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Globins Synthesize the Second Messenger c-di-GMP in *Bacteria*

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Abstract

Globin-coupled sensors (GCS) are heme-binding signal transducers in *Bacteria* and *Archaea* where an N-terminal globin controls the activity of a variable C-terminal domain. Here we report that *Bpe*GReg, a globin-coupled diguanylate cyclase (GCDC) from the whooping-cough pathogen *Bordetella pertussis*, synthesizes the second messenger bis-(3'–5')-cyclic diguanosine monophosphate (c-di-GMP) upon oxygen binding. Expression of *Bpe*GReg in *Salmonella typhimurium* enhances biofilm formation, while knockout of the *Bpe*GReg gene of *B. pertussis* results in decreased biofilm formation. These results represent the first identification of a gaseous ligand for any diguanylate cyclase and provide definitive experimental evidence that a globin-coupled sensor regulates c-di-GMP synthesis and biofilm formation. We propose that the synthesis of c-di-GMP by globin sensors is a widespread phenomenon in bacteria.

Keywords

globin; oxygen sensor; c-di-GMP; diguanylate cyclase; biofilm

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Introduction

Globins are heme-containing proteins found in all three domains of life, *Eukarya*, *Archaea*, and *Bacteria*.[1] The discovery of microbial globins showed that globins participate in many more roles than O_2 metabolism in cells, including sensing of gaseous ligands. The first globin-coupled sensors (GCS) discovered were the HemAT aerotactic transducers that couple a globin-based O_2-sensing domain to a chemotaxis methyl-carrier protein (MCP) domain.[2–4] The GCS proteins form a broad family of sensors with variable transmitter modules, including the GGDEF domain.[3,4] GGDEF domains are widespread in bacteria and possess diguanylate cyclase (DGC) activity.[5–10] DGCs synthesize cyclic dimeric guanosine monophosphate (c-di-GMP), a global second messenger implicated in regulating bacterial motility, exopolysaccharide (EPS) production, and biofilm formation at both translational and post-translational levels.[8,11–14] Given that biofilm formation is important for virulence of many bacterial pathogens,[9,15–17] including *Bordetella pertussis*,[18] these data suggest a role of O_2 sensing in regulating pathogenicity.

Results and Discussion

Our *in silico* analysis of microbial genome sequences identified *Bpe*GReg (*Bordetella pertussis* globin-coupled regulator) from the whooping-cough pathogen *B. pertussis* as a potential globin-coupled diguanylate cyclase (GCDC) (Fig. S1). The conserved domain search initially identified two domains in *Bpe*GReg (475 residues): a globin domain (residues 1–155) and a GGDEF domain (residues 297–475) (Fig. 1a). A hybrid threading/homology modeling technique identified the highest-scoring template for the region spanning residues 167 to 289 as the receiver domain of response regulator PhoB from *Escherichia coli*.[19] Thus, *Bpe*GReg consists of three domains: an N-terminal globin domain, a middle domain, and a C-terminal DGC domain. We created a homology model of *Bpe*GReg based on the crystal structures of HemAT-*Bs* from *Bacillus subtilis* and PleD from *Caulobacter crescentus* (Fig. 1a). In this model *Bpe*GReg is a dimer, with an active site (A-site) in each monomer for binding to one GTP molecule and an inhibitory site (I-site) in each monomer[20,21] for binding to a pair of intercalated c-di-GMP molecules. We therefore postulated that *Bpe*GReg is a heme-binding protein that can synthesize c-di-GMP and influence biofilm formation based on O_2 tension.

To characterize *Bpe*GReg, we purified recombinant His-tagged *Bpe*GReg by metal-affinity chromatography. The recombinant protein had the expected molecular mass of 53.7 kDa (Fig. 1b) and showed characteristic heme protein absorption spectra in the near UV and visible regions (Fig. 1c–e and Fig. S2a and b). Addition of ligands to the ferrous *Bpe*GReg caused the Soret absorption band to shift from 431 nm (deoxy, unliganded Fe^{II} state) to 416 nm for the O_2-bound, 422 nm for the carbon-monoxide bound, and 420 nm for the nitric-oxide bound states. We further measured the O_2, CO and NO binding affinities and kinetics of *Bpe*GReg (Table 1). *Bpe*GReg binds O_2 and CO (K_d = 0.64 and 0.055 μM, respectively) with affinities comparable to those of sperm-whale myoglobin (Table 1). The association rate constant for NO binding to *Bpe*GReg was 16 $\mu M^{-1}s^{-1}$, a value similar to that reported for sperm-whale myoglobin (Table 1). We concluded that *Bpe*GReg is a heme-binding protein.

