GMP structure with the backbone torsion angle plot shown in the inset. (a) Structure of c-di-GMP as observed bound to the I-site of diguanylate cyclases (DgcZ, 3tvk; the second molecule of the symmetric dimer is not shown). This structure is symmetric and representative, as demonstrated by the inset, which shows the mean backbone torsion angles (with standard deviation) obtained by averaging the two GMP halves of the c-di-GMP structures determined in complex with DgcZ (3tvk), tDGC (4urg), and Maqu2607 (3ign) and in a small-molecule crystal structure [19]. (b) Structure of c-di-GMP as observed when bound to a phosphodiesterase [PdeL(YahA), 4lj3] with the scissile bond indicated. The torsion angle plot (inset) of this asymmetric structure is given for the two halves separately in blue and orange (lower left GMP moiety). The plot has been averaged over the c-di-GMP structures observed in complex with PdeL(YahA), 4lj3)) and with PdeA (4hjf). Note that the upper right GMP moiety shows the same conformation, including the C3′-endo sugar pucker, as in panel (a).

GMP moieties (on the right side in Fig. 1b). While the major part of the backbone retains the relaxed conformation as described above, the conformation of the "right" moiety is distinct (indicated in orange in the inset to Fig. 1b), resulting in an orientational change of the phosphate group adjacent to the scissile bond. Whether such pronounced substrate distortion facilitates substrate hydrolysis awaits further investigations.

The repeated observation of dimeric self-intercalated c-di-GMP molecules and polymorphism observed in solution in the presence of monovalent cations [22] prompted an NMR investigation to study c-di-GMP aggregation kinetics and thermodynamics [23]. It was shown that c-di-GMP is in a fast monomer/dimer equilibrium with a K_d of about 1 mM under physiological salt conditions. Considering that the cellular c-di-GMP concentration is in the low μM range, preformed c-di-GMP dimers are thus probably not relevant for c-di-GMP signaling.

Diguanylate Cyclase Structure and Comparison with Adenylate Cyclase

Synthesis of the twofold symmetric c-di-GMP molecule involves the antiparallel alignment of two GTP molecules such that the substrate molecules can perform mutual, intermolecular nucleophilic in-line attacks of their (deprotonated) 3′-hydroxyl group onto the phosphorous atoms of the adjacent substrate. This reaction is catalyzed by the diguanylate cyclase GGDEF domain (Pfam: GGDEF), which can bind one GTP molecule and is believed to homodimerize transiently.

The fold of the GGDEF domain (Fig. 2a) is related to that of the catalytic domain of adenylate/guanylate cyclases (Fig. 2b; Pfam: Guanylate_cyc) as was predicted by Pei and Grishin [24]. Both structures show the same (βααββαβ)-fold (Fig. 2c) with the two central antiparallel β-strands being connected by a β-hairpin carrying the eponymous GGDEF signature in diguanylate cyclases and a more relaxed xGDxF/Y motif in mononucleotide cyclases. The aspartate of the motif (which is sometimes a glutamate in diguanylate cyclases) together with an aspartate from β1 are, in both cases, coordinating the two magnesium ions that are critical for catalysis (for a recent review, see Ref. [25]). Based on the conservation of both the fold and the catalytically important residues, an evolutionary link between the two enzyme families is most likely [24,26]. Notably, the GGDEF domain is longer at the N terminus, starting with a characteristic wide β-turn connecting the short β0 and β0′ strands (see further below) followed by helix α0, whereas the adenylate/guanylate cyclase fold shows some additional decorations at the C terminus (Fig. 2a and b).

In both enzymes, the nucleoside triphosphate binds to an equivalent site, with the β-, γ-phosphates

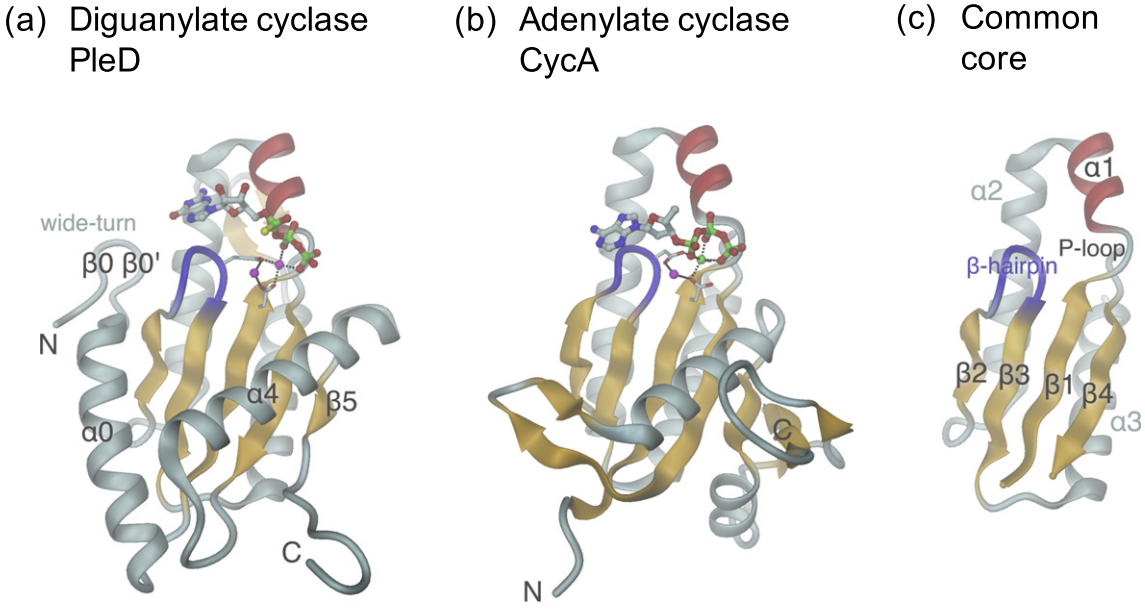

Fig. 2. Structure comparison of diguanylate and adenylate cyclases. (a) Fold of the GGDEF domain (PDB code: 2v0n; Pfam: 00990) with structural elements unique to this fold indicated. (b) Fold of the nucleotide cyclase domain (PDB code: 4wp9; Pfam: 00211). (c) The common core (βααββαβ) fold of the two enzyme classes. Cartoon representation with the characteristic β-hairpin at the substrate-binding site colored in blue and the helix involved in phosphate binding in red. In addition, bound substrate analogs and the two catalytic cations (Mg^{++} in magenta, Ca^{++} in green) coordinated by two conserved carboxylic residues are shown in full.

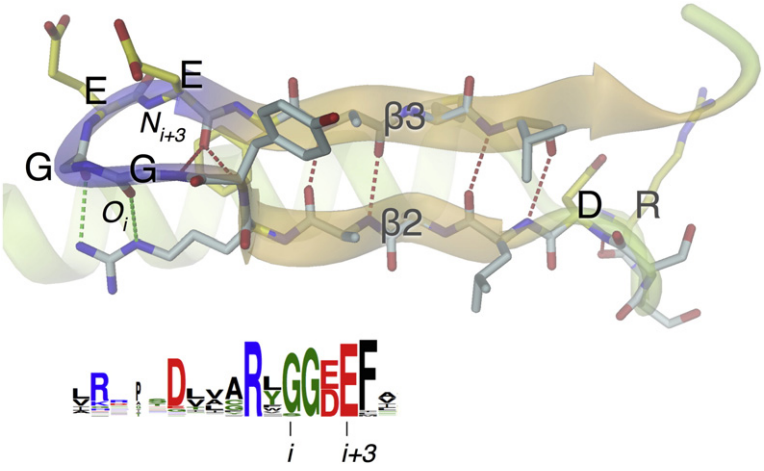

Fig. 3. Active site β-hairpin. Detailed structure of the noncanonical GGDEF β2–β3 hairpin (3rbe) and the corresponding sequence motif (excerpt of Fig. 5).

interacting with the P-loop/N-cap of helix α1 and the ribose moiety hovering above the β-hairpin. Remarkably, the β-hairpin does not form a regular ($O_i \rightarrow NH_{i+3}$) β-turn. Rather, the peptide planes of residues i-2, i-1, and i are oriented perpendicular to the β-sheet plane, resulting in a sharp kink of the C-terminal end of β2 (Fig. 3). Apparently, this distortion is induced by the presence of the invariant arginine side chain in position i-2 (Arg 366 in PleD from *Caulobacter crescentus*; UniProt: Q9A5I5) that forms H-bonds with main-chain carbonyls i and i+1. The peculiar β-hairpin conformation together with the conserved glycine in position i may provide the necessary space for the intermolecular reaction catalyzed by the GGDEF dimer (see below). In adenylylate cyclases, the conformation of the β-hairpin is rather canonical, except that the carbonyl i is rotated out of the β-sheet plane, although toward the opposite side (not shown).

