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Mechanism and Role of Globin Coupled Sensor Signaling

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Abstract

The discovery of the globin coupled sensor family of heme proteins has provided new insights into signaling proteins and pathways by which organisms sense and respond to changing oxygen levels. Globin coupled sensor proteins consist of a sensor globin domain linked to a variety of output domains, suggesting roles in controlling numerous cellular pathways and behaviors in response to changing oxygen concentration. Members of this family of proteins have been identified in the genomes of numerous organisms and characterization of globin coupled sensors with output domains, including methyl accepting chemotaxis proteins, kinases, and diguanylate cyclases, have yielded an understanding of the mechanism by which oxygen controls activity of globin coupled sensor protein output domains, as well as downstream proteins and pathways regulated by globin coupled sensor signaling. Future studies will expand our understanding of these proteins both *in vitro* and *in vivo*, likely demonstrating broad roles for globin coupled sensors in controlling oxygen-dependent microbial physiology and phenotypes.

Keywords

globin; heme; oxygen sensor; c-di-GMP; two-component signaling; second messenger

1. Introduction

Heme sensor proteins are ubiquitous from bacteria to mammals and are used to sense gaseous ligands, such as nitric oxide (NO), carbon monoxide (CO), and oxygen (O₂), to control diverse physiological responses (Gilles-Gonzales, 2005; Martinkova, Kitanishi, & Shimizu, 2013; Shimizu et al., 2015). The diverse families of heme sensor proteins include Heme Nitric oxide/OXygen (H-NOX) binding domains, heme-PAS domains, CooA proteins, and sensor globin domains (for a recent review of heme sensor proteins, please see (Shimizu et al., 2015)). Sensor globin domains are found as the heme domain within globin coupled sensors (GCS), a family of putative O₂ sensors consisting of an N-terminal sensor globin domain linked to a C-terminal output domain (Figure 1) (Hou, Freitas, et al., 2001). Putative GCS proteins are predicted to contain many types of output domains, such as methyl accepting chemotaxis protein (MCP), kinase, diguanylate cyclase (DGC), adenylate cyclase, gene regulatory function, and domains of unknown function.

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GCS proteins were first discovered as heme sensors that controlled aerotaxis in *Bacillus subtilis* and *Halobacterium salinarum* (Hou, Belisle, et al., 2001; Hou, Freitas, et al., 2001; Hou et al., 2000). Further studies have identified roles for GCS proteins in controlling O₂-dependent second messenger production (Burns, Deer, & Weinert, 2014; Burns et al., 2016; Kitanishi et al., 2010; Roy, Sen Santara, Adhikari, Mukherjee, & Adak, 2015; Roy et al., 2014; Sawai et al., 2010; Sen Santara et al., 2013; Tarnawski, Barends, & Schlichting, 2015; Tuckerman et al., 2009; Wan et al., 2009; Wu et al., 2013), phosphorylation cascades (Fojtikova et al., 2015; Jia, Wang, Rivera, Duong, & Weinert, 2016; Kitanishi et al., 2011; Stranova et al., 2016), and biofilm formation (Donné, Van Kerckhoven, Maes, Cos, & Dewilde, 2016; Tagliabue et al., 2010; Wan et al., 2009; Wu et al., 2013). Given that putative GCS proteins are found within the genome sequences of numerous Gram-negative and Gram-positive bacteria, archaea, and even some protozoa (Vinogradov, Tinajero-Trejo, Poole, & Hoogewijs, 2013), GCS proteins likely play important roles in controlling O₂-dependent physiology and phenotypes.

To date, characterized GCS proteins include MCP-containing proteins from *B. subtilis* (Hou et al., 2000), *Bacillus halodurans* C-125 (Hou, Belisle, et al., 2001), and *H. salinarum* (Hou et al., 2000); DGC-containing proteins from *Escherichia coli* (Tuckerman et al., 2009), *Shewanella putrefaciens* (Wu et al., 2013), *Bordetella pertussis* (Wan et al., 2009), *Pectobacterium carotovorum* (Burns et al., 2014), *Desulfotalea psychrophila* (Sawai et al., 2010), and *Azotobacter vinelandii* (Thijs et al., 2007); kinase-containing GCS protein from *Anaeromyxobacter* sp. Fw109-5 (Kitanishi et al., 2011); adenylate cyclase-containing GCS from *Leishmania major* (Sen Santara et al., 2013); putative gene-regulating GCS from *Vibrio brasiliensis* (Jia et al., 2016), and GCS of unknown function from *Geobacter sulfureducens* (Pesce et al., 2009). Through a compilation of biochemical, spectroscopic, and physiological studies, the importance of these proteins in sensing the gaseous environment and regulating key organismal phenotypes is being elucidated.

2. General Structural and Ligand Binding Characteristics

The sensor globin domains within GCS proteins share sequence and structural homology with other globin domains, including mammalian myoglobin, and consist of an α -helical fold (Figures 2 and 3) (Pesce et al., 2009; Tarnawski et al., 2015; Zhang & Phillips, 2003a, 2003b). Compared to myoglobin, sensor globins lack the D helix found in myoglobin (Mb) and hemoglobin (Hb) and typically have a shortened E helix. The heme is bound within a pocket primarily consisting of helices C, E, F, and G, with the histidine that serves as the proximal ligand to the heme residing on the F helix. In sensor globins characterized to date, hydrogen bonding residues are found in the distal pocket and typically consist of a tyrosine located on helix B, and a threonine or serine residue also involved in hydrogen bonding found on helix E within some GCS proteins. All of the GCS proteins characterized so far have been demonstrated to form the expected Fe^{II}-NO, Fe^{II}-CO, and Fe^{II}-O₂ complexes with spectral characteristics (Figure 4) that are very similar to myoglobin and other histidyl-ligated heme proteins (Burns et al., 2014; Hou et al., 2000; Kitanishi et al., 2011; Sawai et al., 2010; Wan et al., 2009; Zhang, Olson, & Phillips, 2005).

Crystal structures have been solved of the sensor globin domain from multiple GCS proteins, including the MCP-containing GCS from *Bacillus subtilis* (HemAT-*Bs*) (Zhang & Phillips, 2003a, 2003b) and the diguanylate cyclase-containing GCS from *Escherichia coli* (EcDosC) (Tarnawski et al., 2015). Within these structures, the globin domains were found to crystallize as dimers, with a large buried dimer interface (1300–1800 Å²). Helices G and H, as well as part of the Z helix in the case of HemAT-*Bs* globin, on each globin monomer form the interface with an anti-parallel four-helix bundle. The heme pocket opening is found away from the dimer interface, although residues from the G helix also line the back of the heme pocket. In addition, the heme is buried deeper than in Mb and Hb, with decreased solvent exposed area and a large heme cavity. While structures of individual domains from GCS proteins and homologous domains have been crystallized, a full-length structure so far has remained elusive.

3. Methyl Accepting Chemotaxis Protein-Containing GCS Proteins

MCP-containing GCS proteins are distributed throughout over 160 sequenced bacterial genomes (Vinogradov et al., 2013) and likely will be found to be more widely spread as additional bacterial genomes are sequenced. Currently, MCP-containing GCSs are the most prevalent GCS class as identified by bioinformatics searches, followed by DGC-containing GCSs. The MCP-containing GCS proteins have been named HemAT for Heme Aerotaxis Transducer. To date, three MCP-containing GCS proteins from *Bacillus subtilis* (HemAT-*Bs*) (Hou et al., 2000), *Halobacterium salinarium* (HemAT-*Hs*) (Hou et al., 2000), and *Bacillus halodurans* C-125 (HemAT-*Bh*) (Hou, Belisle, et al., 2001) have been characterized *in vitro* and/or *in vivo*, with HemAT-*Bs* having been the subject of intensive study.

3.1 HemAT-Bs

HemAT-*Bs* is a 432 amino acid protein and was one of the first two GCS proteins to be identified and characterized. It affects chemotaxis of *B. subtilis* to in response to O₂ levels, resulting in an aerophilic response. However, HemAT-*Bs* is not essential for aerotaxis, as all other putative MCP-like proteins in *B. subtilis* must be deleted to generate a robust HemAT-*Bs*-dependent aerophilic response (Hou et al., 2000). In addition, HemAT-*Bs* does not appear to be methylated by methyltransferase CheR during the O₂ response, which is in contrast to many other bacteria in which methylation of an MCP results in the activation of histidine kinase CheA, part of the two component CheA/CheY signal transduction pathway in bacterial aerotaxis (Collins, Lacal, & Ottemann, 2014; Hou et al., 2000). Despite the lack of observable methylation, HemAT-*Bs* interacts with CheA, which presumably results in the observed O₂-dependent effects on taxis (Szurmant & Ordal, 2004). Furthermore, HemAT-*Bs* has been demonstrated to cluster with transmembrane chemoreceptors at the poles of *B. subtilis*, possibly forming complexes with these chemoreceptors (Cannistraro, Glekas, Rao, & Ordal, 2011), and is involved in pellicle formation of cultures at the air-liquid interface (Hölscher et al., 2015).

3.1.1 Ligand Binding Characteristics—Biochemical and spectroscopic characterization of purified HemAT-*Bs* and the isolated HemAT-*Bs* globin domain have provided insights into protein structure and ligand binding of GCS proteins (Aono et al.,

2002; El-Mashtoly et al., 2008; El-Mashtoly et al., 2012; Hou, Belisle, et al., 2001; Hou, Freitas, et al., 2001; Ohta, Yoshimura, Yoshioka, Aono, & Kitagawa, 2004; Pinakoulaki, Yoshimura, Daskalakis, et al., 2006; Pinakoulaki, Yoshimura, Yoshioka, Aono, & Varotsis, 2006; Yoshida, Ishikawa, Aono, & Mizutani, 2012; Yoshimura et al., 2006; Yoshimura, Yoshioka, Mizutani, & Aono, 2007; Zhang et al., 2005; Zhang & Phillips, 2003b). Full-length HemAT-*Bs* forms a dimer in solution with an elongated, asymmetric shape, while the globin domain forms a monomer in solution but has been crystallized as a dimer (Zhang et al., 2005; Zhang & Phillips, 2003b).

The protein binds O₂ reversibly and exhibits biphasic binding kinetics, as measured by both kinetic measurements (stopped flow and laser flash photolysis) and equilibrium binding measurements (Table 1) (Hou, Freitas, et al., 2001; Zhang et al., 2005). Through resonance Raman and mutagenic studies, biphasic ligand binding kinetics have been demonstrated to originate from interactions with distal pocket residues (Y70 and T95; Figure 3B) and different open/closed forms of the distal pocket (Figure 5) (Ohta et al., 2004; Yoshimura et al., 2006; Zhang et al., 2005). In the full-length, WT protein, three Fe-O stretching frequencies are observed, which, through mutagenesis and isotopic labeling, can be assigned to interactions of the bound O₂ with a water molecule hydrogen bonded to the distal pocket threonine (T95) within a closed distal pocket, interactions with T95 in a hydrogen bonding network with the distal tyrosine, and an open form of the pocket without direct hydrogen bonding interactions with O₂. From these studies, it has been proposed that T95 is required for O₂ sensing, while Y70 is crucial for signal transduction in HemAT-*Bs* (Ohta et al., 2004). In addition, the differences in hydrogen bonding and pocket conformation alter O₂ affinity, allowing HemAT-*Bs* to respond to O₂ over a concentration range of ~300 μM (Yoshimura et al., 2006; Zhang et al., 2005).