To test whether *Bpe*GReg functions as a globin-regulated diguanylate cyclase, we measured the conversion of GTP to c-di-GMP using two independent methods: reversephase liquid chromatography coupled with mass spectrometry (LC-MS) and a thin layer chromatography (TLC) radioactive assay using [α-^{32}P]-GTP as the substrate. The data showed that ferrous *Bpe*GReg can synthesize c-di-GMP from GTP (Fig. 2). We then measured the production of c-di-GMP by different states of *Bpe*GReg, unliganded and liganded. The ferrous O_2-bound form of *Bpe*GReg produced the highest amount of c-di-GMP per mole of protein compared to the unliganded form, and the CO- and NO-bound forms (Fig. 2c). We concluded that *Bpe*GReg is an O_2-switched DGC that cycles between a low-activity unliganded ferrous state and a highly active O_2-bound state.

Our *Bpe*GReg model predicted that c-di-GMP can bind to I-sites, causing feedback inhibition of the enzyme activity as initially noted for PleD.[20] To test the inhibition activity of *Bpe*GReg, we coupled the cyclase reaction with a phosphodiesterase (PDE) that continuously linearized the cyclic nucleotide product to pGpG. Comparison of the reactions with and without the PDE clearly indicates product inhibition (Fig. 3a). The reaction quickly slowed without removal of the c-di-GMP product and stopped long before the GTP substrate was exhausted. By contrast, the reaction was linear and proceeded to completion when it was coupled to the PDE (Fig. 3a). Furthermore, the addition of 20 μM c-di-GMP at the start of a *Bpe*GReg-catalyzed reaction strongly inhibited the reaction (Fig. 3b). The use of a coupled assay allowed us to measure the reaction rates of the liganded and unliganded forms of *Bpe*GReg without the complications of feedback inhibition. Thus we determined that the O_2-bound *Bpe*GReg produces c-di-GMP at an initial rate of 2.5 min^{-1}, a rate 10 times faster than that of the unliganded ferrous form (Fig. 3c–f). In conclusion, O_2 enhances the DGC activity of *Bpe*GReg, but accumulation of the c-di-GMP product inhibits this activity.

It has been shown that in motile bacterial cells, high levels of c-di-GMP suppress motility in favor of EPS production and biofilm formation.[8,25] *Salmonella typhimurium* strain ATCC 14028 produces an rdar (red, dry, and rough) colony morphology on Congo red agar (as indicators of cellulose or biofilm formation) at 28°C, but not at 37°C.[26] However, when the DGC AdrA is overexpressed in this strain at 37°C (leading to high levels of c-di-GMP), the temperature regulation is overcome and the rdar morphotype develops.[25,27] Accordingly, we tested whether the expression of *Bpe*GReg in *S. typhimurium* at 37°C would affect motility, EPS production and biofilm formation by this bacterium. We found that, in cells expressing *Bpe*GReg, motility was suppressed (Fig. 4a) and the rdar morphotype was observed (Fig. 4b). Cells carrying only the vector did not develop the rdar morphotype (Fig. 4b). In parallel, we examined the colonies by scanning electron microscopy (SEM). The SEM micrographs showed that the cells expressing *Bpe*GReg produced layers of biofilm in contrast to the control strain (Fig. 4c). An alternative biofilm assay (liquid culture in glass tubes) showed that *Bpe*GReg also enhances biofilm formation in liquid culture (Fig. 4d). These physiological data demonstrate that *Bpe*GReg, when expressed in *S. typhimurium*, inhibits motility and enhances EPS production and biofilm formation.

Next we investigated the function of the middle domain (Fig. 1a). Bioinformatic analysis of this domain identified one highly conserved residue, His^{225} (Fig. S1). Mutation of His^{225} to alanine resulted in a protein with normal globin absorption spectra (Fig. S4). Neither the

unliganded nor the O_2-bound states, however, produced any detectable c-di-GMP. In addition, physiological assays showed a failure of the *Bpe*GReg H225A mutant to confer an rdar morphotype or support biofilm formation (Fig. 4a–d). These data suggest that the middle domain might be required for proper folding of the enzymatic domain, but not the heme binding domain.

To examine whether *Bpe*GReg is involved in biofilm formation in its native *B. pertussis* host, we constructed a *bpeGReg* knockout mutant of *B. pertussis* strain ATCC 9340. A transcriptional fusion suicide vector pFUS2[28] was used to inactivate the target *bpeGReg* gene. Compared to wild-type *B. pertussis* 9340, the knockout strain formed less biofilm (Fig. 4e). The residual biofilm formation could be due to the fact that, besides *Bpe*GReg, the *B. pertussis* genome encodes four other predicted DGCs and four c-di-GMP PDEs.[7] These proteins likely respond to physiological signals other than O_2 and probably modulate a variety of processes via the second messenger c-di-GMP, including biofilm and virulence-factor production.

It has been reported that *B. pertussis* does not grow anaerobically but will grow in O_2 tensions as low as 6% of atmospheric O_2.[29] Thus the lowest concentration of dissolved O_2 to allow growth, about 78 μM, would be expected to keep *Bpe*GReg (K_d = 0.64 μM) in an O_2-bound active state. Considering that *B. pertussis* colonizes the upper respiratory tract, *Bpe*GReg could serve as a key O_2 sensor for directing *B. pertussis* to colonize or not.