In contrast to aforementioned similarities, di- and mononucleotide cyclases exhibit distinct interactions with the substrate base. In diguanylate cyclases, the guanine base is forming three well-defined H-bonds with Asn335 and Asp344 (PleD) from the helical α1/α2 tower (Fig. 4a). These specific interactions explain why ATP is not a substrate. The relaxed substrate specificity of some diguanylate cyclases [27], which are able to synthesize c-AMP-GMP and c-di-AMP, can be attributed to the presence of a Ser/Thr residue

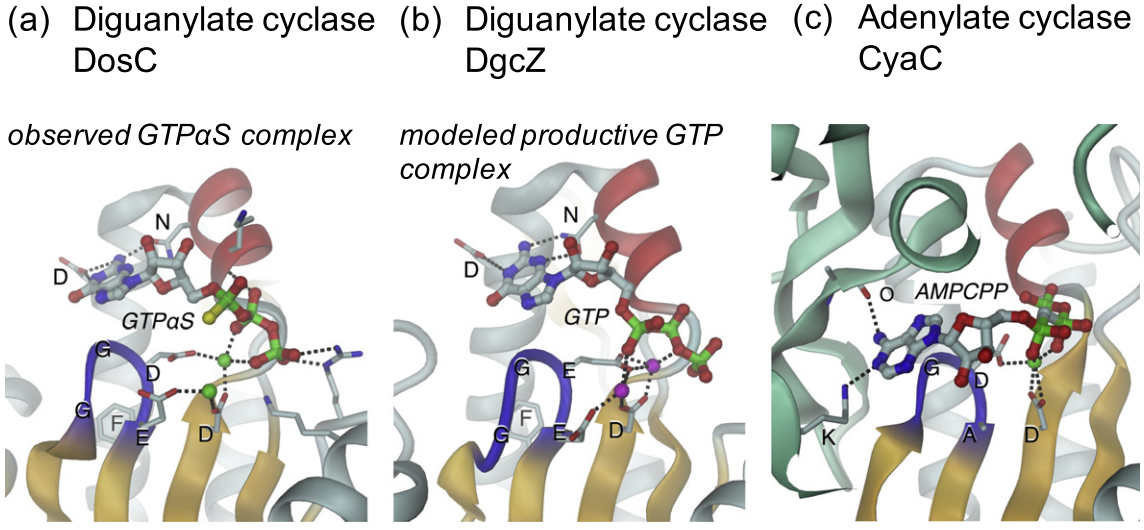

Fig. 4. Substrate (analog) binding mode to nucleotide cyclases. (a) Structure of DosC with bound GTPαS substrate analog (4zvf [30]). (b) Structure of DgcZ (3tvk [28]) complexed with a modeled GTP substrate in productive conformation (γ = +60°). (c) Structure of adenylate cyclase CycA with bound AMPCPP substrate analog (1wc0, [31]), with adjacent subunit colored in aquamarine. Important protein residues are shown in full, and divalent cations are represented as in Fig. 2.

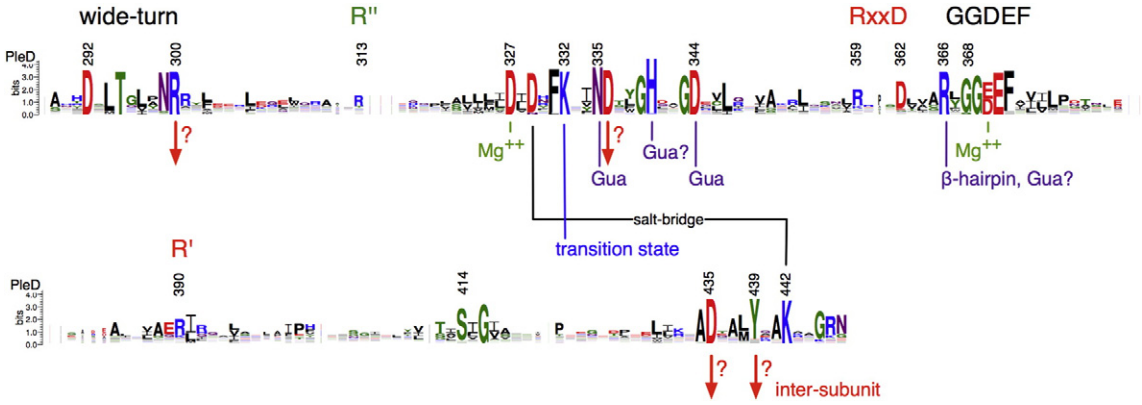

Fig. 5. GGDEF sequence conservation. The sequence logo [77] is based on the 37 sequences of the Pfam seed alignment that have an intact GG(D/E)EF motif and are therefore bona fide cyclases. Conserved sites are annotated with known or predicted function. Mg^{++}, magnesium coordination; Gua, guanine binding. The red arrows indicate residues that may be part of the subunit interface of the competent GGDEF dimer. For the "wide-turn", see Fig. 7; for the GGDEF motif, see Fig. 3; for the Ip (RxxD, R′) the Is (R″) inhibition sites, see Fig. 12. Numbers correspond to PleD.

instead of the Asp. In mononucleotide cyclases, the base is binding across the dimer interface (Fig. 4c), with the subsite more variable and often promiscuous. The GGDEF sequence logo shown in Fig. 5 shows strict conservation of the functional residues discussed above. Other conserved positions will be discussed further below.

Catalytic Mechanism

For intermolecular phosphodiester formation, two GTP molecules have to be aligned in antiparallel orientation (Fig. 6a). This is thought to be accomplished by appropriate dimerization of substrate-loaded GGDEF domains. The corresponding structure has not yet been elucidated, but a model has been put forward [28] (Fig. 6b). This was derived from the structure of the dimeric DgcZ (GGDEF) post-catalysis complex (Fig. 6c), in which the c-di-GMP product symmetrically bridges the two active half-sites [28]. Another dimeric GGDEF/c-di-GMP complex structure is known [29], but with an entire c-di-GMP molecule bound to each of the two half-sites, thus not representing the immediate post-catalysis state.

During modeling, it was realized that the conformation of the GTPαS substrate analog, as seen in complex with DgcZ (GGDEF) [28] and also with

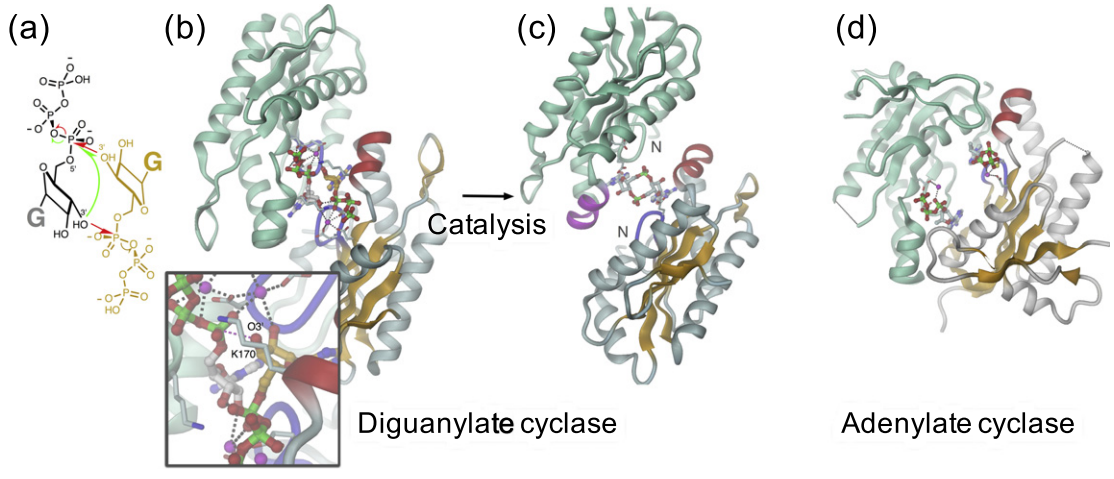

Fig. 6. Diguanylate and adenylate cyclase: catalysis and dimeric structure. (a) Schematic scheme of mono- (green arrow) and dinucleotide (red arrows) cyclization reactions (intra- or intermolecular nucleophilic attack of $O3^-$ onto αP. (b) Putative arrangement of two DgcZ(GGDEF) domains loaded with GTP (productive conformation, see Fig. 4b) ready to form two symmetric intramolecular phosphodiester bonds, as proposed by Zähringer and coworkers [28]. The inset shows an enlargement of the top left binding site. (c) DgcZ(GGDEF)/c-di-GMP product complex (3tvk). (d) Structure of homodimeric adenylate cyclase MA1120 from *Mycobacterium avium* (4wp9) in complex with substrate analog.

DosC [30] (Fig. 2a), is significantly distinct from the conformation that the GMP moiety attains in the cyclic product. Therefore, the observed GTPαS most likely does not represent a productive substrate conformation. Specifically, the conformation differs in the γ torsion angle, which is −60° for the bound GTPαS, and invariably, +60° for c-di-GMP (Fig. 1). It is possible that the nucleotide structure is perturbed by the thiol modification, as has been found for adenylate cyclases, where AMPCPP seems to mimic more faithfully than ATPαS genuine substrate binding [31]. However, within the binding site, the substrate structure can easily be changed to the productive conformation without disrupting the observed interactions of the β- and γ-phosphate and the guanine moieties with the enzyme [28] (Fig. 3b).

