**Globin Domain Interactions Control Heme Pocket Conformation and Oligomerization
of Globin Coupled Sensors**

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Abstract

Globin coupled sensors (GCS) are O₂-sensing proteins used by bacteria to monitor the surrounding gaseous environment. To investigate the biphasic O₂ dissociation kinetics observed for full-length GCS proteins, isolated globin domains from *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*Globin), and *Bordetella pertussis* (*Bpe*Globin), have been characterized. *Pcc*Globin is found to be dimeric, while *Bpe*Globin is monomeric, indicating key differences in the globin domain dimer interface. Through characterization of wild type globin domains and globin variants with mutations at the dimer interface and within the distal pocket, dimerization of the globin domain is demonstrated to correlate with biphasic dissociation kinetics. Furthermore, a distal pocket tyrosine is identified as the primary hydrogen bond donor, while a secondary hydrogen bond donor within the distal heme pocket is involved in conformation(s) that lead to the second O₂ dissociation rate. These findings highlight the role of the globin dimer interface in controlling properties of both the heme pocket and full-length GCS proteins.

Keywords: Globin coupled sensor; oxygen sensor; globin domain; oligomerization state

1. Introduction

Bacteria have evolved sophisticated systems to sense changes in their environment and to respond, optimizing their survival¹⁻². The mechanism by which bacteria sense changing conditions and transmit those signals into metabolic and phenotypic responses is an active area of research. The ability of bacteria to adjust to changing O₂ concentrations³⁻⁸ suggests signaling pathway(s) that can sense extracellular O₂ and adjust intracellular chemistry. A class of heme

proteins, termed globin coupled sensors, is predicted to serve as bacterial environmental O₂ sensors within a large number of bacteria^{5, 9-11}. Globin coupled sensors (GCS) consist of a sensor globin domain linked to an output domain by a variable middle domain. Output domains vary widely and include methyl accepting chemotaxis proteins (MCP)¹²⁻¹⁴, histidine kinases¹⁵, anti-anti-sigma factors¹⁶, and diguanylate cyclases^{5, 17-20}, with methyl accepting chemotaxis proteins and diguanylate cyclases being the most prevalent.

Within the cell, diguanylate cyclases are found fused to many types of regulatory domains and are responsible for cyclizing two GTP to form c-di-GMP, a bacterial secondary messenger that controls biofilm formation²¹⁻²². Diguanylate cyclases have been demonstrated to be catalytically active as dimers, with the cyclization reaction occurring across the dimer interface²³⁻²⁷, regardless of their regulatory domain. Work on the diguanylate cyclase-containing GCS proteins from *E. coli* (*EcDosC*)^{18, 20, 28}, *Desulfotalea psychrophila* (*HemDGC*)¹⁹, *Bordetella pertussis* (*BpeGReg*, the causative agent of whooping cough)^{17, 29}, and *Pectobacterium carotovorum* ssp. *carotovorum* (*PccGCS*, a plant pathogen)¹⁷ has demonstrated that cyclase activity is increased upon binding of O₂ to the globin domain, as compared with the Fe^{II} unligated state. Further characterization of GCS proteins from *B. pertussis* and *P. carotovorum* found that full length GCS proteins form mixtures of oligomeric states (monomer-dimer-tetramer and dimer-tetramer-octamer, respectively)¹⁷. For both *PccGCS* and *BpeGReg*, the tetrameric assemblies were found to exhibit the highest cyclase activity (on a per monomer basis), individual oligomeric assemblies were slow to re-equilibrate (>18 hrs.), and oligomer percentages were not dependent on protein or salt concentration, suggesting that *PccGCS* and *BpeGReg* exist as mixtures of kinetically trapped oligomers¹⁷. Furthermore, a shift toward tetrameric assemblies, which exhibit

increased catalytic activity, was triggered by O₂ binding to the heme ¹⁷, highlighting the role of the sensor globin domain in controlling protein conformation/oligomerization. Characterization of *EcDosC* ²⁸ and HemDGC ¹⁹ also showed that the proteins formed primarily dimeric and tetrameric assemblies, respectively, highlighting that GCS oligomerization may be conserved.

Structures of isolated sensor globin domains from *Bacillus subtilis* (HemAT-*Bs*, MCP output domain)³⁰ and *EcDosC* ²⁸ GCS proteins have been solved and shown to form dimers in the crystals (homology model of *Pcc*Globin based on HemAT-*Bs* is shown in Fig. 1). The globin domains maintain a primarily α -helical globin fold with dimer interfaces that consist of two helices from each monomer, arranged in coiled-coil packing with key hydrophobic and electrostatic interactions helping to stabilize the dimer ^{28, 30}. Within the heme pocket, a conserved proximal histidine ligates to the heme, and a distal tyrosine helps to stabilize bound ligands (Fig. 1A). The HemAT-*Bs* globin distal pocket has a second distal pocket hydrogen bond donor, a threonine, that also interacts with bound ligands ³⁰⁻³², while the *EcDosC* globin only has a distal pocket tyrosine ^{18, 28}. In contrast, the prototypical histidyl-ligated heme protein, myoglobin, contains a distal pocket histidine that coordinates bound ligands. Changes in oxidation/ligation states resulted in subtle rearrangements within the sensor globin structures, such as slight rotation at the dimer interface, which are hypothesized to lead to the observed ligand-dependent changes in catalytic activity ³⁰. However, to date, the effects of dimerization on GCS globin domain structure and conformation are still unknown.