We provided experimental evidence that a globin-based O_2 sensor regulates DGC activity and controls bacterial biofilm formation. Our working hypothesis for *Bpe*GReg is that O_2 binding to the globin domain will reorient the *Bpe*GReg dimer so as to enhance the *Bpe*GReg activity. Alternatively, it is also possible that O_2 binding to the globin domain of *Bpe*GReg will enhance its dimerization to an active enzyme, analogously to the phosphorylation-dependent dimerization needed for the activation of PleD.[30] GCDCs are found in a variety of bacteria besides *B. pertussis*, including several other pathogens and a number of free-living soil and marine organisms (Fig. S1). Analysis of the GCDCs from *Azotobacter vinelandii* and *Chromobacterium violaceum* shows that, like *Bpe*GReg, they also regulate biofilm formation and motility (Fig. S5), indicating that O_2 sensing by globins to enhance biofilm production is a widespread phenomenon that was previously overlooked.

Materials and Methods

Sequence alignment and homology modeling

The multiple sequence alignment of selected GCDCs was created in ClustalX and manually refined in DNAstar before final presentation using Adobe Illustrator. Homology modeling was performed using the Prime package (Schrödinger). A threaded model of the middle domain (residues 156–266) of *Bpe*GReg was produced using Threader 3.5 from University College London. The alignment of the middle domain with pdb 1b00 was modeled using MODELLER Release 8v2 from the Sali Lab at UCSF. Analyses of hydrophobicity and hydrophilicity in the active and inhibitory sites in the GGDEF domain were calculated and illustrated with Site Map 2.0 (Schrödinger).

Bacterial strains and culture conditions

E. coli TOP10 cells (Invitrogen) were used for routine cloning. Rosetta2(DE3)pLysS cells (Novagen) were used for expression of the His-tagged proteins. *S. typhimurium* ATCC 14028, donated by Dr. Susan Ayin (University of Hawaii, Manoa), was used for physiological studies. *S. typhimurium* was cultured in LB without salt. *B. pertussis* ATCC 9340 was grown at 37°C on Bordet-Gengou (BG) agar plates with 15% defibrinated sheep blood or Stainer-Scholte broth (SSB) supplemented with heptakis (2, 6-di-O-methyl)-β-cyclodextrin (Sigma). When appropriate, the antibiotics used were ampicillin (100 μg/ml), chloramphenicol (34 μg/ml), and gentamycin (20 μg/ml). Genomic DNA from *B. pertussis* ATCC 9797D, *A. vinelandii* ATCC 12518, and *C. violaceum* ATCC 12472 were purchased from American Type Culture Collection. Genomic DNA from *B. pertussis* 9340 was extracted using the GNOME DNA Isolation Kit (QBiogene).

Plasmid construction

For protein expression and purification, *Bpe*GReg (GenBank accession no. **NP_882025**) was engineered with an N-terminal hexahistidine tag by PCR (primers listed in Table S1). The PCR product was cloned into the pCR4Blunt-TOPO vector (Invitrogen) and subcloned into the pET-3a expression vector (Novagen). *Av*GReg (GenBank accession no. **ZP_00415257**) and *Cv*GReg (GenBank accession no. **NP_899909**) were engineered with C-terminal hexahistidine tags and cloned into pET-25b and pET-27b, respectively. For expression in *S. typhimurium*, non-tagged versions of *Bpe*GReg, *Av*GReg, and *Cv*GReg were cloned into the pTrc99A vector (Table S1). The QuikChange Site-Directed Mutagenesis protocol (Stratagene) was used to construct the H225A mutant (Table S1).

To inactivate *Bpe*GReg in *B. pertussis* 9340, a 600 bp region (nucleotides 289–888) of the *bpeGReg* gene was amplified by PCR (Table S1) and cloned into the suicide vector pFUS2 (provided by Dr. Camille Locht). Gene inactivation was performed by one-step homologous recombination[28] and confirmed by PCR.

Expression and purification

His-tagged *Bpe*GReg, *Av*GReg, and *Cv*GReg were overexpressed in *E. coli* Rosetta2(DE3)pLysS cells induced with 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at room temperature for 6–8 hours and purified by Co^{2+}-affinity chromatography according to Piatibratov *et al*..[31] Purified proteins were analyzed by SDS-PAGE and the concentration was determined by the Bradford protein assay (BioRad) using a bovine serum albumin standard.[32] The purified proteins were stored at −70°C until use.

Absorption spectra and ligand binding parameters

Unless otherwise noted, all determinations of UV-Vis absorption and ligand binding were for 2–5 μM protein in 0.10 M sodium phosphate, pH 7.5 at 25°C. Absorption spectra were monitored with a Cary 4000 UV-Vis spectrophotometer (Varian). Laser-flash photolysis and stopped-flow measurements were done with an LKS.60 laser kinetic spectrometer fitted with a PiStar stopped-flow drive unit (Applied Photophysics). For sample excitation, the LKS.60 spectrometer was coupled to a Quantel Brilliant B Nd: YAG laser with second-harmonic

generation. Ligand-binding kinetics were followed for 2–5 μM protein at a wavelength of maximum difference between the starting and final species. Each rate constant was calculated from a linear plot of k_{obs} versus ligand concentration including at least four ligand concentrations.

