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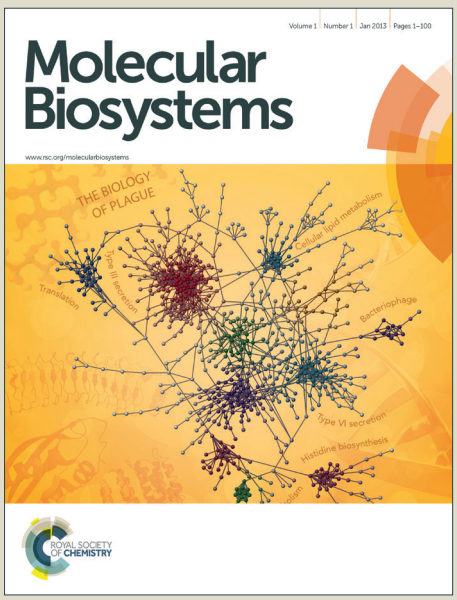

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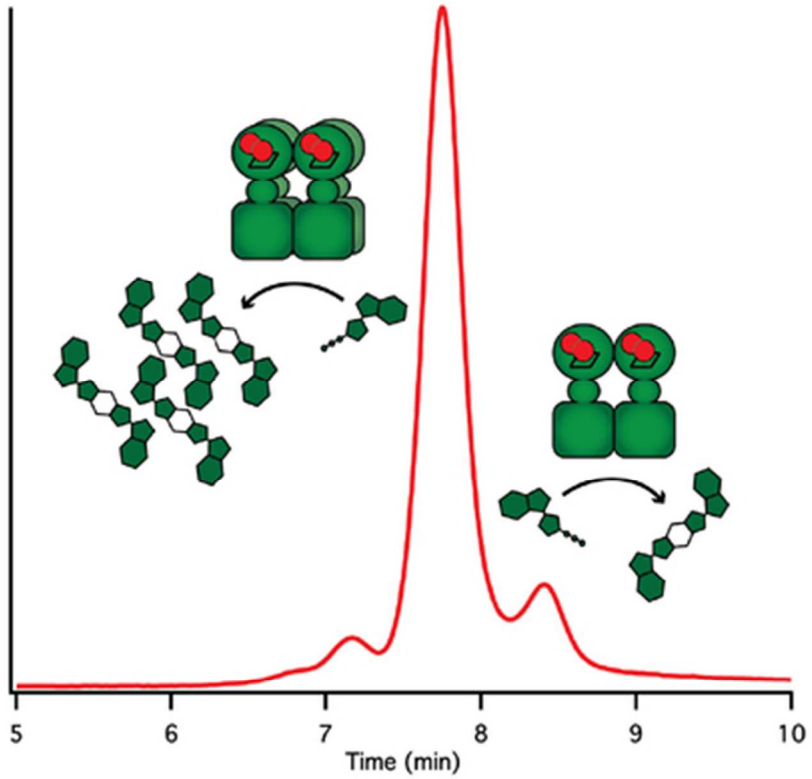

Globin coupled sensors exist as a mixture of oligomeric states, which affect both enzyme catalysis and oxygen binding.
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Oligomeric State Affects Oxygen Dissociation and Diguanylate Cyclase Activity of Globin Coupled Sensors

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Bacterial biofilm formation is regulated by enzymes, such as diguanylate cyclases, that respond to environmental signals and alter c-di-GMP levels. Diguanylate cyclase activity of two globin coupled sensors is shown to be regulated by gaseous ligands, with cyclase activity and O_2 dissociation affected by protein oligomeric state.

In the environment, bacteria are often found as part of complex microbial communities, termed biofilms, which provide protection from predation, environmental stress, and host immune responses, as well as increase their resistance to antibiotics.[1,2] A major regulator of biofilm formation is bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP),[3-5] which is also an important bacterial second messenger. Within the cell, diguanylate cyclases are responsible for cyclizing two molecules of GTP to form c-di-GMP, while phosphodiesterases hydrolyze c-di-GMP to linear pGpG.[6] Downstream effectors bind c-di-GMP and alter protein activity and gene expression, leading to changes in phenotype.[4]