Figure 6b shows the model of the catalytically competent GGDEF dimer. The subunits have been arranged such that the 3′-hydroxyl groups of the two substrates (in productive conformation) are positioned in line with the scissile bond as required for an intermolecular nucleophilic attack onto the α-phosphorous atoms. There is no titratable residue close to the O3′-group such that in adenylate cyclases [25], deprotonation of the hydroxyl-group probably proceeds *via* a water molecule that could be activated by the close-by metal. Lys170 (PleD: 332) is well positioned to stabilize the transition state. Indeed, the corresponding Ala mutant in XAC0610 is 100-fold less active [32]. Since this residue is donated by the subunit adjacent to the one holding the substrate, futile GTP hydrolysis may be prevented before substrate encounter. A similar GGDEF dimer has been observed in the absence of substrate and ions [33], although at the non-physiologically low pH of 4, where the changed electrostatics may permit the approach of the two domains. A few strongly conserved residues are found in the predicted interface of the GGDEF dimer (as indicated in Fig. 5). Adenylate cyclases, as exemplified by the homodimeric CycA (Fig. 6d), show a distinct subunit association, with contacts involving the C-terminal adenylate-cyclase-specific structural elements.

The k_{cat} of diguanylate cyclases is rather low (typically in the order of 1 min^{-1}) [28,30,34,35], which may be linked to the requirement of GGDEF rearrangement to allow product/substrate exchange (Fig. 6b and c). Considerably larger turnover numbers were measured for WspR and its artificial GCN4–GGDEF construct. Possible reasons for the higher efficiency of WspR compared to PleD have been discussed in Ref. [36]. Efficient turnover has also been reported for XAC0610 (k_{cat} = 65 min^{-1}) by Oliveira and coworkers [32], who also carefully examined the sigmoidal dependence of the activity on substrate concentration, which is indeed expected, since the reaction is of second order with respect to the substrate. Still, the authors argue that there is also some cooperativity component, although their noncooperative kinetic model fits the data already satisfactorily. In fact, an increase in the GTP binding affinity for the second molecule (i.e., cooperativity) is hard to rationalize within a mechanistic model that assumes GTP binding to the active half-sites before GGDEF dimer formation. Indeed, this assumption seems reasonable, since the active dimer model does not exhibit any access/exit route for the substrates. A sigmoidal product formation *versus* substrate concentration has also been observed for an isolated (inhibition relieved) GGDEF domain of a diguanylate cyclase from a thermophilic organism [35].

GGDEF Activation

Encounter of substrate-loaded GGDEF domains can be expected to be inefficient due to the slow macromolecular diffusion rate. Indeed, isolated GGDEF domains show very little or no activity [37,38]. Thus, it is not surprising that, almost invariably, diguanylate cyclases carry accessory domains at their N termini, with most of them known or predicted to form dimers (see below). These enzymes would be expected to be constitutively active, since tethering two GGDEF domains to a dimeric stem would increase their local concentration dramatically, enabling productive encounter, even in the absence of any specific (non-covalent) interactions with the stem [11]. This notion was nicely corroborated by a study of Sondermann and colleagues on a GGDEF hybrid construct carrying an N-terminal dimerization segment (GCN4-zipper; see Fig. 8a), which was found to be highly active [37].

Since the formation of a competent GGDEF is required for diguanylate cyclase activity, two simple mechanisms can be envisaged to control the enzyme: (i) signal-induced homodimerization of the input domain or (ii) signal-dependent reorganization of the input domains within a preexisting dimer to change the relative arrangement and/or mobility of the C-terminally appended GGDEF domains.

It appears that both mechanisms are utilized by nature. Before discussing the evidence for this, however, the N-terminal part of the catalytic domain that connects to the input domain deserves a closer look. The GGDEF domain starts with an invariant DxLT motif (Fig. 5), which is folded to a noncanonical turn with an $O_i \rightarrow NH_{i+4}$ H-bond (called "wide-turn" hereafter; see Fig. 7) joining two short β-strands (β0, β0′). The strict conservation of the Asp and the Thr (Fig. 7a, inset), which stabilize the wide-turn by engaging in several H-bonds, suggests the conservation of the turn structure, which is indeed observed (Fig. 7b). A PDB database search using PDBemotif [39] showed that this main-chain conformation with aspartate and threonine/serine at the respective positions exists in several other structures, for example,

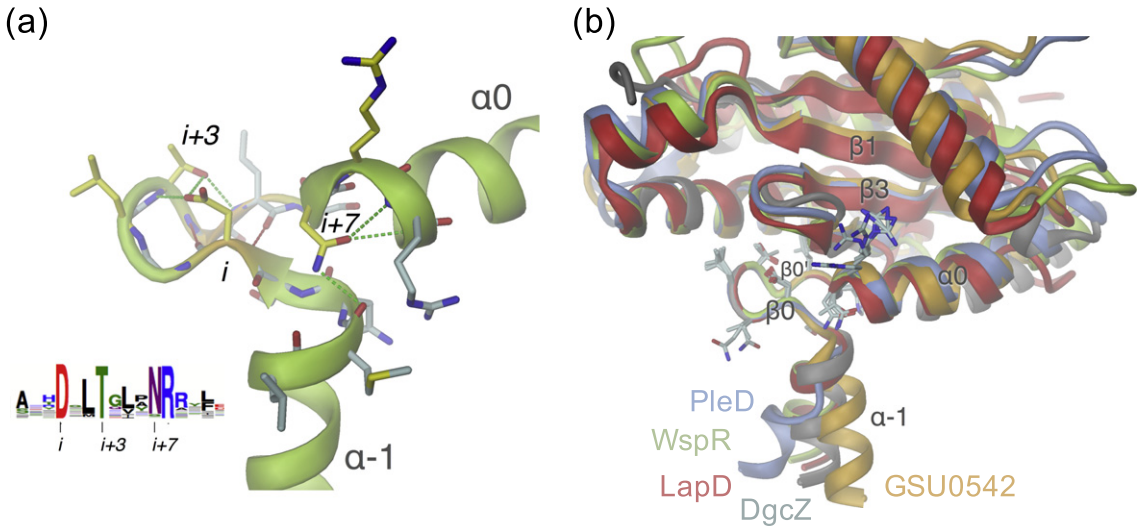

Fig. 7. The wide-turn at the N terminus of the GGDEF domain. (a) Full model of the N-terminal part of GGDEF N and preceding linker helix (3ign) and the corresponding sequence motif (excerpt of Fig. 5). (b) Superposition of the DxLT motif of the various indicated diguanylate cyclase structures. Note the close agreement among the GGDEF domains and the variable orientation of the preceding liker helix α-1.

in adenylate cyclase (2ak3, D149DLS) and Ser/Thr kinases (3uc3, D41KLT). Thus, the wide-turn represents a more widespread and probably rigid structural element.

The superposition of GGDEF domains shown in Fig. 7b demonstrates that the wide-turn and the GGDEF-specific α0 helix are rigidly packed onto the core of the domain, whereas orientation of the (invariably helical) segment (α-1) preceding the wide-turn and connecting to the various kinds of input domains is variable. This appears functionally relevant, since GGDEF mobility relative to (dimeric) input domains is probably required for the encounter of the catalytic domains and for product/substrate exchange. Strikingly, also the conserved asparagine following the wide-turn (position i+7) has a structural role by forming capping H-bonds with both the α-1 and α0 helix. In contrast, the conserved Arg i+8 shows variation in its side chain orientation. Probably, it interacts with the adjacent subunit within the dimer (see below).

By now, several full-length diguanylate cyclase structures have been determined. They are shown in Figs. 8 and 9, and Table 1 lists further details. The structures, complemented with structure predictions (Fig. 10) and sequence alignments (Fig. 11), provide valuable information regarding domain organization and linker structure, the aspects that are most relevant for the mechanisms of signal transduction.

Dimerization

Activation by dimerization has been demonstrated for diguanylate cyclase PleD. (Pseudo-)phosphorylation, that is, BeF_3^- modification, of its Rec domain reduced the dimerization K_d from 100 to below 10 μM [34], accompanied by a 50-fold increase in activity at the employed enzyme concentration [40]. Figure 8c shows the tight dimeric stem formed by the P-Rec and Rec′ input domains and the elongated C-terminal helices of the Rec' domain (colored in magenta) that contact each other at the C-terminal end and connect to the GGDEF domains. The latter domains form a noncompetent dimer being cross-linked at the "backside" by c-di-GMP. More about this allosteric inhibition feature is discussed below.

Although reminiscent of PleD, the activation mechanism of the Rec-GGDEF protein WspR, the other full-length diguanylate cyclase studied in detail, appears to be more complex [37,41]. Here, the C-terminal helix of the Rec domain is considerably longer and forms a parallel coiled coil (stalk) with its symmetry mate (Fig. 8b). The C-terminal ends of the stalk are at a larger distance compared to PleD, keeping the two GGDEF domains apart. A tetramer (not shown) is formed by interdigitation of two such dimers. Activation appears to proceed *via* phosphorylation-induced formation of even higher oligomers [42], but no structural details about the active domain constellation are known.

