Bacteriophytochromes are bacterial photoreceptors that sense red/far red light using the biliverdin chromophore. Most bacteriophytochromes work as photoactivated protein kinases. The *Rhodobacter sphaeroides* bacteriophytochrome BphG1 is unconventional in that it has GGDEF and EAL output domains, which are involved, respectively, in synthesis (diguanylate cyclase) and degradation (phosphodiesterase) of the bacterial second messenger c-di-GMP. The GGDEF-EAL proteins studied to date displayed either diguanylate cyclase or phosphodiesterase activity but not both. To elucidate the function of BphG1, the holoprotein was purified from an *Escherichia coli* overexpression system designed to produce biliverdin. The holoprotein contained covalently bound biliverdin and interconverted between the red (dark) and far red (light-activated) forms. BphG1 had c-di-GMP-specific phosphodiesterase activity. Unexpectedly for a photochrome protein, this activity was essentially light-independent. BphG1 expressed in *E. coli* was found to undergo partial cleavage into two species. The smaller species was identified as the EAL domain of BphG1. It possessed c-di-GMP phosphodiesterase activity. Surprisingly, the larger species lacking EAL possessed diguanylate cyclase activity, which was dependent on biliverdin and strongly activated by light. BphG1 therefore is the first phytochrome with a non-kinase photoactivated enzymatic activity. This shows that the photosensory modules of phytochromes can transmit light signals to various outputs. BphG1 is potentially the first “bifunctional” enzyme capable of both c-di-GMP synthesis and hydrolysis. A model for the regulation of the “opposite” activities of BphG1 is presented.

The genome of the anoxygenic phototrophic bacterium *Rhodobacter sphaeroides* 2.4.1 contains two highly similar (97% identity) proteins, RSP4191 (located in plasmid 2.4.1d) and RSP4111 (located in plasmid 2.4.1c), with an unusual domain architecture (Fig. 1A). Each is composed of the PAS-GAF-PHY photosensory module, typically present in phytochromes, linked to the unconventional GGDEF-EAL output module (Fig. 1B).

Phytochromes represent one of the six principal classes of photoreceptors identified thus far (1). In plants, phytochromes mediate light-dependent growth and development (reviewed in Ref. 2). In bacteria, phytochromes regulate biosynthesis of photosynthetic and nonphotosynthetic pigments (3–6) and motility (7). Most phytochromes covalently bind a linear tetrapyrrole, bilin, and function as receptors of red/far red light (for a recent review, see Ref. 8). Their photoreception is based on the ability of the bilin chromophore to undergo reversible conversion between the red-absorbing (P_r) and far red-absorbing (P_f) forms (9). Phytochromes from bacteria, except for some phytochromes from cyanobacteria, bind biliverdin (BV) and are known as bacteriophytochromes (Bphs) (6).

The N-terminal photoreception modules of Bphs incorporate all features required for chromophore binding and for photocconversion. A photoreception module of a Bph is typically composed of three protein domains, PAS, GAF, and PHY. PAS and GAF are ubiquitous sensory transduction domains involved in the binding of small molecular ligands and/or in protein–protein interactions (10, 11). PHY is likely to belong to the GAF family (12). The GAF domains of phytochromes possess bilin lyase activity required for covalent chromophore attachment (13). Both PAS and PHY domains contribute to proper conformation of the chromophore (13). The PAS domains of Bphs usually contain specific Cys residues, to which BV is covalently attached. In most cyanobacterial phytochromes and in plant phytochromes, the Cys residues are located in the PHY domains (14–16).

The C-terminal output modules of most studied phytochromes are kinases, histidine kinases in Bphs and cyanobacterial phytochromes and putative serine/threonine kinases in plant phytochromes (6, 17–21). In contrast, RSP4191 and RSP4111 contain a GGDEF-EAL module. The GGDEF and EAL domains control the turnover of cyclic dimeric GMP (c-di-GMP), which functions as a ubiquitous second messenger in bacteria. c-di-GMP affects the formation and structure of bacterial biofilms, flagella- and pili-based motility, gene expression, and host-pathogen interactions (reviewed in Ref. 22). We have demonstrated that *R. sphaeroides* can synthesize c-di-GMP (23); however, the functions of c-di-GMP in this organism are yet to be identified.