The distal pocket residues of HemAT-*Bs* (Y70 and T95) adopt distinct conformations depending on the bound ligand, assisting with ligand recognition and discrimination, resulting in low CO affinity and monophasic binding (Pinakoulaki, Yoshimura, Yoshioka, et al., 2006; Zhang et al., 2005). In addition to the distal hydrogen bonding residues, a heme pocket leucine (L92; Figure 3B) serves as a conformational gate that both helps to maintain protein conformations following ligand binding and serves to direct ligand migration. Within the proximal pocket, a cavity near Y133 serves to bind and/or store gas molecules that also can be accessed through the L92 conformational gate once ligands have dissociated from the heme iron (Pinakoulaki, Yoshimura, Daskalakis, et al., 2006; Pinakoulaki, Yoshimura, Yoshioka, et al., 2006).

While considerable attention has been given to the ligand binding characteristics of HemAT proteins, *in vitro* enzyme kinetic assays to probe the effects of O₂ have proved elusive, as the MCP domain does not generate a measurable product and reconstituting methyl transfer pathways *in vitro* can be quite difficult (Collins et al., 2014; Martinkova et al., 2013).

3.1.2 Signal Transduction in HemAT-*Bs*—Based on the aerotaxis data, binding of O₂ to HemAT-*Bs* should result in a conformational change that can transmit the O₂ binding event to the MCP domain and then to other chemotaxis-related proteins. While there is no full-length structural information on HemAT-*Bs*, or any other GCS protein, crystal structures

of the globin domain in different ligation states and a variety of resonance Raman experiments on the full-length protein have been used to probe the global effects of ligand binding to generate models of signal transduction (Aono et al., 2002; El-Mashtoly et al., 2008; El-Mashtoly et al., 2012; Ohta et al., 2004; Pinakoulaki, Yoshimura, Daskalakis, et al., 2006; Pinakoulaki, Yoshimura, Yoshioka, et al., 2006; Yoshida et al., 2012; Yoshimura et al., 2006; Yoshimura et al., 2007; Zhang et al., 2005; Zhang & Phillips, 2003b).

HemAT-*Bs* globin has been crystallized as a dimer in both the Fe^{III}-CN and Fe^{II} states, providing a comparison of GCS globin structures with and without ligand bound to the heme (Figure 3C). From these structures, a higher degree of symmetry within the dimer is observed in the ligand bound state, as compared with the unligated structure. In addition, the two subunits displayed differential structural changes when comparing Fe^{III}-CN and Fe^{II} structures, with Y70 rotating ~100 degrees away from the heme in the Fe^{II} structure, suggesting that heterogeneity or negative cooperativity in HemAT-*Bs* may be a cause of the biphasic O₂ binding kinetics and large sensing concentration range. Comparison of the ligand bound and unbound structures also demonstrates changes throughout the globin, with the G helix at the dimer interface being displaced relative to the H helix on each subunit, and the G and H helices undergoing small rotational movements, in addition to the globin monomers rotating relative to each other (Zhang & Phillips, 2003b).

Further differences observed upon ligand binding in the full-length protein include changes around the heme propionate (Figure 3B), which are propagated through hydrogen bonding between a heme-peripheral histidine (H86) and a heme propionate, resulting in conformational changes of the CE loop and E helix (which contains T95) (Yoshimura et al., 2006). Furthermore, mutation of H86 or T95 to alanine, preventing key hydrogen bonding interactions, disrupts the conformational changes of the B helix (which contains Y70) and the G helix, disrupting signal transduction from the globin domain to the middle domain (El-Mashtoly et al., 2008). Overall, ligand binding has been demonstrated to result in small rearrangements throughout the globin domain that alter symmetry and are propagated through the middle domain to control interactions of the MCP output domain.

3.2 HemAT-*Hs* and HemAT-*Bh*

While HemAT-*Hs* and HemAT-*Bh* have been the subject of considerably fewer studies, characterizations suggest that both proteins behave in a similar fashion to HemAT-*Bs* (Hou, Belisle, et al., 2001; Hou, Freitas, et al., 2001; Hou et al., 2000). HemAT-*Hs* is essential for aerotaxis in *H. salinarium* and controls an aerophobic response. In addition, HemAT-*Hs* is methylated by CheR *in vivo*, thereby enhancing CheA activation and downstream aerotactic signaling, but direct interaction partners of HemAT-*Hs* are still unknown (Hou et al., 2000; Schlesner et al., 2012). Both HemAT-*Hs* and HemAT-*Bh* exhibit similar ligand binding properties and reasonable amino acid similarity to HemAT-*Bs* (HemAT-*Bs* vs. HemAT-*Bh* = 56% similarity; HemAT-*Bs* vs. HemAT-*Hs* = 38% similarity), suggesting that the proteins may utilize similar sensing mechanisms (Hou, Belisle, et al., 2001; Hou, Freitas, et al., 2001; Hou et al., 2000).

4. Diguanylate Cyclase-Containing GCS Proteins

To date, GCS proteins with diguanylate cyclase (DGC) output domains have been characterized from *E. coli* (*EcDosC*), *Shewanella putrefaciens* (*DosD*), *Bordetella pertussis* (*BpeGReg*), *Pectobacterium carotovorum* (*PccGCS*), *Desulfotalea psychrophila* (*HemDGC*), and *Azotobacter vinelandii* (*AvGReg*) (Burns et al., 2014; Burns et al., 2016; Donné et al., 2016; Kitanishi et al., 2010; Lambry et al., 2016; Nakajima et al., 2012; Sawai et al., 2010; Tagliabue et al., 2010; Tarnawski et al., 2015; Tuckerman et al., 2009; Wan et al., 2009; Wu et al., 2013). All of these DGC-containing GCS proteins have a linking middle domain between the N-terminal sensor globin and C-terminal DGC domain. However, the middle domains vary greatly in length, from ~40 amino acids in *HemDGC* to ~140 amino acids for *EcDosC*, *BpeGReg*, *DosD*, *PccGCS*, and *AvGReg* (Figure 6). In addition, the proteins exhibit modest sequence similarity (30–60%), supporting the subtle differences observed experimentally.

The C-terminal DGC domain in this subclass of GCS proteins synthesizes cyclic dimeric 3', 5'-guanosine monophosphate (c-di-GMP) from two molecules of guanosine triphosphate (GTP). The DGC domain exerts requirements on the overall protein structure, as DGC domains are catalytically active as dimers, with each monomer binding one molecule of GTP and the cyclization occurring across the DGC dimer interface. Catalysis occurs within the GGDEF active site of the conserved domain, with the GTP monomers being arranged in an anti-parallel alignment. DGC domains also often contain a product-binding inhibitory site motif (RxxD) that binds c-di-GMP and inhibits enzyme activity. Therefore, within DGC-containing GCS proteins, cyclase activity potentially can be controlled by both ligand binding to the globin domain and c-di-GMP binding to the cyclase domain (Schirmer, 2016).

In bacteria, c-di-GMP has been demonstrated to serve as a second messenger that controls numerous cellular pathways and processes, including biofilm formation (Burns et al., 2016; Hengge, 2009; Schirmer, 2016; Tuckerman et al., 2009). Given the importance of biofilm formation in bacterial growth and survival, including being of great import in infections by various bacterial pathogens, the roles of DGC-containing GCS proteins in controlling O₂-dependent c-di-GMP production have garnered considerable interest (Donné et al., 2016; Tagliabue et al., 2010; Wu et al., 2013). In addition, as c-di-GMP production can be monitored *in vitro*, DGC-containing GCS proteins have provided an opportunity to dissect the effects of ligand binding on catalysis, linking these effects to structural rearrangements (Burns et al., 2014; Burns et al., 2016; Lambry et al., 2016; Rivera, Burns, Vansuch, Chica, & Weinert, 2016; Shimizu et al., 2015; Tarnawski et al., 2015; Wu et al., 2013).

4.1 *E. coli* *DosC*

The *E. coli* GCS, termed *EcDosC* (for *E. coli* Direct oxygen sensor Cyclase), has an ~150 amino acid middle domain linking the N-terminal globin and C-terminal diguanylate cyclase domains (Kitanishi et al., 2010; Tuckerman et al., 2009). The proximal histidine is conserved within the heme pocket; however only a tyrosine (Y43) is found as a distal hydrogen bond donor (a hydrogen bonding residue is not present at the homologous position of T95 in *HemAT-Bs*; Figure 2) (Kitanishi et al., 2010). *EcDosC* is able to bind all of the typical diatomic ligands and can stabilize O₂ binding ($K_d = 15 \mu\text{M}$), although the binding

affinity is decreased by ~10-fold as compared to HemAT-*Bs*, potentially due to the altered hydrogen bonding interactions. Mutation of Y43 to alanine or leucine results in lack of measurable O₂ binding, while the Y43F and Y43W variants significantly weaken O₂ affinity, further highlighting the importance of Y43 in stabilizing O₂ bound to the heme. In addition, in contrast to HemAT-*Bs*, *EcDosC* does not exhibit biphasic O₂ dissociation kinetics, suggesting differences in the heme pocket, likely due to the single hydrogen bond donor, and/or protein conformation (Kitanishi et al., 2010).

While a second distal pocket hydrogen bonding residue is not present in *EcDosC*, a distal pocket leucine (L65) plays an important role in stabilizing bound O₂. Mutation of L65 results in a spin state change for Fe^{III}, from 5-coordinate high spin for WT to 6-coordinate low spin for L65G/Q/T variants with H₂O as the axial ligand. L65 also serves to limit access to the distal pocket and ligand rebinding within the pocket, with mutations resulting in increased ligand association rates. In addition, blocking water access to the distal pocket decreases auto-oxidation of the heme, stabilizing the Fe^{II}-O₂ state of *EcDosC* (Nakajima et al., 2012).

