Identification and Characterization of a Cyclic di-GMP-specific Phosphodiesterase and Its Allosteric Control by GTP*

Received for publication, April 22, 2005, and in revised form, June 23, 2005
Published, JBC Papers in Press, July 1, 2005, DOI 10.1074/jbc.M504429200

Matthias Christen, Beat Christen, Marc Folcher, Alexandra Schauerte, and Urs Jenal‡
From the Division of Molecular Microbiology, Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland

Cyclic diguanosine monophosphate (c-di-GMP) is a global second messenger controlling motility and adhesion in bacterial cells. Synthesis and degradation of c-di-GMP is catalyzed by diguanylate cyclases (DGC) and c-di-GMP-specific phosphodiesterases (PDE), respectively. Whereas the DGC activity has recently been assigned to the widespread GGDEF domain, the enzymatic activity responsible for c-di-GMP cleavage has been associated with proteins containing an EAL domain. Here we show biochemically that CC3396, a GGDEF-EAL composite protein from Caulobacter crescentus is a soluble PDE. The PDE activity, which rapidly converts c-di-GMP into the linear dinucleotide pGpG, is confined to the C-terminal EAL domain of CC3396, depends on the presence of Mg2+ ions, and is strongly inhibited by Ca2+ ions. Remarkably, the associated GGDEF domain, which contains an altered active site motif (GEDEF), lacks detectable DGC activity. Instead, this domain is able to bind GTP and in response activates the PDE activity in the neighboring EAL domain. PDE activation is specific for GTP (K_D = 4 μm) and operates by lowering the K_m for c-di-GMP of the EAL domain to a physiologically significant level (420 nM). Mutational analysis suggested that the substrate-binding site (A-site) of the GGDEF domain is involved in the GTP-dependent regulatory function, arguing that a catalytically inactive GGDEF domain has retained the ability to bind GTP and in response can activate the neighboring EAL domain. Based on this we propose that the c-di-GMP-specific PDE activity is confined to the EAL domain, that GGDEF domains can either catalyze the formation of c-di-GMP or can serve as regulatory domains, and that c-di-GMP-specific phosphodiesterase activity is coupled to the cellular GTP level in bacteria.

The cyclic nucleotides cAMP and cGMP are universally used as second messengers in intracellular signal transduction pathways. They mediate cellular processes such as vision, electrolyte homeostasis, or smooth muscle relaxation by modulating the activity of protein kinases, GTPases, or ion channels (1, 2). The intracellular levels of cAMP and cGMP are tightly controlled by their rate of synthesis (catalyzed by adenyl cyclases or guanylyl cyclases) and hydrolysis (catalyzed by phosphodiesterases). Phosphodiesterases (PDE) play a major role in the cellular response mediated by cyclic nucleotides and are used as primary therapeutic targets for several diseases (3). They act as effectors of signal transduction, function as homeostatic regulators of cyclic nucleotide levels, have been implicated in desensitization and termination of stimulation, and may also play an important role in controlling the diffusion of cyclic nucleotides and in channeling cyclic nucleotide signals (4, 5). Moreover, photoreceptor in human rod cells is mediated by rhodopsin and light signal transduction results from a dramatic reduction in cGMP concentrations, catalyzed by cGMP-specific PDE (1).

Whereas cAMP signaling is common to both prokaryotes and eukaryotes, cGMP does not seem to be used by bacterial cells. However, there is accumulating evidence that the cyclic dimer of GMP, c-di-GMP, plays a critical role in bacterial signal transduction (6, 7). c-di-GMP is synthesized from two GTP molecules by diguanylate cyclases (DGCs), and hydrolyzed by PDEs via the linear intermediate pGpG to GMP (Fig. 1A). Even though c-di-GMP was discovered almost two decades ago (8), its global role in bacterial signaling has become apparent only recently in the view of the growing bacterial genome sequence information available. In recent years, a rapidly increasing number of genetic studies have linked proteins involved in c-di-GMP synthesis or turnover to the ability of different bacteria to switch between a motile, single-cell state and a multicellular behavior associated with the production of extracellular matrix components and surface adhesion (9–21). Biochemical studies have associated the DGC activity with the readout domain of the Caulobacter crescentus PleD response regulator protein (22).

This domain, termed GGDEF (after its signature amino acid motif Gly-Gly-Asp-Glu-Phe), is widespread in bacteria but is not found outside the bacterial kingdom (23). The observation that GGDEF domains are often associated with domains involved in signal perception or signal transduction, argued for the existence of a dedicated regulatory network that converts a variety of different signals into the production of the second messenger c-di-GMP (6, 23). The resolution of the three-dimensional structure of the PleD response regulator in complex with c-di-GMP has not only revealed that the overall fold of the GGDEF domain is virtually identical to the adenylate cyclase, but has also proposed a catalytic mechanism for the condensation of two GTP molecules into c-di-GMP (24). In contrast to the molecular nature of the DGC, the c-di-GMP-specific PDE activity has remained somewhat of a mystery. Initial genetic and biochemical studies have linked PDE activity to proteins that contain both GGDEF and EAL domains (18, 19, 25, 26). Like

1 The abbreviations used are: PDE, phosphodiesterase; c-di-GMP, cyclic diguanosine monophosphate; pGpG, linear diguanosine monophosphate; MeOH, methanol; DGC, diguanylate cyclase; H6, hexahistidine tag; HPLC, high performance liquid chromatography; ESI-MS, electrospray ionization-mass spectrometry.

‡ To whom correspondence should be addressed: Division of Molecular Microbiology, Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland. Tel.: 41-61-267-2135; Fax: 41-61-267-2118; E-mail: urs.jenal@unibas.ch.

