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Globin domain interactions control heme pocket conformation and oligomerization of globin coupled sensors

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ABSTRACT

Globin coupled sensors (GCS) are O_2-sensing proteins used by bacteria to monitor the surrounding gaseous environment. To investigate the biphasic O_2 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 O_2 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.

1. Introduction

Bacteria have evolved sophisticated systems to sense changes in their environment and to respond, optimizing their survival [1–2]. 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 O_2 concentrations [3–8] suggests signaling pathway(s) that can sense extracellular O_2 and adjust intracellular chemistry. A class of heme proteins, termed globin coupled sensors, is predicted to serve as bacterial environmental O_2 sensors 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 kinases [15], *anti*-anti-sigma factors [16], 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 formation [21–22]. Diguanylate cyclases have been demonstrated to be catalytically active as dimers, with the cyclization reaction occurring across the dimer interface [23–27], regardless of their regulatory domain. Work on the diguanylate cyclase-containing GCS proteins from *E. coli* (*Ec*DosC) [18,20,28], *Desulfotalea psychrophila* (HemDGC) [19], *Bordetella pertussis* (*Bpe*GReg, the causative agent of whooping cough) [17,29], and *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*GCS, a plant pathogen) [17] has demonstrated that cyclase activity is increased upon binding of O_2 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 *Pcc*GCS and *Bpe*GReg, 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 h.), and oligomer percentages were not dependent on protein or salt concentration, suggesting that *Pcc*GCS and *Bpe*GReg exist as mixtures of kinetically trapped oligomers [17]. Furthermore, a shift toward tetrameric assemblies, which exhibit increased catalytic activity, was triggered by O_2 binding to the heme [17], highlighting the role of the sensor globin domain in controlling protein conformation/oligomerization. Characterization of *Ec*DosC [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 *Ec*DosC [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

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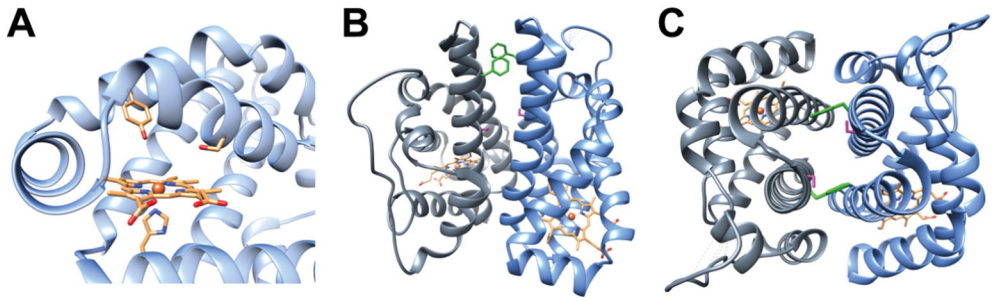

Fig. 1. Homology model of *Pcc*Globin (based on PDB ID: 1OR4 [30]). A) Heme pocket with proximal histidine and distal tyrosine and serine highlighted. B) Dimer interface with F148R shown in green and S155R shown in pink. C) Alternate view of the putative dimer interface.

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 *Ec*DosC 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.

Previous spectroscopic characterization of HemAT-*Bs* observed biphasic O_2 dissociation kinetics [32], which, through resonance Raman studies [31], were attributed to multiple open and closed conformations of the heme pocket. In addition, O_2 dissociation from dimeric and tetrameric assemblies of *Pcc*GCS and *Bpe*GReg was found to be biphasic [17], 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 O_2 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 μm, 150 Å, 4.6 × 300 mm and SEC 3 μm, 300 Å, 7.8 × 300 mm HPLC columns).

2.2. Mutagenesis

Codon-optimized genes for *Bpe*GReg and *Pcc*GCS in pET-20b [17] 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 μg/mL ampicillin and the DNA sequences were confirmed by sequencing. Globin constructs were generated based on the length of the globin domain predicted by BLAST [33] and based on homology models. Stop codons were introduced at position 176 of *Pcc*GCS and position 161 of *Bpe*GReg. A *Bpe*Globin 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 described [17], with minor modifications. Globin domain expressions were induced with 100 μM IPTG for ~20 h 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 h. 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 hexahistidine 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 × 300 mm, 300 Å and 4.6 mm × 300 mm, 150 Å) and diode array detector (simultaneous detection at 214, 416, and 431 nm). Proteins were reduced in an anaerobic chamber and then allowed to bind O_2 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 μM) while the globin mutants were analyzed at 100 μM.

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