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The abbreviations used are: P_r, red-absorbing form of a phytochrome; P_f, far red-absorbing form of a phytochrome; Bph, bacteriophytochrome; BV, biliverdin; c-di-GMP, cyclic dimeric GMP; DGC, diguanylate cyclase; PDE, phosphodiesterase; HPLC, high-pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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The GGDEF domains possess diguanylate cyclase (DGC) activity; i.e. they synthesize c-di-GMP from two GTP molecules (23–25). DGCs function as homodimers, where each GGDEF domain apparently binds one GMP moiety (26). c-di-GMP is degraded into linear dimeric GMP by the EAL domains that possess phosphodiesterase (PDE) activity (24, 27–29). The activity of EAL domains does not require dimerization (27). Although the GGDEF and EAL domains present in bacterial genomes are linked to various sensory domains (30, 31), to date, only regulation by oxygen and by phosphorylation have been studied in these proteins (23, 25, 32). We designated RSP4191 and RSP4111, respectively, BphG1 and BphG2, where “G” stands for c-di-GMP (Fig. 1A).

The BphG proteins contain GGDEF and EAL domains arranged in tandem (GGDEF-EAL). Theoretically, these proteins could possess either DGC or PDE activity or both. However, prior to this study, no proteins containing both activities have been identified. Therefore, it was unclear whether the “opposite” activities, i.e. c-di-GMP synthesis and c-di-GMP hydrolysis, could coexist in a single protein, and if they could, how they would be regulated. To gain insights into these questions, we undertook biochemical and photochemical analyses of the *R. sphaeroides* BphG proteins. We found that (i) similar to conventional Bphs, these proteins undergo reversible photoconversion between the P_6 forms, i.e. they are photochromic; (ii) they possess two enzymatic activities; however, only one activity is light-dependent; and (iii) they may employ an apparently unique switch between the opposite enzymatic activities.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—The *bphG1* gene was PCR-amplified from the *R. sphaeroides* 2.4.1 genomic DNA using primers FbphG1-Xbal (5’-GCTCTAGAGGTGTCATGCAGCTC-TCTGGG-3’) and RbphG1-HindIII (5’-CCCAAGCTTATGATGGTATGTTCCTTTCGTGATGCCGCGATC-3’) (restriction sites are underlined). The Xbal/HindIII-digested 2.8-kb PCR product was ligated into the NheI/HindIII-digested pET23a vector (Novagen) to yield plasmid pBphG1::His10-expressing *R. sphaeroides* BphG1::His10 fusion (Fig. 1B). The 3.15-kb *bphG2* fragment was PCR-amplified from the genomic DNA of the *R. sphaeroides* bphG1 null mutant (to be described elsewhere) using primers FbphG2-Xbal and RbphH-KpnI (5’-GGCGGCTTATGATGGTATGTTCCTTTCGTGATGCCGCGATC-3’) (restriction sites are underlined). The Xbal/HindIII-digested fragment containing *bphG2* was cloned into pET23a to yield pBphH15 expressing pBphG2::His10. All constructs were verified by DNA sequencing.

For BphG holoprotein expression, plasmid pPL-PCB (KmR) expressing Synechocystis sp. heme oxygenase and biliverdin reductase genes *ho1* and *pcyA* (33) was modified as follows. The *pcyA* gene was deleted, and the *P lac* promoter was replaced with the *P T7* promoter. The transformants of *Escherichia coli* BL21(DE3) (pLysS) (Novagen) carrying *ho1* under the *P T7* promoter proved to be heterogeneous in regard to the *ho1* levels. One transformant accumulated *ho1* protein to higher levels compared with others, as judged by the light green color of the cells following induction with isopropyl 1-thio-β-D-galactopyranoside and SDS-PAGE. Restriction analysis of the plasmid isolated from this strain suggested that the *ho1*-containing plasmid was integrated into pLysS. The resulting hybrid plasmid (KmR) expressing high levels of Ho1 was designated pT7-ho1-1. The DNA sequence of the coding region of the *ho1* gene from pT7-ho1-1 remained intact, which allowed us to use this plasmid as a source of BV IXα, the product of *Ho1* reaction (33). pBphG8–10 and pBphH15 were transformed into BL21(DE3) (pLysS) and BL21(DE3) (pLysS; pT7-ho1-1) to produce the apo- and holoproteins, respectively. The PAS-GAF-PHY-GGDEF fragment was PCR-amplified using primers FbphG1-Xbal and RphyGGDEF-HindIII (5’-CCCAAGCTTATGATGGTATGTTCCTTTCGTGATGCCGCGATC-3’) and cloned into pET23a to generate plasmid pPgyGGDEF (Fig. 1B).