* This work was supported by Swiss National Science Foundation Fellowships 31–59050.99 and 3100A0–108186/1 (to U. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
GGDEF, the EAL (after its signature amino acid motif Glu-Ala-Leu) domain is found only in bacteria and its distribution more or less mirrors that of the GGDEF domains (23, 27).

Together, this has led to the proposal that the c-di-GMP-specific PDE activity might reside in the EAL domain (23).

The PleD response regulator is required for pole development during the *C. crescentus* cell cycle (11). During Caulobacter cell differentiation PleD specifically sequesters to one pole of the cell, where the morphological changes take place (22). Polar sequestration of PleD is coupled to the activation of the C-terminal GGDEF output domain via phosphorylation of the N-terminal receiver domain (22). This observation was lending support for the idea that synthesis of c-di-GMP by PleD might be limited to one cell pole may be to locally activate downstream targets or to restrict c-di-GMP production to one compartment during Caulobacter asymmetric cell division (22).

One would imagine that in both cases, a potent cellular PDE activity is required to rapidly counteract the DGC activity over time and to maintain spatial gradients established by PleD. To monitor and characterize the c-di-GMP-specific PDE activity in *C. crescentus*, we first developed an assay based on the hydrolysis of 32P-radiolabeled c-di-GMP. We then showed that the soluble fraction of *C. crescentus* cell extracts indeed contains a strong PDE activity. To characterize this activity more closely, we concentrated on EAL proteins encoded in the *C. crescentus* chromosome. A mutant lacking gene CC3396, which codes for a GGDEF-EAL composite protein, showed a more than 80% reduction of the soluble PDE activity (Table I). Enzymatic assays and UV cross-link experiments with purified full-length protein and single domain fragments confirmed that the PDE activity is contained within the EAL domain of CC3396. Remarkably, EAL-based PDE activity of CC3396 is strictly required for the GTP-specific activity of the EAL-borne PDE activity (Fig. 1B).

**Materials and Methods**

**Strains, Plasmids, and Media—** *C. crescentus* strains were grown in complex peptone yeast extract or in minimal glucose media (28). *Escherichia coli* strains were grown in Luria broth (LB) supplemented with antibiotics for selection, where necessary. The exact procedure of strain and plasmid construction is available on request.

**Purification of CC3396 and Preparation of C. crescentus Cell Extracts—** *E. coli* BL21 cells carrying the respective expression plasmid were grown in LB medium with ampicillin (100 mg/ml), and expression was induced by adding isopropyl-1-thio-D-galactopyranoside at A600 0.4 to a final concentration of 0.4 mM. After harvesting by centrifugation, cells were resuspended in buffer containing 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 5 mM β-mercaptoethanol, lysed by passage through a French pressure cell, and the suspension was clarified by centrifugation for 10 min at 5,000 × *g*. Soluble and insoluble protein fractions were separated by a high-spin centrifugation step (100,000 × *g*, 1 hr). The supernatant was loaded onto nickel-nitrilotriacetic acid affinity resin (Qiagen), washed with buffer, and eluted with an imidazol gradient. Protein preparations were examined for purity by SDS-PAGE, and fractions containing pure protein were pooled and dialyzed for 12 h at 4 °C.

*C. crescentus* CB15 cells were grown in peptone yeast extract and harvested by centrifugation at an A600 of 0.4. Cells were resuspended in buffer containing 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 5 mM mercaptoethanol, and 5 mM EDTA. Soluble and insoluble protein fractions were separated by a high-spin centrifugation step (100,000 × *g*, 1 hr). The supernatant was dialyzed for 4 h in buffer containing EDTA and then for 8 h in the same buffer without EDTA. Protein concentrations were measured by UV absorption.

**Synthesis and Purification of [32P]-c-di-GMP—** [32P]-Labeled c-di-GMP was produced enzymatically using α-labeled [32P]GTP (3000 Ci/mmol, Amersham Bioscience) and purified hexahistidine-tagged PleD, a phosphorylation independent constitutive active form of the PleD diguanylate cyclase (22). To a mixture of 87.5 μl of reaction buffer (250 mM NaCl, 25 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 5 mM β-mercaptoethanol, and 10.5 μM PleD-H6), 12.5 μl of α-labeled [32P]GTP (125 μCi, 41.66 pmol, 3000 Ci/mmol) was added. After 5 min at 25 °C, the reaction was stopped by adding an equal volume of 0.5 M EDTA, pH 8.0. The protein was precipitated by heating for 5 min at 95 °C followed by centrifugation for 2 min at 10,000 × *g*. The supernatant was loaded on a batch RP-18 column, salt was removed by washing 5 times with 200 μl of 25 mM triethylammonium carbonate buffer, pH 7.0, containing 1% (v/v) MeOH. c-di-GMP was eluted with 2 × 200 μl of triethylammonium carbonate containing 5% (v/v) MeOH. The buffer was subsequently removed in the SpeedVac and the purity of the compound was tested by separation on polyethyleneimine-cellulose plates (1:1.5 (v/v) saturated NH4SO4, 1.5 M KH2PO4, pH 3.6).

**Phosphodiesterase Assay—** c-di-GMP-specific phosphodiesterase activity was measured by monitoring the decrease of [32P]-c-di-GMP and the increase of [32P]GppGp by thin-layer chromatography. The PDE reaction buffer for the 100,000 × *g* supernatant of *C. crescentus* cell extracts or purified preparations of hexahistidine-tagged protein contained 10 mM NaCl, 50 mM Tris, pH 8.0, 10 mM β-mercaptoethanol. The GTP/protein mixtures were preincubated for 2 min prior to the addition of c-di-GMP. The reactions were carried out at 30 °C, aliquots were removed at different time points, and the reaction was stopped by adding an equal volume of 0.5 M EDTA, pH 8.0.