Many Rec-GGDEF proteins have a considerably shorter linker and likely conform to a simpler regulatory mechanism. A prominent example is Rrp1, the only diguanylate cyclase of *Borrelia burgdorferi*, which has been shown to become activated by phosphorylation [43] (for more information about diguanylate cyclase covered in this review, see Table 2). Whether activation

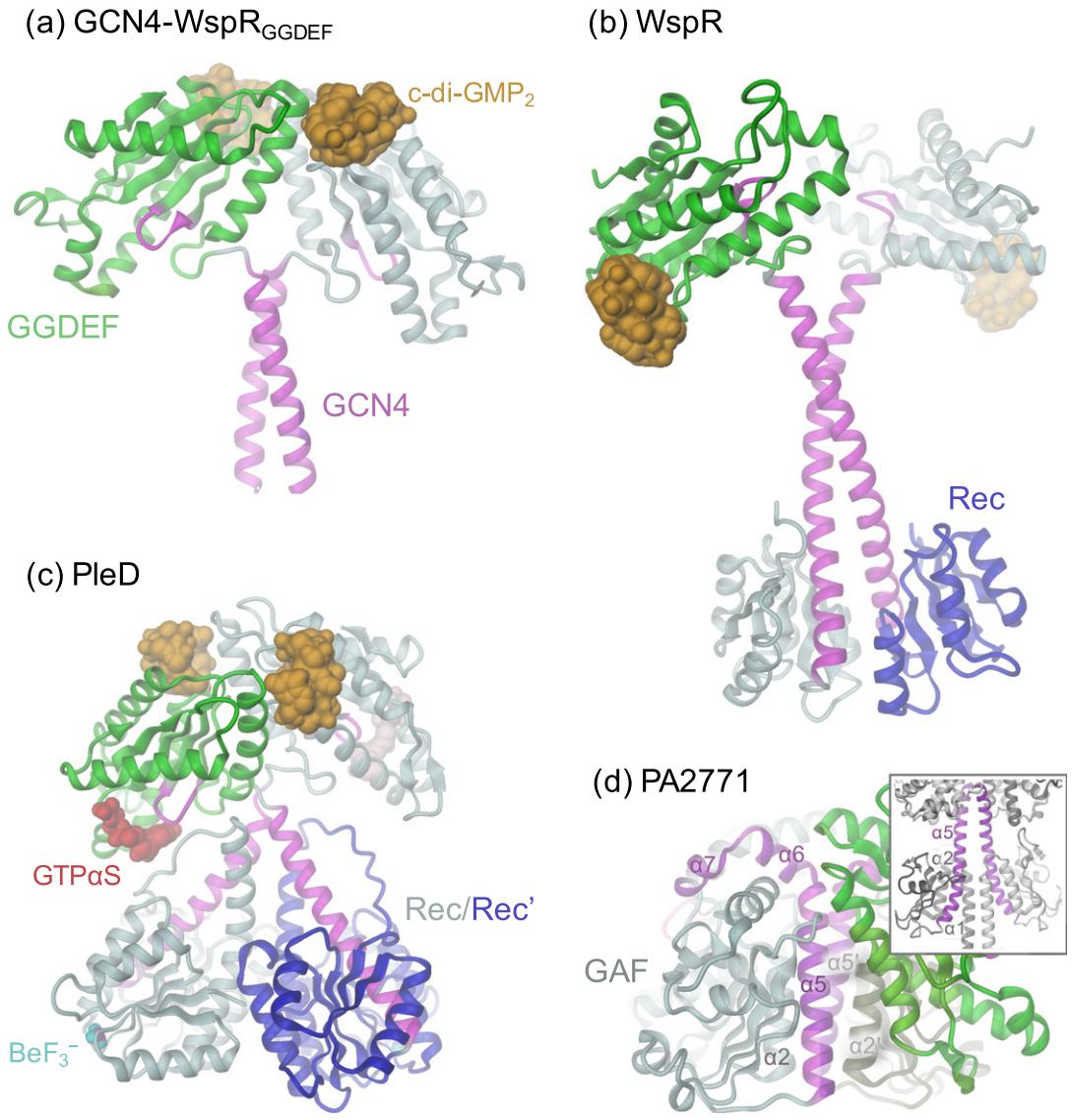

Fig. 8. Diguanylate cyclase structures (I). (a–d) The indicated crystal structures correspond to PDB codes 3i5c, 3bre, 2v0n, and 4zmu; see also Table 1. Domains and bound ligands (surface representation) are labeled. The inset to panel (d) shows a canonical GAF dimer (as part of PDEA2, 3ibj) with interface helices labeled.

proceeds *via* dimerization or a change in subunit arrangement remains to be investigated. The structure and profile-based alignment of Rec-linked GGDEF domains (Fig. 11) show a similar heptad repeat pattern at the C terminus of the linker. Strikingly, the linker lengths differ by 2 or 3 × 7 residues, which would be well tolerated within a coiled coil and, thus, provides additional corroboration for such an organization of the stalk.

Change in Domain Arrangement or Mobility

In contrast to PleD, many diguanylate cyclases appear to be constitutive dimers or tetramers but need an input signal for activation (or relief of auto-inhibition). To this group belong the enzymes with associated globin, PAS, PYP, and GAF domains. These enzymes are discussed in the following.

Globin-coupled sensors

Five globin-coupled sensors [44], which carry heme as prosthetic group, have been characterized in detail; see Ref. [30] and references therein. The structurally best-characterized globin-coupled sensor is DosC from *Escherichia coli* that is composed of three consecutive domains (globin-MID(middle domain)-GGDEF) and for which crystal structures of all three individual domains, but not for the full-length

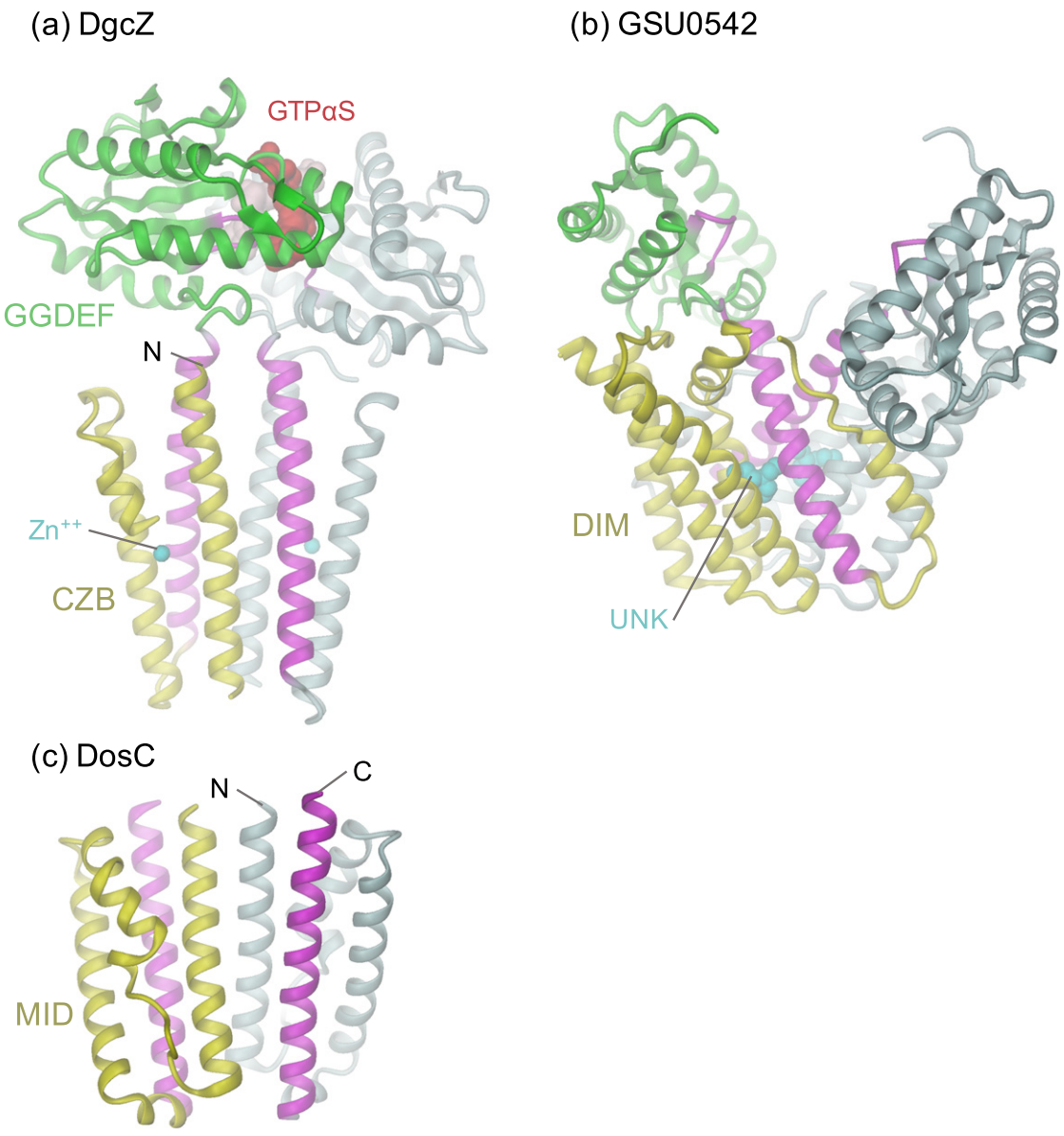

Fig. 9. Diguanylate cyclase structures (II). (a–c) The indicated crystal structures correspond to PDB codes 4h54, 3ezu, and 4zvc; see also Table 1.

protein, are available [30]. A small quaternary change of the globin dimer induced by a change in the redox state of the heme (which may mimic O2 binding) was observed. This reorganization is probably transmitted directly to the adjacent MID dimer (Fig. 9c), which in turn would pass the signal (*via* a change in the packing of the MID helices) to the GGDEF dimer. Interestingly, the structure of the DosC(Mid) domain is similar to that of the CZB (chemoreceptor zinc-binding) domain of DgcZ, but with the N-terminal, instead of the C-terminal helices, being in contact with each other (cf. Fig. 9a and c). This may reflect the intrinsic plasticity of this domain, which could potentially be used to change the mutual distance of the C termini linking to the GGDEF domain and thereby control activity, as also discussed in Ref. [30], and by Zähringer *et al.* [28] for DgcZ.