Figure 1. Homology model of *Pcc*Globin (based on PDB ID: 1OR4³⁰). A) Heme pocket with proximal histidine and distal tyrosine and serine highlighted. B) Dimer interface with F148R shown in green and S155R shown in pink. C) Alternate view of the putative dimer interface.

Previous spectroscopic characterization of HemAT-*Bs* observed biphasic O₂ dissociation kinetics³², which, through resonance Raman studies³¹, were attributed to multiple open and closed conformations of the heme pocket. In addition, O₂ dissociation from dimeric and tetrameric assemblies of *Pcc*GCS and *Bpe*GReg was found to be biphasic¹⁷, again suggesting multiple conformations, and possibly hydrogen bonding patterns, within GCS heme pockets. To investigate the role of sensor globin domains in dimerization of full-length GCS proteins and the effects of globin-globin interactions on O₂ binding kinetics and hydrogen bonding interactions, isolated globin domains from *Pcc*GCS and *Bpe*GReg were investigated to determine if globin domains are important determinants of full-length GCS oligomerization and the effects of oligomerization on the heme pocket.

2. Materials & Methods

2.1. Materials. All chemicals, PCR-related enzymes, and desalting columns used in these experiments were purchased from Sigma-Aldrich, RPI, Bio-Life, GE Healthcare, and Bio-Rad.

Analytical gel filtration columns were manufactured by Agilent (SEC 3 μ m, 150 Å, 4.6x300 mm and SEC 3 μ m, 300 Å, 7.8x300 mm HPLC columns).

2.2. Mutagenesis. Codon-optimized genes for *BpeGReg* and *PccGCS* in pET-20b¹⁷ served as templates for site-directed mutagenesis (mutagenic primers are listed in Table S1). Positive transformants of all constructs were screened on LB plates containing 100 μ g/mL ampicillin and the DNA sequences were confirmed by sequencing. Globin constructs were generated based on the length of the globin domain predicted by BLAST³³ and based on homology models. Stop codons were introduced at position 176 of *PccGCS* and position 161 of *BpeGReg*. A *BpeGlobin* construct truncated at position 156 also was generated and analyzed; however, this construct was unstable, suggesting that the globin domain extends further than predicted by BLAST. PCR mutagenesis conditions were as follows: 95 °C for 1:00 min; (95 °C for 0:50 min, 55 °C for 0:40 min, 68 °C for 6:00 min), repeated 16 times; 68 °C for 7:00 min.

2.3. Expression and Purification. Proteins were expressed and purified as previously described¹⁷, with minor modifications. Globin domain expressions were induced with 100 μ M IPTG for ~20 hours at 25 °C before being harvested via centrifugation. Cell pellets were resuspended in Buffer A (50 mM Tris, 300 mM NaCl, 5% Glycerol (v/v), 20 mM imidazole, pH 7.0), lysed using a homogenizer (Avestin, Inc.) and the resulting lysates were centrifuged at 130,000 x g in a Beckman Optima L-90X ultracentrifuge at 4 °C for 1 hour. Supernatants were applied to a pre-equilibrated HisPur Ni-column (Fisher Scientific) and proteins were eluted with Buffer B (buffer A with 250 mM imidazole, pH 7.0). Purified proteins (with intact hexa-histidine tag) were desalted using a S200 gel filtration column (GE Healthcare) into Buffer C (50 mM Tris, 50 mM NaCl, 1 mM DTT, 5% Glycerol (v/v), pH 7.0). Proteins were collected and concentrated via ultrafiltration (YM-10, 10 kDa MWCO filter, Millipore), aliquoted, flash frozen, and stored at -

80 °C until use. Protein identity and purity were verified by SDS-PAGE (Fig. S1) and MALDI mass spectrometry (Table S2).

2.4. Electronic Absorption Spectroscopy. All spectra were recorded on an Agilent Cary 100 with Peltier accessory. Preparation of complexes was carried out as previously described except that the proteins were prepared in Buffer D (50 mM Tris, 50 mM NaCl, 1 mM DTT, pH 7.0) ^{17, 34-35}.

2.5. Analytical Gel Filtration. Samples were analyzed as previously described ¹⁷. Briefly, globin constructs were detected via size exclusion chromatography using an Agilent 1200 infinity system with an Agilent SEC-300 (7.8 mm X 300 mm, 300Å and 4.6 mm X 300 mm, 150Å) and diode array detector (simultaneous detection at 214, 416, and 431 nm). Proteins were reduced in an anaerobic chamber and then allowed to bind O₂ following mixing with aerobic buffer before injection onto SEC-300 column. The mobile phase for all experiments consisted of 150 mM sodium phosphate, pH 7.0, with varying concentrations of NaCl (0-300 mM). Spectra were collected for each peak during the SEC run to confirm that the heme remained in the appropriate ligation state. Globular proteins (Sigma-Aldrich) consisting of thyroglobulin (669 kDa), ferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and myoglobin (16.7 kDa) were used as molecular weight standards for calibration curves. *Pcc*Globin and *Bpe*Globin were analyzed at various concentrations (5, 10, 50, 100, 200 μ M) while the globin mutants were analyzed at 100 μ M.