The *Bpe*GReg O_2 association rate constant (k_{on} (O_2)) was measured by laser-flash photolysis at 436 or 414 nm using 64–1024 μM O_2. The *Bpe*GReg O_2 dissociation rate constant (k_{off} (O_2)) was measured by stopped-flow at 436 nm by mixing 2–5 μM oxy-*Bpe*GReg with 1 mM sodium dithionite. The *Bpe*GReg CO association rate constant (k_{on} (CO)) was measured by laser-flash photolysis at 419 nm using 30–480 μM CO. The *Bpe*GReg CO dissociation rate constant (k_{off} (CO)) was measured by ligand displacement in the stopped-flow at 423 nm by mixing 3.5 μM carbonmonoxy-*Bpe*GReg (in 20 μM CO) with 500 μM nitric oxide. The *Bpe*GReg NO association rate constant (k_{on} (NO)) was measured by laser-flash photolysis at 435 nm using 15–60 μM nitric oxide.

Enzymatic diguanylate cyclase assays

Deoxy-*Bpe*GReg was produced by reduction of the purified protein with 10 mM DTT in an anaerobic chamber (Coy). Oxy-*Bpe*GReg was prepared by diluting deoxy-*Bpe*GReg into air-saturated reaction buffer. Generally, reactions contained 1–5 μM *Bpe*GReg in 20 mM sodium phosphate, pH 7.5, 10 mM $MgCl_2$, and 2 mM DTT at 23°C. For the DGC-PDE coupling assays, *Ec*Dos was added to a three-fold molar excess to the *Bpe*GReg. Reactions were started by adding 500 μM GTP ([α-^{32}P], Perkin-Elmer). Aliquots were removed from the reaction at the indicated times and mixed with one-fourth volume of 0.50 M EDTA, pH 8.0. The stopped reactions were then heated at 100°C for 5 minutes, and precipitated proteins were removed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was analyzed by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), or liquid chromatography coupled with mass spectrometry (LC-MS). For TLC analysis, 2 μl of the supernatant was spotted onto PEI-cellulose F TLC plates (Merck KGaA). The plates were developed in a 1.5:1 solution of KH_2PO_4 (1.5 M, pH 3.6): $(NH_4)_2SO_4$ (4 M, pH 3.6). Under these conditions GTP migrates with R_f = 0.58, c-di-GMP with R_f = 0.18, and pGpG with R_f = 0.34. Data acquisition was by phosphorimaging with a storage phosphor screen (Kodak K-HD) and a Typhoon 9200 variable mode imager (Amersham Pharmacia Biotech). Data analysis was with Image Quant 5.2 software (Molecular Dynamics). Known amounts of [α-^{32}P]-GTP were used for standardization. For HPLC analysis shown in Fig. 3f, 10 μl of the stopped reactions were injected onto an Adsorbosphere Nucleotide-Nucleoside reverse-phase C18 HPLC column (Alltech, 250 × 4.6 mm) equipped with an Adsorbosphere Nucleotide-Nucleoside guard column (Alltech/Grace, 7.5 × 4.6 mm). The mobile phase consisted of (A) 0.15 M NaH_2PO_4, pH 5.2 and (B) 40% acetonitrile (balance A eluent). A linear gradient from 0–35% B for 10 min at 1 ml/min was used to separate GTP, c-di-GMP, and pGpG. Known quantities of all three molecules were used for standardization. For LC-MS analyses shown in Fig. 2a and b, the samples were injected into a Dionex C-18 column (150 × 4.6 mm) in an Agilent 1100 series LC/MS system and separated with a gradient from 2% acetonitrile/98% trifluoroacetic acid (vol/vol) to 95% aceotonitrile/5% trifluoroacetic acid at a flow rate of 1 ml/min. Nucleotides were

detected at a wavelength of 254 nm for LC, as shown in Fig. 2a; c-di-GMP was detected in negative single-ion monitoring mode at *m/z* 689 with MS, as shown in Fig. 2b.