The iron(III) form of the enzyme shows a k_{cat} of 0.8 min^{-1}, whereas the iron(II) form is catalytically inactive, consistent with the relief of auto-inhibition upon oxygen binding. However, a truncated MID-GGDEF construct was found to be only partially active. Possibly, this is due to a non-native MID domain organization in the absence of the N-terminal globin domain. Although the regulatory model proposed by Tarnawski *et al.* [30] appears attractive, it should be considered that the two DosC homologs appear to be fully active only in the tetrameric state (a

Table 1. Crystal structures of full-length diguanylate cyclases or individual domains thereof

PBD code	Protein	Organism	Domain Organization*	Ligands	Res.	Reference
3i5c	WspR hybrid	*P. aeruginosa*	GCN4-GGDEF	c-di-GMP	1.9 Å	[37]
3bre	WspR	*P. aeruginosa*	Rec-GGDEF	c-di-GMP	2.4 Å	[74]
2v0n	PleD	*C. crescentus*	Rec-Rec′-GGDEF	GTPαS, c-di-GMP	2.7 Å	[34]
3ign	MAQU2607	*M. hydrocarbon*	(PAS)-GGDEF	c-di-GMP	1.8 Å	[75]
4zmu	PA2771	*P. aeruginosa*	GAF-GGDEF	-	2.5 Å	Chen *et al.* (to be unpublished)
4kg1	XC0249	*X. campestris*	cNMP-binding-(GGDEF)	cGMP	2.4 Å	[55]
4wxo	SadC	*P. aeruginosa*	(TM)-GGDEF	-	2.8 Å	Liu *et al.* (to be unpublished)
4h54	DgcZ	*E. coli*	CZB-GGDEF	GTPαS, c-di-GMP	3.9 Å	[28]
4zvc	DosC	*E. coli*	(globin)-MID-(GGDEF)	-	1.5 Å	[30]
3ezu	GSU0542	*G. sulfurreducens*	DIM-GGDEF	UNK	2.0 Å	Joint Center for Structural Genomics (to be published)

* Complete domain organization, with domains that are not part of the structure given in brackets.

state not observed for DosC) [45] and that DosC has been reported to form a functional complex with DosP, a c-di-GMP phosphodiesterase that is co-expressed with DosC [46].

A diguanylate cyclase with the simpler globin-GGDEF organization (HemDGC) has been shown to be active only in the presence of oxygen (k_{cat} = ~6 min^{-1}), but not CO [47]. The enzyme is a constitutive tetramer and it may be speculated that its domain arrangement might be similar to tetrameric WspR, since the two domains are linked by a segment with high coiled-coil propensity (Fig. 11).

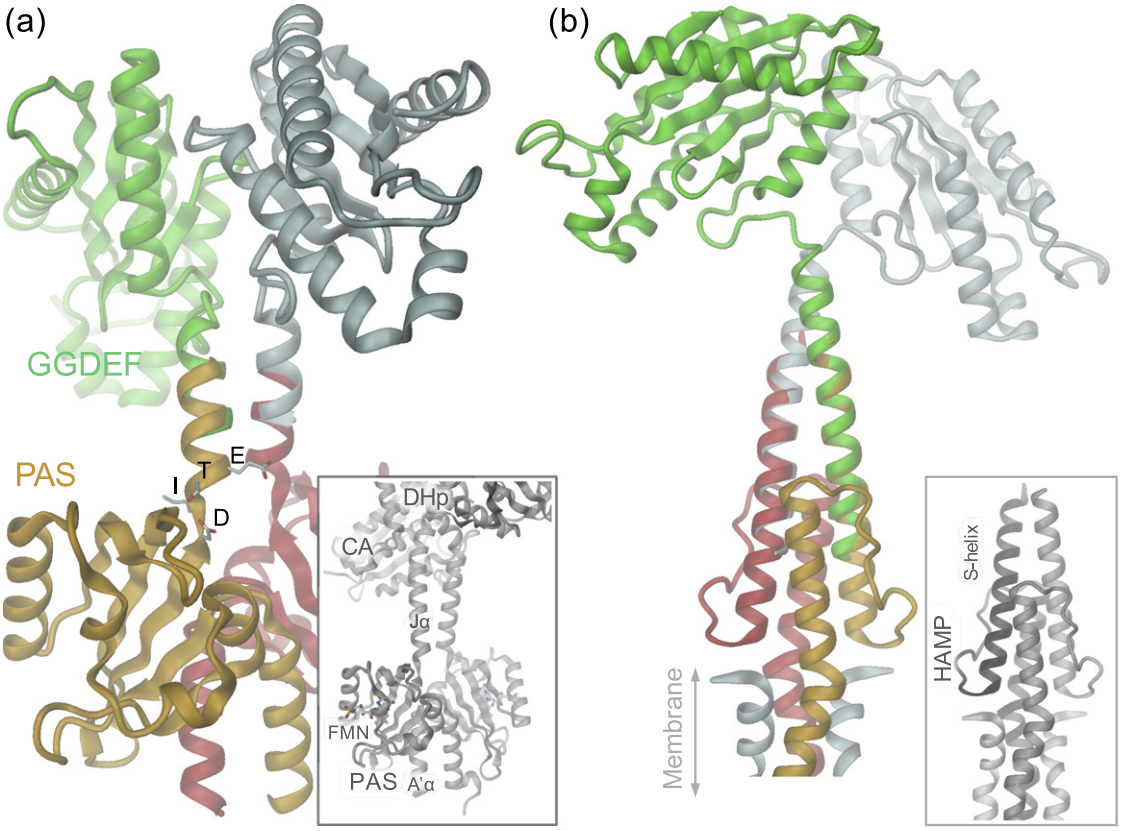

Fig. 10. Predicted domain arrangements in PAS-GGDEF and HAMP-GGDEF proteins. Shown are the assembled, overlapping template structures as identified by HHPred profile–profile matching. (a) Domain arrangement in AxDGC2 as represented by the dimeric template structures of Yxxx/FixL(PAS) [16] (4gcz; red and orange) and GCN4-GGDEF [37] (3i5c; green and gray). The characteristic DITE motif at the start of the 4gcz J-helix is shown in full. In AxDGC2, the corresponding sequence is EITE. (b) Domain arrangement in YfiN as represented by the dimeric template structures of an Af1503 construct [69] (4cq4; red and orange) and GCN4-GGDEF [37] (3i5c; green and gray). The approximate position of the membrane is indicated by the double arrow.