4.1.1 *EcDosC* Domain Characterization and Structures—Similar to HemAT-*Bs*, full-length *EcDosC* was found to be primarily dimeric in solution and to form a moderately elongated shape, with small percentages of larger oligomeric species. Characterization of individual domain constructs of *EcDosC*, including crystal structures of each domain, have provided the first insights into the possible three dimensional structure of a full-length GCS protein (Figures 7 and 8) (Tarnawski et al., 2015). The *EcDosC* globin domain forms a dimer both in solution and in the crystal structure, suggesting a role in dimerization of the full-length protein. Overall, the *EcDosC* globin structure is quite similar to the HemAT-*Bs* globin, with a sensor globin fold and buried heme pocket; however, there are a few subtle differences between the globin domains. In contrast to the HemAT-*Bs* globin dimer, the *EcDosC* globin dimer has a smaller buried surface area (1300 Å² vs. 1800 Å²) and does not have any dimer contacts between the Z helices. Given that HemAT-*Bs* globin forms monomeric species in solution while *EcDosC* globin forms dimers, subtle differences in sequence at the interface, rather than total buried surface area, must lead to the altered dimer affinity. In addition, the size of the heme pocket also is decreased for *EcDosC* globin, as compared to HemAT-*Bs* globin (685 Å³ and 995 Å³, respectively) (Tarnawski et al., 2015).

A truncation of the *EcDosC* middle domain was crystallized to yield structures of middle domain dimers, which consisted of nearly entirely α -helical content (Figure 7B) (Tarnawski et al., 2015). The dimeric middle domains formed an anti-parallel four-helix bundle, with the A and E helices lining the interface and forming a coiled-coil structure, and the N- and C-termini of the middle domain on the same of the assembly. While the middle domain structures were solved in two crystal forms (P12₁1 and P2₁2₁2₁), only minor differences were observed, with a small rotation (~1.5°) and shift of the E-D-C-B helices of one monomer, as related to the other monomer, which did not provide significant insight into changes upon O₂ binding and activation. However, as the middle domains of DGC-containing GCS proteins are not homologous to previously characterized domains, these structures provide information that has allowed for generation of full-length GCS protein models (Tarnawski et al., 2015).

Overall, the structure of the *Ec*DosC DGC domain is quite similar to previously reported structures of unrelated DGC-containing proteins and consists of five strands of anti-parallel β -sheets, surrounded by five α -helices (Figure 7C). The DGC domain forms a monomer both in solution and in the crystal structure, which is also analogous to DGC domains from PleD and WspR. Furthermore, the conserved GGDEF active site is maintained on the $\beta 2$ - $\beta 3$ loop and the c-di-GMP-binding inhibitory site, located on a nearby loop, also is conserved (Tarnawski et al., 2015).

From the individual domain structures and solution data on the full-length protein, a model of the full-length *Ec*DosC protein has been proposed (Figure 8). In this model, which was developed using docking protocols and manual positioning, each individual domain is depicted as a homo-dimer, and the globin domain is proposed to bind to one end of the middle domain, with the DGC domain bound to the other end of the middle domain. The resultant full-length *Ec*DosC model depicts a dimer that exhibits an elongated shape, as was observed for the full-length protein in solution. However, as 21 amino acids of the middle domain (residues 155–176) are not present in any of the structures and were modeled as unstructured linker, future studies will be required to refine the current understanding of *Ec*DosC structure. Nevertheless, the individual domain structures and full-length *Ec*DosC model provide the first experimental evidence regarding the architecture of DGC-containing GCS proteins and suggest that contacts within the middle domain may be required for signal transduction (Tarnawski et al., 2015).

4.1.2 Heme Ligand-Dependent Diguanylate Cyclase Kinetics—The production of a measurable product by *Ec*DosC, c-di-GMP, has allowed for in depth interrogation of the effects of various ligation and oxidation states on activity of the catalytic domain. Both the Fe^{II} unliganded and Fe^{II} -NO forms of *Ec*DosC resulted in nearly undetectable cyclase activity, while Fe^{III} yielded the greatest rate of catalysis, and Fe^{II} - O_2 and Fe^{II} -CO resulted in intermediate rates of c-di-GMP production (Table 2). These results raised the possibility that *Ec*DosC senses cellular O_2 levels through a change in redox state of the heme iron, with O_2 binding and auto-oxidation resulting in formation of Fe^{III} *Ec*DosC. However, the auto-oxidation rate of *Ec*DosC was found to be quite slow (0.0092 min^{-1} ; sperm whale myoglobin = 0.001 min^{-1}), suggesting that O_2 binding to *Ec*DosC in *E. coli* likely does not cause oxidation of the heme on the time scales relevant for O_2 -dependent c-di-GMP production and signaling and that *Ec*DosC reversibly senses diatomic O_2 (Kitanishi et al., 2010).

Truncation of *Ec*DosC to yield a middle domain-DGC domain construct resulted in a protein that dimerizes in solution and exhibited ~4-fold lower diguanylate cyclase activity, as compared to full-length Fe^{III} *Ec*DosC. However, the isolated DGC domain construct (lacking both the globin and middle domains) forms monomeric species in solution and does not exhibit catalytic activity (DGC domains must dimerize for cyclase activity), suggesting that globin/middle domains increase dimer affinity and are required to activate cyclase activity, rather than solely serving to repress activity of the cyclase domain (Tarnawski et al., 2015).

4.1.3 *EcDosC* Signaling in *E. coli*—*EcDosC* (also annotated as *yddV*) is located within the DOS operon with *EcDosP*, a heme-PAS domain protein with c-di-GMP phosphodiesterase activity. *EcDosC* has been demonstrated to be predominantly expressed during the entry into stationary phase and is one of the most highly expressed DGC-containing proteins in *E. coli*. Furthermore, overexpression of *EcDosC* results in increased intracellular c-di-GMP levels and increased biofilm mass. *EcDosC* and *EcDosP* have been shown work in concert to alter c-di-GMP levels in response to O₂ concentration, resulting in altered *E. coli* biofilm formation through regulation of curli-encoding genes and the operon that produces extracellular poly-*N*-acetylglucosamine. Both curli and poly-*N*-acetylglucosamine are key components of *E. coli* biofilms, demonstrating direct control of O₂-dependent biofilm formation by *EcDosC* (Kitanishi et al., 2010; Tagliabue et al., 2010).

In addition to roles in biofilm formation, *EcDosC* and *EcDosP* have been demonstrated to associate *in vivo*, forming hetero-oligomers. Furthermore, *EcDosC*/*EcDosP* hetero-oligomers interact with part of the *E. coli* RNA processing machinery, termed the degradosome, and co-purify as a ~1.3 MDa complex. Proteins and RNA were found within the complex, including RNase E, enolase, and PNPase. PNPase exhibits both 3'-polynucleotide polymerase activity and 3'-to-5'-exoribonuclease activity, depending on the other proteins within the degradosome complex. When isolated in the *EcDosC*/*EcDosP* complex, PNPase was demonstrated to synthesize RNA tails under anaerobic conditions, but not under aerobic conditions. Furthermore, purified PNPase activity is directly regulated by c-di-GMP levels. Given the differential O₂-dependent activation of *EcDosC* and *EcDosP*, these data strongly suggest that *EcDosC* and *EcDosP* together regulate O₂-dependent levels of c-di-GMP within the immediate vicinity of PNPase to control PNPase activity and RNA processing. While the molecular details of the protein-protein interactions and particular mRNAs processed by the *EcDosC*/*EcDosP* complex are unknown, these studies suggest that O₂-dependent c-di-GMP production by GCS proteins likely controls downstream pathways, including mRNA degradation, in addition to biofilm formation (Tuckerman, Gonzalez, & Gilles-Gonzalez, 2011).

4.2 *Shewanella putrefaciens* DosD

A DGC-containing GCS protein, termed DosD (for Direct Oxygen Sensor Diguanylate cyclase), was identified in *S. putrefaciens* due to similarity to *EcDosC*. Purified DosD was demonstrated to bind heme and O₂, with O₂ binding resulting in an increase in c-di-GMP production. Deletion of the *dosD* gene was demonstrated to not affect growth, but did result in a significant loss in biofilm mass and cellular attachment. Furthermore, DosD was found to regulate intracellular c-di-GMP levels in response to O₂; DosD is responsible for nearly 50% of intracellular c-di-GMP when *S. putrefaciens* is grown aerobically, but does not affect anaerobic c-di-GMP levels, demonstrating a clear role in controlling c-di-GMP related pathways in *S. putrefaciens* (Wu et al., 2013).

The downstream effects of O₂-dependent c-di-GMP production by DosD include regulation of genes involved in adhesion, including *bpfA*, which encodes an RTX adhesin, and *aggA*, which is a component of the type I secretion system in *S. putrefaciens* and is involved in exporting BpfA. Both BpfA and AggA are required for DosD-dependent effects on biofilm

formation, suggesting that, similarly to *EcDosC*, *DosD* serves to sense O₂ levels and regulate expression of key proteins involved in bacterial biofilm formation by regulating c-di-GMP levels. Given the broad importance of c-di-GMP in regulating biofilm formation, it is likely that all DGC-containing GCS proteins will be found to play some role in O₂-dependent expression of biofilm-related genes (Cheng et al., 2016; Wu et al., 2013).

4.3 *Bordetella pertussis* *BpeGReg*

Characterization of the GCS from *B. pertussis*, the causative agent of whooping cough (termed *BpeGReg*; *B. pertussis* Globin Regulator), has demonstrated that the GCS protein is involved in regulating biofilm formation, similar to the homologous proteins in *E. coli* and *S. putrefaciens*. *BpeGReg* exhibits sequence similarity to *EcDosC*, with the conserved proximal histidine, but contains two distal pocket hydrogen bonding residues (Y43 and S68) in homologous positions to the hydrogen bonding residues in HemAT-*Bs* (Figure 2). Mutational analysis of the *BpeGReg* globin domain has demonstrated that Y43 provides the primary hydrogen bond stabilizing bound O₂, while S68 provides secondary hydrogen bonding contacts. These results suggest a heme pocket conformation of the distal tyrosine similar to the *EcDosC* pocket with a single hydrogen bond donor, but are in contrast to the arrangement of the HemAT-*Bs* pocket that contains two hydrogen bonding residues. As HemAT-*Bs* and *BpeGReg* globins exhibit modest amino acid sequence similarity (32%), differences in the globin domains and/or the middle and output domains likely are responsible for altering the globin domain conformations/structure and affecting ligand binding (Burns et al., 2014; Burns et al., 2016; Rivera et al., 2016; Wan et al., 2009).

Within the *BpeGReg* middle domain, mutation of a histidine (H225) that shows sequence conservation within DGC-containing GCS proteins that have ~140 amino acid middle domains, such as *EcDosC* and *DosD*, resulted in loss of diguanylate cyclase activity. From the *EcDosC* middle domain crystal structures, H225 likely is not involved in packing of the four helix bundle, but could potentially be interacting with the DGC domain, if the full-length *EcDosC* model is correct and *BpeGReg* adopts a similar conformation (Burns et al., 2014; Burns et al., 2016; Rivera et al., 2016; Wan et al., 2009). If so, these putative interactions could be important for correct orientation of the DGC domains. However, insights from the full-length *EcDosC* model may not be directly applicable to *BpeGReg* because, in contrast to *EcDosC*, *BpeGReg* forms a mixture of oligomeric states in solution, consisting of monomeric, dimeric, and tetrameric species. In addition, altering protein or salt concentration does not affect the equilibrium between the oligomeric states and isolated oligomers are slow to re-equilibrate, suggesting that maintaining the ratio of oligomeric states may be involved in protein function (Burns et al., 2014).