2.6. Stopped Flow UV-Visible Spectroscopy. O₂ dissociation rates were performed as previously described ^{17, 35}. Globins were reduced with dithionite in an anaerobic chamber (Coy), desalted into Buffer D, and then mixed with O₂ saturated buffer to yield final concentrations of 5-10 μ M. Protein samples were rapidly mixed with a solution of sodium dithionite in Buffer D (final

dithionite concentration = 5 mM; dithionite concentration of 0.5 mM also was tested and dithionite concentration was found to not affect the O₂ dissociation rate) in an SX20 stopped flow apparatus. The dissociation of O₂ from globin constructs at 25 °C was monitored using the SX20 stopped flow equipped with a diode array detector and temperature controlled bath, and fit globally using Pro-KII software (Applied Photophysics). Additional fitting analysis was performed using Igor Pro (Wavemetrics).

2.7. Laser Flash Photolysis. Oxygen association rates were measured as previously described^{16, 35-36}. Fe^{II}-O₂ complexes of globin constructs were generated in Buffer D as described above (Section 2.4). Transient visible spectra were collected at room temperature on a home-built nanosecond transient infrared system described elsewhere with minor modifications³⁵⁻³⁶. Briefly, a SurelightTM Nd:YAG (Continuum, Inc.) 1064 nm fundamental was frequency doubled and the resulting 532 nm pump power adjusted to 65 μJ/pulse and the light focused to a ~1.3 mm diameter spot. The 405 nm probe (Thor Labs, Inc.) was focused onto a silicon avalanche photodetector (Thor Labs, Inc.). To calculate ΔA, the pre-photolysis signal was averaged and used as I₀, and the pumped signal was used as I (ΔA = -log(I/ I₀)). Transient visible spectra were analyzed with IGOR Pro 5.00 (Wavemetrics, Inc.). Reported rates are the average of three rates from triplicate measurements and the error reported as +/- the standard deviation of the three rates.

2.8. Protein Crosslinking. Proteins were incubated with BS³ crosslinker (Thermo Scientific) for 45 minutes at room temperature in 1:1, 1:10, 1:20, 1:50, 1:100 and 1:200 (protein:crosslinker) concentration ratios. Reactions were quenched by adding 0.5 M ammonium bicarbonate and letting the reaction incubate for an additional 20 minutes. The protein samples were then mixed

with 6X SDS loading buffer and heat denatured at 90 °C for 15 minutes before being run on a Criterion TGX 4-20% gradient gel (Bio-Rad).

3. Results and Discussion

Previous studies found that GCS proteins from *P. carotovorum* and *B. pertussis* exist as mixtures of oligomeric states, with the dimeric and tetrameric assemblies having different catalytic activity, but consistent O₂ dissociation rates, for each respective GCS¹⁷. However, the interfaces between domains and molecular effects of oligomerization are currently unknown. To identify the effects of oligomerization on the heme pocket and ligand binding, truncations were made to *PccGCS* and *BpeGReg* to yield isolated globin domains (*PccGlobin* and *BpeGlobin*, respectively). In addition, mutations of putative distal pocket residues (tyrosine and serine; *BpeGlobin*(S68A) and (Y43A), *PccGlobin*(S82A) and (Y57A)) were generated to investigate the roles of hydrogen bonding patterns in O₂ dissociation kinetics (Fig. 1A). Finally, mutations were introduced at the putative globin dimer interface (*PccGlobin*(F148R) and (S155R)) to weaken globin-globin interactions and further investigate the role(s) of globin dimerization on ligand binding properties (Fig. 1B and 1C).

3.1. Heme spectra of globin constructs

PccGlobin and *BpeGlobin* WT and mutant constructs were expressed, purified, and characterized by UV-visible spectroscopy. Both proteins exhibit spectra that are characteristic of histidyl-ligated heme proteins and are nearly identical to the respective full-length proteins^{17, 29} (Fig. 2A, 2B) and to other previously characterized sensor globins^{14, 19-20, 29}. Similarly, ferrous *PccGlobin*(S82A), *BpeGlobin*(S68A), and *PccGlobin*(F148R) were able to bind all of the

Figure 2. UV-visible spectra of *PccGlobin* and *BpeGlobin* proteins (50 mM Tris, 50 mM NaCl, 1 mM DTT, pH 7.0; 5-10 μM protein. Black, Fe^{II} ; red, $\text{Fe}^{\text{II}}\text{-O}_2$; blue, $\text{Fe}^{\text{II}}\text{-CO}$; green, $\text{Fe}^{\text{II}}\text{-NO}$. A) *PccGlobin* WT. B) *BpeGlobin* WT. C) *PccGlobin*(S82A). D) *BpeGlobin*(S68A). E) *PccGlobin*(F148R). Absorbance maxima and extinction coefficients are listed in Table S3.

diatomic ligands (CO, NO, O₂) and exhibited spectra nearly identical to their respective WT globin constructs (Fig. 2C-E). In contrast, both tyrosine mutants, *Pcc*Globin(Y57F) and *Bpe*Globin(Y43F), rapidly oxidized when exposed to O₂, with no detectable stable O₂ binding, identifying the distal pocket tyrosine residues as the predominant hydrogen bond donors.