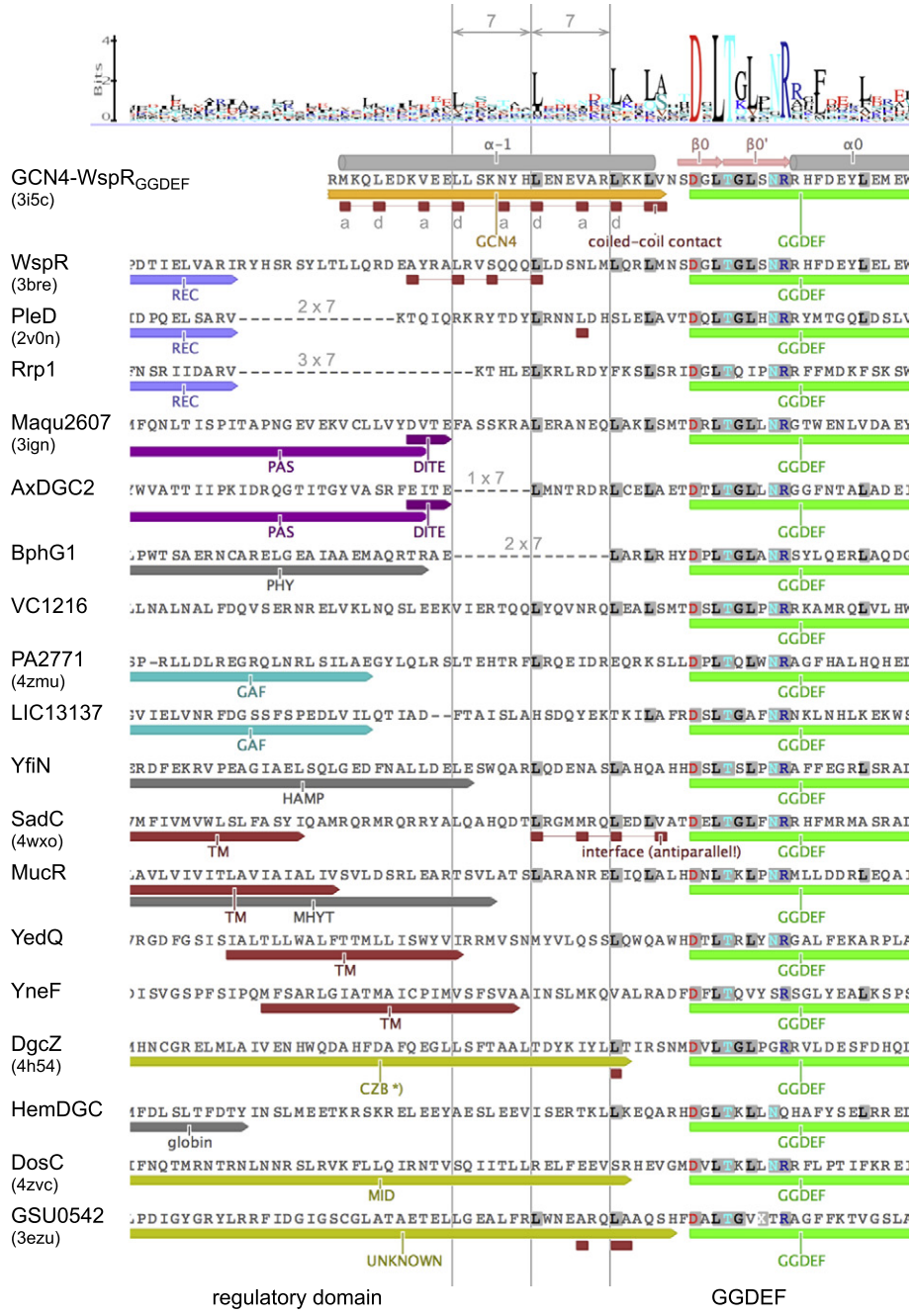

Fig. 11. Sequence alignment of selected diguanylate cyclases with various input domains. The alignment is based on structure comparisons and HHPred [51] profile–profile matching. Note that the various linker segments comply largely with a hydrophobic heptad repeat (the a and d positions are indicated at the top), indicating coiled-coil formation, with the exception of LIC13137. Observed coiled-coil interactions are indicated by brown rectangles. Secondary structures of the GCN4-WspRGGDEF hybrid structure (top) are indicated; compare with Fig. 8a.

VC1216 is a bacteriohemerythrin-GGDEF protein, again with a domain linker showing a leucine heptad repeat (Fig. 11). Its catalytic activity is approximately 10 times higher in the diferrous than in the diferric form [48].

PAS domain

PAS domains, which are known to serve as universal signal sensors or interaction modules [15], are often found N-terminally to GGDEF domains. A

Table 2. Domain organization of selected diguanylate cyclases

Protein	Organism	Domain Organization	UniProt Code	Reference
Rrp1	*B. burgdorferi*	Rec-GGDEF	Q0MYT4	[43]
AxDGC2	*A. xylinus*	PAS-GGDEF-EAL	O87377	[50]
BphG1	*R. sphaeroides*	GAF-GGDEF-EAL	Q8VRN4	[56]
PelD	*P. aeruginosa*	TM-TM-TM-GAF-GGDEF	Q9HZE7	[60,61]
VC1216	*V. cholerae*	Hemerythrin-GGDEF	Q9KSP0	[48]
LIC13137	*L. interrogans*	GAF-GGDEF	Q72MQ6	C. Guzzo, personal communication
YfiN (TpbB)	*P. aeruginosa*	TM-CHASE8-TM-HAMP-GGDEF	Q9I4L5	[65]
LapD	*P. fluorescens*	TM-PAS-like-TM-HAMP-GGDEF*-EAL*	Q3KK31	[68]
HemDGC	*D. psychrophila*	globin-GGDEF	Q6ARU5	[47]

well-studied example is diguanylate cyclase AxDGC2 that carries as redox sensor a flavin adenine dinucleotide (FAD) cofactor bound to its PAS domain. Upon oxidation, the cyclase activity is enhanced about eightfold, while the K_m remains largely unaffected [49].

Insight into the mechanism of PAS to GGDEF signal transduction can be gained by resorting to known structures of PAS-controlled histidine kinases, such as the well-studied dimeric NifL protein [50] or the full-length, light-sensing YtvA/FixL histidine kinase hybrid [16] (PDB code: 4gcz; Fig. 10a, inset). The PAS domain (also classified as LOV) of the latter structure reveals that not only the N-terminal Aα helices but also the C-terminal Jα helices form a (coaxial) coiled coil and that the Jα helix is contiguous with the first helix of the DHp domain, providing firm coupling with the effector domain. Thus, any quaternary change in the PAS dimer induced upon the excitation of the flavin mononucleotide (FMN) chromophores would alter the packing of the DHp bundle and thus affect autophosphorylation [16].

Using the dimeric structures of YtvA/FixL and GCN4-WspR(GGDEF) as templates and sequence alignment of the query sequence given by the profile–profile match determined by HHPred [51], a homology model of AxDGC2 was derived with an extended Jα stalk linking the PAS and GGDEF domains (Fig. 10a). Note that in the coiled-coil region, the two templates overlap such that also the relative arrangement of the domains is unambiguously defined. Assuringly, the leucine heptad repeat pattern of the AxDGC2 linker sequence is found properly aligned with the corresponding pattern of the GCN4 coiled coil of the WspR hybrid (Fig. 11). This indicates the conservation not only of the input domain fold but also of the coiled-coil organization of Jα helices among AxDGC2 and the template histidine kinase. Thus, for PAS-GGDEF enzymes, an analogous signal transmission mechanism as in PAS-linked histidine kinases can be proposed, with signal transduction mediated by a quaternary change in the Jα stalk.

GAF domain

Another prominent regulatory input domain of diguanylate cyclases is the GAF domain that is typically found associated to a homotypic dimer in full-length proteins, such as in the full-length eukaryotic PDEA2 [52] (see Fig. 8d, inset). Here, α1 and the C-terminal α5 helix form coaxial coiled coils, very similar to the situation in the PAS dimer (Fig. 10a, inset). It should be noted, however, that the dimerization propensity of GAF domains seems to be not very high, since in isolation or even in tandem, they have been found dissociated [53]. Dimerization may also depend on the presence of the ligand; for a review, see Ref. [54].

LIC13137 from *Leptospira interrogans* is a GAF-GGDEF protein that gets activated upon cyclic adenosine monophosphate cAMP binding to its dimeric GAF domain (C. Guzzo, personal communication), thus establishing a functional link between cyclic nucleoside monophosphate (cNMP) and c-di-GMP-mediated signaling pathways. The full-length structure of a bona fide GAF-GGDEF diguanylate cyclase (PA2771) has been determined by structural genomics (Fig. 9d). It shows a tight GAF dimer with α2 (not α1) and α5 helix mediating the subunit contact. Unusually, however, α5 is not protruding from the domain as a continuous helix. Rather, the GAF-GGDEF linker is broken into two further helical pieces (α6 and α7), and the GGDEF domains are making lateral contacts with the GAF dimer. Obviously, this represents a nonproductive constellation. The unknown input signal event, probably ligand/ion binding to the GAF pocket, may cause the detachment of the GGDEF domains and the straightening up of the linker segment to generate a canonical coiled coil (as suggested by the sequence; Fig. 11) and thus activate the enzyme.

cNMP-binding domain

XC0249 from *Xanthomonas campestris* is a cNMP-binding GGDEF protein that gets activated by cGMP binding [55]. The structure of the dimeric cNMP-binding domain shows a coiled coil formed by the C-terminal C-helices that would connect to the output domain. For the *E. coli* catabolite gene activator protein, the lengthening of the C-terminal C-helix of its cNMP-binding domain upon cAMP binding has been demonstrated by NMR and X-ray crystallography

[56,57]. This was accompanied by a drastic change in the relative arrangement of the helix-turn-helix output domains, explaining the mechanism of this cAMP-regulated transcription factor. It would be most interesting to see whether a similar mechanism operates in cNMP-binding GGDEF proteins.

PAS-GAF-PHY domains

A light-sensitive diguanylate cyclase/phosphodiesterase (BphG1) has been studied several years ago [58], demonstrating that the GGDEF domain can be controlled by a PAS (with bound biliverdin)-GAF-PHY input module. In a synthetic biology approach, this protein has been recently converted to an efficient, near-infrared light-responsive diguanylate cyclase for optogenetic application [59]. Yang *et al.* [60] were the first to report the structure of a complete bacterial phytochrome input module of a histidine kinase. Very recently, the full-length structure of a bacteriophytochrome with a PAS output domain was reported [61]. Both dimeric structures show again (as in YtvA/FixL) the coaxial coiled coils, formed in succession by the GAF A-helices, by the GAF E-helices, which are continuous with the PHY A-helices, and by the PHY E-helices. Since the GAF and PYP E-helices are thus topologically equivalent to the PAS J-helix, a similar signal transduction mechanism as described above for PAS domains appears likely indeed.