Bacteria have evolved sophisticated systems to sense changes in their environment and respond to optimize their survival.[5] The ability of bacteria to adjust to changing O_2 tensions[7-11] suggests signalling pathway(s) that can sense extracellular O_2 and adjust intracellular chemistry. A class of heme proteins, termed globin coupled sensors, is predicted to serve as bacterial environmental O_2 sensors.[12-14] Globin coupled sensors (GCS) consist of a sensor globin domain linked to an output domain by a variable middle domain. Types of output domains include methyl accepting chemotaxis proteins (MCP),[13-15] histidine kinases,[16] anti-anti-sigma factors, and diguanylate cyclases,[17-20] with methyl accepting chemotaxis proteins and diguanylate cyclases being the most prevalent.[12]

Recent work has focused on characterizing GCSs with diguanylate cyclase catalytic domains. In particular, work on the diguanylate cyclase-containing proteins from *Escherichia coli*, *Bordetella pertussis*, and *Desulfotalea psychrophila* has found that cyclase activity is increased upon binding of O_2 to the globin domain, as compared with the Fe^{II} unligated state.[17-20] Furthermore, a study published on the diguanylate cyclase-containing GCS from *Shewanella putrefaciens* found that the protein was responsible for generating nearly 50% of the cellular c-di-GMP concentration, as well as increased biofilm formation, when grown aerobically but had no effect under anaerobic conditions.[7] These studies suggest that diguanylate cyclase-containing GCSs are important O_2 sensors in bacteria that control c-di-GMP levels.

The mechanism by which O_2 binding to the heme domain activates the cyclase domain of GCSs is not yet fully understood. Previous work has focused on signal transduction in the GCS from *Bacillus subtilis* (HemAT-*Bs*), which contains a MCP output domain.[14] Both X-ray crystallography and resonance Raman spectroscopy have found that ligand binding causes reorganization of heme pocket residues that is propagated to the middle domain and is likely responsible for changes in output domain activity.[21-24]

In the case of diguanylate cyclases, cyclase activation has primarily been studied in proteins unrelated to GCSs, such as WspR from *Caulobacter crescentus*[25-28] and PleD from *Pseudomonas fluorescens*.[29,30] These studies have shown that both PleD and WspR are active as dimers and that oligomerization state is controlled by activation of a receiver or sensing domain.[25-31] Intriguingly, a study on the diguanylate cyclase-containing GCS from *D. psychrophila* only observed a tetrameric configuration, which was catalytically active.[18] These data pose the question of whether cyclase activity of other GCSs correlates with that of paradigmatic diguanylate cyclases, such as PleD and WspR, or if the tetrameric assembly is universally active for diguanylate cyclase-containing GCSs.

To further understand how O_2 binding activates diguanylate cyclase activity in GCSs, a novel putative GCS from the plant pathogen *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*GCS) was characterized and compared to the previously described GCS, termed *Bpe*GReg, from *B. pertussis*,[20] the causative agent of whooping cough. *Bpe*GReg was found to increase c-di-GMP

production in the Fe^{II}-O_2 ligation state, with Fe^{II}-CO and Fe^{II}-NO yielding intermediate activity, and Fe^{II} unligated state resulting in the lowest cyclase activity.[25] The two proteins display 35% amino acid sequence identity and 51% similarity (ESI Fig. S1), which suggests that they may provide insight as to whether subtle changes in amino acid sequence affect GCS activation.

P. carotovorum is a plant pathogen that is a major cause of bacterial soft rot (maceration of plant tissues).[32, 33] The bacterium infects a number of plants, including potatoes, carrots, lettuce, and cabbage and exhibits increased virulence under low O_2 conditions.[32-36] As c-di-GMP levels and biofilm formation are inversely related to virulence in numerous bacteria,[1] *Pcc*GCS may be responsible for synthesizing c-di-GMP in response to O_2 and altering pathogenicity. To investigate the role of *Pcc*GCS in O_2 sensing within *P. carotovorum*, detailed characterization of the protein biochemistry was performed. Michaelis-Menten (MM) parameters of both proteins were determined to understand how O_2 affects catalysis. In addition, oligomerization states of the proteins were determined to identify the active form of the enzyme, as well as the effect of oligomerization on heme ligand affinity. The comparison to *Bpe*GReg was used to further inform our understanding of GCS activation and signal transduction.