3.2. Oligomeric states of WT globin constructs

Due to previous studies demonstrating that GCS proteins oligomerize¹⁷, analytical gel filtration (AGF) was performed to determine the accessible oligomerization states. Both WT globin proteins were found to form predominantly (>90%) one oligomeric state, dimer for *Pcc*Globin (with <5% tetramer) and monomer for *Bpe*Globin (with <5% dimer) (Fig. 3A). Similar to full-length *Pcc*GCS and *Bpe*GReg proteins, *Pcc*Globin and *Bpe*Globin (36% identical, 61% similar) access different oligomeric states, despite the lack of obvious differences in amino acid similarity at the putative dimer interface (Fig. S2).

The dimerization affinities of WT *Pcc*Globin and *Bpe*Globin were interrogated by changing protein and salt concentrations. From 5-200 μ M protein concentration, only minor shifts (<10%) in the oligomerization state of the proteins were observed and *Bpe*Globin did not form significant amounts of dimeric species even at the highest protein concentrations (Fig. 3B, 3C). Furthermore, a small change was observed for *Pcc*Globin at different salt concentrations (~6 kDa based on retention time), but the *Pcc*Globin dimer interface could not be disrupted, even at 300 mM NaCl (Fig. 4A).

Figure 3. *PccGlobin* and *BpeGlobin* oligomerization states (50 mM Tris, 50 mM NaCl, 1 mM DTT, pH 7.0). Retention times for globular standards (molecular weight listed) are plotted above each graph. A) Comparison of $\text{Fe}^{\text{II}}\text{-O}_2$ *PccGlobin* (dashed, dimer) and $\text{Fe}^{\text{II}}\text{-O}_2$ *BpeGlobin* (solid, monomer). B) *PccGlobin* ($\text{Fe}^{\text{II}}\text{-O}_2$) oligomerization state does not depend on concentration. C) *BpeGlobin* ($\text{Fe}^{\text{II}}\text{-O}_2$) oligomerization state does not exhibit concentration dependence.

These results show that the globin domains exhibit similar properties to the full-length GCS proteins, namely that oligomerization

state is not significantly modulated by protein concentration or ionic strength¹⁷. Additionally, neither *PccGlobin* nor *BpeGlobin* shifted oligomeric states when examined under anaerobic conditions; therefore O_2 binding is not responsible for altering protein conformation to allow globin dimerization (Fig. 4B). Similarly, anaerobic AGF analysis of full-length *PccGCS* demonstrated that O_2 binding controlled the dimer-tetramer-octamer/high molecular weight

(HMW) equilibrium, but Fe^{II} *Pcc*GCS did not yield any measureable monomer formation (J. Burns, S. Rivera, and E.E. Weinert; manuscript under review). Therefore, it is likely that O_2 binding causes conformational changes that propagated through the full-length GCS proteins, altering protein-protein interactions and altering oligomer affinity without changing globin domain dimerization.

Figure 4. Salt, O_2 , and mutation-dependent oligomerization. Retention times for globular standards (molecular weight listed) are plotted above each graph. A) *Pcc*Globin $\text{Fe}^{\text{II}}\text{-O}_2$ remains dimeric at a range of salt concentrations. B) *Pcc*Globin Fe^{II} exists as a dimer and *Bpe*Globin Fe^{II} exists as a monomer. C) Mutation of the distal pocket tyrosine does not affect oligomerization state (*Pcc*Globin and *Bpe*Globin Fe^{III} , due to rapid oxidation in air). D) $\text{Fe}^{\text{II}}\text{-O}_2$ *Pcc*Globin and *Bpe*Globin distal pocket serine mutants maintain WT oligomerization states. E) *Pcc*Globin(F148R) ($\text{Fe}^{\text{II}}\text{-O}_2$) mutation weakens dimer affinity. F) Non-crosslinked *Pcc*Globin runs as a monomer by denaturing PAGE (left), while addition of BS³ crosslinking reagent results in formation of a clear dimer band (right).

These data suggest that the globin domains are key determinants of the ability of full-length GCS proteins to exist as monomeric proteins. *Bpe*Globin is monomeric and *Bpe*GReg is observed in the monomeric state and exists as a monomer-dimer-tetramer mixture¹⁷. In contrast, *Pcc*Globin is dimeric, and *Pcc*GCS has not been observed as a monomer; instead, *Pcc*GCS exists as a dimer-tetramer-octamer/HMW mixture¹⁷. Strong interactions between globin domains in *Pcc*GCS likely preclude formation of monomeric *Pcc*GCS, while weak interactions in *Bpe*Globin readily allow *Bpe*GReg monomer formation. As less than 5% of *Pcc*Globin forms tetrameric assemblies, additional interactions clearly are required to generate higher order oligomers (tetramers and octamers); however, globin domain dimer affinity controls the accessibility of monomeric full-length proteins.

3.3. Characterization of globin mutants

As the oligomerization states of *Pcc*Globin and *Bpe*Globin could not be altered by protein or salt concentration, weakening the globin-globin interface was probed through mutagenesis. Using a homology model based on crystal structures of other sensor globins^{28, 30, 37}, residues putatively lining the interface were identified (Fig. 1B, 1C, S2). Phenylalanine148 in *Pcc*Globin was chosen for mutagenesis as it is proposed to face directly into the coiled-coil of the dimer interface. Introduction of the F148R mutation in *Pcc*Globin resulted in a shift to monomeric protein, as assessed by AGF (Fig. 4E). However, further analysis using protein-protein cross-linking found that *Pcc*Globin F148R still was able to form a dimer in solution (>35% dimer, based on cross-linking gel integration), although with significantly reduced dimer affinity (Fig. 4F).