**Diguanylate Cyclase Assay—** The reaction mixtures with purified hexahistidine-tagged protein contained 25 mM Tris-HCl, pH 8.0, 250 mM NaCl, 10 mM MgCl2, and were started by the addition of 100 μM [32P]GTP (Amersham Biosciences; 3000 Ci/mmol). At regular time intervals the reaction was stopped with an equal volume of 0.5 M EDTA, pH 8.0.

**Polyethyleneimine-cellulose Chromatography—** Samples were dissolved in 5 μl of running buffer containing 1.15 (v/v) saturated NH4SO4 and 1.5 M KH2PO4, pH 3.6, and blotted on Polygram® CEL 300 polyethyleneimine-cellulose (Macherey-Nagel). Plates were developed in 1:1.5 (v/v) saturated NH4SO4 and 1.5 M KH2PO4, pH 3.6 (R(c-di-GMP) 0.2, Rj(GppG) 0.4), dried, and exposed on a Storage PhosphorScreen (Amersham Biosciences). The intensity of the various radioactive species was calculated by quantifying the intensities of the relevant spots using ImageJ software, version 1.33.

**Limited Tryptic Proteolysis—** To 90 μl of purified hexahistidine-tagged protein samples (1.5 mg/ml) dissolved in PDE Reaction Buffer (see above), 10 μl of trypsin solution (2 μg/ml trypsin in 1 mM HCl and 250 mM NaCl) was added. After incubation for 5 min at 37 °C, 2 μl of freshly prepared phenylmethylsulfonyl fluoride (Applichem) solution (0.1% in ethanol) was added, and the reaction was filtered though a 0.45-μm syringe filter (Whatman) before the digest products were gel filtered by gel filtration. Gel filtration experiments were performed on a SMART System using a Superdex 75 column (Amersham Biosciences) at a flow rate of 80 μl/min. The buffer contained 250 mM NaCl, 25 mM Tris, pH 8.0, 10 mM MgCl2, and 5 mM β-mercaptoethanol. Fractions of 80 μl were collected for the phosphodiesterase activity assay and for UV cross-linking experiments.

**UV Cross-linking with [32P]GTP and [32P]-c-di-GMP—** Protein samples were incubated for 10 min on ice in PDE reaction buffer containing 10 μM c-di-GMP, 100 μM GTP, and [32P]-c-di-GMP (0.75 μCi, 6000 Ci/mmol) or [32P]GTP (0.75 μCi, 3000 Ci/mmol). Samples were irradiated at 254 nm for 20 min on an ice-cooled, parafilm-wrapped 96-well aluminum block in an KR-100 photochemical reactor with a RPR-3500 UV lamp (The Southern New England Ultraviolet Co.). After irradiation, samples were mixed with 2× SDS-PAGE sample buffer (250 mM Tris-HCl, pH 6.8, 40% glycerol, 8% SDS, 2.4 M β-mercaptoethanol, 0.06% bromphenol blue, 40 mM EDTA) and heated for 5 min at 95 °C. Labeled proteins were separated by SDS-PAGE and stained for 95 °C by autoradiography.

**HPLC Analysis and ESI-MS Mass Spectrometry—** Reaction products were analyzed on an Agilent 1100 analytical reverse phase high performance liquid chromatography system with a diode array detector at 254 nm. Macherey-Nagel C125/3 LiChrospher 100 RP-18, 5-μm particle size, was used at 30 °C with 25 mM triethylammonium carbonate buffer, pH 7.0, containing 5% (v/v) methanol as mobile phase and a flow rate of 0.3 ml/min. ESI-MS mass spectra were measured on an Esquire 3000plus (Bruker Daltonics) and on a TQ-S7000 (Finngan) mass spectrometer. Matrix-assisted laser desorption ionization spectra were measured on a Reflex III spectrometer (Bruker Daltonics).
RESULTS

PDE Activity in the Soluble Fraction of C. crescentus Cell Extracts—To analyze the C. crescentus protein fractions for c-di-GMP-specific PDE activity, we developed an enzymatic assay, which is based on the hydrolysis of radiolabeled c-di-GMP and separation of the products on thin layer chromatography plates (see “Materials and Methods”). The constitutive active PleD mutant form, PleD*-H6 (22), was purified to homogeneity and used to enzymatically convert [33P]GTP to [33P]-c-di-GMP. When purified [33P]-c-di-GMP was added to aliquots of the dialyzed 100,000 g supernatant of cell extracts of C. crescentus wild-type strain CB15, the dicyclic nucleotide was rapidly hydrolyzed (Table I), arguing for the presence of a potent PDE activity in the soluble fraction of these cells.

A total of five genes encoding soluble EAL proteins were found on the C. crescentus chromosome. To identify a candidate PDE protein and to verify that it contributes to the enzymatic activity found in cell extracts, we selected CC3396 for further analysis. This decision was mainly based on the relatively small size and simple domain architecture of CC3396 (Fig. 1B). An in-frame deletion mutation of gene CC3396 was generated, and extracts of the resulting mutant strain UJ2812 were assayed for PDE activity in vitro. As shown in Table I, PDE activity of strain UJ2812 was reduced by about 80% as compared with wild-type, arguing that under the conditions tested, CC3396 is responsible for a major fraction of the PDE activity of the cell.