4.3.1 Effect of Ligand Binding on Oligomerization—Similar to HemAT-*Bs*, full-length *BpeGReg* exhibits biphasic O₂ dissociation kinetics, yielding O₂ binding constants of 117 and 900 nM, and potentially allowing *B. pertussis* to respond to a wide range of environmental O₂ concentrations. Characterization of individual oligomeric states yielded differences in the O₂ dissociation rates (Table 1), although no significant differences in the amplitudes of each rate were observed (Burns et al., 2014). Furthermore, the *BpeGReg* globin domain construct exclusively forms monomers in solution and exhibited monophasic

O₂ dissociation kinetics, as well as an increased rate of O₂ association. Taken together, these data support a model in which different oligomerization states result in differences in structure and/or conformation of the globin heme pocket, thereby changing O₂ binding affinity (Rivera et al., 2016).

4.3.2 Ligand-Dependent Cyclase Activation—*BpeGReg* contains the conserved RxxD motif that binds c-di-GMP and results in product inhibition; however, *in vitro* enzyme kinetics could be measured by including a c-di-GMP phosphodiesterase in the reaction mixture to eliminate product inhibition. Analysis of enzyme assays demonstrated that *BpeGReg* Fe^{II}-O₂ yields the greatest rate of c-di-GMP production, with Fe^{II} resulting in the lowest rate, and Fe^{II}-NO and Fe^{II}-CO yielding intermediate rates. In contrast to *EcDosC*, *BpeGReg* Fe^{II} results in measurable c-di-GMP production ($k_{\text{cat}} = 0.18 \text{ min}^{-1}$) and O₂ binding only yields ~3-fold increase in c-di-GMP synthesis, demonstrating that the conformational changes associated with O₂ dissociation are not sufficient to fully inactivate the cyclase domain. Binding of O₂ also resulted in a decreased K_M for GTP, which suggests that the O₂ binding event is propagated throughout the protein, potentially altering both the orientation of the active sites and the GTP binding pocket (Burns et al., 2014; Burns et al., 2016).

Binding of O₂ within the *BpeGReg* globin domain results in changes to the ratio of oligomeric states of the protein (Figure 9A), suggesting a mechanism for O₂-dependent regulation of cyclase activity. The different oligomeric states of *BpeGReg* exhibit differential rates of c-di-GMP production; tetrameric assemblies have ~5-fold greater cyclase activity than dimeric assemblies, when standardized to monomer concentration. Furthermore, the inhibitory (RxxD) site also affects oligomerization state; c-di-GMP binding shifts the equilibrium away from tetrameric assemblies, while mutations (AxxD) that eliminate product binding allow *BpeGReg* to access higher oligomeric states and alter O₂ dissociation kinetics. Therefore, the shifts in oligomeric state upon O₂ or c-di-GMP binding alter both O₂ affinity and c-di-GMP production, which may allow the organism to adapt its response based on environmental O₂ and intracellular c-di-GMP concentrations (Burns et al., 2014; Burns et al., 2016).

4.4 *Pectobacterium carotovorum* PccGCS

P. carotovorum is a plant pathogen that displays O₂-dependent virulence and results in rotting of infected plant hosts. The GCS protein within *P. carotovorum*, termed *PccGCS* (*P. carotovorum* ssp. *carotovorum* Globin Coupled Sensor) displays 51% amino acid sequence similarity to *BpeGReg* (35% amino acid identity), with intact DGC active site (GGDEF) and inhibitory site (RxxD) motifs, as well as heme pocket proximal histidine, distal tyrosine, and distal serine residues within the globin domain. Heme ligand-dependent activation of cyclase activity follows the same trend as for *BpeGReg*, with *PccGCS* Fe^{II}-O₂ exhibiting the greatest activity, and *PccGCS* also was demonstrated to exhibit biphasic O₂ dissociation rates (Burns et al., 2014).

However, despite these similarities, *PccGCS* exhibits key differences when compared to other DGC-containing GCS proteins. *PccGCS* exists as a mixture of dimer-tetramer-

octamer/high molecular weight (HMW) species, in contrast to *BpeGReg* (monomer-dimer-tetramer) and *EcDosC* (primarily dimer). The globin domain likely affects the differences in the smallest accessible oligomeric state; *BpeGReg* globin is monomeric in solution, while *PccGCS* and *EcDosC* globin domains are dimeric in solution. The various GCS proteins also exhibit differences in O₂ binding kinetics, with *EcDosC* exhibiting markedly slower O₂ association and faster O₂ dissociation rates, as compared to *BpeGReg* and *PccGCS*. However, ~1.5–2-fold differences in O₂ dissociation rates were observed for *BpeGReg* and *PccGCS*, suggesting that poorly understood differences in the protein structures/conformations result in pronounced differences in O₂ affinity and O₂-dependent cyclase activation (Burns et al., 2014; Rivera et al., 2016).

4.4.1 *PccGCS* Ligand-Dependent Activity—The different oligomeric states of *PccGCS* were demonstrated to exhibit different cyclase activities, similar to *BpeGReg*, with tetrameric assemblies exhibiting greater activity than dimeric assemblies. As both O₂ binding to the globin domain and c-di-GMP binding to the DGC domain shifted the *PccGCS* oligomer equilibrium (Figure 9B), as was observed for *BpeGReg*, ligand-dependent changes in GCS oligomerization may be a mechanism used to control activity of a subset of DGC-containing proteins. Signal-dependent changes in oligomerization of unrelated DGC-containing proteins previously also has been observed, suggesting that oligomerization state changes likely are a more general strategy to control c-di-GMP production. The lack of O₂-dependent changes in oligomerization observed for *EcDosC* might be due to formation of hetero-oligomers with *EcDosP* or involve changes in the hetero-oligomer composition, suggesting that DGC-containing GCS proteins may have evolved different activation mechanisms depending on their cellular partners. (Burns et al., 2014; Burns et al., 2016).

4.5 *Desulfotalea psychrophila* HemDGC

The DGC-containing GCS protein from *Desulfotalea psychrophila* (HemDGC) exhibits the conserved GCS domain architecture (N-terminal globin – middle domain – C-terminal DGC) but contains a truncated middle domain of ~40 amino acids (as compared to ~140 amino acids for the other characterized DGC-containing GCS proteins; Figure 6). Additional differences are found within the HemDGC heme pocket, which has been demonstrated through mutagenesis, ligand binding, and resonance Raman studies to contain distal tyrosine (Y55) and glutamine (Q81) residues that interact with bound ligands. Within the distal pocket, Y55 is required to stabilize O₂ binding, while Q81 provides secondary interactions that limit auto-oxidation. In contrast, heme-bound CO only interacts with Q81, suggesting a mechanism for discriminating between O₂ and CO (Sawai et al., 2010).

Cyclase activity was observed only for HemDGC Fe^{II}-O₂, with all other ligation states resulting in lack of measurable activity, further supporting the ability of HemDGC to discriminate between heme ligands. HemDGC was found to exist as tetrameric assemblies; however, the oligomeric state did not change upon changing ligation state. These data suggest that HemDGC exhibits a different mechanism to propagate the O₂ binding event, as compared to *BpeGReg* and *PccGCS*, and further supports the possibility that GCS proteins have evolved for a particular role in each organism. While the *in vivo* effects have not been investigated, given that *D. psychrophila* is an obligate anaerobe, production of c-di-GMP and

biofilm formation in response to O₂ may be a mechanism to protect the organism from toxic effects of O₂ and/or oxidative stress (Sawai et al., 2010).

4.6 *Azotobacter vinelandii* AvGReg

A GCS protein with putative DGC activity was identified in the genome of *A. vinelandii*, termed AvGReg (*A. vinelandii* Globin Regulator), with similar domain lengths and conserved heme pocket residues (proximal histidine; distal tyrosine and threonine) to EcDosC, DosD, BpeGReg, and PccGCS (Thijs et al., 2007). In contrast to other previously GCS proteins, *in vitro* characterization of full-length AvGReg found that the protein formed a six-coordinate, low spin Fe^{II} complex, whereas the AvGReg globin domain truncation formed a five-coordinate, high spin Fe^{II} complex, similar to what has been observed in other GCS proteins. The differences in ligation state between the full-length and globin constructs is surprising, and suggest that the middle/DGC domains might cause rearrangements within the heme pocket in full-length AvGReg or that expression/purification conditions (AvGReg and globin domain were isolated from inclusion bodies and refolded) affected the final protein structures. AvGReg Fe^{II}-O₂ also was demonstrated to rapidly react with NO, and therefore was suggested to potentially play a role in NO detoxification *in vivo* (Thijs et al., 2007). More recently, the Dewilde group has deposited a structure of AvGReg globin within the Protein Data Bank (PDB ID: 4UII) that overlays well with the structure of HemAT-*Bs* globin, but does not have electron density for the loop containing residues 48–67 (containing the distal tyrosine residue), suggesting that the distal pocket exhibits substantial flexibility (Figure 10). To date, the activity of the DGC domain and potential control by heme ligand binding to AvGReg have not been investigated.

5. Kinase-Containing GCS Proteins

Within bacteria, two-component signaling pathways, consisting of a sensor histidine kinase and response regulator, are very prevalent and many examples have been characterized to understand how bacteria sense and respond to changes in their environment (Szurmant & Ordal, 2004). A subset of GCS protein sequences contain histidine kinase domains, suggesting a mechanism by which O₂ binding to a globin domain heme can directly control activity of a downstream partner protein.

5.1 *Anaeromyxobacter* sp. FW109-5

Characterization of the GCS protein from *Anaeromyxobacter* sp. Fw109-5, termed A/GcHk (*Anaeromyxobacter* sp. FW109-5 Globin coupled Histidine kinase), has provided insight into signal transmission between globin and kinase domains in GCS proteins (Fojtikova et al., 2016; Fojtikova et al., 2015; Kitanishi et al., 2011; Stranova et al., 2016). Within the heme pocket, a distal tyrosine (Y45) is required for stable O₂ binding, forming a direct hydrogen bond, and H99 serves as the proximal ligand (Figure 2). In addition, A/GcHk O₂ association exhibits biphasic kinetics, while O₂ dissociation yields monophasic kinetics, yielding a wide range of O₂ K_d values (77 nM and 670 nM) and further supporting the possibility that many GCS proteins may have evolved biphasic O₂ binding kinetics to yield physiological response over a wide O₂ concentration range (Kitanishi et al., 2011).