**Spectral Analysis**—Absorbance spectra were recorded with a UV-1601 PC UV-visible spectrophotometer (Shimadzu) at room temperature. Protein solution (100 μl) in a 10-mm light path quartz cuvette was irradiated directly in the spectropho...
Unorthodox Bacteriophytochrome Involved in c-di-GMP Turnover

R. sphaeroides BphG1 Is a Photochromic Bph—We anticipated that BphG1 and BphG2, whose sequences are almost identical, would have very similar characteristics (Fig. 1A). Therefore, we chose to characterize BphG1 and subsequently to test the key findings on BphG2. We constructed a C-terminal BphG1::His$_{10}$ fusion and overexpressed it under the P$_{f0}$ promoter in E. coli BL21(DE3) (pLysS). We purified the apoprotein using Ni$^{2+}$ affinity chromatography followed by size exclusion chromatography (Fig. 2A, lanes 2–5). To overexpress the BphG1 holoprotein, we used BL21(DE3) (pLysS) containing a constructed plasmid, pT7-ho1–1, which expressed the Synecochystis sp. heme oxygenase gene ho1 (33) and therefore produced BV (Fig. 2A, lanes 6–9).

Enzymatic Assays—All enzymatic assays were performed at 30°C in a water bath. A standard reaction mixture (1 ml) contained 0.5–4.0 μM enzyme in the PDE or DGC assay buffer (23). The protein kept in the dark was irradiated through a flexible light guide for 5 min prior to reaction with red or far red light, and the light was kept on for the duration of the assay. The reaction was started by the addition of 1:100 (v/v) of c-di-GMP for PDE assays or GTP for DGC assays. Aliquots (100 μl) were withdrawn at different time points, mixed with 10 μl of 0.2 M CaCl$_2$ (PDE reactions only), and boiled for 5 min. The precipitated protein was removed by centrifugation at 15,000 × g for 5 min. The supernatant was filtered through a 0.22-μm pore size filter (Millipore) and analyzed by reversed-phase high-pressure liquid chromatography (HPLC) as described earlier (23). Protein assays were performed using the BCA method (Pierce) with bovine serum albumin as the protein standard. Proteins were analyzed using SDS-PAGE or the Protein 200 Plus LabChip kit on an Agilent 2100 Bioanalyzer (Agilent Technologies).

Chromatography—Nucleotides were separated and quantified by reversed-phase HPLC (Summit HPLC System, Dionex, CA) as described earlier (23) using the 15 × 4.6-cm Supelcosil LC-18-'T' column (Supelco). Proteins were purified and their oligomeric state was determined using a Superdex 200, 10/300 GL gel filtration column (Amersham Biosciences) equilibrated with the PDE assay buffer as described earlier (23).

Mass Spectroscopy—The 31-kDa band corresponding to the protein copurified with BphG1 was purified by size exclusion chromatography and run on SDS-PAGE. The protein band was excised from the gel and sent to Proteomic Research Services, Inc. (Ann Arbor, MI) for peptide fingerprinting and protein identification. There, the protein was subjected to in-gel trypsin digestion and MALDI-TOF mass spectroscopy analysis on a Voyager DE-STR instrument (Applied Biosystems). The m/z values of the tryptic peptide products were analyzed by ProFound software.

RESULTS

R. sphaeroides BphG1 Is a Photochromic Bph—We anticipated that BphG1 and BphG2, whose sequences are almost identical, would have very similar characteristics (Fig. 1A). Therefore, we chose to characterize BphG1 and subsequently to test the key findings on BphG2. We constructed a C-terminal BphG1::His$_{10}$ fusion and overexpressed it under the P$_{f0}$ promoter in E. coli BL21(DE3) (pLysS). We purified the apoprotein using Ni$^{2+}$ affinity chromatography followed by size exclusion chromatography (Fig. 2A, lanes 2–5). To overexpress the BphG1 holoprotein, we used BL21(DE3) (pLysS) containing a constructed plasmid, pT7-ho1–1, which expressed the Synecochystis sp. heme oxygenase gene ho1 (33) and therefore produced BV (Fig. 2A, lanes 6–9).