Purified CC3396 Is a c-di-GMP-specific PDE, Which Converts c-di-GMP into the Linear Form pGpG—The above experiments suggested that CC3396 is a prime candidate for a soluble PDE in C. crescentus. A hexahistidine-tagged version of the CC3396 protein was expressed in E. coli and purified to homogeneity on a nickel affinity column. When used in the PDE assay described above, purified fractions of the CC3396 protein could readily hydrolyze radiolabeled c-di-GMP (Table I). Separation of the reaction mixture on TLC plates revealed that the labeled c-di-GMP was rapidly converted into a new nucleotide species (Fig. 2B). HPLC analysis (Fig. 2A) and mass spectrometry identified this compound as the linearized diguanylate derivative pGpG (Fig. 2C, m/z—699.0, for c-di-GMP and m/z—707.0 for pGpG). Although the conversion of c-di-GMP into pGpG was relatively rapid (turnover rate: 2.42 ± 0.28 min⁻¹), GMP appeared as a secondary product of the reaction at an about 10-fold slower rate (Table I). Thus, CC3396 specifically and rapidly cleaves c-di-GMP into its linear form, whereas the formation of GMP might be a nonspecific byproduct of the enzymatic reaction. The PDE activity of CC3396 is highly specific for the cyclic dimer of GMP and showed no significant affinity for monomeric nucleotides cGMP and cAMP (data not shown). Also, whereas Mg²⁺ ions were critical for PDE activity, Ca²⁺ showed a strong inhibitory effect on the hydrolysis of c-di-GMP (Table II). Under no conditions were we able to detect DGC activity of the purified protein, arguing that the GGDEF domain of CC3396 is not a DGC (Table I).

Stimulation of the c-di-GMP-specific PDE Activity of CC3396 by GTP—The activity of monomeric PDEs is controlled by binding small effector molecules (including cAMP or cGMP) to N-terminal regulatory domains (5). To test the possibility that CC3396 could also be allosterically regulated, we measured the c-di-GMP-specific PDE activity of CC3396 in the presence or absence of different nucleotides (Table II). cAMP, cGMP, and dibutyryl-cGMP did not affect PDE activity of CC3396. Similarly, AMP, ATP, GMP, and GDP showed no effect. However, when the reaction mixture was supplemented with GTP (100 μM), the initial rate of the reaction increased by about 40-fold to 106.8 ± 1.5 μmol of c-di-GMP formed per μmol of protein and minute (Table II, Fig. 2B). The same positive effect was observed for an equimolar mixture of either GTP and GDP or GTP and GMP, arguing that both GDP and GMP do not counteract the positive effect of GTP. Interestingly, the GTP-activated form of CC3396 quantitatively converted c-di-GMP into the linear form pGpG, but failed to produce substantial amounts of GMP (Fig. 2, A and B, Table I). Together this suggested that the enzymatic activity of CC3396 responsible for the cleavage of c-di-GMP into pGpG is positively controlled.

| Strain/protein | c-di-GMP-specific | pGpG-specific | DGC activitya |
|---------------|-----------------|---------------|---------------|
| C. crescentus CB15b | 0.12 ± 0.02 μmol/(mg min) | 0.054 ± 0.004 μmol/(mg min) | ND/c |
| UJ2812 (ACC3396)b | 0.02 ± 0.01 μmol/(mg min) | ND/c |
| CC3396-His6c,d | 2.42 ± 0.28 μmol/(mg min) | 0.12 ± 0.06 μmol/(mg min) | 10 ± 5 pmol/μmol min |
Fig. 2. C. crescentus protein CC3396 is a phosphodiesterase. A, HPLC analysis of the PDE reaction products. Purified CC3396 protein (5 μM) was incubated for 1 min with 100 μM c-di-GMP, and 4 μM GTP. Nucleotides were separated on a RP-18 column before (top panel) and after the enzymatic reaction (bottom panel), and fractions were analyzed by ESI-MS. GTP, which was added to activate the reaction, was not hydrolyzed.

B, PDE activity of CC3396 in the absence (open symbols) or presence of GTP (4 μM GTP, closed symbols). The c-di-GMP hydrolysis activity of purified CC3396 is indicated as a function of the absolute concentrations of c-di-GMP (circles) and pGpG (squares) as determined by thin layer chromatography. Reactions included 150 nM purified CC3396 protein and 20 μM c-di-GMP and were incubated at 30 °C in buffer as described under “Materials and Methods.” The polyethyleneimine-cellulose thin layer chromatogram with the raw data is shown below the graph with each time point spotted in triplicate (upper panel, with GTP; lower panel without GTP).

C, mass spectrometry analysis of the reaction products of the CC3396 PDE. Mass spectrometry analysis of the reaction product of the PDE (top panel) and c-di-GMP (bottom panel) as shown in A and B. Top panel, ESI-MS of pGpG (m/z − 352.9 (pGpG)$^+$, and 707.0 (pGpG)$^2$. Bottom panel, ESI-MS of c-di-GMP $^m$− 689.0 [c-di-GMP$^+$H]$^-$, m/z − 699.9 [c-di-GMP]$^2$H$^-$Na$^+$, m/z − 710.9 [c-di-GMP$^+$Na]$^2$. D, determination of the equilibrium constant for GTP. Initial velocities of the PDE reaction were measured at increasing concentrations of GTP and $V_{max}/2$ was determined to be 4 μM.
TABLE II
Activation of c-di-GMP-specific PDE by GTP

| Nucleotide | PDE activity (μmol c-di-GMP/μmol min) |
|------------|--------------------------------------|
| AMP        | 1.93 ± 0.08                          |
| ATP        | 2.09 ± 0.22                          |
| cAMP       | 1.17 ± 0.46                          |
| cGMP       | 2.13 ± 0.32                          |
| dibu-cGMP  | 1.79 ± 0.18                          |
| GMP        | 1.87 ± 0.20                          |
| GDP        | 1.92 ± 0.40                          |
| GTP        | 106.8 ± 1.5                          |
| GTP + GDP  | 113.2 ± 1.9                          |
| GTP + GMP  | 97.2 ± 1.5                           |
| GTP, no Mg | 0.52 ± 0.10                          |
| GTP + Ca   | 1.61 ± 0.37                          |

*a* 200 μM.