Phenotypic assays

To detect cellulose biosynthesis, *S. typhimurium* was grown on LB without salt plates containing Congo red (40 μg/ml) for 40 hours at 37°C. Swimming motility was assayed on 0.3% agar plates (1% tryptone, 0.5% NaCl, 1 μM thiamine) at 28°C for 6 hours. Biofilm formation was observed in glass tubes for *S. typhimurium* and *B. pertussis*. *S. typhimurium* strains were diluted to an OD_{600} of 0.01 in LB without salt. Five milliliters were aliquoted into glass tubes and sealed with parafilm. The tubes were incubated at 37°C with shaking (250 rpm) for 36 hours. Adherence to the glass and cell clumping were visually compared. *B. pertussis* strains were incubated at 37°C without shaking for 7 days and adherent cells were stained with 0.1% crystal violet. *S. typhimurium* colonies grown on Congo red plates were observed by scanning electron microscopy (SEM). Sections of colonies were fixed with 4% glutaraldehyde and 0.05% ruthenium red in 0.1 M cacodylate buffer (pH 7.4), post fixed with 1% osmium tetroxide, dehydrated in ethanol, and critical point dried. Samples were coated with gold/palladium and examined with a Hitachi S-800 field emission scanning electron microscope at 15 kV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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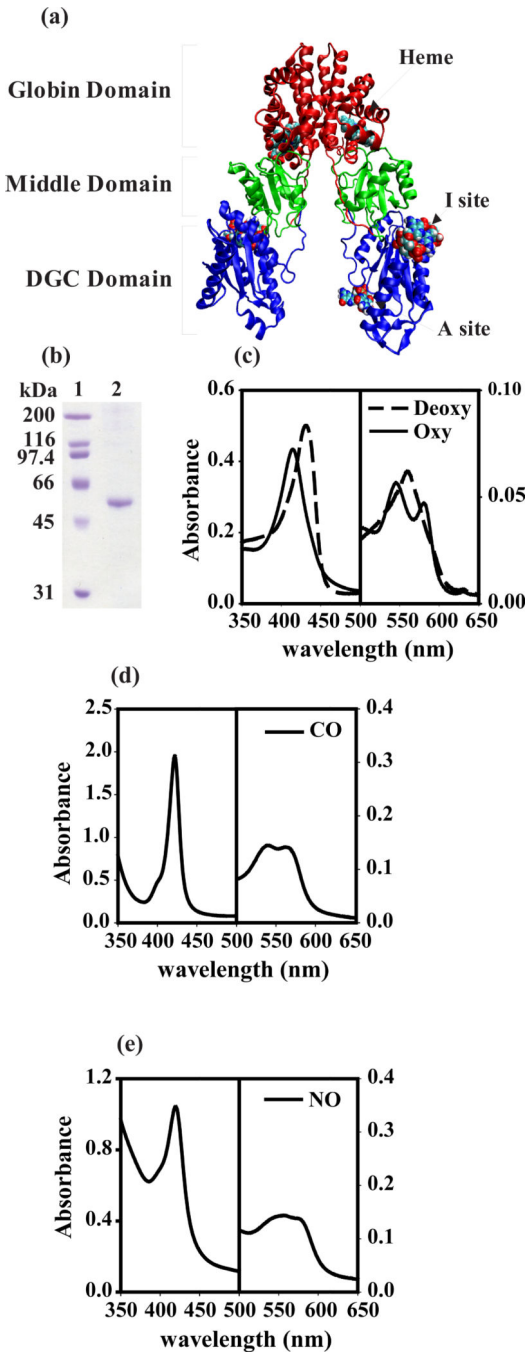

Figure 1.

Identification of *Bpe*GReg from *B. pertussis* as a globin. (a) Model of a possible complete *Bpe*GReg dimer in ribbon representation with globin (red), middle (green), and DGC (blue) domains. (b) Coomassie-stained SDS-PAGE of purified *Bpe*GReg. Lane 1, molecular weight markers; lane 2, *Bpe*GReg$_{6\times\text{-His}}$. (c–e) Absorption spectra of *Bpe*GReg in ferrous state and bound to various ligands. All measurements were done at 25°C and pH 7.5 in 100 mM sodium phosphate buffers.