When purified, the BphG1 apoprotein was colorless, whereas the holoprotein had a blue-green color indicative of bound BV. By size exclusion chromatography, we determined that BphG1 was present in several forms, i.e. oligomers of the apparent molecular masses >443 kDa, and a presumed tetramer of the apparent mass of 390 kDa. A very small amount of BphG1 was present as an apparent monomer (92 kDa) (Fig. 2B).

The UV-visible spectrum of the BphG1 holoprotein in the dark was dominated by the absorption maximum of 709 nm, characteristic of the P$_{f0}$ form (Fig. 3A). This suggests that P$_{f0}$ is the ground state of BphG1. Upon irradiation with red light (694 nm) or with white light, we observed decreased absorption at 709 nm and a new absorption maximum at 755 nm (Fig. 3A). The peak at 755 nm corresponds to the P$_{f0}$ form. When the red light-irradiated BphG1 was exposed to the far red light (768 nm), the P$_{f0}$ form quickly reverted to the P$_{f0}$ form. The difference spectrum (P$_{f0}$ – P$_{f0}$) was similar to the difference spectra of the Bphs that function as protein histidine kinases (3, 6, 15, 19) (Fig. 3A). It is known that the photoexcited forms of many phytocromes can convert to the ground state in the dark, and so could BphG1. It converted from the P$_{f0}$ to the P$_{f0}$ form in the dark slowly, taking ~40 min for a 95% conversion (Fig. 3B).

The PAS domain of BphG1 contains a cysteine residue, Cys-18, which is positionally conserved among Bphs and is predicted to serve as the site of BV attachment (14, 15). To test whether or not BV in the BphG1 holoprotein was bound covalently, we separated the holo- and apoproteins by SDS-PAGE, stained the gel with a Zn$^{2+}$ solution, and exposed it to UV light. We observed the zinc-dependent fluorescence of the holoprotein (but not the apoprotein), which indicated that BV was bound covalently (Fig. 3C). Therefore, in all respects, BphG1 behaved as a typical photochromic Bph.

BphG1 Has an Essentially Light-independent c-di-GMP-specific PDE Activity—To test for enzymatic activity of BphG1, we incubated the holoprotein with GTP and c-di-GMP, which are the substrates of the DGC and PDE activities, respectively. Incubation with GTP did not result in the formation of c-di-GMP, whether the reaction was performed in the dark or light (Fig. 2C). Longer term incubation with GTP yielded some GDP and P$_{i}$ (not shown), which is characteristic of the enzymatically inactive GGDEF domains (23). The incubation of BphG1 with c-di-GMP resulted in formation of linear dimeric GMP, the
expected product of the PDE reaction (Fig. 2D). No hydrolysis of cyclic mononucleotides (cAMP or cGMP) was detected (not shown). Therefore, BphG1 functions as a c-di-GMP-specific PDE.

We tested the effect of irradiation on the PDE activity of the holoprotein but found no such effect. We were puzzled as to why the activity of the photochromic protein would be light-independent. Therefore, we undertook a significant effort to identify conditions where the PDE activity would show light dependence. We modified the composition and pH of the reaction buffer, varied the protein-to-substrate ratios, tested the effects of additional nucleotides on enzymatic activity, examined the light dependence of individual oligomeric forms of BphG1, purified and assayed the activity of the BphG2 holoprotein using plasmid pBphH15, replaced the Synechocystis sp. heme oxygenase with the R. sphaeroides heme oxygenase BphO1, and tested the light dependence of the BphG1-BphO1 complex. The maximum difference in the rate of PDE reaction between the Pfr and Pr forms that we have observed was with the tetramer fraction of BphG1. However, even there the difference was marginal, i.e. ~25%, which is unlikely to be physiologically significant (Fig. 2E). It appears that photoreception is decoupled from the output activity of BphG1. Therefore,