*Pcc*Globin(S82A) and *Bpe*Globin(S68A) remained dimeric and monomeric, respectively, with no change as compared to their corresponding WT globin (Fig. 4D). In addition, from the AGF traces, both Tyr mutations remained in the same oligomerization state (dimer and monomer, respectively) as the respective WT globin (Fig. 4C). Taken together, these studies demonstrate that mutations within the heme pocket that alter hydrogen-bonding patterns do not affect oligomerization state of the globin domains. In addition, *Pcc*Globin dimer affinity can be modulated through introduction of charged residues at the putative dimer interface, which supports the homology model structure and further highlights the high affinity of *Pcc*Globin dimers.

3.4. Effects of Dimerization on Oxygen Binding Kinetics

Previous work on full-length *Pcc*GCS and *Bpe*GReg found oligomerization-dependent effects on both cyclase activity and the two O₂ dissociation rates (Table 1)¹⁷. The different oligomerization states of the isolated globin domains provided a model system to interrogate the role of the globin-globin interface in altering O₂ binding kinetics. Comparing k_{off} rates (k_1 and k_2) previously obtained for *Pcc*GCS and *Bpe*GReg¹⁷ to *Pcc*Globin and *Bpe*Globin, respectively, it can be noted that dissociation rates do not significantly change from full-length protein to globin truncation (Table 1, Fig. S3). However, *Bpe*Globin exhibits a loss of the fast rate (k_2), while *Pcc*Globin exhibits a large decrease in fast rate percentage (44% vs. 21% for *Pcc*GCS and *Pcc*Globin, respectively). The complete loss of k_2 for *Bpe*Globin, compared to the biphasic O₂ dissociation kinetics of *Pcc*Globin, suggests that the globin domain dimer interface is required for effective formation of the conformation(s) that lead to the fast rate. In addition, the decrease in the amplitude of k_2 observed for *Pcc*Globin demonstrates that the remaining two domains in the full-

length GCS proteins play important roles in altering heme pocket conformation by contributing to a state with decreased O₂ stabilization, thereby contributing to the fast rate. Removal of the middle and output domains of HemAT-*Bs* also resulted in changes in O₂ dissociation and loss of the heme pocket closed conformation³¹⁻³², supporting roles for these domains in affecting the heme pocket.

Table 1. O₂ binding kinetics (k_{on} , O₂ association rate; k_1 and k_2 , O₂ dissociation rates ; % k_1 and % k_2 , amplitude of each respective O₂ dissociation rate). Significant oligomeric states ($\geq 10\%$) in the solution of each protein during binding measurements are listed.

Protein	Oligomeric state(s)	k_{on} ($\mu\text{M}^{-1} \text{s}^{-1}$)	k_1 (s^{-1})	k_2 (s^{-1})	% k_1^a	% k_2^a	K_D (nM)	
<i>Bpe</i> GReg	Monomer, dimer, tetramer	7.0 ^b	0.82 ± 0.01^c	6.3 ± 0.1^c	39 ^c	60 ^c	117	900
<i>Bpe</i> Globin	Monomer	17.6 ± 0.8	1.18 ± 0.04	-	100	-	67	-
<i>Bpe</i> Globin (S68A)	Monomer	20.3 ± 0.5	2.08 ± 0.01	-	100	-	102	-
<i>Bpe</i> Globin (Y43F)	Monomer	-	-	-	-	-	-	-
<i>Pcc</i> GCS	Dimer, tetramer, HMW	7.2 ± 0.3^d	0.56 ± 0.01^c	3.87 ± 0.08^c	56 ^c	44 ^c	78	538
<i>Pcc</i> GCS Dimer	Dimer	N.D.	0.67 ± 0.02^c	4.7 ± 0.3^c	56 ^c	44 ^c	N.D.	N.D.
<i>Pcc</i> Globin	Dimer	6.1 ± 0.3	0.66 ± 0.01	5.8 ± 0.1	79	21	108	944
<i>Pcc</i> Globin (S82A)	Dimer	7.9 ± 0.5	3.02 ± 0.01	-	100	-	382	-
<i>Pcc</i> Globin (Y57F)	Dimer	-	-	-	-	-	-	-
<i>Pcc</i> Globin (F148R)	Monomer, dimer	7.4 ± 0.1	1.22 ± 0.01	6.98 ± 0.07	86	14	165	943

- Not observed

N.D., not determined

HMW, high molecular weight (octamer and larger)

^a All errors for % k_1 and % k_2 are $\leq 5\%$

^b Ref. [29]

^c Ref. [17]

^d Ref. [16]

The effect of salt concentration on *Pcc*Globin O₂ dissociation rates also was measured to determine if the higher salt concentrations, which can be accessed using stopped flow as compared to AGF (1 M vs. 300 mM, respectively), would weaken salt bridges putatively stabilizing the globin dimer interface. Despite the high salt concentration, no change in O₂ dissociation rates or rate percentages was observed (Table 2). These data further support the AGF data that show that the *Pcc*Globin dimer interface has high affinity and is a key determinant of dimer formation in full-length *Pcc*GCS.