The PDE Activity of CC3396 Resides in the C-terminal EAL Domain—The observation that CC3396 harbors PDE but lacks DGC activity raised the question of whether the enzymatic activity is entirely comprised within the GGDEF or the EAL domain, or is maybe the result of a catalytic interaction between the two domains. To distinguish between these possibilities, we attempted to separate the two domains by a limited tryptic digest of the full-length CC3396 protein and to determine the enzymatic activities of the individual domains. Treatment with trypsin resulted in the specific cleavage of CC3396 into two distinct peptide fragments of ~30 and 27 kDa in size, according to their migration behavior in polyacrylamide gels (Fig. 3A). Separation of these two cleavage products by gel filtration, followed by mass spectrometry analysis (Fig. 3, C and D) revealed that the slightly larger peptide corresponds to the N-terminal portion of CC3396, which includes the entire GGDEF domain (amino acids 1–279; fractions 8 and 9 in Fig. 3C), whereas the smaller peptide corresponds exactly to the C-terminal EAL domain (amino acids 280–554; fractions 10 and 11 in Fig. 3C). The cleavage site mapped to the Arg279 residue positioned in the center of the linker that connects the GGDEF and the EAL domain (Fig. 3D). It is reasonable to assume that the two domains can be separated by proteolysis because this charged residue is easily accessible for the protease because of its position in the flexible inter-domain linker.

PDE activity was found exclusively in fractions 10 and 11 of the gel filtration column used to separate the tryptic digest of CC3396 (Fig. 3C). Because fractions 10 and 11 contain the C-terminal EAL fragment, this strongly supported the view that the c-di-GMP-specific PDE activity is fully contained within the EAL domain. As shown in Table III, the specific activity of the separated EAL domain is similar to the activity found for the full-length CC3396 protein, arguing that the overall PDE activity is not significantly reduced upon separation of the catalytically active EAL from the GGDEF domain. Interestingly, when analyzing a CC3396 mutant with a mutation in the highly conserved aspartic acid residue of the EAL motive (E323Q), we found that both PDE activity and induction by GTP was not affected by this change (Table III). In vivo studies with the *Vibrio cholerae* EAL protein VieA had shown that a glutamate to alanine exchange at this position resulted in loss of activity (19). It is possible that the more conservative mutation chosen for CC3396 might still support PDE activity.

Allosteric Activation of the PDE Activity in EAL Through Binding of GTP to the GGDEF Domain—Whereas the EAL signature sequence of the C-terminal EAL domain is conserved in CC3396, the GGDEF domain has one of the highly conserved Gly residues of the active site (A-site) motif (24) replaced by Glu (GEDEF) (Fig. 3D). It is possible that this altered A-site in GGDEF is still able to bind GTP but cannot catalyze the diguanylate cyclase reaction. Such an altered domain might have been recruited as a regulatory module for the PDE activity residing in the C-terminal EAL domain. This would be in agreement with the observation that CC3396 has no apparent DGC activity (Table I). Also, a regulatory role for the GGDEF domain would be consistent with the finding that the isolated EAL domain almost fully retained the specific PDE activity of full-length CC3396, but in contrast to the intact protein could not be activated by GTP (Table III).

To obtain evidence in support of this idea we performed UV cross-link experiments with [33P]c-di-GMP and [33P]GTP using purified full-length CC3396 and the two individual domains separated by trypsin treatment (Fig. 3A). [33P]c-di-GMP specifically bound to full-length CC3396 and to the C-terminal EAL domain, but not to the N-terminal GGDEF domain fragment (Fig. 4A). In contrast, [33P]GTP, while also cross-linking to the full-length protein, did not bind to the EAL domain fragment but instead specifically reacted with the N-terminal GGDEF domain fragment (Fig. 4B). This suggested that GTP imposes allosteric control on the PDE enzyme activity of CC3396 by binding to its regulatory GGDEF domain.

Catalytically active GGDEF domains bind GTP in their A-site pocket, which in part is formed by a loop structure consisting of the highly conserved GGDEF (often GGGEF) motif (24). One possibility is that the slightly altered A-site motif (GEDEF) of the N-terminal domain of CC3396 has retained the ability to bind GTP and in response activates the associated EAL domain. To test this we generated a mutant CC3396 protein with the A-site motif changed to GQNEF. As shown in Table III, the mutant fully retained its PDE activity. But in contrast to the wild-type protein, the PDE activity was more or less constitutive with a 10-fold higher basal level activity in the absence of GTP as compared with wild-type (Table III). The
FIG. 3. The PDE activity of CC3396 resides in the C-terminal EAL domain. A, SDS-polyacrylamide gel with purified full-length CC3396 (FL), CC3396 after trypsin treatment (see “Materials and Methods”) (TT), and elution fractions 8–11 of the gel filtration column used to separate the tryptic fragments (see “Materials and Methods” and panel C). Note that lane TT was pasted from an independent gel. Samples of undigested (B) and trypsin-digested CC3396 protein (C) were separated by gel filtration (see “Materials and Methods”), and the PDE activity of fractions 5–18 eluting from the column was determined as described under “Materials and Methods.” The TLC plates with the resolved reaction products originating from each fraction are shown below the graphs. The bars in the graphs indicate the relative activity measured for each fraction, and the curve shows the protein concentration as determined by UV spectrometry. The protein peak of fractions 8 and 9 in panel C corresponds to the N-terminal GGDEF domain of CC3396, and the protein peak of fractions 10 and 11 corresponds to the C-terminal EAL domain of CC3396. Note that on the gel filtration column, the N-terminal GGDEF fragment runs at the position of a dimer, whereas the full-length CC3396 protein and the N-terminal EAL fragment run at the equivalent position of monomers. Fraction 15 corresponds to the cleaved C-terminal His tag, as determined by antibody staining with anti-His antibody. D, mass spectrometry analysis of the peptides originating from a tryptic digest of fractions 9 and 10 from panel C. A total of seven fragments of the GGDEF domain of CC3396 could be assigned to fraction 9, and a total of six fragments of the EAL domain could be assigned to fraction 10 digest. The corresponding fragments are highlighted in capital letters. LC-MS analysis of the undigested sample of fraction 10 revealed a mass of 29650.86 (Da 4.16) (amino acids 280–554, theoretical mass: 29655.0). The proposed trypsin cleavage site (Arg279) is highlighted.
of the several thousand bacterial GGDEF and EAL proteins listed in the non-redundant data bases have either a GGDEF or an EAL domain fused to other signaling domains, a large fraction combines both domains in the same polypeptide (31). A similar heterogeneity is found for cNMP (cAMP or cGMP)-specific cyclases and PDEs in eukaryotic cells, where several families of each enzyme class vary in ligand and co-factor specificities, in regulatory properties, and in tissue distribution (1, 4). This raised several important questions: Are the enzymatic activities responsible for the “make and break” of c-di-GMP really confined to these highly modular single domains? And if so, do all multidomain proteins that contain both a GGDEF and an EAL domain harbor both activities or have some of these proteins “specialized” in that they catalyze only the synthesis or degradation of c-di-GMP, respectively? The few examples studied so far have either been associated with DGC or PDE activity (25). No bifunctional enzyme has been described as yet. And finally, how would these activities be controlled if no obvious regulatory domains are fused to GGDEF or EAL?