3 M. Tarutina and M. Gomelsky, unpublished data.

FIGURE 2. Biochemical characterization of the BphG1 protein. A, overexpression in E. coli and purification of the apoform (lanes 2–5) and holoform (lanes 6–9) of BphG1. Lane 1, molecular mass markers, in kDa; lanes 2 and 6, uninduced cells; lanes 3 and 7, cells after induction with isopropyl 1-thio-β-D-galactopyranoside; lanes 4 and 8, proteins eluted from the Ni²⁺ column; lanes 5 and 9, proteins after size exclusion chromatography showing oligomeric states of the BphG1 holoprotein. Log MW (molecular mass) is plotted on the y-axis. The following molecular mass standards (Sigma) were used: equine spleen apoferritin (443 kDa), Saccharomyces cerevisiae alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), bovine erythrocytes carbonic anhydrase (29 kDa), equine heart cytochrome c (12 kDa), and cyanocobalamin (1.35 kDa). B, size exclusion chromatography showing oligomeric states of the BphG1 holoprotein. C, HPLC chromatogram showing that BphG1 does not react with GTP. Incubation times are shown. D, HPLC chromatogram showing products of incubation of BphG1 with c-di-GMP. NAD was used as a loading control. E, kinetics of the PDE activity of the purified tetramer fractions of the Pr and Pfr forms of the BphG1 holol- and apoproteins (0.5 μM protein). Error bars are based on three independent measurements.
BphG1 functions as an essentially light-independent c-di-GMP-specific PDE.

The lack of light dependence prompted us to investigate the possibility that the PDE activity responds to the presence or absence of BV, which would make BphG1 a sensor of BV, not light (12). To test this possibility, we compared the PDE activities of the apo- and holoforms of BphG1. These activities turned out to be very similar (Fig. 2E). The lack of BV dependence confirmed our conclusion that the PDE activity is decoupled from photoreception. This remained at odds with photochromicity of the BphG1 holoprotein until a serendipitous discovery described below revealed the light-dependent properties of BphG1, which were completely unexpected.

The Protein Copurified with BphG1 Is Its EAL Domain—We noticed that an ≈31-kDa protein was copurified with BphG1 through the Ni²⁺ affinity chromatography, whether it was expressed as an apo- or holoprotein (Fig. 2A, lanes 4 and 8). We decided to explore the nature of the copurified protein. We separated the 31-kDa protein from BphG1 by size exclusion chromatography (Fig. 4A) and subjected it to trypsin digestion followed by peptide fingerprinting by MALDI-TOF mass spectroscopy. To our surprise, the copurified protein was found to perfectly match (16 peptides matched; expectation 5 × 10⁻¹⁰) the sequence of BphG1. The utmost N-terminal peptide was identified as 664GELFRPSLYEETTQLVELDNDMR. It corresponds to the linker between the GGDEF and EAL domains as determined by the Pfam protein domain data base. If we assume that Gly⁶⁶⁴ is the utmost N-terminal residue in the copurified protein, then the expected size of the copurified protein would be 273 amino acids. This correlates with 31 kDa, the observed molecular mass of the copurified protein. The copurified protein is therefore composed of the linker between the GGDEF and EAL domains as determined by the Pfam protein domain data base. If we assume that Gly⁶⁶⁴ is the utmost N-terminal residue in the copurified protein, then the expected size of the copurified protein would be 273 amino acids. This correlates with 31 kDa, the observed molecular mass of the copurified protein. The copurified protein is therefore composed of the linker between the GGDEF and EAL domains as determined by the Pfam protein domain data base. If we assume that Gly⁶⁶⁴ is the utmost N-terminal residue in the copurified protein, then the expected size of the copurified protein would be 273 amino acids. This correlates with 31 kDa, the observed molecular mass of the copurified protein. 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Knowing that EAL domains are sufficient for PDE activity (27), we tested the activity of the 31-kDa fragment. We found that it indeed retained a c-di-GMP-specific PDE activity. It is noteworthy that, on the size exclusion column, the EAL domain fragment ran with an apparent mass of 58 kDa, corresponding to that of a dimer (Fig. 4B). The apparent dimerization is unlikely to be required for PDE activity, because individual EAL domains that we analyzed earlier existed predominantly in the monomeric forms (27). Therefore, the tendency to homodimerize must be somewhat specific to the EAL from BphG1 and/or to the linker between GGDEF and EAL. The relative specific PDE activity of the EAL domain fragment was ~30% of that of the full-length BphG1 (Fig. 4C). Puzzled by the observation that the enzymatic activity of BphG1 was concentrated in its EAL domain, we decided to investigate the function of the remainder of the protein PAS-GAF-PHY-GGDEF.