Table 2. *Pcc*Globin WT salt-dependent O₂ dissociation rates.

Salt concentration	k_1 (s ⁻¹)	k_2 (s ⁻¹)	% k_1	% k_2
50 mM NaCl	0.681 ± 0.002	5.48 ± 0.08	82.88 ± 0.5	17.12 ± 0.5
300 mM NaCl	0.598 ± 0.001	3.5 ± 0.1	79.39 ± 0.8	20.61 ± 0.8
500 mM NaCl	0.584 ± 0.002	4.64 ± 0.08	82.88 ± 0.4	17.12 ± 0.4
750 mM NaCl	0.543 ± 0.002	4.0 ± 0.2	82.27 ± 1.2	17.73 ± 1.2
1000 mM NaCl	0.563 ± 0.001	5.01 ± 0.05	80.67 ± 0.5	19.33 ± 0.5

Measurement of *Pcc*Globin(F148R) O₂ dissociation rates in varying salt concentrations found that 0 mM NaCl resulted in k_1 and k_2 percentages similar to WT *Pcc*Globin, likely due to increased dimer formation, while inclusion of salt decreased the percentage of k_2 (Tables 1 and 3), potentially by stabilizing monomeric *Pcc*Globin(F148R). These data highlight the importance of the globin dimer interface in controlling conformations of the heme pocket and O₂ affinity within globin coupled sensors. An additional mutation (S155R) putatively located at the center of the dimer interface was generated to further disrupt *Pcc*Globin dimerization. However, this mutation resulted in unstable protein that readily aggregated, prohibiting analysis of the effects of dimerization on O₂ binding kinetics.

To further characterize the effects of globin dimerization on the heme pocket, O₂ association rates were measured (Table 1). *Pcc*Globin exhibited an association rate (k_{on}) almost identical to full-length *Pcc*GCS (6.1 $\mu\text{M}^{-1}\text{s}^{-1}$ and 7.2 $\mu\text{M}^{-1}\text{s}^{-1}$ ¹⁶, respectively), demonstrating that removing the middle and diguanylate cyclase domains, but maintaining the globin dimer interface, does not significantly affect O₂ entry into the heme pocket. In contrast, *Bpe*Globin exhibited a markedly faster O₂ association rate, as compared to full-length *Bpe*GReg (17.6 $\mu\text{M}^{-1}\text{s}^{-1}$ vs. 7.0 $\mu\text{M}^{-1}\text{s}^{-1}$ ²⁹, respectively). The biphasic O₂ dissociation rates yield calculated O₂ affinities of 108 nM and 944 nM for *Pcc*Globin, as compared to 78 nM and 538 nM for full-length *Pcc*GCS. In contrast, *Bpe*Globin yields a single 67 nM O₂ affinity, while O₂ affinities of 117 nM and 900 nM were calculated for full-length *Bpe*GReg. Dual O₂ affinities in both full-length proteins may allow the organisms to sense a wide range of O₂ concentrations, allowing the bacteria to modulate their responses to adapt to moderate and then low O₂ levels.

Table 3. *Pcc*Globin(F148R) salt-dependent O₂ dissociation rates.

Salt Concentration	k_1 (s ⁻¹)	k_2 (s ⁻¹)	% k_1	% k_2
0 mM NaCl	1.06 ± 0.002	6.38 ± 0.04	80.5 ± 2.4	19.5 ± 2.4
50 mM NaCl	1.224 ± 0.002	6.98 ± 0.07	85.9 ± 0.5	14.1 ± 0.5
150 mM NaCl	1.203 ± 0.002	7.43 ± 0.09	86.2 ± 1.1	13.8 ± 1.4
300 mM NaCl	1.226 ± 0.003	6.97 ± 0.08	85.5 ± 1.9	14.5 ± 1.9

As truncating *Pcc*GCS did not result in a change in O₂ association rate, it is unlikely that the change in rate for *Bpe*Globin is due to the loss of the middle and cyclase domains. Instead, loss of the globin dimerization interface may be responsible for the 2.5-fold increase in the rate of O₂ binding. Given that crystal structures of homologous sensor globins solved as dimers found that

entry to the heme pocket was away from the globin-globin interface^{28, 30} (Fig. 1), the changes in k_{on} are unlikely to be due to O₂ access being blocked by the dimer interface. Instead, both the O₂ association and dissociation rate data for *Bpe*Globin suggest that loss of the globin dimer interface results in significant conformational changes to the heme pocket, increasing accessibility and altering conformations/dynamics within the pocket.

3.5. Involvement of Distal Pocket Residues in Biphasic O₂ Dissociation Rates

Based on crystal structures and sequence alignments, *Pcc*Globin and *Bpe*Globin are proposed to have two amino acids (Y57/S82 and Y43/S68, respectively) within the distal pocket that serve as hydrogen bond donors and stabilize O₂ binding (Fig. 1A, S2)^{17-18, 28, 30}. Within HemAT-*Bs*, both the distal pocket tyrosine and threonine contribute to O₂ stabilization, while *Ec*DosC only has a distal tyrosine to provide hydrogen bonding^{18, 30-32}. Therefore, mutations of both distal pocket residues within *Pcc*Globin and *Bpe*Globin were analyzed to determine which residue is involved in the fast O₂ dissociation rate (k_2).