Recent biochemical and structural studies have proposed a catalytic and regulatory mechanism for the synthesis of c-di-GMP by the GGDEF protein PleD (22, 24). Here we show that CC3396, a GGDEF-EAL protein of C. crescentus harbors c-di-GMP-specific PDE activity but lacks DGC activity. Analysis of the catalytic activities of the individual domains strongly suggested that the PDE activity of CC3396 is confined to the C-terminal EAL domain, and does not depend on the physical presence of the N-terminal GGDEF domain. To our knowledge, this is the first report that directly links an isolated EAL domain with the ability to catalyze the hydrolysis of c-di-GMP in vitro. Our data further propose a regulatory role for the N-terminal GGDEF domain of CC3396. The in vitro PDE activity of CC3396 is increased about 40-fold upon addition of GTP. Activation of the PDE activity seems to occur via the reduction of the $K_D$ for c-di-GMP from above 100 $\mu$M in the absence of GTP to 420 nM when GTP was present. Several lines of evidence suggest that GGDEF mediates this allosteric control through an interaction with the associated EAL domain. (i) Whereas the basal level PDE activity of full-length CC3396 and the isolated EAL domain are comparable, GTP activation could only be detected if the GGDEF domain was present. (ii) Compared with the bona fide DGC PleD (22), the GGDEF domain of CC3396 has a slightly altered consensus sequence A-site motif (GEDEF). Consistent with this, CC3396 does not seem to possess diguanylate cyclase activity in vitro. (iii) GTP specifically binds to the GGDEF but not to the associated catalytic EAL domain. (iv) A defined mutation in the A-site motif of the GGDEF domain (GQNEF) abolished allosteric activation and resulted in a constitutive activity of the associated EAL domain. This last observation implies that the GGDEF domain of CC3396 is a GGDEF-like domain, which is still able to bind GTP in the A-site cavity with a relatively high affinity ($K_D$ 4 $\mu$M) but does not catalyze the formation of c-di-GMP. If so, an original GGDEF domain might have been recruited as sensory domain for GTP through the loss of its catalytic function and the evolution of a regulatory interaction with EAL. If such a regulatory role of a GGDEF domain has indeed evolved from an enzymatically active GGDEF domain, two scenarios are possible. Either the GGDEF domain has lost DGC activity because key catalytic residues are missing, or because, in the context of the GGDEF-EAL composite protein, it is no longer able to form a dimeric structure required to condense two GTP molecules into c-di-GMP (24).

Thus, we propose that GGDEF domains, depending on their sequence conservation or on their oligomeric status, can have

---

**Table III**

| Protein   | c-di-GMP-specific PDE activity$^a$ | 10 $\mu$M c-di-GMP | 4 $\mu$M GTP |
|-----------|-----------------------------------|---------------------|--------------|
| CC3396    | 2.42 ± 0.28                       | 57.9 ± 5.9          |              |
| EAL domain$^b$ | 1.32 ± 0.33                      | 2.54 ± 1.10         |              |
| E323Q     | 1.2 ± 4.2                        | 76.2 ± 8.9          |              |
| ED123Q    | 26.9 ± 3.8                       | 77.3 ± 7.7          |              |

$^a$ PDE activity (initial velocities) was measured in the presence of 10 $\mu$M c-di-GMP and in the presence or absence of 4 $\mu$M GTP.

$^b$ The isolated EAL domain of CC3396 corresponds to fraction 10 of Fig. 3C.

---

**Fig. 4. GGDEF of CC3396 is a GTP binding regulatory domain.**

Full-length CC3396 (FL) and protein from elution fractions 8–11 of the gel filtration column used to separate the tryptic fragments were separated by SDS-PAGE (see Fig. 3A) and were used to UV cross-link with diguanylate cyclase activity in vitro.
two alternative biological activities and can play different roles in the controlled formation and hydrolysis of c-di-GMP. It is conceivable that at least a subgroup of the large family of bacterial GGDEF-EAL composite proteins represents PDEs with an associated regulatory GGDEF domain that can act as GTP sensor. At the same time, GGDEF-EAL proteins may exist that combine both a GGDEF-born DGC and an EAL-associated PDE activity. And finally it is equally possible that the EAL domain of GGDEF-EAL composite proteins also engages in a regulatory function by controlling the N-terminal DGC activity in response to the prevailing c-di-GMP concentration. Such a regulatory mechanism has been proposed recently for the DCG activity of the PleD response regulator, which is under tight negative allosteric control by its own product, c-di-GMP (24). A direct consequence of our findings is that each GGDEF or EAL domain will first have to be carefully analyzed biochemically before it can be assigned a catalytic or regulatory role.

