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# Evidence for fast conformational change upon ligand dissociation in the HemAT class of bacterial oxygen sensors

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**Abstract** **Here we report the results of transient absorption and photoacoustic calorimetry studies of CO photodissociation from the heme domain of the bacterial oxygen sensor HemAT-Bs. The results indicate that CO photolysis is accompanied by an overall $\Delta H$ of $-19$ kcal mol$^{-1}$ and $\Delta V$ of $+4$ ml mol$^{-1}$ as well as a red-shifted kinetic difference spectrum all occurring in <50 ns. Analysis of the $\Delta H/\Delta V$ reveals that a conformational change takes place with a $\Delta H_{conf}$ of $-40$ kcal mol$^{-1}$ and $\Delta V_{conf}$ of $-22$ ml mol$^{-1}$. These thermodynamic changes are consistent with an increase in the solvent accessible surface area of the protein upon ligand dissociation, as observed in the X-ray structure of the ferric CN-bound and CN free forms of HemAT-Bs.**


*Keywords:* HemAT-Bs; Photoacoustic calorimtery; Heme; Signaling; Aerotaxis

## 1. Introduction

Globin coupled sensors (GCS) represent a unique class of oxygen sensing heme proteins found in both Archaea and Bacteria [1]. Unlike the more ubiquitous PAS domain sensors found in Bacteria, Archaea and Eukaryotes, which contain an α/β type fold with an anti-parallel β-barrel structure that encapsulates the sensor element, GCS proteins contain an N-terminal heme sensor domain reminiscent of a myoglobin (Mb) type fold [1–4]. These proteins also contain a C-terminal domain homologous to the cytoplasmic signaling domain of various eukaryotic type chemoreceptors. The first two members discovered in the GCS class of sensor were HemAT-Hs from the archaea *Halobacterium salinarum* and HemAT-Bs from the gram positive prokaryote *Bacillus subtilis* [2]. Both HemAT-Hs and HemAT-Bs share structural homology with the globin family. Specifically, both proteins have conserved His (F8), Pro (C2), and Phe (CD1) corresponding to amino acids Pro56, His123, Phe69 (HemAT-Bs numbering). In addition, both proteins participate in aerotaxis responses in their respective organisms. HemAT-Hs has been implicated in an aerophobic response while HemAT-Bs is involved in an aerophylic response [2,5]. HemAT-Hs also undergoes methylation-dependent adaptation via CheR methyltransferase.

A recent crystal structure of the HemAT-Bs heme domain (HemAT-BsHD) revealed a Mb-like heme sensing domain with a more traditional 'three over two' helical fold typical of globins that is structurally distinct from PAS-domain heme sensors [6]. The crystal structure also showed a dimer in the asymmetric unit similar to the EcDosH [7]. Similar to the FixL heme domains, structural differences between liganded and unliganded forms was modest. The most significant changes were: rotation of the proximal His (His123) and displacement of a distal Tyr residue (Tyr70). These results suggest that structural changes associated with the signaling event are quite small or that larger *transient* conformational changes are not detected by X-ray structures.

Like other heme oxygen sensors, conformational changes associated with ligand binding/dissociation to heme sensing moieties are thought to initiate signaling in the GCS. Thus, characterizing those conformational changes is critically important to understanding the signal transduction process. We have utilized transient absorption spectroscopy and time-resolved photoacoustic calorimetry (PAC) to examine the thermodynamics and conformational dynamics following CO photodissociation from Fe(2+)HemAT-BsHD.

## 2. Materials and methods

### 2.1. Protein expression, isolation and purification

The open reading frame corresponding to the N-terminal 180 amino acid heme binding domain of HemAT (HemAT-BsHD) was amplified from *B. subtilis* genomic DNA (ATCC 23857D-5). The primers employed for PCR were 5′ATGTTATTTAAAAAAGAC3′ (forward) and 5′AAACGCTTCAAGGACAAGCAG3′ (reverse). The resulting PCR product was subsequently amplified and cloned into pDEST17 (Invitrogen) using Gateway technology (Invitrogen) according to the manufacturer's instructions. The primers used for amplification were 5′GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATCGAGGGACGAATGTTATTTAAAAAAGAC3′ (forward) and 5′CCCCACCACTTTGTACAAGAAAGCTGGGTTTAAAACGCTTCAAGGACAAGCAG3′ (reverse). The resulting plasmid was named pHemAT$_{180}$ and contains an N-terminal six residue histidine tag. The integrity of the HemAT open reading frame was verified by sequencing in the Washington State University LBB1.

pHemAT$_{180}$ was established in *Escherichia coli* strain BL21(DE3) and was used to express the HemAT histidine-tagged N-terminal domain protein. This strain also contained pHPEX3 which expresses the hemin transporter ChuA (a kind gift from Professor D.C. Goodwin (2004), Auburn University. Protein Expr. Purif. 35, 76–83). The expression strain with two plasmids was maintained under dualampicillin (pHemAT$_{180}$) and chloramphenicol (pHPEX) selection. For protein expression, "Terrific Broth" (Current Protocols in Molecular Biology, Ausubel et al., Eds.) was inoculated to $A_{560} = 0.05$ and incubated with shaking at 37 °C until $A_{560}$ increased to 0.6–0.8. Protein expression was induced by addition of 0.5 mM IPTG and hemin was added to a final concentration of 50 μM. The temperature was reduced to 25 °C and incubation was continued for 15 h.