5.1.1 AfGcHk Kinase Activity—Within AfGcHk, H183 was identified as the site of autophosphorylation (Figure 11A), with AfGcHk Fe^{III}-OH, Fe^{III}-CN, Fe^{II}-O₂, and Fe^{II}-CO exhibiting robust autophosphorylation activity, while AfGcHk Fe^{II} yielded low rates of autophosphorylation. However, as the Fe^{II}-O₂ complex is stable for multiple days at room temperature, AfGcHk most likely serves as an O₂ sensor *in vivo*, rather than a redox sensor (Kitanishi et al., 2011). As expected for histidine kinases, which require dimerization for catalytic activity, AfGcHk was found to exist as a dimer in solution (Kitanishi et al., 2011; Stranova et al., 2016). In addition to autophosphorylation activity, AfGcHk was demonstrated to phosphorylate two aspartic acid residues (D52 and D169; Figure 11A) within a cognate response regulator, which is located adjacent to the AfGcHk gene in the *Anaeromyxobacter* genome (Kitanishi et al., 2011).

5.1.2 Interaction of AfGcHk and Cognate Response Regulator—Binding of the AfGcHk to its cognate response regulator (RR) forms a transient 2:1 complex ($K_d = 18 \mu\text{M}$) in solution, although 2:2 complexes ($158 \mu\text{M } K_d$) also could be observed (Stranova et al., 2016). Hydrogen-deuterium exchange mass spectrometry (HDX-MS) of AfGcHk, RR, and the complex were utilized to provide information regarding accessibility of residues throughout the proteins, thereby reporting on conformational changes and protein-protein interactions. As there is no structural information on AfGcHk, HDX-MS data also provided information regarding the accuracy of the structural models and insight into the individual domain structures (Stranova et al., 2016).

Within the AfGcHk globin domain, the helices predicted to form the hydrophobic core and dimer interface exhibited very low rates of deuterium exchange, as expected for residues with low solvent accessibility and flexibility. In contrast, the residues surrounding the heme distal pocket were highly solvent accessible, suggesting significant flexibility. The flexibility in the distal pocket likely is related to requirements for O₂ binding and transmission of the ligand binding signal (Stranova et al., 2016). In the current model, the sensor globin domain is located at the end of the H8/H9 helices of the kinase domain in a linear arrangement (Figure 11B). Within the histidine kinase domain, AfGcHk also formed dimer interactions through helices H8 and H9, likely further stabilizing the dimer. In addition, these helices provided a binding site for the Rec1 domain of RR, positioning H183 on AfGcHk within ~11 Å of D52 and potentially allowing for phosphate transfer. While the current model of AfGcHk and its interactions with RR is low resolution, these data provide key insights into the structure and interactions of AfGcHk, and provide a basis for future investigations aimed at understanding the rearrangements that occur upon O₂ binding (Stranova et al., 2016).

6. Adenylate Cyclase-Containing GCS Proteins

As organisms utilize second messenger signaling to control numerous important functions, the occurrence of DGC-containing GCS proteins suggested that production of other second messengers also might be linked to O₂ levels. Among both prokaryotes and eukaryotes, cAMP controls vital functions, including carbon utilization and virulence of a number of pathogens, and is synthesized by adenylate cyclase domains (AC), which share a conserved structural motif (Botsford & Harman, 1992; Schirmer, 2016; Sen Santara et al., 2013).

6.1 *Leishmania major* HemAC-*Lm*

To date, only characterization of the AC-containing GCS protein in *Leishmania major*, termed HemAC-*Lm*, has been reported. In contrast to the prokaryotic organisms from which other characterized GCS are derived, HemAC-*Lm* was discovered in the genome of the eukaryotic pathogen that causes leishmaniasis. HemAC-*Lm* consists of an N-terminal globin-A domain, followed by a globin-B domain, and a C-terminal AC domain (Figure 12). Both globin domains bind heme and can bind O₂ and CO, with the globin-A proximal histidine located at H161 and globin-B proximal histidine at position H311. Given that both globin domains can bind O₂, it is possible that the domains work in concert to control AC activity (Roy et al., 2015; Roy et al., 2014; Sen Santara et al., 2013).

Cyclase activity was observed for HemAC-*Lm* Fe^{II}-O₂ and Fe^{II}-CO, with a K_M of 1.9 mM for ATP, and *in vivo* cAMP levels were correlated with O₂ consumption by *L. major*. Furthermore, both overexpression and decreased expression of HemAC-*Lm* resulted in changes in cell shape and decreased growth under aerobic conditions, but resulted in cell death under anaerobic conditions. Taken together, these studies have suggested key roles for both cAMP signaling and O₂ sensing in *L. major*, including control of antioxidant gene expression, as well as the importance of HemAC-*Lm* signaling in parasite survival (Sen Santara et al., 2013).

7. Stressosome-Related GCS Proteins

Within *B. subtilis*, a protein, termed RsbR, has been characterized that contains a non-heme globin domain linked to a STAS (sulfate transport and anti-anti- σ factor) domain as part of the *rsb* operon involved in stressosome formation and signaling (Chen, Yudkin, & Delumeau, 2004; Marles-Wright & Lewis, 2010; Murray, Delumeau, & Lewis, 2005). The non-heme globin maintains the sensor globin structure, but mutations within the globin heme pocket (including mutation of the proximal histidine) yielded a protein with a sensor globin fold that is unable to bind heme (Murray et al., 2005). The stressosome was demonstrated to be an ~1.8 MDa complex in *B. subtilis* formed by RsbR, RsbS (STAS domain that makes protein-protein interactions with the RsbR STAS domain), and RsbT (kinase, phosphorylates RsbR/RsbS) that senses environmental stress and, through a phosphorylation cascade involving additional *rsb* proteins, eventually resulting in activation of σ^B and transcription of over 150 genes (Eymann et al., 2011; Gaidenko & Price, 2014; Kim, Gaidenko, & Price, 2004a, 2004b; Marles-Wright et al., 2008; Price et al., 2001). However, the ligand(s) that bind and activate the *B. subtilis* non-heme globin-containing RsbR are unknown. Therefore, bioinformatic searches that predicted the occurrence of GCS proteins with STAS output domains suggested that these proteins could provide insights into both stressosome signaling and function of additional GCS proteins (Chen et al., 2004; Gaidenko & Price, 2014; Murray et al., 2005).

7.1 *Vibrio brasiliensis* GCS

RsbR protein sequences that contained the heme pocket proximal histidine and distal tyrosine were identified in the genomes of multiple *Vibrio* species (Pane-Farre, Lewis, & Stulke, 2005), including *V. vulnificus*, which causes skin and soft tissue infections with a

~50% mortality rate (Williams, Blackman, Morrison, Gibas, & Oliver, 2014). Expression of the RsbR protein from *V. brasiliensis* (*VbRsbR*), a non-pathogenic relative, found that the protein was heme bound and able to bind diatomic ligands (Jia et al., 2016). When *VbRsbR* was incubated with *VbRsbS* and *VbRsbT*, the other components of the stressosome complex, the proteins were able to associate, although complex formation did not depend on heme ligation state. Similar to HemAT-*Bs* and DGC-containing GCS proteins, *VbRsbR* exhibited biphasic O₂ dissociation kinetics. In addition, O₂ dissociation from *VbRsbR* was affected by formation of the *VbRsbR/S/T* complex; however, in contrast to *BpeGReg* and *PccGCS*, the amplitudes of each rate were altered upon complex formation (Table 1) but the dissociation rates were not affected. While the molecular details behind these differences are unknown, these data further support the roles of subtle differences between sensor globin domains, as well as the middle and output domains of GCS proteins, in affecting globin ligand binding kinetics and affinity (Jia et al., 2016).

Phosphorylation activity of *VbRsbT* was demonstrated to be controlled by ligation/oxidation state of *VbRsbR*; Fe^{II}-O₂ and Fe^{III} *VbRsbR* inhibited *VbRsbT* phosphoryl transfer to *VbRsbR* and *VbRsbS*, while inclusion of *VbRsbR* Fe^{II}-CO and Fe^{II}-NO exhibited intermediate activity, and *VbRsbR* Fe^{II} resulted in maximum phosphoryl transfer (Jia et al., 2016). As phosphorylation of both RsbR and RsbS was absolutely required for activation of the *B. subtilis* Rsb signaling pathway (Gaidenko & Price, 2014; Kim et al., 2004a), these studies suggest that *VbRsbR* controls O₂-dependent stressosome signaling in *V. brasiliensis* (Figure 13). These data also suggest that the conformational changes that occur in *VbRsbR* upon O₂ association/dissociation are sufficient for signal propagation to multiple binding partners (Jia et al., 2016).

The sites of phosphorylation on *VbRsbR* were found to occur on the short α -helical linker connecting the globin and STAS domains (*VbRsbR* S178, T182) (Jia et al., 2016). Previous mutational studies on the homologous linker from *B. subtilis* RsbR had demonstrated that linker is required for activation of downstream responses (Gaidenko, Bie, Baldwin, & Price, 2012), suggesting that phosphorylation of the *VbRsbR* linker may lead to the changes in protein conformation that activate *VbRsbT*. Additional phosphorylation sites were identified in *VbRsbT* on an α -helix (*VbRsbT* Y30, S31, S39) that, in homologous proteins, is involved in kinase dimerization and interactions with downstream kinases, highlighting a possible mechanism to transmit the O₂ binding event to downstream proteins (Jia et al., 2016).

8. GCS Proteins of Unknown Function

In addition to GCS proteins with potential functions that can be ascertained based on the sequence of the output domain, a number of genes encoding putative GCS proteins do not encode an output domain with readily identifiable activity. The gene sequence for the GCS from *Geobacter sulfurreducens*, *GsGCS*, contains a transmembrane signal-transduction domain of unknown function (Pesce et al., 2009). Structural characterization of *GsGCS* Fe^{III} globin domain yielded a sensor globin fold, forming a similar dimer structure to HemAT-*Bs*, but with bis-histidine coordination of the heme through H66 and H93. In addition, the distal pocket tyrosine (Y40) was found to be pointed away from the distal pocket, in contrast to structures of HemAT-*Bs*. *GsGCS* was demonstrated to bind O₂, NO, and CO, and exhibited

monophasic O₂ dissociation kinetics, potentially due to the altered heme pocket (Desmet et al., 2010; Pesce et al., 2009). However, as yet the *in vivo* function of GsGCS and the mechanism of activation require further study.

9. Conclusions

The diversity of output domains and physiological functions regulated by GCS proteins suggests that future studies will continue to find broad roles for these proteins in controlling O₂-dependent signaling pathways and phenotypes. The recent characterization of GCS proteins with catalytically active output domains has provided the basis for our understanding of how the O₂ binding signal is transmitted from the globin domain to control output domain function and has laid the groundwork for future studies into the rearrangements that occur upon ligand binding. Given that characterized GCS proteins have been demonstrated to play roles in biofilm formation, motility, and cell growth, the GCS protein family likely will be found to control additional important pathways and may serve as targets for novel tools to control O₂-dependent signaling.