The model that we propose for catalysis and regulation of the CC3396 PDE is shown in Fig. 1B. The protein architecture with an N-terminal regulatory and a C-terminal catalytic domain is reminiscent of cNMP-specific PDEs found in eukaryotes (e.g. PDE5, a phosphodiesterase highly specific for cGMP has two non-catalytic CGMP binding sites located at the N terminus). Binding of cGMP to these allosteric sites stimulates PDE activity, increases cGMP hydrolysis, and thus forms a negative feedback mechanism regulating the cellular cGMP concentration (32). Other N-terminal regulatory domains of cNMP-specific PDEs can serve as phosphorylation sites, can interact with transducing proteins, or act as an allosteric binding site for Ca2+/calmodulin effectors (5). It is reasonable to assume that c-di-GMP-specific PDEs in bacteria are also tightly controlled and that the allosteric control of CC3396 reported here represents a general phenomenon of this class of enzymes.

PDE activity is likely to be a critical component of c-di-GMP signaling in bacterial cells. But why would phosphodiesterase activity be coupled to the cellular concentration of GTP? Römling and colleagues (18) have reported that upon expression of the DGC protein AdrA in Salmonella typhimurium, the cellular GTP to c-di-GMP ratio reverses from about 100:1 to 1:10 (18). Thus, it is possible that when c-di-GMP synthesis is fully induced, uncontrolled hydrolysis of c-di-GMP to pGpG and GMP would deplete the cellular GTP pool. A massive reduction of the cellular GTP concentration has been reported as a consequence of the increased production of the “alarmone” pppGpp upon amino acid starvation in Bacillus subtilis (33). Similarly, the GTP concentration decreases considerably upon nitrogen starvation in C. crescentus (34). It is possible that to prevent drainage of the cellular GTP pool, specific PDEs are quickly turned off when the GTP concentration drops under a threshold level. Considering that the Kₚ for GTP of CC3396 is about 4 μM, one would expect such a threshold GTP concentration to be in the low micromolar range. Together with the observation that DGCs can be subject to tight allosteric feedback inhibition by their own product (24), this could be interpreted as a simple means for flux-controlled sensitivity, which would allow breaching the threshold for signal transduction by either increased production or decreased degradation of the second messenger. Alternatively, the prevailing GTP level of the cell itself could be used as a physiological signal to control the internal concentration of c-di-GMP through the controlled activity of PDEs. A drastic drop of the GTP concentration to the low micromolar range could lead to a rapid and substantial increase of the cellular c-di-GMP concentration through the inhibition of one or several key PDEs, which respond to GTP in a similar manner as observed for CC3396. Whereas such a regulatory role for GTP remains speculative, cellular GTP pools are known to affect developmental transitions in bacteria. A decrease in the cellular GTP concentration, but not of other purine or pyrimidine nucleotides, correlates with the initiation of morphological differentiation during nutrient starvation of B. subtilis and Streptomyces griseus (29, 35, 36). The signal responsible for the induction of sporulation is the reduced GTP pool, rather than pppGpp, which is formed under the same starvation conditions (29). The cellular GTP concentration is sensed by CooY, a transcriptional repressor of several sporulation and motility genes, whose repression activity depends on binding of GTP with a Kₚ in the physiologically relevant millimolar range (37, 38). It remains to be shown if the GTP concentration plays a similar regulatory role in cellular c-di-GMP signaling.

Finally, what is the physiological role of CC3396? CC3396 substantially contributes to the PDE activity in the soluble fraction of actively growing C. crescentus cells. It is possible that this protein adds to a more or less constant and rapid degradation of the freely diffusible cytoplasmic pool of c-di-GMP and would only be turned off under severe depletion of GTP. The cellular concentration of c-di-GMP has been determined to be about 1 μM in growing C. crescentus cells (this study) or 5–10 μM in cell culure producing Acetobacter xylinum (39). This is in good agreement with a Kₚ for c-di-GMP of 420 nM, which was determined for the PDE activity of CC3396 in the presence of GTP. It has been argued that specifically localized DGCs might act as “local pacemakers” of metabolic reactions resulting in cellular gradients of c-di-GMP (6, 40). In such a model, c-di-GMP synthesis and signaling would be locally confined and one would imagine that a strong and constitutive PDE activity is critical to spatially confine different c-di-GMP signaling pathways. Further studies are needed to test this idea more thoroughly.

Acknowledgments—We thank Timlan Schirmer and Helma Wennenmers for helpful discussions, Thomas Augst and Paul Jeno for mass spectrometry analysis, and Nicholas Amiot for providing a sample of c-di-GMP.

