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A globin-coupled oxygen sensor from the facultatively alkaliphilic *Bacillus halodurans* C-125

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Abstract We have recently discovered heme-containing signal transducers from the archaeon *Halobacterium salinarum* (HemAT-*Hs*) and the gram-positive bacterium *Bacillus subtilis* (HemAT-*Bs*). These proteins bind diatomic oxygen and trigger aerotactic responses. We identified that HemAT oxygen-sensing domains contain a globin-coupled sensor (GCS) motif, which exists as a two-domain transducer, having no similarity to the PAS domain (Period circadian protein, Ah receptor nuclear translocator protein, Single-minded protein) superfamily transducers. Using the GCS motif, we predicted that a 439-amino-acid protein annotated as a methyl-accepting chemotaxis protein (MCP) in the facultatively alkaliphilic bacterium *Bacillus halodurans* is a globin-coupled oxygen sensor. We cloned, expressed, and purified GCS_{Bh} and performed its spectral analysis. GCS_{Bh} binds heme and shows myoglobin-like spectra. This suggests that GCS_{Bh} acts as an oxygen sensor and transmits a conformational signal through a linked signaling domain to trigger an aerotactic response in *B. halodurans*.

Key words Heme sensor · Transducer · Globin · Alkaliphile

Introduction

We recently discovered myoglobin-like heme-containing transducers in the archaeon *Halobacterium salinarum* (HemAT-*Hs*) and the gram-positive bacterium *Bacillus subtilis* (HemAT-*Bs*). These proteins bind diatomic oxygen and mediate an aerotactic response (Hou et al. 2000). The N-termini of these transducers resemble myoglobin, and their C-termini are homologous to the cytoplasmic signaling domain of bacterial chemoreceptors. We have localized the minimal heme-binding region that retains the heme and oxygen-binding properties of the respective native proteins. We also determined the effect of replacing each histidine in the minimal heme-binding region with alanine and identified His-123 as the proximal heme-binding residue in both HemATs. Using this information, we constructed a 90-residue myoglobin-like domain transducer motif (Hou et al. 2001). Using the globin-coupled sensor (GCS) motif, we have predicted that a 439-amino-acid protein (BH0505) annotated as a methyl-accepting chemotaxis protein (MCP) is a globin-coupled two-domain oxygen sensor in the alkaliphilic bacterium *Bacillus halodurans* C-125 (JCM9153).

Among all alkaliphilic *Bacillus* isolates, *B. halodurans* C-125, isolated by Horikoshi in 1970 (Takami et al. 2001), is the most thoroughly characterized strain, physiologically, biochemically, and genetically (Takami et al. 1999; Nakasone et al. 2000; Takami and Horikoshi 2000; Takami et al. 2000). The recently completed genome sequence of *B. halodurans* offers a wealth of basic information regarding gene conservation and diversity (Takami et al. 2000). This paper reports the cloning, expression, and characterization of a globin-coupled oxygen sensor (GCS_{Bh}) not previously identified in this alkaliphilic bacterium. We postulate that GCS_{Bh} acts as an aerotactic transducer and helps *B. halodurans* find the optimum oxygen concentration in a highly alkaline environment.

Materials and methods

Chemicals and enzymes

All chemicals were reagent grade. Imidazole, dimethoxybenzidine, and sodium dithionite were purchased from Sigma (St. Louis, MO, USA). Isopropyl thiogalactoside

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(IPTG) was purchased from Fisher Scientific (Houston, TX, USA). *Pfu* DNA polymerase used in the polymerase chain reaction (PCR) analysis was purchased from Stratagene (La Jolla, CA, USA). Restriction enzymes were from Promega (Madison, WI, USA).

Bacterial strains

Escherichia coli competent cells were purchased commercially: TOP 10 cells from Invitrogen (Carlsbad, CA, USA), PlysS cells from Novagen (Madison, WI, USA), and JM109 cells from Promega (Madison, WI, USA).

Homology modeling, sequence extraction, and alignment

Homology modeling of GCS_{Bh} was performed according to Hou et al. (2001). Extracted sequences were initially aligned using ClustalX (Windows-based version of ClustalW; ftp://ftp.ebi.ac.uk/pub/software/dos/clustalx/) and were manually adjusted using MegAlign (DNAStar).