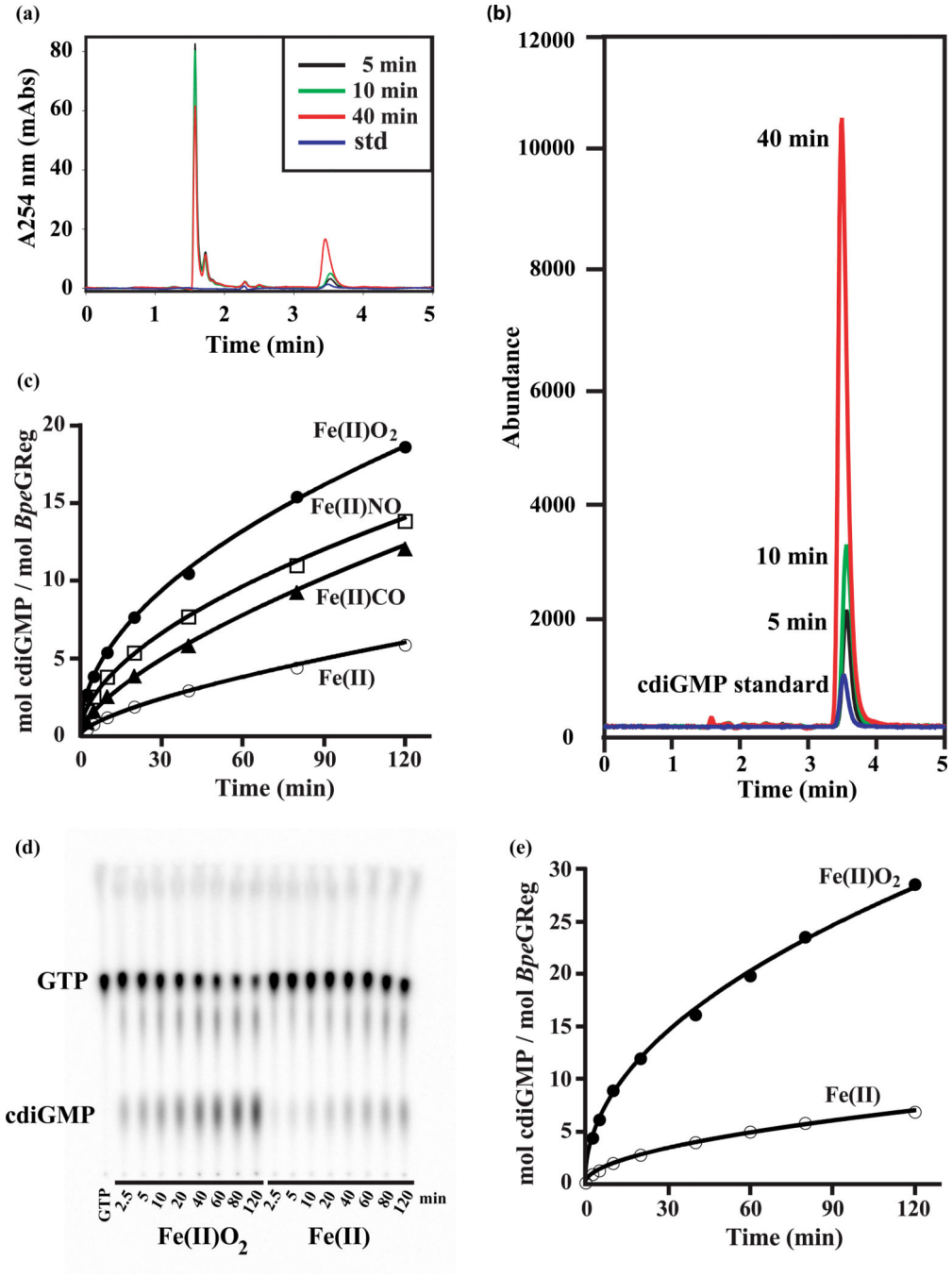

Figure 2.

Identification and quantification of c-di-GMP production *in vitro* by different liganded states of *Bpe*GReg. All the experiments shown here measure the conversion of GTP (100–500 μM) to c-di-GMP. (a) Activity of *Bpe*GReg at 5 min (black), 10 min (green) and 40 min (red), as assayed by reverse-phase HPLC. The GTP elutes at 1.8 min and the c-di-GMP at 3.6 min; a c-di-GMP standard is shown (blue). (b) Mass spectra coupled to the HPLC shown in panel A; the c-di-GMP is detected with single-ion mode (SIM, negative) at *m/z* 689. (c) Influence of heme ligands on *Bpe*GReg activity; activity data for the deoxy, O_2-bound, CO-bound, and

NO-bound states are based on HPLC-MS and TLC assays that gave identical results. Levels of c-di-GMP were measured for reactions stopped at 2.5, 5, 10, 20, 40, 80, and 120 min. (d) Influence of O_2 on *Bpe*GReg activity; levels of c-di-GMP were measured by TLC for reactions stopped at 2.5, 5, 10, 20, 40, 60, 80, and 120 min. The reactions correspond to the curves shown in panel (e). The differences between the respective oxy and deoxy curves in panels (c) and (e) are due to the different degrees of feedback inhibition in the starting preparations of enzyme when the DGC assay is not coupled to a PDE (see Fig. 3).