References

- Aono S, Kato T, Matsuki M, Nakajima H, Ohta T, Uchida T, & Kitagawa T (2002). Resonance Raman and ligand binding studies of the oxygen-sensing signal transducer protein HemAT from *Bacillus subtilis*. *Journal of Biological Chemistry*, 277(16), 13528–13538. [PubMed: 11821422]
- Botsford JL, & Harman JG (1992). Cyclic AMP in prokaryotes. *Microbiological Reviews*, 56(1), 100–122. [PubMed: 1315922]
- Burns JL, Deer DD, & Weinert EE (2014). Oligomeric state affects oxygen dissociation and diguanylate cyclase activity of globin coupled sensors. *Molecular Biosystems*, 10(11), 2823–2826. [PubMed: 25174604]
- Burns JL, Rivera S, Deer DD, Joynt SC, Dvorak D, & Weinert EE (2016). Oxygen and c-di-GMP Binding Control Oligomerization State Equilibria of Diguanylate Cyclase-Containing Globin Coupled Sensors. *Biochemistry*, 55, 6642–6651. [PubMed: 27933792]
- Cannistraro VJ, Glekas GD, Rao CV, & Ordal GW (2011). Cellular stoichiometry of the chemotaxis proteins in *Bacillus subtilis*. *Journal of Bacteriology*, 193(13), 3220–3227. doi:10.1128/jb.01255-10 [PubMed: 21515776]
- Chen CC, Yudkin MD, & Delumeau O (2004). Phosphorylation and RsbX-dependent dephosphorylation of RsbR in the RsbR-RsbS complex of *Bacillus subtilis*. *Journal of Bacteriology*, 186(20), 6830–6836. doi:10.1128/jb.186.20.6830-6836.2004 [PubMed: 15466036]
- Cheng Y-Y, Wu C, Wu J-Y, Jia H-L, Wang M-Y, Wang H-Y, . . . Xiao Y-Z (2016). FlrA represses transcription of biofilm-associated bpfA operon in *Shewanella putrefaciens*. *Applied and Environmental Microbiology*. doi:10.1128/aem.02410-16
- Collins KD, Lacal J, & Ottemann KM (2014). Internal sense of direction: sensing and signaling from cytoplasmic chemoreceptors. *Microbiology and Molecular Biology Reviews*, 78(4), 672–684. doi: 10.1128/mmbr.00033-14 [PubMed: 25428939]
- Desmet F, Thijs L, El Mkami H, Dewilde S, Moens L, Smith G, & Van Doorslaer S (2010). The heme pocket of the globin domain of the globin-coupled sensor of *Geobacter sulfurreducens*--an EPR study. *Journal of Inorganic Biochemistry*, 104(10), 1022–1028. doi:10.1016/j.jinorgbio.2010.05.009 [PubMed: 20605218]
- Donné J, Van Kerckhoven M, Maes L, Cos P, & Dewilde S (2016). The role of the globin-coupled sensor YddV in a mature *E. coli* biofilm population. *Biochimica et Biophysica Acta*, 1864(7), 835–839. doi:10.1016/j.bbapap.2016.04.005 [PubMed: 27083533]
- El-Mashtoly SF, Gu Y, Yoshimura H, Yoshioka S, Aono S, & Kitagawa T (2008). Protein conformation changes of HemAT-Bs upon ligand binding probed by ultraviolet resonance Raman

- spectroscopy. *Journal of Biological Chemistry*, 283(11), 6942–6949. doi:10.1074/jbc.M709209200 [PubMed: 18162468]
- El-Mashtoly SF, Kubo M, Gu Y, Sawai H, Nakashima S, Ogura T, . . . Kitagawa T (2012). Site-specific protein dynamics in communication pathway from sensor to signaling domain of oxygen sensor protein, HemAT-Bs: Time-resolved Ultraviolet Resonance Raman Study. *Journal of Biological Chemistry*, 287(24), 19973–19984. doi:10.1074/jbc.M112.357855 [PubMed: 22528495]
- Eymann C, Schulz S, Gronau K, Becher D, Hecker M, & Price CW (2011). In vivo phosphorylation patterns of key stressosome proteins define a second feedback loop that limits activation of *Bacillus subtilis* sigmaB. *Molecular Microbiology*, 80(3), 798–810. doi:10.1111/j.1365-2958.2011.07609.x [PubMed: 21362065]
- Fojtikova V, Bartosova M, Man P, Stranova M, Shimizu T, & Martinkova M (2016). Effects of hydrogen sulfide on the heme coordination structure and catalytic activity of the globin-coupled oxygen sensor AfGcHK. *Biometals*, 29(4), 715–729. doi:10.1007/s10534-016-9947-z [PubMed: 27395436]
- Fojtikova V, Stranova M, Vos MH, Liebl U, Hranicek J, Kitanishi K, . . . Martinkova M (2015). Kinetic Analysis of a Globin-Coupled Histidine Kinase, AfGcHK: Effects of the Heme Iron Complex, Response Regulator, and Metal Cations on Autophosphorylation Activity. *Biochemistry*, 54(32), 5017–5029. doi:10.1021/acs.biochem.5b00517 [PubMed: 26212354]
- Gaidenko TA, Bie X, Baldwin EP, & Price CW (2012). Two surfaces of a conserved interdomain linker differentially affect output from the RST sensing module of the *Bacillus subtilis* stressosome. *Journal of Bacteriology*, 194(15), 3913–3921. doi:10.1128/jb.00583-12 [PubMed: 22609918]
- Gaidenko TA, & Price CW (2014). Genetic evidence for a phosphorylation-independent signal transduction mechanism within the *Bacillus subtilis* stressosome. *PLoS One*, 9(3), e90741. doi: 10.1371/journal.pone.0090741 [PubMed: 24599254]
- Germani F, De Schutter A, Pesce A, Berghmans H, Van Hauwaert M-L, Cuypers B, . . . Dewilde S (2016). Crystal structure of the *Azotobacter vinelandii* globin-coupled oxygen sensor in the aquomet form.
- Gilles-Gonzales M-A, Gonzalez G (2005). Heme-based sensors: defining characteristics, recent developments, and regulatory hypotheses. *Journal of Inorganic Biochemistry*, 99, 1–22. [PubMed: 15598487]
- Hengge R (2009). Principles of c-di-GMP signalling in bacteria. *Nature Reviews Microbiology*, 7(4), 263–273. [PubMed: 19287449]
- Hölscher T, Bartels B, Lin Y-C, Gallegos-Monterrosa R, Price-Whelan A, Kolter R, . . . Kovács ÁT (2015). Motility, Chemotaxis and Aerotaxis Contribute to Competitiveness during Bacterial Pellicle Biofilm Development. *Journal of Molecular Biology*, 427(23), 3695–3708. doi:10.1016/j.jmb.2015.06.014 [PubMed: 26122431]
- Hou S, Belisle C, Lam S, Piatibratov M, Sivozhelezov V, Takami H, & Alam M (2001). A globin-coupled oxygen sensor from the facultatively alkaliphilic *Bacillus halodurans* C-125. *Extremophiles*, 5(5), 351–354. [PubMed: 11699649]
- Hou S, Freitas T, Larsen RW, Piatibratov M, Sivozhelezov V, Yamamoto A, . . . Alam M (2001). Globin-coupled sensors: a class of heme-containing sensors in Archaea and Bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 98(16), 9353–9358. doi: 10.1073/pnas.161185598 [PubMed: 11481493]
- Hou S, Larsen RW, Boudko D, Riley CW, Karatan E, Zimmer M, . . . Alam M (2000). Myoglobin-like aerotaxis transducers in Archaea and Bacteria. *Nature*, 403(6769), 540–544. doi: 10.1038/35000570 [PubMed: 10676961]
- Jia X, Wang J. b., Rivera S, Duong D, & Weinert EE (2016). An O₂-Sensing Stressosome from a Gram Negative Bacterium. *Nature Communications*, 7, 12381.
- Kim TJ, Gaidenko TA, & Price CW (2004a). In vivo phosphorylation of partner switching regulators correlates with stress transmission in the environmental signaling pathway of *Bacillus subtilis*. *Journal of Bacteriology*, 186(18), 6124–6132. doi:10.1128/jb.186.18.6124-6132.2004 [PubMed: 15342582]