PelD receptor

PelD is a transmembranous c-di-GMP receptor with the ligand being recognized by its C-terminal GGDEF-like domain, which is linked N-terminally to a GAF domain. The structure of this two-domain fragment has been reported and shows a monomeric structure with interacting domains and a rather short, disordered linker [62,63]. This noncanonical organization is in line with the absence of coiled-coil motif for the linker and the absence of a DxLT motif at the N terminus of the GGDEF-like domain. Still, it could also be caused by the absence of the transmembrane segment of this protein.

Transmembrane Signaling

Many GGDEF proteins appear to be controlled by external signals as indicated by associated membrane-spanning domains with large extracytosolic segments and are known or predicted to be constitutive dimers.

A well-studied example is the YfiBNR system of *Pseudomonas aeruginosa* (also found in other bacteria; see Ref. [64]), which comprises YfiN, a dimeric membrane-bound GGDEF protein. Its TM–CHASE8-TM-HAMP-GGDEF domain composition is reminiscent of histidine kinases, which carry DHp and CA domains instead of the GGDEF domain (for a recent review, see Ref. [17]). It has been shown for several sensors that the C-terminal helix of the HAMP domain is followed without interruption by the S-helix [65], which forms a parallel, dimeric coiled coil with its symmetry mate. Signal perception by the periplasmic domain is thought to result in a change in the organization/dynamics of this stalk (that is merged with the DHp bundle in histidine kinases) [66], very similar to the situation in soluble PAS, PYP, or GAF proteins discussed above.

A similar signal transduction mechanism can be envisioned for YfiN. The periplasmic YfiR protein represses the cytosolic GGDEF activity [67], most likely by affecting the dimeric organization of the periplasmic CHASE8 domains. This, in turn, would affect the relative arrangement of the two GGDEF domains *via* the TM-HAMP-S-helix segment.

A detailed structural model of full-length YfiN has been constructed by Giardina *et al.* [38], and possible structural changes have been discussed, best appreciated in their deposited movie. However, since then, new structural information has become available, which allows the refinement of this model. First, the LapD template structure for its periplasmic domain has been revised [68], now showing canonical (non-swapped) dimer organization. Second, the structure of an extended HAMP-S-helix construct (4cq4) has been determined [69], revealing in detail the predicted C-terminal coiled coil. Figure 10b shows a model for the organization of the cytosolic segment of YfiN, based on the 4cq4 construct and the WspR-GCN4 structure. As for AxDGC2 discussed above, the templates are overlapping, such that the position of the stalk sequence with respect to WspR-GCN4 is defined, and the hydrophobic patterns of the linkers match nicely (Fig. 11). Apparently, the S-helix bundle mediates signal transduction the same way as the Jα bundle in PAS-GGDEF and the α5 bundle in GAF-GGDEF proteins as suggested above.

Interestingly, there is another thoroughly studied regulatory system in *P. aeruginosa*, LapDG, that is organized similarly as YfiNR but mediates inside-out signaling [70] with c-di-GMP recognized by its cryptic EAL domain. C-di-GMP binding to the EAL domain activates the receptor and makes its PAS domain competent for sequestration of the periplasmic LapG protease (thereby inhibiting the LapG-catalyzed cleavage of a cell-surface-bound adhesin). Both a cytosolic S-helix-GGDEF-EAL fragment and a periplasmic PAS fragment have been analyzed structurally in the apo and in the c-di-GMP complexed state [68,70], and both are showing large structural changes upon complexation. C-di-GMP binding to the EAL domain causes its detachment from the S-helix and the formation of a canonical EAL/EAL dimer, whereas LapG binding to the PAS dimer induces asymmetry that is consistent with the

observed 1:2 stoichiometry. It should be noted that in the context of the full-length protein, apo-EAL binding to the S-helix would be possible only upon dissociation of the coiled coil and an azimuthal rotation of the S-helix. Indeed, disulfide mapping on full-length LapD appears to be consistent with such a scenario (H. Sondermann, personal communication).

GGDEF Inhibition

With GGDEF-mediated catalysis relying on the productive encounter of two substrate-loaded domains, principles of enzyme repression could be based on—apart from substrate binding competition or classical allosteric changes of active site structure—-signal-induced subunit dissociation or the impediment of active half-sites encounter. All hitherto unraveled inhibitory mechanisms operate according to the last mechanism.

Signal-Mediated Inhibition

The molecular organization of the transmembranous diguanylate cyclase YfiN and the inferred signal transduction mechanism have been discussed above. YfiN activity is inhibited by YfiR as has been shown *in vivo* [67] and, most recently, *in situ*, that is, with native membrane patches (S. Kauer *et al.*, to be published).

DgcZ from *E. coli* is a constitutive dimer with each subunit composed of a zinc-binding domain (CZB) and a catalytic GGDEF domain. The crystal structure of the full-length protein (Fig. 9a) has been determined in the presence of zinc showing the two active half-sites juxtaposed, but at a distance too far to allow catalysis [28], which is consistent with the fact that the enzyme is inactive in the presence of zinc. Although the apoprotein structure is not known, it has been suggested that the removal of zinc may either change the packing of the C-terminal helices of the CZB input domains or increase their mobility to allow the formation of a competent GGDEF dimer. Indeed, distinct helix packing is observed in the structurally related MID domain of DosC (compare Fig. 9a and d); see Ref. [30].

Negative Feedback

Many diguanylate cyclases show strong allosteric product inhibition ($K_i < 1$ μM) [20,71], which can be relieved by the addition of a c-di-GMP-specific phosphodiesterase [71,72]. This effect overrules any input-mediated activation and sets an upper limit for the product concentration. Most GGDEF crystal structures have been determined in complex with c-di-GMP and almost invariably show an intercalated c-di-GMP dimer bound to a tight turn between α2 and β2 (for examples, see Fig. 8a–c) that provides an arginine and an aspartate for the ligand interaction (for a detailed description, see also Ref. [13]). On the sequence level, the site can be easily recognized by its RxxD motif (preceding the GGDEF motif; Fig. 5), with R and D in positions i and i+3 of the turn, respectively (in DgcZ, the motif is degenerated to RxxE but is still competent for c-di-GMP binding). An arginine (R′) provided by helix α3 completes the binding site close to the α2, β2 turn (Fig. 12).

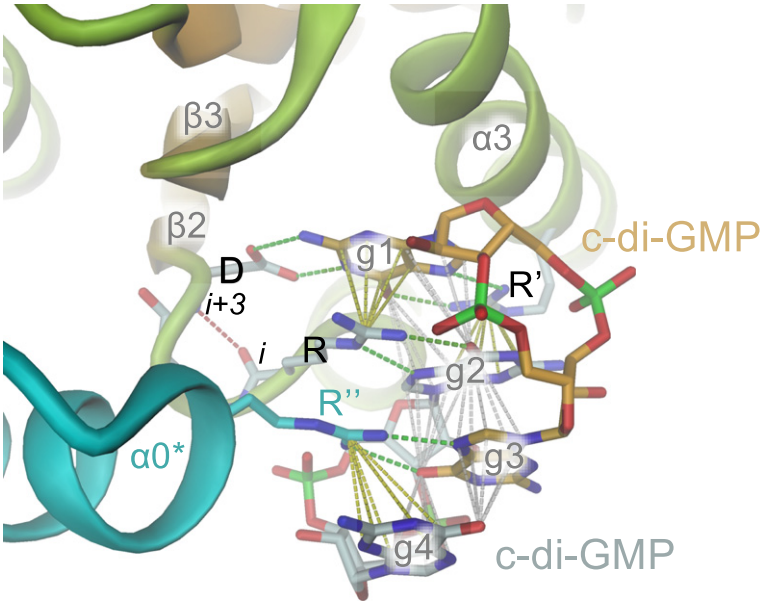

Fig. 12. Intercalated c-di-GMP dimer bound to the allosteric GGDEF inhibition site. The guanine bases are labeled g1 to g4 according to their position in the dimer. The two c-di-GMP molecules are distinguished by color. The dimeric ligand is bound to R and D of the RxxD motif, R′ of helix α3 (comprising together the Ip site) and R″ from α0* of the adjacent GGDEF domain. H-bonds are indicated in green and red, cation–π interactions in yellow, and base–base stacking in gray.