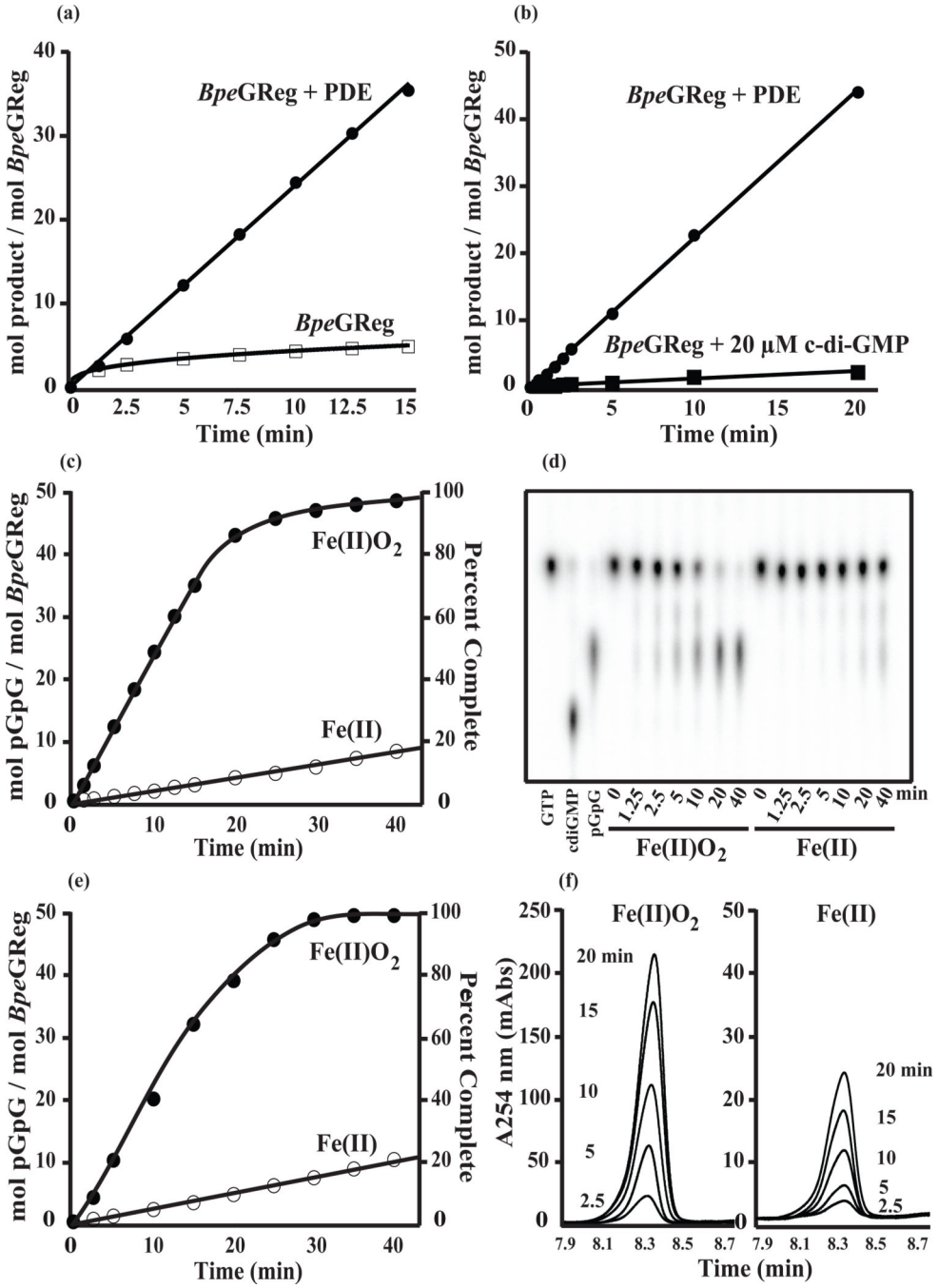

Figure 3.

Activation of *Bpe*GReg by O_2 and feedback inhibition of the enzyme by c-di-GMP. Rates of conversion of 500 µM GTP to c-di-GMP were followed either by measuring c-di-GMP directly or by measuring formation of pGpG from a PDE-coupled reaction. (a) Activity of 5 µM oxy-*Bpe*GReg without coupling to a PDE, or with coupling to a PDE. (b) Activity of 1 µM oxy-*Bpe*GReg in a reaction started in the presence of 20 µM c-di-GMP, or coupled to a PDE. (c) Activity of 5 µM *Bpe*GReg in air ($Fe(II)O_2$), or under anaerobic conditions (Fe(II)). Note the 13-fold drop in the activity in the absence of O_2, and note that in air the

reaction is essentially complete within 30 min. (d) TLC showing representative time points from the reactions shown in panel (c); the first three lanes are shown as references, with the R_f values being: GTP (0.58), c-di-GMP (0.18), and pGpG (0.34). (e) Repeat of the reactions shown in (c) for analysis by HPLC. (f) HPLC traces of representative pGpG peaks from the reactions shown in panel (e); the peaks are from the 2.5, 5.0, 10, 15, and 20 min time points.