- Kim TJ, Gaidenko TA, & Price CW (2004b). A multicomponent protein complex mediates environmental stress signaling in *Bacillus subtilis*. *Journal of Molecular Biology*, 341(1), 135–150. doi:10.1016/j.jmb.2004.05.043 [PubMed: 15312768]
- Kitanishi K, Kobayashi K, Kawamura Y, Ishigami I, Ogura T, Nakajima K, . . . Shimizu T (2010). Important roles of Tyr43 at the putative heme distal side in the oxygen recognition and stability of the Fe(II)-O₂ complex of YddV, a globin-coupled heme-based oxygen sensor diguanylate cyclase. *Biochemistry*, 49(49), 10381–10393. doi:10.1021/bi100733q [PubMed: 21067162]
- Kitanishi K, Kobayashi K, Uchida T, Ishimori K, Igarashi J, & Shimizu T (2011). Identification and functional and spectral characterization of a globin-coupled histidine kinase from *Anaeromyxobacter* sp. Fw109–5. *Journal of Biological Chemistry*, 286(41), 35522–35534. doi:10.1074/jbc.M111.274811 [PubMed: 21852234]
- Lambry J-C, Stranova M, Lobato L, Martinkova M, Shimizu T, Liebl U, & Vos MH (2016). Ultrafast Spectroscopy Evidence for Picosecond Ligand Exchange at the Binding Site of a Heme Protein: Heme-Based Sensor YddV. *The Journal of Physical Chemistry Letters*, 7(1), 69–74. doi:10.1021/acs.jpclett.5b02517 [PubMed: 26651267]
- Marles-Wright J, Grant T, Delumeau O, van Duinen G, Firbank SJ, Lewis PJ, . . . Lewis RJ (2008). Molecular architecture of the “stressosome,” a signal integration and transduction hub. *Science*, 322(5898), 92–96. doi:10.1126/science.1159572 [PubMed: 18832644]
- Marles-Wright J, & Lewis RJ (2010). The stressosome: molecular architecture of a signalling hub. *Biochemical Society Transactions*, 38(4), 928–933. doi:10.1042/bst0380928 [PubMed: 20658979]
- Martinkova M, Kitanishi K, & Shimizu T (2013). Heme-Based Globin-Coupled Oxygen Sensors: Linking Oxygen Binding to Functional Regulation of Diguanylate Cyclase, Histidine Kinase and Methyl-accepting Chemotaxis. *Journal of Biological Chemistry*, 288, 27702–27711. doi:10.1074/jbc.R113.473249 [PubMed: 23928310]
- Murray JW, Delumeau O, & Lewis RJ (2005). Structure of a nonheme globin in environmental stress signaling. *Proceedings of the National Academy of Sciences of the United States of America*, 102(48), 17320–17325. doi:10.1073/pnas.0506599102 [PubMed: 16301540]
- Nakajima K, Kitanishi K, Kobayashi K, Kobayashi N, Igarashi J, & Shimizu T (2012). Leu65 in the heme distal side is critical for the stability of the Fe(II)-O₂ complex of YddV, a globin-coupled oxygen sensor diguanylate cyclase. *Journal of Inorganic Biochemistry*, 108, 163–170. doi:10.1016/j.jinorgbio.2011.09.019 [PubMed: 22005448]
- Ohta T, Yoshimura H, Yoshioka S, Aono S, & Kitagawa T (2004). Oxygen-Sensing Mechanism of HemAT from *Bacillus subtilis*: A Resonance Raman Spectroscopic Study. *Journal of the American Chemical Society*, 126(46), 15000–15001. doi:10.1021/ja046896f [PubMed: 15547976]
- Pane-Farre J, Lewis RJ, & Stulke J (2005). The RsbRST stress module in bacteria: a signalling system that may interact with different output modules. *Journal of Molecular Microbiology and Biotechnology*, 9(2), 65–76. doi:10.1159/000088837 [PubMed: 16319496]
- Pesce A, Thijs L, Nardini M, Desmet F, Sisinni L, Gourlay L, . . . Dewilde S (2009). HisE11 and HisF8 provide bis-histidyl heme hexa-coordination in the globin domain of *Geobacter sulfurreducens* globin-coupled sensor. *Journal of Molecular Biology*, 386(1), 246–260. doi:10.1016/j.jmb.2008.12.023 [PubMed: 19109973]
- Pinakoulaki E, Yoshimura H, Daskalakis V, Yoshioka S, Aono S, & Varotsis C (2006). Two ligand-binding sites in the O₂-sensing signal transducer HemAT: implications for ligand recognition/discrimination and signaling. *Proceedings of the National Academy of Sciences of the United States of America*, 103(40), 14796–14801. doi:10.1073/pnas.0604248103 [PubMed: 17003124]
- Pinakoulaki E, Yoshimura H, Yoshioka S, Aono S, & Varotsis C (2006). Recognition and Discrimination of Gases by the Oxygen-Sensing Signal Transducer Protein HemAT As Revealed by FTIR Spectroscopy. *Biochemistry*, 45(25), 7763–7766. doi:10.1021/bi0604072 [PubMed: 16784227]
- Price CW, Fawcett P, Ceremonie H, Su N, Murphy CK, & Youngman P (2001). Genome-wide analysis of the general stress response in *Bacillus subtilis*. *Molecular Microbiology*, 41(4), 757–774. [PubMed: 11532142]
- Rivera S, Burns JL, Vansuch GE, Chica B, & Weinert EE (2016). Globin domain interactions control heme pocket conformation and oligomerization of globin coupled sensors. *Journal of Inorganic Biochemistry*, 164, 70–76. doi:10.1016/j.jinorgbio.2016.08.016 [PubMed: 27614715]

- Roy J, Sen Santara S, Adhikari A, Mukherjee A, & Adak S (2015). Control of catalysis in globin coupled adenylate cyclase by a globin-B domain. *Archives of Biochemistry and Biophysics*, 579, 85–90. doi:10.1016/j.abb.2015.06.007 [PubMed: 26095616]
- Roy J, Sen Santara S, Bose M, Mukherjee S, Saha R, & Adak S (2014). The ferrous-dioxy complex of Leishmania major globin coupled heme containing adenylate cyclase: the role of proximal histidine on its stability. *Biochimica et Biophysica Acta*, 1844(3), 615–622. doi:10.1016/j.bbapap.2014.01.004 [PubMed: 24424241]
- Sawai H, Yoshioka S, Uchida T, Hyodo M, Hayakawa Y, Ishimori K, & Aono S (2010). Molecular oxygen regulates the enzymatic activity of a heme-containing diguanylate cyclase (HemDGC) for the synthesis of cyclic di-GMP. *Biochimica et Biophysica Acta*, 1804(1), 166–172. doi:10.1016/j.bbapap.2009.09.028 [PubMed: 19818878]
- Schirmer T (2016). C-di-GMP Synthesis: Structural Aspects of Evolution, Catalysis and Regulation. *Journal of Molecular Biology*, 428(19), 3683–3701. doi:10.1016/j.jmb.2016.07.023 [PubMed: 27498163]
- Schlesner M, Miller A, Besir H, Aivaliotis M, Streif J, Scheffer B, . . . Oesterhelt D (2012). The protein interaction network of a taxis signal transduction system in a Halophilic Archaeon. *BMC Microbiology*, 12(1), 272. doi:10.1186/1471-2180-12-272 [PubMed: 23171228]
- Sen Santara S, Roy J, Mukherjee S, Bose M, Saha R, & Adak S (2013). Globin-coupled heme containing oxygen sensor soluble adenylate cyclase in Leishmania prevents cell death during hypoxia. *Proceedings of the National Academy of Sciences of the United States of America*, 110(42), 16790–16795. doi:10.1073/pnas.1304145110 [PubMed: 24082109]
- Shimizu T, Huang D, Yan F, Stranava M, Bartosova M, Fojtikova V, & Martinkova M (2015). Gaseous O₂, NO, and CO in signal transduction: structure and function relationships of heme-based gas sensors and heme-redox sensors. *Chemical Reviews*, 115(13), 6491–6533. doi:10.1021/acs.chemrev.5b00018 [PubMed: 26021768]
- Stranava M, Martinek V, Man P, Fojtikova V, Kavan D, Vanek O, . . . Martinkova M (2016). Structural characterization of the heme-based oxygen sensor, AfGcHK, its interactions with the cognate response regulator, and their combined mechanism of action in a bacterial two-component signaling system. *Proteins*, 84(10), 1375–1389. doi:10.1002/prot.25083 [PubMed: 27273553]
- Szurmant H, & Ordal GW (2004). Diversity in chemotaxis mechanisms among the bacteria and archaea. *Microbiology and Molecular Biology Reviews*, 68, 301–319. [PubMed: 15187186]
- Tagliabue L, Antoniani D, Maciag A, Bocci P, Raffaelli N, & Landini P (2010). The diguanylate cyclase YddV controls production of the exopolysaccharide poly-N-acetylglucosamine (PNAG) through regulation of the PNAG biosynthetic *pgaABCD* operon. *Microbiology*, 156(Pt 10), 2901–2911. doi:10.1099/mic.0.041350-0 [PubMed: 20576684]
- Tarnawski M, Barends TR, & Schlichting I (2015). Structural analysis of an oxygen-regulated diguanylate cyclase. *Acta Crystallographica Section D*, 71(Pt 11), 2158–2177. doi:10.1107/S139900471501545X
- Thijs L, Vinck E, Bolli A, Trandafir F, Wan X, Hoogewijs D, . . . Dewilde S (2007). Characterization of a globin-coupled oxygen sensor with a gene-regulating function. *Journal of Biological Chemistry*, 282(52), 37325–37340. doi:10.1074/jbc.M705541200 [PubMed: 17925395]
- Tuckerman JR, Gonzalez G, & Gilles-Gonzalez MA (2011). Cyclic di-GMP activation of polynucleotide phosphorylase signal-dependent RNA processing. *Journal of Molecular Biology*, 407(5), 633–639. doi:10.1016/j.jmb.2011.02.019 [PubMed: 21320509]
- Tuckerman JR, Gonzalez G, Sousa EH, Wan X, Saito JA, Alam M, & Gilles-Gonzalez MA (2009). An oxygen-sensing diguanylate cyclase and phosphodiesterase couple for c-di-GMP control. *Biochemistry*, 48(41), 9764–9774. doi:10.1021/bi901409g [PubMed: 19764732]
- Vinogradov SN, Tinajero-Trejo M, Poole RK, & Hoogewijs D (2013). Bacterial and archaeal globins - A revised perspective. *Biochimica et Biophysica Acta*, 1834, 1789–1800. doi:10.1016/j.bbapap.2013.03.021 [PubMed: 23541529]
- Wan X, Tuckerman JR, Saito JA, Freitas TA, Newhouse JS, Denery JR, . . . Alam M (2009). Globins synthesize the second messenger bis-(3'-5')-cyclic diguanosine monophosphate in bacteria. *Journal of Molecular Biology*, 388(2), 262–270. doi:10.1016/j.jmb.2009.03.015 [PubMed: 19285985]

- Williams TC, Blackman ER, Morrison SS, Gibas CJ, & Oliver JD (2014). Transcriptome sequencing reveals the virulence and environmental genetic programs of *Vibrio vulnificus* exposed to host and estuarine conditions. *PLoS One*, 9(12), e114376. doi:10.1371/journal.pone.0114376 [PubMed: 25489854]
- Wu C, Cheng Y-Y, Yin H, Song X-N, Li W-W, Zhou X-X, . . . Yu H-Q (2013). Oxygen promotes biofilm formation of *Shewanella putrefaciens* CN32 through a diguanylate cyclase and an adhesin. *Scientific Reports*, 3, 1945. doi:10.1038/srep01945 [PubMed: 23736081]
- Yoshida Y, Ishikawa H, Aono S, & Mizutani Y (2012). Structural dynamics of proximal heme pocket in HemAT-Bs associated with oxygen dissociation. *Biochimica et Biophysica Acta*, 1824(7), 866–872. doi:10.1016/j.bbapap.2012.04.007 [PubMed: 22564695]
- Yoshimura H, Yoshioka S, Kobayashi K, Ohta T, Uchida T, Kubo M, . . . Aono S (2006). Specific Hydrogen-Bonding Networks Responsible for Selective O₂ Sensing of the Oxygen Sensor Protein HemAT from *Bacillus subtilis*. *Biochemistry*, 45(27), 8301–8307. doi:10.1021/bi060315c [PubMed: 16819829]
- Yoshimura H, Yoshioka S, Mizutani Y, & Aono S (2007). The formation of hydrogen bond in the proximal heme pocket of HemAT-Bs upon ligand binding. *Biochemical and Biophysical Research Communications*, 357(4), 1053–1057. doi:10.1016/j.bbrc.2007.04.041 [PubMed: 17459338]
- Zhang W, Olson JS, & Phillips GN Jr. (2005). Biophysical and kinetic characterization of HemAT, an aerotaxis receptor from *Bacillus subtilis*. *Biophysical Journal*, 88(4), 2801–2814. doi:10.1529/biophysj.104.047936 [PubMed: 15653746]
- Zhang W, & Phillips GN Jr. (2003a). Crystallization and X-ray diffraction analysis of the sensor domain of the HemAT aerotactic receptor. *Acta Crystallographica Section D*, 59(Pt 4), 749–751. doi:S0907444903003299
- Zhang W, & Phillips GN Jr. (2003b). Structure of the oxygen sensor in *Bacillus subtilis*: signal transduction of chemotaxis by control of symmetry. *Structure*, 11(9), 1097–1110. doi:S0969212603001692 [PubMed: 12962628]

Figure 1.
GCS proteins consist of a N-terminal globin domain containing a heme linked to a C-terminal output domain.