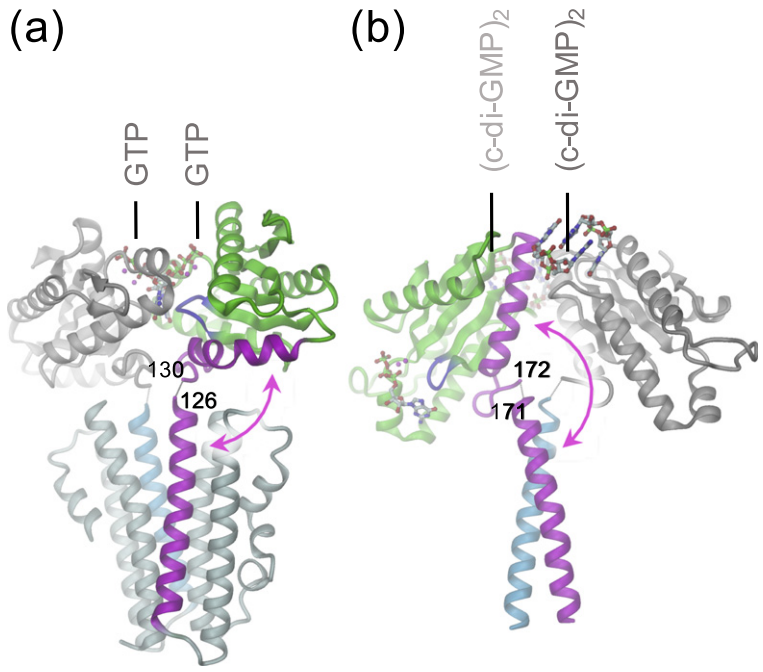

Fig. 13. Comparison of the GGDEF dimer in (a) competent and (b) product-inhibited constellation. (a) Side view of competent DgcZ; for a top view, see Fig. 6b. (b) C-di-GMP-inhibited GCN4-WspRGDDEF hybrid structure. For clarity, both structures have been idealized such that the dyad axes (not shown) of the input and output domains are colinear. Note that to convert both GGDEF dimers into each other, mainly the tilt angle (double arrow) between GGDEF domain and stalk would have to be changed.

The binding mode reflects the multivalent nature of the c-di-GMP dimer. The orientation and position of the RxxD motif residues projecting from the β-turn are well suited to allow the lateral H-bonding of the aspartate to the Watson–Crick (N1-H, N2-H2) edge of the proximal guanine base g1 and of the arginine to the Hoogsteen (O6, N7) edge of g2. Finally, arginine R′ forms H-bonds with the Hoogsteen edge of g1. In addition, and overlooked in the early reports, the two guanidinium groups form favorable cation–π interaction with the proximal bases (R with g1, R′ with g2). Such combination of H-bonding, cation–π interaction, and base–base stacking (Fig. 12) is frequently observed in protein–DNA interactions, where it is responsible for stair motif formation [73]. Most likely, the two c-di-GMP molecules bind sequentially (which appears sterically possible), since the concentration of preformed dimer can be neglected at cellular concentration [23].

Binding of c-di-GMP to the RxxD/R′ site of PleD (called primary inhibition site Ip), has only a minor inhibitory effect [34] and induces no significant allosteric structural changes [33]. Thus, enzyme inhibition relies on the observed (non-covalent) cross-link of the ligand to a secondary inhibition site (Is) on a separate domain (i.e., there would be "inhibition by domain immobilization") [20,34]. Figure 12 shows in detail how arginine R″, provided by helix α0* from the second GGDEF domain, is H-bonded to the Hoogsteen edge of g3 and stacks with g4, whereas Fig. 13b demonstrates how the (two symmetry-related) intercalated c-di-GMP dimers cross-link the backside of the two GGDEF domains. Note that the Is site is not uniquely defined; rather, an arginine from the last (PleD, MAQU2607) or from the last but one turn (WspR) of α0* can fulfill this function.

Side-by-side comparison of the competent and the inhibited GGDEF dimer structures (Fig. 13) is illuminating, since it suggests that the two forms can easily interconvert, mainly through a change in the tilt angle of the catalytic domains with respect to the stalk. Such mobility has already been inferred from the structure superpositions shown in Fig. 7b and would explain that negative feedback overrides input-domain-mediated activation.

For a diguanylate cyclase that lacks an RxxD motif (XCC3486 from *X. campestris*), competitive product inhibition has been demonstrated (K_i = 6 μM) and a peculiar binding mode has been unraveled with two partly intercalated c-di-GMP molecules occupying the two half-sites of a dimer [29]. Whether this binding mode is of physiological importance is difficult to judge, because no mutations were found that specifically abolished inhibition but not activity.

Concluding Remarks

During the last decade, a large body of structural and functional data has been acquired on diguanylate cyclases and has been reviewed here also in relation to the corresponding knowledge on mononucleotide cyclases, which catalyze a similar reaction, and on histidine kinases, which are controlled by similar signals. The large group of GGDEF-EAL proteins, some of them true bifunctional enzymes (for example,

see Ref. [74]), has not been covered; for a review, see Ref. [10]. Whether and how these two activities can be regulated reciprocally remains an important question. A corresponding structure has been determined recently [75]. Likewise, the group of catalytically inactive GGDEF receptors that use the inhibition site for ligand recognition has only be touched; see the recent review by Chou and Galperin [13].

The synthesis of the peculiar c-di-GMP compound with 2-fold symmetry requires the antiparallel association of two GTP-loaded GGDEF domains to allow the formation of two intermolecular phosphodiester bonds. Most diguanylate cyclases are constitutive dimers with accessory domains mediating homotypic subunit contacts. Strikingly, the various kinds of input domains (Rec, PAS, GAF, HAMP, etc.), which are specific for distinct signals, show a common protruding C-terminal helix that forms a parallel coiled coil (stalk) with its symmetry mate.

For many of the input domains, which also control other output domains like c-di-GMP-specific EAL phosphodiesterases (which are active only in the dimeric state [76]), histidine kinases, or eukaryotic phosphodiesterases, the rearrangement of the subunits within the dimer has been observed upon ligand binding or signal perception (light, oxygen, redox potential, etc.) by prosthetic groups. Such rearrangement could thus potentially change the crossing angle and/or the azimuthal orientation of the helices in the coiled-coil input–output domain linker (Fig. 14), which we would like to compare to the actions that can be exerted with a pair of chopsticks (in contrast to a pair of scissors with their fixed pivot and blade orientations; an analogy that is used for aspartate receptor and histidine kinase regulation).

In diguanylate cyclase, such a quaternary change would affect the relative distance/orientation of the two catalytic GGDEF domains that are linked to the C termini of the stalk. By this mechanism, productive encounter of the GTP-loaded GGDEF domains can be enabled/impeded, and correspondingly, enzyme activity can be controlled in response to signal perception. To attain the definite, competent dimer configuration (defined by GGDEF/GTP/Mg^{++} charge and surface complementarity), and for product release, mobility of the two catalytic domains relative to the stalk is probably required as indicated in Fig. 14 ("tilt"). Such mobility has the additional benefit wherein a range of stalk configurations would be compatible with activity, relieving evolutionary pressure on that signal transduction element.

Since, apart from the connecting coiled-coil, there is no need for any specific interactions between input and output domains, the domains may easily be recombined in a modular way without compromising the mechanism. Obviously, this opens a large playground for evolution to combine various input and output functions. Hopefully, it also legitimizes the conclusions drawn to a large extent in this review by analogy with the recent findings on histidine kinase regulation.

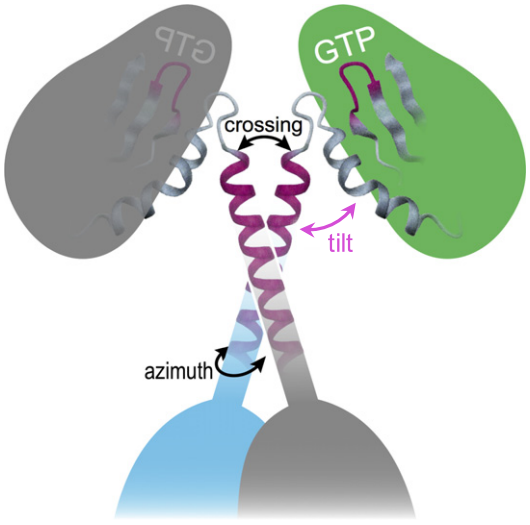

Fig. 14. Chopstick model of diguanylate cyclase regulation. The rigid extensions (red helices) of the regulatory dimer (bottom) form a coiled coil. A signal-induced change in the packing of the regulatory subunits thus affects the geometry (crossing and azimuth angles) of the coiled coil. Depending on the relative distance and orientation of the stalk termini, the intrinsic mobility of the appended GGDEF domains (tilt angle) will allow productive encounter of the two GTP-loaded catalytic domains to ensure catalysis.

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regulation

Abbreviations used:
c-di-GMP, cyclic di-guanosine monophosphate; GTP, guanosine triphosphate; cAMP, cyclic adenosine monophosphate; cNMP, cyclic nucleoside monophosphate; GGDEF domain, GGDEF motif-containing diguanylate cyclase domain; EAL domain, EAL motif-containing c-di-GMP phosphodiesterase; PAS, Per-Arnt-Sim; GAF, cGMP phosphodiesterase/adenylate cyclase/FhlA transcriptional activator; PHY, phytochrome specific; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; Ip, primary inhibition site; Is, secondary inhibition site; MID, middle domain; CZB, chemoreceptor zinc-binding; HAMP, present in histidine kinases, adenylate cyclases, methyl accepting proteins and phosphatases; TM, transmembrane domain.

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