To probe the enzymatic activity and activation of *Pcc*GCS, the codon optimized full-length gene (GenScript) was cloned into expression vector pET-20b and overexpressed in *E. coli* Tuner (DE3) pLysS cells. The protein was purified by Ni-NTA affinity chromatography, followed by dialysis to remove any residual GTP and c-di-GMP, and subsequent gel filtration chromatography (see ESI† Methods for gene sequence and detailed experimental methods). *Bpe*GReg was expressed and purified in an analogous manner. Purity of both proteins was assayed by SDS-PAGE and identities confirmed by mass spectrometry (ESI† Fig. S2).

UV-visible spectroscopy of purified *Pcc*GCS resulted in spectra that correspond to a histidyl-ligated heme protein, as expected. The spectra in various ligation and oxidation states are similar to previously characterized GCSs (Fig. 1, Soret and α/β band maxima in legend).[13-20, 22] In addition, the spectra of purified *Bpe*GReg are identical to those reported previously.[20]

To determine the ligand(s) responsible for activating *Pcc*GCS diguanylate cyclase activity, enzyme kinetic assays were performed. Diguanylate cyclase activity was quantified in real time through measurement of pyrophosphate production (EnzCheck Pyrophosphate Kit, Life Technologies). The real time assay was compared and validated with previously described HPLC-based assays.[20] EcDosP,[37] a c-di-GMP phosphodiesterase, was included in all kinetic reactions to hydrolyze c-di-GMP to linear pGpG and eliminate product inhibition (ESI† Methods). Enzyme kinetic assays

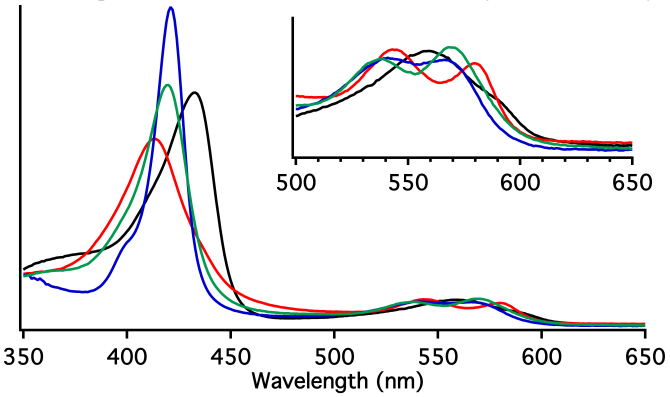

Figure 1. UV-visible spectra of *Pcc*GCS. Black, Fe^{II} (433, 562 nm); red, Fe^{II}-O_2 (415, 548, 583 nm); blue, Fe^{II}-CO (422, 545, 570 nm); green, Fe^{II}-NO (421, 540, 573 nm).

Table 1. Ligation state-dependent enzyme kinetics.

Protein	Ligation state	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}min^{-1}$)
*Bpe*GReg	Fe^{II}	0.18	120 ± 11	1500
	Fe^{II}-O_2	0.59	57 ± 8	10350
	Fe^{II}-CO	0.23[a]	ND	---
	Fe^{II}-NO	0.38[a]	ND	---
*Pcc*GCS	Fe^{II}	0.29	62 ± 3	4677
	Fe^{II}-O_2	0.73	31 ± 6	23548
	Fe^{II}-CO	0.43[a]	ND	---
	Fe^{II}-NO	0.51[a]	ND	---

Standard deviations for k_{cat} are less than 10% of the measured values. ND = not determined.
[a] Fe^{II}-CO and Fe^{II}-NO rates were measured under v_{max} conditions.

were performed at four enzyme concentrations (0.5-3 μM) and eight GTP concentrations (0-500 μM) to obtain accurate MM parameters for the Fe^{II} and Fe^{II}-O_2 ligation states (Table 1, ESI† Fig. S3). In addition to measuring the catalytic rates for *Pcc*GCS, MM parameters for *Bpe*GReg were also determined, as they had not been previously reported.

Similar to previous work on *Bpe*GReg[20] and the *E. coli* GCS,[17, 19] cyclase activity of *Pcc*GCS was lowest for the Fe^{II} unligated state and highest for the Fe^{II}-O_2 ligation state, with Fe^{II}-NO and Fe^{II}-CO yielding intermediate activity (Table 1). The k_{cat} for both enzymes increased upon binding O_2 (3.2 and 2.5 fold for *Bpe*GReg and *Pcc*GCS, respectively) while the K_M decreased approximately 2-fold, suggesting that O_2 binding at the heme is propagated into a rearrangement of the catalytic domains that results in improved substrate binding and increased catalytic turnover.