FIGURE 3. Photochemical characterization of the BphG1 protein. A, UV visual spectra of the Pₐ and Pₖ forms of the BphG1 holoprotein and the difference (Pₐ – Pₖ) spectrum. B, Pₐ → P, recovery in the dark. C, zinc-induced fluorescence of chromoproteins separated by SDS-PAGE showing BV binding to the BphG1 holoprotein but not the apoprotein (45). Protein amounts in adjacent lanes differ by 3-fold.

FIGURE 4. Biochemical characterization of the EAL domain fragment of BphG1. A, protein purification. Lane 1, molecular mass markers, in kDa; lane 2, the EAL domain fragment after size exclusion chromatography of the protein mixture eluted from the Ni²⁺ column (see Fig. 1A, lane 4). B, size exclusion chromatography showing an apparent EAL domain dimer. C, kinetics of the PDE activity.
The PAS-GAF-PHY-GGDEF Fragment of BphG1 Is a Light-dependent DGC—The PAS-GAF-PHY-GGDEF fragment was not copurified along with BphG1 as was the EAL domain fragment, because the His tag used for affinity purification is located at the C terminus of BphG1. We therefore engineered a C-terminal His tag to the PAS-GAF-PHY-GGDEF protein fragment (Fig. 1B, plasmid pHGDEF) and overexpressed and purified this fragment in the holo- and apoforms (Fig. 5A and not shown). We found that, similar to the full-length BphG1, the PAS-GAF-PHY-GGDEF holoprotein contained covalently bound BV and was photochromic. Its spectral parameters were identical to those of the full-length BphG1 (not shown). We tested the enzymatic activity of this fragment. Intriguingly, it displayed DGC activity (Fig. 5B). Furthermore, the DGC activity was strongly activated by light (Fig. 5C). Therefore, the PAS-GAF-PHY-GGDEF fragment functions as a bona fide photoactivated DGC. The PAS-GAF-PHY-GGDEF apoprotein showed no DGC activity suggesting that BV is essential for this activity (not shown).

Based on the observations presented above, we hypothesize that BphG1 has a potential to work as a “bifunctional” enzyme. Depending on conditions, it may act either as PDE, which degrades c-di-GMP essentially independently of light, or as DGC, which synthesizes c-di-GMP in the light-dependent manner. This makes BphG1 the first photochromic Bph that has a non-kinase light-dependent enzymatic output(s) and the first potentially bifunctional enzyme involved in c-di-GMP synthesis and hydrolysis. The switch in “enzymatic identity” from PDE to DGC employed by BphG1 may involve a cleavage of the C-terminal EAL domain.

DISCUSSION

In this report, we have presented spectroscopic and biochemical characterization of BphG1, the unconventional Bph from R. sphaeroides. The second, highly similar (97% identity) Bph protein present in this bacterium, BphG2, proved to be indistinguishable from BphG1 in vitro. We found that BphG1, which is different from the majority of Bphs in that it contains the GGDEF-EAL output module, responds to light in a manner similar to that of the Bphs containing kinase outputs. We also found that one of the two enzymatic activities of BphG1, DGC, is light-dependent in vitro, whereas the other one, PDE, is essentially light-independent.

Until recently, it has remained unclear to what extent the complex photosensory modules present in phytochromes can operate with the non-kinase outputs (8). A study by Chen et al. (34) on a plant phytochrome PhyB has shown that light-induced conformational changes transduced to an output PAS domain result in the exposure of the cryptic nuclear localization signal, which promotes translocation of PhyB to the nucleus. Our data on the PAS-GAF-PHY-GGDEF fragment of BphG1 suggest that light-induced conformational changes are efficiently transduced to the GGDEF domain to result in activation of the DGC activity. Therefore, photosensory modules of phytochromes can regulate different outputs. Hence, phytochromes are no exception to modular organization, which is common in sensory transduction proteins (35). It is reasonable to anticipate that many Bph-like proteins identified through microbial and environmental sequencing and containing non-kinase outputs, e.g. methyl-accepting domain, phosphatase 2C domain, GGDEF, and EAL, will prove to function as bona fide photoreceptors (36). These advances and the recent progress with structural characterization of the photosensory core of the Deinococcus radiodurans Bph (16) provide an exciting possibility to rationally design phytochrome-based photoswitches with desired outputs. It is noteworthy that the PAS-GAF-PHY-GGDEF fragment from BphG1 can already be employed for photoactivated c-di-GMP synthesis. It may be useful for studying the molecular mechanisms of action of this novel second messenger in bacteria or for investigating pharmacological effects of c-di-GMP in mammalian cell cultures and tissues (37–39).