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Following induction and expression the cells were collected by centrifugation and resuspended in lysis/wash buffer (50 mM Na phosphate pH 8, 300 mM NaCl, 20 mM imidazole), lysed by two passages through a French pressure cell operated at 12000 psi and cleared by centrifugation. The dark red supernatant was then applied to a $Ni^{2+}$-NTA column (Qiagen) and the column was washed extensively with buffer. Protein was eluted from the column with a 20–300 mM imidazole gradient in buffer. Protein fractions were pooled then concentrated and exchanged into other buffers using a centrifugal concentrator (Amicon Ultra). Protein purity was monitored by SDS–PAGE, MALDI-TOF Mass Spectrometry (WSU LBB2) and UV–Vis spectroscopy.

### 2.2. Sample preparation

Samples for PAC were prepared by diluting HemAT-BsHD from an ~150 μM stock into a buffer containing 50 mM sodium phosphate (pH 8.0) and 100 mM NaCl (the protein is predominantly in the homodimeric state under these conditions). The deoxy form of the protein was formed by placing the oxy form of HemAT-BsHD in a quartz optical cuvette that was then sealed with a septum cap and purged with Ar. Dithionite was added from a buffered stock solution to give a final concentration of ~100 μM. The CO-bound form was obtained by saturating solutions of the deoxy HemAT-BsHD with CO resulting in a final solution CO concentration of 1 mM (1 atm pressure). The protein concentration for PAC samples was ~75 μM while those for transient absorption were ~25 μM. The equilibrium binding of CO to deoxy HemAT-BsHD was performed by titrating aliquots of CO saturated buffer (1 mM) into a solution containing ~10 μM deoxy HemAT-BsHD. The change in absorption at 420 nm was monitored and plotted versus [CO]. The $K_a$ value for CO binding was obtained using:

$$\Delta A_{420\ \mathrm{nm}} = K_a \Delta\varepsilon_{420\ \mathrm{nm}}[\mathrm{HemAT\text{-}BsHD}]_0[\mathrm{CO}]/(1 + K_a[\mathrm{CO}]) \quad \text{(I)}$$

where $\Delta\varepsilon_{420\ \mathrm{nm}}$ is the change in molar extinction at 420 nm (obtained from the fit) and $[\mathrm{HemAT\text{-}BsHD}]_0$ is the initial concentration of deoxy HemAT-BsHD. Equilibrium UV–Vis spectra were obtained using a Shimadzu UV2401 spectrophotometer.

### 2.3. PAC methods

PAC measurements were performed (as described previously [8]) by placing a 1 × 1 cm quartz cuvette containing 1.0 mL of a sample in a temperature controlled sample holder (Quantum Northwest) housing a Panametrics V103 transducer. Contact between the cuvette and the detector was facilitated with a thin layer of vacuum grease. Photodissociation of CO was achieved with a 532 nm laser pulse (Continuum Minilite I frequency double Q-switched Nd:YAG laser, 6 ns pulse, <80 μJ). The acoustic signal was amplified with an ultrasonic preamp (Panametrics) and recorded using an NI 5102 Oscilloscope (15 MHz) controlled by VirtualBench software (National Instrument). The PAC data were analyzed using the multiple temperature method in which sample and calorimetric reference acoustic traces are obtained as a function of temperature. The ratio of the amplitudes of the sample and reference acoustic signals ($\phi$) are then plotted versus $C_p\rho/\beta$ according to:

$$\phi E_{hv} = Q + \Delta V_{\mathrm{con}}(C_p\rho/\beta) \quad \text{(II)}$$

where $\phi$ is the ratio of the acoustic amplitudes for the sample and reference (i.e., $\phi = \{A_S/A_R\}$) Q is the heat released to the solvent, $\beta$ is the coefficient of thermal expansion of the solvent ($K^{-1}$), $C_p$ is the heat capacity (cal $g^{-1}$ $K^{-1}$), $\rho$ is the density (g $mL^{-1}$) and $\Delta V_{\mathrm{con}}$ represents conformational/electrostriction contributions to the solution volume change. A plot of $\phi E_{hv}$ versus $C_p\rho/\beta$ (varied by changing the solution temperature) gives a straight line with a slope equal to $\Delta V_{\mathrm{con}}$ and an intercept equal to the released heat ($Q$). Subtracting $Q$ from $E_{hv}$ and dividing by the quantum yield gives $\Delta H$ (i.e., $\Delta H = \{E_{hv-Q}\}/\Phi$) for processes occurring faster than the time resolution of the instrument (<20 ns). The $Q/\Phi$ values for subsequent kinetic processes represent $-\Delta H$ for that step (i.e., heat released). The value of $\Phi$ for CO photolysis from the ferrous form of HemAt-BsHD has previously been determined to be 0.90 ± 0.1 (Alam and Larsen, unpublished results).

### 2.4. Transient absorption methods

Transient absorption (TA) experiments were performed by monitoring the change in intensity of light from a Xe arc lamp (Oriel) emerging from the sample followed by passage through a 1/4 m single monochromator equipped with an Oriel R928 photo-multiplier tube. The signal was amplified using a home-built pre-amplifier (1 MHz bandwidth) followed by a Stanford Instruments SR445A 350 MHz post amplifier. The signal was digitized using a Tektronix TDS7404 4 GHz digital oscilloscope. The sample was excited with the second harmonic of a Continuum Leopard I Q-switched mode-locked Nd:YAG laser (<20 ps, 20 mJ/pulse, 20 Hz). Signal traces are the average to 20 laser pulses.

## 3. Results

The recombinant heme domain of HemAT-Bs displays an optical spectrum with a Soret maximum at ~410 nm and visible bands at 578 nm (α-band) and 538 nm (β-band) (Fig. 1). Upon deoxygenation the Soret band shifts to 425 nm with a broad visible band centered at 555 nm. Incubation of the deoxy protein in the presence of CO results in a Soret band at ~418 nm and visible bands at 573 nm and 535 nm. These spectral changes were utilized to determine the association constant for the recombinant HemAT-BsHD (Fig. 2). Fitting a plot of $\Delta A_{420\ \mathrm{nm}}$ versus CO concentration to a single binding equilibrium yielded a $K_a$ value of $(1.5 \pm 0.5) \times 10^6$ $M^{-1}$ consistent with previous studies of this construct (Alam and Larsen, unpublished results) but is slightly lower than that determined previously by Zhang et al. who reported a value of $6 \times 10^6$ $M^{-1}$ [9].

Photolysis of the CO bound HemAT-BsHD results in the formation of a five coordinate high spin heme complex which decays back to the pre-flash CO bound complex with a pseudo first-order rate constant of 59 $s^{-1}$ (the corresponding second-order rate constant is $5.9 \times 10^4$ $M^{-1}$ $s^{-1}$ with a solution CO concentration of 1 mM). This value is close to that reported by Zhang et al. ($4.3 \times 10^5$ $M^{-1}s^{-1}$) suggesting that the difference in $K_a$ values for CO between Zhang et al. and the present study is due to the values of $k_{\mathrm{off}}$ (0.07 $s^{-1}$ vs. 0.04 $s^{-1}$ from Zhang et al. and the present study, respectively) [9]. In addition, the kinetic optical difference spectrum obtained ~4 μs subsequent to photolysis displays a bathochromic shift relative to the equilibrium optical difference spectrum suggesting an un-relaxed heme pocket geometry subsequent to photolysis (Fig. 3). Since the concentration of transient five coordinate

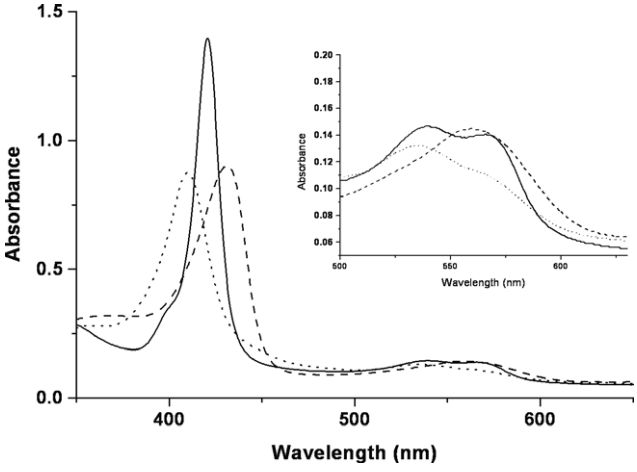


Fig. 1. Equilibrium optical absorption spectra of the HemAT-BsHD: as isolated (dotted line), deoxyHemAT-BsHD (dashed line) and COHemAT-BsHD (solid line). HemAt-BsHD concentration: ~10 μM in 50 mM sodium phosphate (pH 8.0) and 100 mM NaCl.