To further probe the mechanism of O_2-dependent cyclase activation, the oligomerization state(s) of the enzymes in the Fe^{II}-O_2 ligation state were determined using analytic gel filtration (AGF) HPLC (Fig. 2) and Blue-Native PAGE (ESI† Fig. S4). Both *Pcc*GCS and *Bpe*GReg were found to be a mixture of three or more oligomerization states. Surprisingly, despite the high similarity between the two enzymes, *Bpe*GReg exists as a mixture of monomer, dimer, and tetramer, while *Pcc*GCS exists as a mixture of dimer, tetramer and octamer (as well as potentially additional larger species). The two major oligomeric states of both *Bpe*GReg and *Pcc*GCS (dimer and tetramer for both enzymes) were isolated by gel filtration chromatography to >75% purity for each oligomer and found to re-equilibrate very slowly (more than 18 hrs. to observe

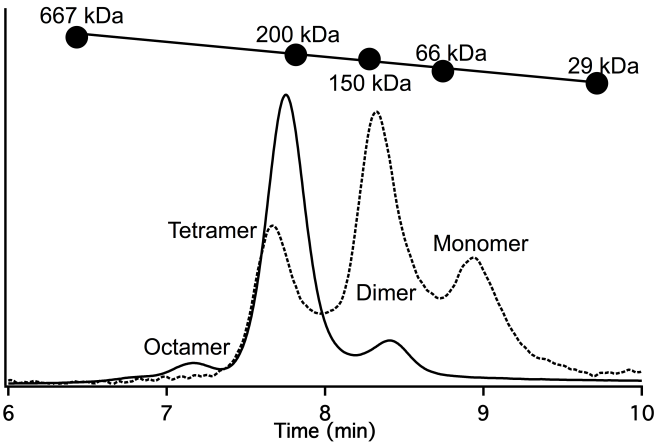

Figure 2. Analytical gel filtration chromatograms of *Bpe*GReg (dashed line) and *Pcc*GCS (solid line). Proteins used for the calibration curve can be found in the ESI Methods.

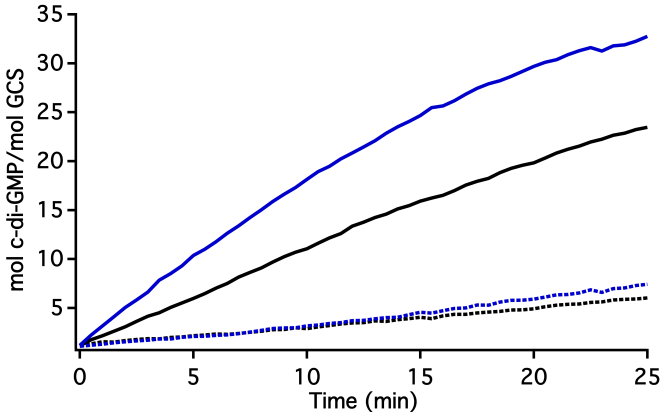

Figure 3. Single oligomeric state enzyme kinetics. Rates of c-di-GMP production for *Pcc*GCS dimer (blue, dashed line) and *Bpe*GReg dimer (black, dashed line) are significantly lower than for *Pcc*GCS tetramer (blue, solid line) and *Bpe*GReg tetramer (black, solid line).

~10% re-equilibration, ESI† Fig. S5).

The slow equilibration between different oligomeric states allowed for analysis of the effects of oligomerization on catalytic activity and heme ligand kinetics. The catalytic activities of the isolated dimer and tetramer fractions of *Bpe*GReg and *Pcc*GCS were tested under v_{max} conditions with protein concentrations normalized for each enzyme (Fig. 3). Surprisingly, both *Bpe*GReg and *Pcc*GCS were more active as tetramers than as dimers (~4-fold increase in rate). This result is in contrast to the previously characterized diguanylate cyclases PleD and WspR,[25, 28-30] which are active as dimers, but similar to the GCS from *D. psychrophila*,[18] which was active as a tetramer. However, the *D. psychrophila* GCS was observed to exist solely as a tetramer, suggesting that subtle differences in amino acid sequences between GCSs, such as linker domain length, result in significant changes in oligomerization state and, potentially, mechanisms of activation.