Figure 2. Globin domain sequence of GCS with key residues highlighted: (a) Distal pocket Tyr. (b) Distal pocket Leu. (c) Distal pocket Thr/Ser. (d) Proximal His. The helix labels can be seen above sequence and is based on HemAT-Bs (PDB: 1OR4).

Figure 3.

HemAT-*Bs* globin domain structure (PDB ID: 1OR4, 1OR6) (Zhang & Phillips, 2003b). (a) Labeling of globin domain helices. (b) Residues of the heme pocket: distal side (Y70, L92, T95); proximal side (H123, Y133); and heme propionate-interacting H86. (c) Globin dimer structure with overlay of two ligation states: cyano-liganded form (gray) - 1OR4, unliganded form (gold) - 1OR6. The labels GA and HA, and GB and HB represent G- and H-helices of subunit A and G- and H-helices of subunit B, respectively. The arrows indicate the movement of the helices when the unliganded dimer binds cyanide.

Figure 4.

Representative UV-visible spectra of a GCS protein (*PccGCS*) (Burns et al., 2014). Fe(II) exhibits a solet band at 430 nm with α/β around 550 nm; Fe(II)-O₂ exhibits a solet band at 416 nm with α/β at 540 and 580 nm; Fe(II)-CO presents a sharp solet band at 422 nm with α/β at 540 and 560 nm; Fe(II)-NO exhibits a solet band of 421 nm with α/β 540 and 570 nm.

Figure 5. Schematic diagram of the conformations of O₂-bound HemAT-*Bs* based on resonance Raman data from Ohta et al., 2004. The left panel of the closed form shows the hydrogen bonding of T95 to water and water to O₂. The middle and right panels of the open forms show the hydrogen bonding of T95 with Y70 and O₂, and T95 with Y70 only, respectively.

Figure 6. Full length sequence alignment of known DGC GCS with highlighted residues/regions: (a) Globin Distal Pocket Tyr; (b) distal Pocket Leu of *EcDosC*; (c) globin Distal pocket Ser/Thr; (d) proximal histidine His; (e) middle domain His; (f) RxxD motif ; (g) GGDEF motif.

Figure 7.

The isolated domains of EcDosC structures (Tarnawski et al., 2015). (a) Globin domain dimer, residues 8-154 (PDB: 4ZVA). (b) Front and top view of middle domain dimer, residues 177-292 (PDB: 4ZVC). (c) Monomeric DGC output domain with GGDEF domain highlighted in blue and RxxD domain highlighted in red, residues 297-460 (PDB: 4ZVG)

Figure 8.

Full length, dimer model determined by docking experiments and reprinted with permission from Tarnawski, et al. 2015. Based on sedimentation data indicating an elongated dimer protein, EcDosC is shown in a linear model. Globin domains are shown with O₂ bound to the heme (O₂ was modeled as bound to the iron). Missing residues 155-176 are indicated by the yellow random coil. Only the two front α -helices of each middle domains (a 4 α - helix bundle) are shown. The DGC output domains are shown with GTP- γ S bound.

Figure 9.

O_2 and c-di-GMP binding alter oligomerization of *BpeGReg* (dashed line, Fe^{II} ; solid line, $\text{Fe}^{\text{II}}\text{-O}_2$) (A) and *PccGCS* (white bar, dimer; grey bar, tetramer; striped bar, octamer) (B) (Burns et al., 2016).

Figure 10. Overlay of HemAT-*Bs* globin (PDB ID: 1OR4) (Zhang & Phillips, 2003b) and AνGReg globin (PDB ID: 4UII) (Germani et al., 2016). There is no density for the helix containing the distal tyrosine in AνGReg globin.

Figure 11.

Histidine kinase *AfGcHK* and cognate response regulator *RR* and the model of their complex based on results from Kitanishi et al., 2011 and Stranova et al., 2016. (a) *AfGcHK* - N-terminus globin domain and C-terminus histidine kinase domain with H183 autophosphorylation site. *RR* - N-terminus Rec1 with D52 phosphorylation site and C-terminus Rec2 with D169 phosphorylation site. (b) The *AfGcHK:RR* complex with 2:1 stoichiometry. The globin domain and helices 8 and 9 of the kinase domain of *AfGcHK* make up the dimer interface. One of the *RR* domains binds to the helices 8/9 bundle of *AfGcHK* where the phosphoryl group is transferred from *AfGcHK* to *RR*.

Figure 12.

HemAC-*Lm* consists of a N-terminal globin-A domain, followed by a globin-B domain, and a C-terminal adenylate cyclase domain (Roy et al., 2015; Roy et al., 2014; Sen Santara et al., 2013).

Figure 13.

Proposed O_2 -sensing *V. brasiliensis* stressosome-regulated signaling pathway. RsbR (purple) binds O_2 under aerobic conditions (right), and RsbT (turquoise) only phosphorylates RsbR, resulting in basal transcription level (top). When the bacteria enter environments with low O_2 levels (left), RsbT is activated and leads to phosphorylation of RsbR at multiple sites; increasing levels of phosphorylated RsbR result in phosphorylation of RsbS (blue) by RsbT. Phosphorylated RsbS reduces the binding affinity of RsbT to the stressosome core, causing RsbT to activate downstream stress response gene transcription (bottom). Identified

phosphorylation sites are listed. Figure reproduced from (Jia et al., 2016) with permission from Creative Commons CC/Nature Publishing Group.

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Table 1.

Oxygen binding kinetics.

Protein	k_{on} ($\mu\text{M}^{-1}\text{s}^{-1}$)	$k_{off,1}$ (s^{-1})	$k_{off,2}$ (s^{-1})	K_d (μM)	Reference
HemAT- <i>Bs</i>	19	1900	87	0.010 0.22	(Zhang et al., 2005)
<i>Ec</i> DosC (YddV)	1.4	21	N.O.	15	(Nakajima et al., 2012)
<i>Ec</i> DosC L65G	11	25	N.O.	2.3	(Nakajima et al., 2012)
<i>Ec</i> DosC L65M	0.41	65	N.O.	160	(Nakajima et al., 2012)
<i>Ec</i> DosC L65Q	0.34	9.8	N.O.	29	(Nakajima et al., 2012)
<i>Ec</i> DosC L65T	7.5	31	N.O.	4.1	(Nakajima et al., 2012)
<i>Ec</i> DosC Y43F	4.6	53	N.O.	12	(Kitanishi et al., 2010)
<i>Ec</i> DosC Y43W	4.6	>150	N.O.	>33	(Kitanishi et al., 2010)
<i>Bpe</i> GReg	7 ^a	0.82 ^b	6.30 ^b	0.117 ^c 0.900 ^c	^a (Wan et al., 2009) ^b (Burns et al., 2014) ^c (Rivera et al., 2016)
<i>Bpe</i> GReg + c-di-GMP	N.R.	0.95	6.72	N.R.	(Burns et al., 2016)
<i>Bpe</i> GReg Tetramer	N.R.	1.33	6.16	N.R.	(Burns et al., 2014)
<i>Bpe</i> GReg Dimer	N.R.	1.23	7.50	N.R.	(Burns et al., 2014)
<i>Bpe</i> GReg R364A	N.R.	0.627	3.88	N.R.	(Burns et al., 2016)
<i>Bpe</i> Globin	17.6	1.18	N.O.	0.067	(Rivera et al., 2016)
<i>Bpe</i> Globin S68A	20.3	2.08	N.O.	0.102	(Rivera et al., 2016)
<i>Pcc</i> GCS	7.2 ^a	0.56 ^b	3.87 ^b	0.078 ^a 0.538 ^a	^a (Rivera et al., 2016) ^b (Burns et al., 2014)
<i>Pcc</i> GCS + c-di-GMP	N.R.	0.575	4.65	N.R.	(Burns et al., 2016)
<i>Pcc</i> GCS Dimer	N.R.	0.668	4.68	N.R.	(Burns et al., 2014)
<i>Pcc</i> GCS Tetramer	N.R.	0.641	3.98	N.R.	(Burns et al., 2014)
<i>Pcc</i> GCS R377A	N.R.	0.97	5.50	N.R.	(Burns et al., 2016)
<i>Pcc</i> Globin	6.1	0.66	5.8	0.108 0.944	(Rivera et al., 2016)
<i>Pcc</i> Globin S82A	7.9	3.02	N.O.	0.382	(Rivera et al., 2016)
<i>Pcc</i> Globin F148R	7.4	1.22	6.98	0.165 0.943	(Rivera et al., 2016)
<i>At</i> GcHK	1.3 0.15	0.10	N.O.	0.077 0.67	(Kitanishi et al., 2011)
<i>At</i> GReg	N.R.	10.6	0.73	0.12	(Thijs et al., 2007)
<i>Vb</i> RsbR	5.2	7.0	67.4	1.4 13.0	(Jia et al., 2016)
<i>Gs</i> GCS	N.R.	N.R.	0.24	N.R.	(Pesce et al., 2009)

N.O. – not observed

N.R. – not reported

Table 2.

Diguanilate cyclase GCS ligand-dependent activity.

Protein	k_{cat} Fe(II)	k_{cat} Fe(III)	k_{cat} Fe(II)-O ₂	k_{cat} Fe(II)-CO	k_{cat} Fe(II)-NO	Reference
<i>Ec</i> DosC (YddV)	<0.001 ^a	0.066 ^a 0.124 ^b	0.022 ^a 0.066 ^b	0.022 ^a	<0.001 ^a	^a (Kitanishi et al., 2010) ^b (Tarnawski et al., 2015)
DosD	0.52	N.R.	1.68	N.R.	N.R.	(Wu et al., 2013)
<i>Bpe</i> GReg	0.18	N.R.	0.59	0.23	0.38	(Burns et al., 2014)
<i>Bpe</i> GReg R364A	0.05	N.R.	0.38	N.R.	N.R.	(Burns et al., 2016)
<i>Bpe</i> GReg R364A (Dimer)	N.R.	N.R.	0.09	N.R.	N.R.	(Burns et al., 2016)
<i>Bpe</i> GReg R364A (Tetramer)	N.R.	N.R.	0.35	N.R.	N.R.	(Burns et al., 2016)
<i>Pcc</i> GCS	0.29	N.R.	0.73	0.43	0.51	(Burns et al., 2014)
<i>Pcc</i> GCS R377A	N.R.	N.R.	0.10	N.R.	N.R.	(Burns et al., 2016)
HemDGC	0.06	0	6.9	0.04	0	(Sawai et al., 2010)

N.R. – not reported