Given the rapid autoxidation, O₂ association and dissociation rates for *Pcc*Globin(Y57F) and *Bpe*Globin(Y43F) could not be obtained. In contrast, both serine mutants, *Pcc*Globin(S82A) and *Bpe*Globin(S68A), were able to bind O₂; however, even though *Pcc*Globin(S82A) forms a dimer (Fig. 4D), the O₂ dissociation rate simplified to a single exponential (Table 1), suggesting that the distal pocket serine is involved in interactions with bound O₂ and/or distal pocket residues that lead to biphasic kinetics and the fast O₂ dissociation rate (k_2). Mutation of *Pcc*Globin distal pocket serine also leads to an increase in k_1 , which is not surprising, given that a secondary

Figure 5. Overview of the effects of globin dimerization on O₂ binding kinetics. Monomeric *BpeGlobin* (top) exhibits a single O₂ dissociation rate, with the distal pocket tyrosine serving as the primary hydrogen bond donor. In contrast, dimeric *PccGlobin* (bottom) exhibits two O₂ dissociation rates, the faster rate requiring the presence of the distal pocket serine. Dimerization causes a conformational change in the distal pocket, positioning the serine such that it affects O₂ dissociation through interactions with bound O₂ and/or the distal pocket tyrosine.

hydrogen-bonding residue has been removed, and is similar to the increase observed for *BpeGlobin*(S68A). The O₂ association rates for the serine mutants of *PccGlobin* and *BpeGlobin* did not change relative to the WT proteins^{16, 29} (Table 1), further confirming that the mutations did not significantly affect the heme pocket. These data support the hypothesis that globin domain dimerization leads to conformation(s) of the distal pocket that adjust the position of the hydrogen bonding residues, leading to increased interaction with the distal pocket serine and resulting in biphasic O₂ dissociation (Fig. 5).

4. Conclusions

In conclusion, globin domains from *Pcc*GCS and *Bpe*GReg exist in different oligomeric states, despite the relatively high amino acid sequence similarity at the putative dimer interface. These differences in oligomerization result in altered properties of the heme domains. Globin domain dimerization within globin coupled sensors leads to biphasic O₂ dissociation, likely through changes in the conformation of the heme pocket. Of the two distal pocket hydrogen bond donors, tyrosine provides direct hydrogen bonding, while a serine residue serves as a secondary hydrogen bond donor. In addition, *Pcc*Globin distal pocket serine makes key interactions involved in the second, fast O₂ dissociation rate observed in dimeric sensor globins, suggesting that dimerization changes the accessible heme pocket conformations and position of the serine. These data provide insight into the oligomerization determinants of the full-length GCS proteins and information about the oligomerization-dependent changes to the heme pocket GCS globin domains.

Abbreviations:

AGF – Analytical gel filtration

*Bpe*Globin – Globin domain from *Bpe*GReg

*Bpe*GReg – Globin Coupled Sensor from *B. pertussis*

C-di-GMP – cyclic diguanosine monophosphate

GCS – Globin Coupled Sensor

GTP – Guanosine triphosphate

HPLC – High Pressure Liquid Chromatography

O₂ – Oxygen

*Pcc*GCS – Globin Coupled Sensor from *P. carotovorum*

*Pcc*Globin – Globin domain from *Pcc*GCS

WT – Wild type

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References

- [1] E. Karatan, P. Watnick, *Microbiol. Mol. Biol. Rev.* 73 (2009), 2, 310-47.
- [2] D. Srivastava, C.M. Waters, *J. Bacteriol.* 194 (2012), 17, 4485-93.
- [3] H. Cypionka, *Annu. Rev. Microbiol.* 54 (2000), 827-48.
- [4] H. Sass, M. Berchtold, J. Branke, H. Konig, H. Cypionka, H.D. Babenzien, *Syst. Appl. Microbiol.* 21 (1998), 2, 212-219.
- [5] C. Wu, Y.Y. Cheng, H. Yin, X.N. Song, W.W. Li, X.X. Zhou, L.P. Zhao, L.J. Tian, J.C. Han, H.Q. Yu, *Sci. Rep.* 3 (2013), 1945.
- [6] C.E. Bauer, S. Elsen, T.H. Bird, *Annu. Rev. Microbiol.* 53 (1999), 495-523.
- [7] M.A. Gilles-Gonzalez, *IUBMB Life* 51 (2001), 3, 165-73.
- [8] J. Green, J.C. Crack, A.J. Thomson, N.E. LeBrun, *Curr. Opin. Microbiol.* 12 (2009), 2, 145-51.
- [9] T. Shimizu, D. Huang, F. Yan, M. Stranova, M. Bartosova, V. Fojtikova, M. Martinkova, *Chem. Rev.* 115 (2015), 13, 6491-533.