In addition to oligomerization-dependent cyclase activity, both *Bpe*GReg and *Pcc*GCS exhibited oligomer-dependent differences in O_2 dissociation rate. Both as-purified mixtures of oligomeric states exhibit bi-exponential dissociation kinetics that are independent of protein batch and trap concentration tested (Table 2, ESI† Fig. S6). Carbon monoxide was not used in the trap as inclusion of CO affected O_2 dissociation rates, potentially due to biphasic association of CO, as was previously observed for *Bpe*GReg.[20] O_2 dissociation from as-purified *Bpe*GReg was found to be more rapid than from *Pcc*GCS (0.82 s^{-1} and 6.30 s^{-1} vs. 0.56 s^{-1} and 3.87 s^{-1}, respectively). Previous studies have shown that O_2 dissociation from various GCSs can occur with either mono or bi-exponential kinetics, depending on the protein and report.[22, 38] The observed biphasic kinetics are consistent with work that identified multiple conformations of the heme pocket using resonance Raman spectroscopy and X-ray crystallography.[21, 23]

Table 2. O_2 dissociation kinetics.

Protein	Oligomer(s)	k_1 (s^{-1})	k_2 (s^{-1})
*Bpe*GReg	As purified	0.82 ± 0.01	6.30 ± 0.11
	Dimer	1.33 ± 0.07	6.16 ± 0.97
	Tetramer	1.23 ± 0.01	7.50 ± 0.06
*Pcc*GCS	As purified	0.56 ± 0.01	3.87 ± 0.08
	Dimer	0.668 ± 0.018	4.68 ± 0.25
	Tetramer	0.641 ± 0.003	3.98 ± 0.03

For both *Bpe*GReg and *Pcc*GCS, the fast rate (k_2) of O_2 dissociation is affected by the oligomerization state, while the slow rate (k_1) is nearly unchanged. In the case of *Bpe*GReg, the fast rate is increased for the tetramer, as compared to the dimer (7.50 s^{-1} and 6.16 s^{-1}, respectively). However, the opposite trend is observed for *Pcc*GCS, with the tetramer exhibiting a decreased k_2 relative to the dimer (3.98 s^{-1} and 4.68 s^{-1}, respectively). The percentages of the fast and slow rates did not vary significantly between the dimer and tetramer of either *Bpe*GReg or *Pcc*GCS (ESI† Table S1).

Taken together with the differences in oligomeric states between *Bpe*GReg and *Pcc*GCS, the O_2 dissociation data further suggest that despite high similarity, subtle differences in amino acid composition between GCSs result in altered biophysical and kinetic characteristics. As the regions of lowest sequence homology exist in the N-terminal region of the globin domain and the middle domain (ESI Fig. 1), these sequences may be responsible for the divergent oligomerization and O_2 kinetic findings. Additionally, the ~15 residue sequences at the N- and C-termini of *Pcc*GCS and *Bpe*GReg, respectively, may play a role in observed differences between the proteins.

Conclusions

In summary, our results demonstrate that *Pcc*GCS is a globin coupled sensor that exhibits heme ligand-dependent diguanylate cyclase activity. Both *Pcc*GCS and *Bpe*GReg exist in multiple oligomeric states that differ between the proteins, despite high amino acid sequence similarity. In contrast to a number of previously characterized diguanylate cyclases, the tetrameric assemblies of *Pcc*GCS and *Bpe*GReg display the highest enzymatic activity. Furthermore, oligomeric state affects the rate of O_2 dissociation from the sensor domain. Thus, GCS cyclase activity may be regulated through a mechanism involving oligomerization state changes. In addition, *Pcc*GCS may be serving as an O_2 sensor to regulate c-di-GMP levels and biofilm formation in *P. carotovorum*.

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Notes and references

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† Abbreviations used: c-di-GMP, bis-(3',5')-cyclic dimeric guanosine monophosphate; DGC, diguanylate cyclase; GCS, globin coupled sensor; *Bpe*GReg, *B. pertussis* globin coupled sensor; *Pcc*GCS, *P. carotovorum* ssp. *carotovorum* globin coupled sensor; MM parameters, Michaelis-Menten parameters; AGF, analytical gel filtration. Electronic Supplementary Information (ESI) available: Experimental methods and supplemental figures. See DOI: 10.1039/c000000x/

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