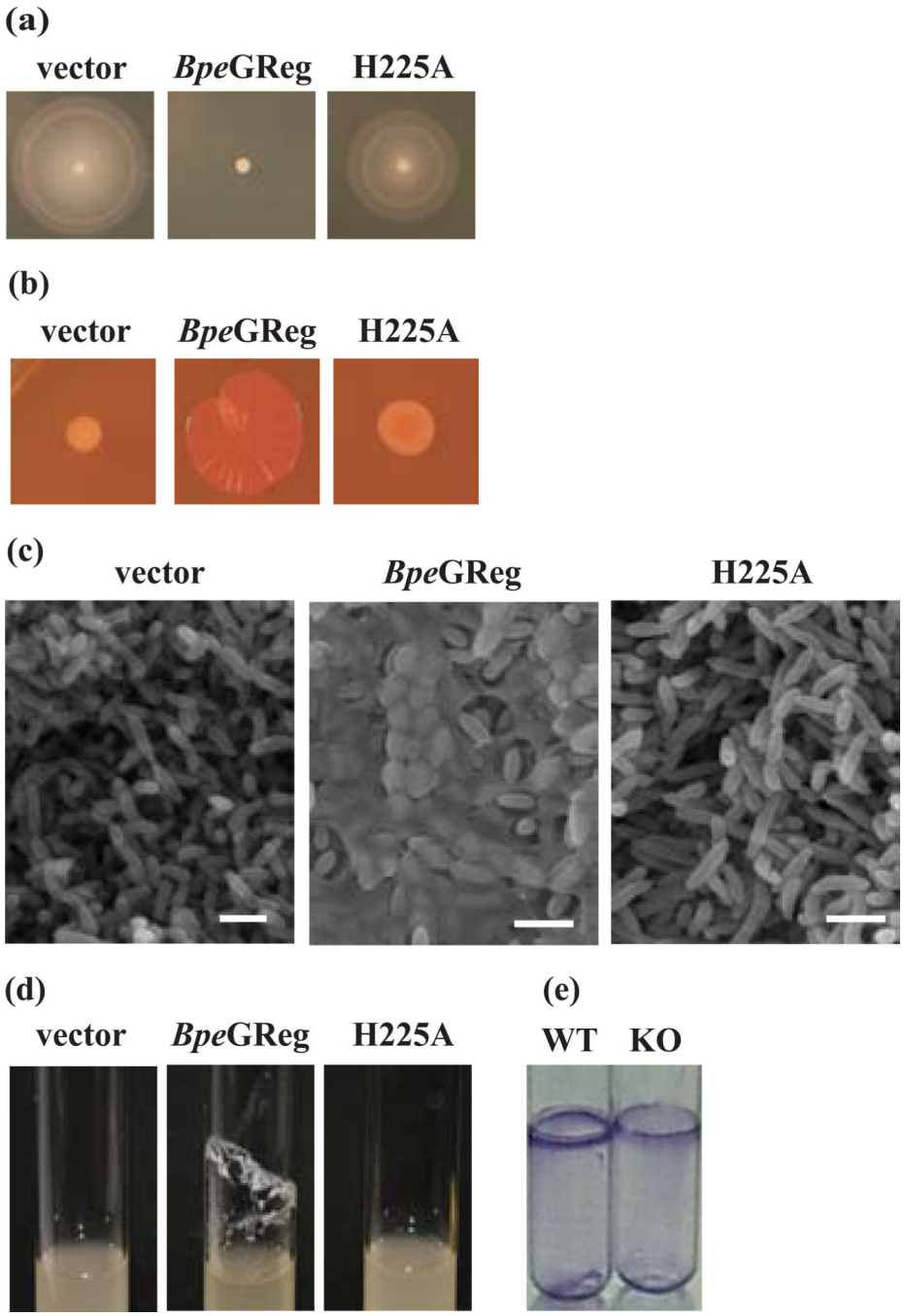

Figure 4.

Phenotypic effects of *Bpe*GReg and its H225A mutant. (a–d) Phenotypes of *S. typhimurium* cells either harboring an empty vector or expressing *Bpe*GReg and the H225A mutant. (a) Swimming motility on 0.3% tryptone agar plates. (b) rdar morphotype development on Congo red plates. (c) SEM micrographs of the colonies shown in (b). Scale bars, 2 μm. (d) Biofilm formation in liquid culture. (e) Biofilm assay of wild-type (WT) *B. pertussis* and the *Bpe*GReg knockout strain (KO). Adherent cells were stained with crystal violet.

Table 1

Ligand-binding parameters of *Bpe*GReg[a] compared to HemAT-*Bs* and sperm-whale myoglobin

	O_2			CO			NO
	k_{on} μM^{-1} s^{-1}	k_{off} s^{-1}	K_d μM	k_{on} μM^{-1} s^{-1}	k_{off} s^{-1}	K_d μM	k_{on} μM^{-1} s^{-1}
***Bpe*GReg**	7.0	4.5	0.64	1.03	0.056	0.055	16
				0.12		0.45	
HemAT-*Bs*[b]	19	1,900	100	0.34	0.067	0.20	
		87	4.6				
SWMb[c]	17	15	0.88	0.51	0.019	0.037	22

[a] Binding parameters for *Bpe*GReg were measured at 25°C in 100 mM NaPi, pH 7.5. The association of CO to *Bpe*GReg was bimodal and manifested two different on-rate constants.

[b] HemAT-*Bs* data were from Zhang *et al*..[22] The dissociation of O_2 from HemAT-*Bs* was bimodal and manifested two-different rate constants.

[c] Sperm-whale myoglobin (SWMb) O_2 and CO binding data are from Springer *et al*.;[23] NO binding data are from Brucker *et al*..[24]