Cloning and construction of C-His-tagged GCS_{Bh}

A recombinant gene fragment encoding the C-terminal six-His-tagged GCS_{Bh} was constructed by a two-step PCR strategy (Hou et al. 2000). Briefly, *B. halodurans* genomic DNA was used to amplify the GCS_{Bh} gene by PCR using top primer 5′-cccatatgcgatagtatagataggtctc-3′ and bottom primer 5′-gtggtggtggtggtggtgtccaccactttctacctcagt-3′. The first PCR amplicon, which contained six His-tagged codons, was used as the template for a second PCR using primers 5′-cccatatgcgatagtatagataggtctc-3′ and 5′-gctctagaggatccttagtg gtggtggtggtggtg-3′. This PCR amplicon was cloned into a TOPO cloning vector and subcloned into *E. coli* expression vector pET3a using *Nde*I and *Bam*HI restriction enzymes.

Construction of C-His-tagged GCS_{Bh} truncations

Gene fragments encoding GCS_{Bh} truncations were constructed by a two-step PCR strategy as described above. An *Nde*I top primer and a site-specific primer were used in the first PCR to amplify different lengths of gene fragments. Site-specific primer sequences are as follows. Four different gene fragments encoding polypeptide fragments were created: GCS_{Bh170} (5′-gtggtggtggtggtggtgctttagcaatgagcgggtggc caagac-3′), GCS_{Bh180} (5′-gtggtggtggtggtggtgcagcacgagctgttgt tcaagattaag-3′), GCS_{Bh190} (5′-gtggtggtggtggtggtgaatccgcttat tctcgttttcatacg-3′), and GCS_{Bh200} (5′-gtggtggtggtggtggtgttc tctttttgacgctcatgttcag-3′). The PCR products were subcloned into the *E. coli* expression vector pET3a.

Protein expression and purification

Recombinant protein expression and purification by Co-affinity chromatography were performed according to Piatibratov et al. (2000).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and heme staining

The full length and truncated variants of GCS_{Bh} were analyzed by modified SDS-PAGE and dimethoxy-benzidine (DMB) heme-specific staining as described by Piatibratov et al. (2000).

Results and discussion

Prediction of protein BH0505 from *B. halodurans* as a globin-coupled sensor

BH0505 was identified in the *B. halodurans* C-125 genome-sequencing project as the product of an open reading frame encoding a 439-amino-acid protein with marked similarities to methyl-accepting chemotaxis proteins (MCP) (Takami et al. 2000). The predicted translation product exhibits two striking features: (a) its amino-termini (residues 1 to 184) display high homology to HemAT-*Bs* (Fig. 1); and (b) residues 222 to 439 are 30% identical to the cytoplasmic signaling domain of Tsr, a MCP from *E. coli* (data not shown). Furthermore, N-terminal residues 1–184 contain a 90-residue GCS motif of a globin-coupled sensor (Hou et al. 2001). We generated a 3-D model of the putative oxygen-

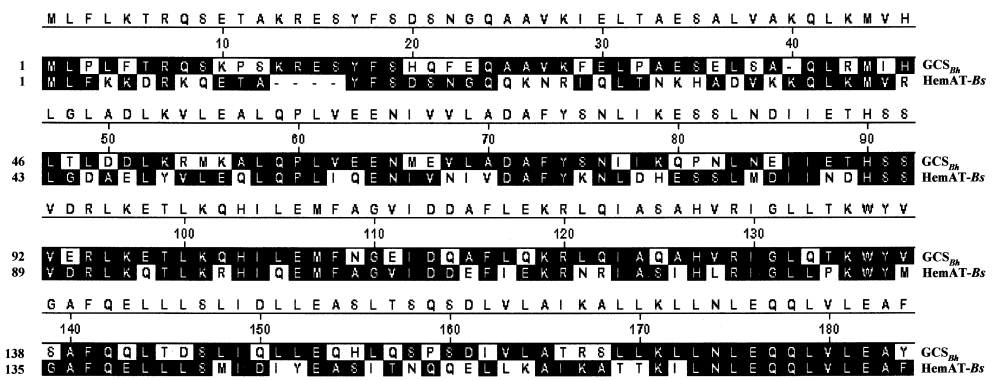

Fig. 1. Sequence alignment of the N-terminus of the globin-coupled oxygen sensor protein from *Bacillus halodurans* (*GCS_{Bh}*) with a heme-containing signal transducer protein from *Bacillus subtilis* (*HemAT-Bs*). Sequences were aligned using the Clustal program of MegAlign/DNAStar. *Black boxes* indicate positions at which the residues are identical