In addition to extending our knowledge of phytochromes, characterization of the BphG1 protein provided new insights into the function and regulation of proteins containing tan-
demly arranged GGDEF-EAL domains. These proteins represent a large fraction of all GGDEF/EAL domain proteins encoded in bacterial genomes, e.g. 38% in *E. coli*. To date, only a handful of them have been assayed in *vitro* (28, 40), and all showed one enzymatic activity, either DGC or PDE. On the genetic level, many more GGDEF-EAL proteins were analyzed (41, 42), yet there was no evidence that any of them were bifunctional. The lack of bifunctionality in a subset of the GGDEF-EAL proteins can be explained by our hypothesis that one of the two domains in the GGDEF-EAL tandem is enzymatically inactive due to mutations (27). However, BphG1 shows that potentially bifunctional enzymes involved in c-di-GMP turnover may also exist.

How are opposite enzymatic activities (c-di-GMP synthesis and hydrolysis) regulated in a bifunctional protein? BphG1 holds an unexpected answer to this question. It possesses an essentially light- and BV-independent PDE activity. However, it can be converted into a DGC upon removal of the EAL domain. Why is DGC activity not expressed in the full-length BphG1? How does removal of EAL reveal the cryptic DGC activity? Why is the PDE activity light-independent, while the DGC activity of a truncated protein light-dependent? Although precise answers to these questions will require additional experimentation, below we propose the “EAL lock” model that is consistent with all *in vitro* observations (Fig. 6).

Our model is based on three elements. (i) BphG1 functions as homotetramer. It is expected that the output domains, not the photosensory module, contribute the most to protein-protein interactions (43). (ii) The observation that the EAL domain plus the upstream linker forms a dimer identifies one of the protein-protein interfaces in the BphG1 homotetramer. Note that dimerization is not required for PDE activity (27). (iii) The DGCs are known to work as homodimers. According to the x-ray structure of the DGC from *Caulobacter crescentus*, PleD, each GGDEF domain monomer is likely to bind one GMP moiety of c-di-GMP (26). Our earlier observations revealed that the individual GGDEF domains readily form homodimers. However, these homodimers are nonproductive, i.e. they have no or low enzymatic activity (23). A conversion of the nonproductive GGDEF homodimer into the productive one requires conformational changes in the input (sensory) domains (23, 25). Based on this information, we propose that protein-protein interactions between the EAL domains of BphG1 restrict mobility of the upstream GGDEF domains, prevent the nonproductive-to-productive conversion of the GGDEF domain homodimer, and therefore preclude expression of the DGC activity. In effect, EAL domain interactions lock BphG1 in the PDE mode. We speculate that the EAL lock also explains the lack of light responsiveness of the full-length BphG1. When the EAL domain is removed, the GGDEF domains gain the mobility necessary to undergo the nonproductive-to-productive homodimer conversion. This conversion is subject to regulation by light.

Whether or not the EAL domain of BphG1 is cleaved off in its native host, *R. sphaeroides* has yet to be tested. Theoretically, additional ways to unlock the EAL lock may exist. For example, one may envision an additional protein that has high affinity to EAL and that may break the EAL-EAL interactions, which would allow greater mobility of the GGDEF domains. Such protein, if it existed, would be expected to inhibit the PDE activity of EAL upon binding. Otherwise, the PDE activity of EAL would cancel the newly released DGC activity. Currently, we know of only one protein that interacts with BphG1, *i.e.* its cognate heme oxygenase BphO1, which supplies BV to BphG1. We tested light responsiveness and PDE-to-DGC conversion of the BphO1-BphG1 complex and found no significant differences compared with the behavior of the BphG1 protein alone. Therefore, the currently available data favor cleavage as a mechanism of unlocking the EAL lock. Does BphG1 have an autoproteolytic activity as was observed for the cyanobacterial Cph1 (44)? Is a protease involved, or does the EAL domain cleavage occur through a nonenzymatic mechanism?
common are cleavage-based switches in the bifunctional GGDEF-EAL proteins? How common are bifunctional GGDEF-EAL proteins? These questions and those raised above form the basis for our future studies.

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