- [10] S.N. Vinogradov, M. Tinajero-Trejo, R.K. Poole, D. Hoogewijs, *Biochim. Biophys. Acta* 1834 (2013), 9, 1789-800.
- [11] M. Martinkova, K. Kitanishi, T. Shimizu, *J. Biol. Chem.* 288 (2013), 39, 27702-11.
- [12] S. Hou, C. Belisle, S. Lam, M. Piatibratov, V. Sivozhelezov, H. Takami, M. Alam, *Extremophiles* 5 (2001), 5, 351-4.
- [13] S. Hou, T. Freitas, R.W. Larsen, M. Piatibratov, V. Sivozhelezov, A. Yamamoto, E.A. Meleshkevitch, M. Zimmer, G.W. Ordal, M. Alam, *Proc. Natl. Acad. Sci. U.S.A.* 98 (2001), 16, 9353-8.
- [14] S. Hou, R.W. Larsen, D. Boudko, C.W. Riley, E. Karatan, M. Zimmer, G.W. Ordal, M. Alam, *Nature* 403 (2000), 6769, 540-4.
- [15] K. Kitanishi, K. Kobayashi, T. Uchida, K. Ishimori, J. Igarashi, T. Shimizu, *J. Biol. Chem.* 286 (2011), 41, 35522-34.
- [16] X. Jia, J.B. Wang, S. Rivera, D. Duong, E.E. Weinert, *Nature Commun.* 7 (2016), 12381.
- [17] J.L. Burns, D.D. Deer, E.E. Weinert, *Mol. Biosyst.* 10 (2014), 11, 2823-6.
- [18] K. Kitanishi, K. Kobayashi, Y. Kawamura, I. Ishigami, T. Ogura, K. Nakajima, J. Igarashi, A. Tanaka, T. Shimizu, *Biochemistry* 49 (2010), 49, 10381-93.
- [19] H. Sawai, S. Yoshioka, T. Uchida, M. Hyodo, Y. Hayakawa, K. Ishimori, S. Aono, *Biochim. Biophys. Acta* 1804 (2010), 1, 166-72.
- [20] J.R. Tuckerman, G. Gonzalez, E.H. Sousa, X. Wan, J.A. Saito, M. Alam, M.A. Gilles-Gonzalez, *Biochemistry* 48 (2009), 41, 9764-74.
- [21] R. Hengge, *Nature Rev. Microbiol.* 7 (2009), 4, 263-73.
- [22] U. Romling, M.Y. Galperin, M. Gomelsky, *Microbiol. Mol. Biol. Rev.* 77 (2013), 1, 1-52.

- [23] C. Chan, R. Paul, D. Samoray, N.C. Amiot, B. Giese, U. Jenal, T. Schirmer, J. Deisenhofer, *Proc. Natl. Acad. Sci. U.S.A.* 101 (2004), 49, 17084-17089.
- [24] A. Deepthi, C.W. Liew, Z.-X. Liang, K. Swaminathan, J. Lescar, *PloS One* 9 (2014), 10, e110912.
- [25] N. De, M.V.A.S. Navarro, R.V. Raghavan, H. Sondermann, *J. Mol. Biol.* 393 (2009), 3, 619-633.
- [26] R. Paul, S. Abel, P. Wassmann, A. Beck, H. Heerklotz, U. Jenal, *J. Biol. Chem.* 282 (2007), 40, 29170-29177.
- [27] P. Wassmann, C. Chan, R. Paul, A. Beck, H. Heerklotz, U. Jenal, T. Schirmer, *Structure* 15 (2007), 8, 915-927.
- [28] M. Tarnawski, T.R. Barends, I. Schlichting, *Acta Crystallogr. D Biol. Crystallogr.* 71 (2015), Pt 11, 2158-77.
- [29] X. Wan, J.R. Tuckerman, J.A. Saito, T.A. Freitas, J.S. Newhouse, J.R. Denery, M.Y. Galperin, G. Gonzalez, M.A. Gilles-Gonzalez, M. Alam, *J. Mol. Biol.* 388 (2009), 2, 262-70.
- [30] W. Zhang, G.N. Phillips, *Structure* 11 (2003), 9, 1097-1110.
- [31] T. Ohta, H. Yoshimura, S. Yoshioka, S. Aono, T. Kitagawa, *J. Am. Chem. Soc.* 126 (2004), 46, 15000-1.
- [32] W. Zhang, J.S. Olson, G.N. Phillips, Jr., *Biophys. J.* 88 (2005), 4, 2801-14.
- [33] C. Camacho, G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, T.L. Madden, *BMC Bioinformatics* 10 (2009), 421.
- [34] E.E. Weinert, C.M. Phillips-Piro, R. Tran, R.A. Mathies, M.A. Marletta, *Biochemistry* 50 (2011), 32, 6832-40.

- [35] E.E. Weinert, L. Plate, C.A. Whited, C. Olea, Jr., M.A. Marletta, *Angew. Chem. Int. Ed. Engl.* 49 (2010), 4, 720-3.
- [36] B.L. Greene, C.H. Wu, P.M. McTernan, M.W. Adams, R.B. Dyer, *J. Am. Chem. Soc.* 137 (2015), 13, 4558-66.
- [37] L.A. Kelley, S. Mezulis, C.M. Yates, M.N. Wass, M.J. Sternberg, *Nature Protoc.* 10 (2015), 6, 845-58.

Graphical Abstract and Synopsis

Bacteria use globin coupled sensors (GCS) to sense and response to changing oxygen levels. Globin domains with high amino acid similarity display differential oligomerization and oxygen binding kinetics. Dimerization of the globin domain within GCS proteins results in changes to the heme pocket, altering the conformation and oxygen dissociation kinetics.