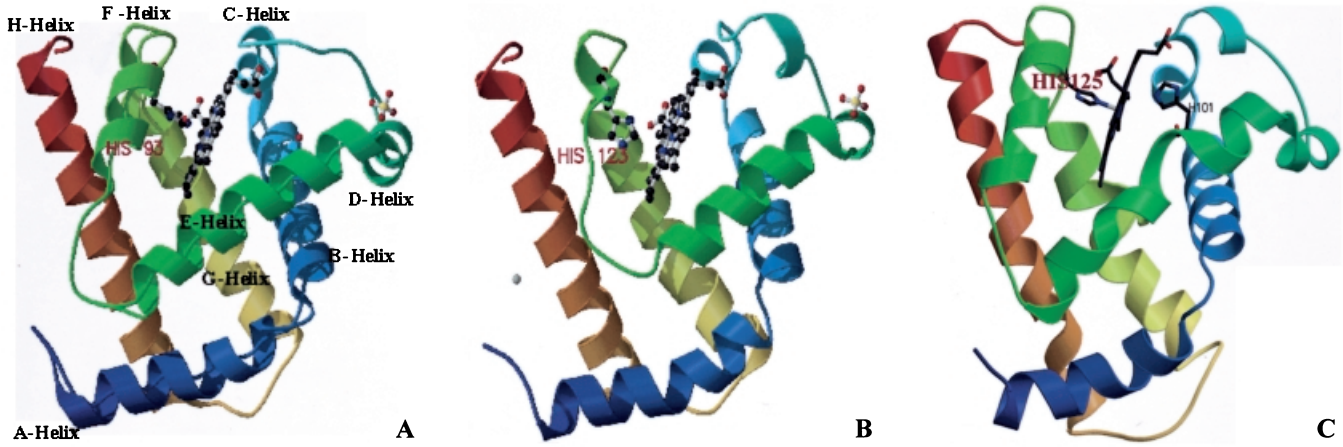

Fig. 2. Homology models of the heme-binding domains of GCS_{Bh} and HemAT-*Bs* compared to the known structure of sperm-whale myoglobin (SWMb). The homology models are of putative heme-binding domains of GCS_{Bh180} (**C**) and HemAT-Bs_{176} (**B**), and the crystal structure is of the heme-binding domain of SWMb (**A**)

sensing domain of GCS_{Bh} through homology modeling using secondary structure predictions along with sequence homology (Fig. 2). The GCS_{Bh} model (Fig. 2C) preserves the overall globin topology, including heme prosthetic group orientation, hydrophobic core of the heme pocket, and electrostatic stabilization of the CD interhelical region.

Identification of GCS_{Bh} as a heme-binding protein

To study the structure and function of this potential globin-coupled sensor transducer, the gene encoding the 439-residue GCS_{Bh} was cloned and expressed in *E. coli* as a His-tagged recombinant protein. Recombinant GCS_{Bh} was purified using co-affinity chromatography according to Piatibratov et al. (2000). The presence of heme in the purified recombinant GCS_{Bh} was determined by dimethoxybenzidine (DMB) heme staining after native PAGE and showed that purified recombinant GCS_{Bh} is indeed a heme-binding protein (data not shown).

The purified recombinant GCS_{Bh} displayed absorption spectra that are characteristic of heme proteins. The absorption spectra in the near-ultraviolet and visible regions of the oxy-, deoxy-, and reoxy- forms of the purified GCS_{Bh} were similar to HemAT-*Bs* (Hou et al. 2000). In the oxy form, the absorption maxima occurred at 413 nm (Soret), 580 nm (α-band), and 544 nm (β-band). In the deoxy form, the Soret peak shifted to 433 nm, and the α- and β-bands converged to 564 nm. The deoxy form of the protein was induced by addition of sodium dithionite. Upon reoxygenation, the absorption maxima of the protein reverted back to their initial oxy form. These results demonstrate that GCS_{Bh} is a heme-containing protein that can reversibly bind oxygen.

Identification of the oxygen-binding domains of GCS_{Bh}

To identify the minimal length of globin-fold domain that can bind heme, we began by generating fragments of gcs_{Bh} that encoded polypeptides spanning the first 170, 180, 190, and 200 residues, respectively, of the normal gene product. These recombinant proteins were constructed with C-terminal His-tags and were purified by cobalt affinity chromatography (data not shown). The effect of C-terminal truncation was explored by examining their absorption spectra in the near-UV and visible region for peaks characteristic of oxygen-bound heme proteins. Fragments GCS_{Bh180}, GCS_{Bh190}, and GCS_{Bh200} bound heme. Expressed-fragment GCS_{Bh170} formed inclusion bodies and was not analyzed by spectrophotometry. A similar phenomenon has been observed with truncated HemAT-Bs_{171} (Hou et al. 2001).