REFERENCES
1. Lucas, K. A., Pitari, G. M., Kazercenian, S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenuk, K. P., and Waldman, S. A. (2000) Pharmacol. Rev. 52, 375–414
2. Kaupp, U. B., and Seifert, R. (2002) Physiol. Rev. 82, 769–824
3. Essayan, D. M. (2001) J. Allergy Clin. Immunol. 108, 671–680
4. Houslay, M. D., and Milligan, G. (1997) Trends Biochem. Sci. 22, 217–224
5. Conti, M., and Jin, S. L. (1999) Prog. Nucleic Acids Res. Mol. Biol. 63, 1–38
6. Jenal, U. (2004) Curr. Opin. Microbiol. 7, 185–191
7. D'Argenio, D. A., and Miller, S. I. (2004) Microbiology 150, 2497–2502
8. Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinerberger-Ohana, P., Mayer, R., Braun, S., de Wreede, E., van der Marel, G. A., van Boom, J. H., and Benziman, M. (1987) Nature 325, 279–281
9. Spiers, A. J., Kahn, S. G., Bohnannon, J., Travisano, M., and Rainey, P. B. (2002) Genetics 161, 33–46
10. Drenkard, E., and Ausubel, P. M. (2002) Nature 416, 740–743
11. Aldridge, P., Paul, R., Goymer, P., Rainey, P., and Jenal, U. (2003) Mol. Microbiol. 47, 1069–1078
12. Huang, B., Whitechurch, C. B., and Mattick, J. S. (2003) J. Bacteriol. 185, 7068–7076
13. Bomech, N., Watnick, P., and Kolter, R. (2003) J. Bacteriol. 185, 1384–1390
14. Guvener, Z. T., and McCarter, L. L. (2003) J. Bacteriol. 185, 5431–5441
15. Tsimas, C., Anderson, C. R., Canales, S. R., and Golden, S. S. (2004) Microbiology 150, 1031–1040
16. Garcia, B., Latasa, C., Solano, C., Garcia-del Portillo, F., Gamazo, C., and Lasa, I. (2004) Mol. Microbiol. 54, 264–277
17. Choy, W. K., Zhou, L., Syn, C. K., Zhang, L. H., and Swarup, S. (2004) J. Bacteriol. 186, 7221–7228
18. Simm, R., Morr, M., Kader, A., Nimtz, M., and Römling, U. (2004) Mol. Microbiol. 53, 1123–1134
19. Tischler, A. D., and Camilli, A. (2004) Mol. Microbiol. 53, 857–869
20. Kirillina, O., Fetherston, J. D., Bobrow, A. G., Abney, J., and Perry, R. D. (2004) Mol. Microbiol. 54, 75–88
21. Johnson, M. R., Montero, C. L., Conners, S. B., Shockley, K. R., Bridger, S. L., and Kelly, R. M. (2005) Mol. Microbiol. 55, 664–674
22. Paul, R., Weiser, S., Amiot, N. C., Chan, C., Schirmer, T., Giuse, B., and Jenal, U. (2004) Genes Dev. 18, 715–727
23. Galperin, M. Y., Nikolskaya, A. N., and Koonin, E. V. (2001) EMBO J. 20, 11–21
24. Chan, C., Paul, R., Somaray, D., Amiot, N. C., Giuse, B., Jenal, U., and...
Allosteric Control of a c-di-GMP-specific Phosphodiesterase

25. Tal, R., Wong, H. C., Calhoon, R., Gelfand, D., Fear, A. L., Volman, G., Mayer, R., Ross, P., Amikam, D., Weinhouse, H., Cohen, A., Sapir, S., Ohana, P., and Benziman, M. (1998) J. Bacteriol. 180, 4416–4425
26. Chang, A. L., Tuckerman, J. R., Gonzalez, G., Mayer, R., Weinhouse, H., Volman, G., Amikam, D., Benziman, M., and Gilles-Gonzalez, M. A. (2001) Biochemistry 40, 3420–3426
27. Galperin, M. Y., Natale, D. A., Aravind, L., and Koonin, E. V. (1999) J. Mol. Microbiol. Biotechnol. 1, 303–305
28. Ely, B. (1991) Methods Enzymol. 204, 372–384
29. Lopez, J. M., Marks, C. L., and Freese, E. (1979) Biochim. Biophys. Acta 587, 238–252
30. Bochner, B. R., and Ames, B. N. (1982) J. Biol. Chem. 257, 9759–9769
31. Galperin, M. Y. (2004) Environ. Microbiol. 6, 543–545
32. Corbin, J. D., and Francis, S. H. (1999) J. Biol. Chem. 274, 13729–13732
33. Ochi, K., Kandala, J., and Freese, E. (1982) J. Bacteriol. 151, 1062–1065
34. Chiaverotti, T. A., Parker, G., Gallant, J., and Agabian, N. (1981) J. Bacteriol. 145, 1463–1465
35. Freese, E., Heine, J. E., and Galliers, E. M. (1979) J. Gen. Microbiol. 115, 193–205
36. Ochi, K. (1997) J. Bacteriol. 169, 3608–3616
37. Ratnayake-Lecamwasam, M., Serror, P., Wong, K. W., and Sonenshein, A. L. (2001) Genes Dev. 15, 1093–1103
38. Bergara, F., Ibarra, C., Iwamasa, J., Patarroyo, J. C., Aguillera, R., and Marquez-Magana, I. M. (2003) J. Bacteriol. 185, 3118–3126
39. Weinhouse, H., Sapir, S., Amikam, D., Shilo, Y., Volman, G., Ohana, P., and Benziman, M. (1997) FEBS Lett. 416, 207–211
40. Ross, P., Mayer, R., and Benziman, M. (1991) Microbiol. Rev. 55, 35–58
41. Ryjenkov, D. A., Tarutina, M., Moskvin, O. V., and Gomelsky, M. (2005) J. Bacteriol. 187, 1792–1798
Identification and Characterization of a Cyclic di-GMP-specific Phosphodiesterase and Its Allosteric Control by GTP
Matthias Christen, Beat Christen, Marc Folcher, Alexandra Schauerte and Urs Jenal

J. Biol. Chem. 2005, 280:30829-30837.
doi: 10.1074/jbc.M504429200 originally published online July 1, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M504429200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 41 references, 18 of which can be accessed free at http://www.jbc.org/content/280/35/30829.full.html#ref-list-1