The absorption spectra for the GCS_{Bh180}, GCS_{Bh190}, and GCS_{Bh200} fragments are displayed in Fig. 3. All three truncated GCS_{Bh} could bind oxygen reversibly, as was demonstrated for the full length of GCS_{Bh}. For GCS_{Bh180} (Fig. 3A), the absorption maxima of the oxy form were found at 410 nm (Soret), 547 nm (β band), and 580 nm (α band). The oxy form of GCS_{Bh190} (Fig. 3B) and GCS_{Bh200} (Fig. 3C) showed absorption maxima similar to those of the oxy GCS_{Bh180} form. The Soret peak shifted to 428 nm, and the α and β bands converged to a broad peak at 560 nm in the deoxy forms of all three truncated GCS_{Bh}. This shift in the Soret peak and the convergence of the α and β bands upon deoxygenation is similar to other globin proteins. After exposure of deoxy GCS_{Bh} fragments to oxygen, the absorption spectra reverted back to the oxy form.

These data indicate that fragment GCS_{Bh180} behaves like other well-characterized heme-containing proteins and exhibits a reversible oxygen-binding capacity, as seen with its counterpart HemAT-Bs_{176}. Further experiments will identify the minimal heme-binding domain in GCS_{Bh}.

We have previously demonstrated that HemAT-*Bs* from neutrophilic bacterium *B. subtilis* is involved in aerophilic responses (Hou et al. 2000). Considering the high homology between GCS_{Bh} and HemAT-*Bs* and their structural similarity, we postulate that GCS_{Bh} functions as an aerotaxis heme-based transducer in facultatively alkaliphilic *B. halo-*

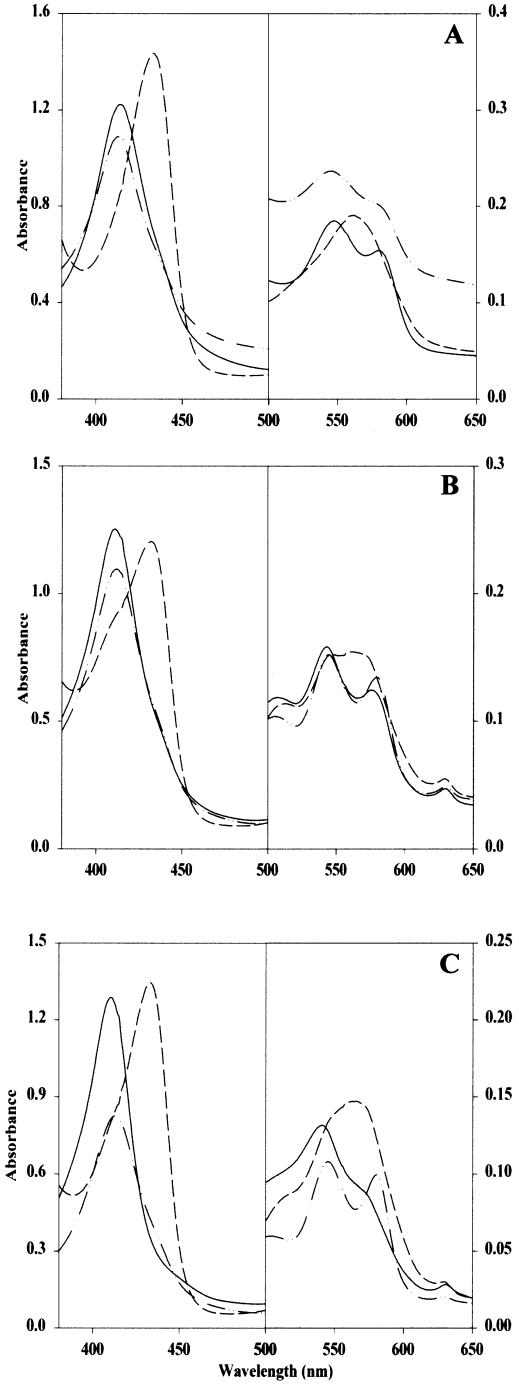

Fig. 3. Absorption spectra of oxygenated (*solid lines*), deoxygenated (*dashed lines*), and reoxygenated (*dot–dash lines*) truncated His-tagged GCS_{Bh} for **A** GCS_{Bh180}; **B** GCS_{Bh190}; and **C** GCS_{Bh200}. Spectra were measured in 200 mM NaCl, 50 mM Na_2HPO_4, pH 8.0. Deoxygenated samples were prepared by the addition of sodium dithionite to the protein solutions

durans. We propose that the amino-terminal domains of GCS_{Bh} act as a sensor by binding diatomic oxygen. This oxygen binding triggers a conformational change in the sensor domain that, in turn, alters the activity of the carboxyl-terminal signaling domain to trigger aerotactic responses.

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