HemAT-Bs is a heme-containing signal transducer protein responsible for aerotaxis of *Bacillus subtilis*. The recombinant HemAT-Bs expressed in *Escherichia coli* was purified as the oxy form in which oxygen was bound to the ferrous heme. Oxygen binding and dissociation rate constants were determined to be \( k_{\text{on}} = 32 \mu M^{-1} s^{-1} \) and \( k_{\text{off}} = 23 s^{-1} \), respectively, revealing that HemAT-Bs has a moderate oxygen affinity similar to that of sperm whale myoglobin (Mb). The rate constant for autoxidation at 37 °C was 0.06 h \(^{-1}\), which is also close to that of Mb. Although the electronic absorption spectra of HemAT-Bs were similar to those of Mb, HemAT-Bs showed some unique characteristics in its resonance Raman spectra. Oxygen-bound HemAT-Bs gave the \( \nu_{\text{Fe-O}} \) band at a noticeably low frequency (560 cm \(^{-1}\)), which suggests a unique hydrogen bonding between a distal amino acid residue and the proximal atom of the bound oxygen molecule. Deoxy HemAT-Bs gave the \( \nu_{\text{Fe-His}} \) band at a higher frequency (225 cm \(^{-1}\)) than those of ordinary His-coordinated deoxy heme proteins. CO-bound HemAT-Bs gave the \( \nu_{\text{Fe-CO}} \) band at 225 cm \(^{-1}\), which fall on the same \( \nu_{\text{Fe-CO}} \) versus \( \nu_{\text{Fe-CO}} \) correlation line as that of Mb. Based on these results, the structural and functional properties of HemAT-Bs are discussed.

Motile bacteria are known to swim toward or away from specific environmental stimuli such as nutrients, oxygen, or light (1, 2). This behavior, termed chemotaxis, is mediated by a signal transduction system consisting of methyl-accepting chemotaxis proteins (MCPs) \(^3\), a histidine kinase CheA, a response regulator CheY, a coupling protein CheW, and the two enzymes that mediate sensory adaptation by covalently modifying the MCPs, CheR and CheB (3–6). Typical MCP is an integral membrane protein with an N-terminal periplasmic substrate binding domain and a C-terminal cytoplasmic signaling domain (7, 8). The binding of a substrate, a repellent, or attractant to the periplasmic substrate binding domain is believed to induce a change in the MCP conformation that allows it to activate CheA (7, 8). The activated CheA phosphorylates CheY, and, consequently, the phosphorylated CheY binds to the switch complex at the base of the flagella to control the direction of flagellar rotation (3–6). In this signal transduction system, MCP acts as a sensor for the external signal and as a signal transducer.

Hou et al. (9) have recently reported that *Bacillus subtilis* and *Halobacterium salinarum* have a signal transducer protein, HemAT-Bs and HemAT-Hs, respectively, for aerotaxis, the migratory response toward or away from oxygen. HemAT-Bs and HemAT-Hs are soluble proteins, and their C-terminal regions, residues 198–432 of HemAT-Bs, are 30% identical to the cytoplasmic signaling domain of Tsr, an MCP from *Escherichia coli* (9). Their N-terminal regions, residues 1–184 in HemAT-Hs and 1–175 in HemAT-Bs, show limited homology to myoglobin (9). Recombinant HemAT-Bs and HemAT-Hs are hemoproteins containing a b-type heme as a prosthetic group and show similar electronic absorption spectra to those of myoglobin (9). HemAT-Bs and HemAT-Hs bind oxygen reversibly as myoglobin does. The residues 1–195 for HemAT-Hs and 1–176 in HemAT-Bs retain the heme- and oxygen-binding properties of the respective native proteins, which represent a globin-coupled sensor motif (10). These results suggest that the heme in HemAT acts as an oxygen sensor and that the binding of oxygen to the heme in HemAT triggers the signal transduction for aerotaxis in *B. subtilis* and *H. salinarum*.

Sensing gas molecules such as \( \text{O}_2 \), \( \text{NO} \), and \( \text{CO} \) is a novel function of hemoproteins (11–14), whereas hemoproteins exhibit a wide variety of functions such as oxygen storage/transport, electron transfer, and redox reactions of various substrates. Recently, reports on hemoprotein sensors in which a heme prosthetic group acts as a sensor for a gas molecule such as \( \text{O}_2 \), \( \text{NO} \), or \( \text{CO} \) are on the increase. FixL (15), direct oxygen sensor (16), and phosphodiesterase A1 (17) for \( \text{O}_2 \) sensors, soluble guanylate cyclase (sGC) (18) for a \( \text{NO} \) sensor, and CooA (19–21) for a \( \text{CO} \) sensor are typical examples for hemoprotein sensors. In these sensor proteins, the binding of \( \text{O}_2 \), \( \text{NO} \), or \( \text{CO} \) to the heme regulates the function of these proteins. The hemes in these sensor proteins play a central role not only for sensing their effector molecules but also for regulating the functional properties with a conformational change induced by the ligand binding.

The heme in HemAT is thought to be the active site for sensing \( \text{O}_2 \), and the binding of \( \text{O}_2 \) to the heme will be respon-
sible for triggering the aerotaxis signal transduction. Therefore, the elucidation for the coordination structure of the heme, especially in the O$_2$-bound form and for the interaction between the bound O$_2$ and the heme pocket, are required to understand the mechanism of the O$_2$ sensing and signal transduction for HemAT. Although the electronic absorption spectra of HemAT-Hs and HemAT-Bs are reported (9), very little information is available for the structure and ligand binding properties of the heme in HemAT. In this work, we present results of the kinetic analysis for oxygen binding with HemAT-Bs, autodissociation kinetics of oxygen-bound HemAT-Bs, and resonance Raman measurements with the deoxy, oxygen, and CO-bound forms of HemAT-Bs. Using resonance Raman spectroscopy, we show a unique heme environment for HemAT-Bs.

**EXPERIMENTAL PROCEDURES**

**Expression of HemAT-Bs in E. coli**—An expression vector for HemAT-Bs was constructed as follows. The hemAT-Bs gene was prepared by polymerase chain reaction (PCR) with the chromosomal DNA of *B. subtilis* and two synthetic deoxyoligonucleotides (5′-gaagggggatgatctattcgagaaagaacggaactgctgc-3′ and 5′-gaagggcattcaggtgattctattcgagaaagaacggaactgctgc-3′ for the sense and antisense primers, respectively) as the template and primers, respectively. Although the original translational initiation codon for HemAT-Bs is TTG in *b. subtilis* (9), the translational initiation codon was changed to ATG in the sense primer, which is underlined in the above sequence. The PCR product was cloned into a pCR4-TOPO vector with the TOPO TA cloning kit (Invitrogen). The plasmid containing the hemAT-Bs gene with the correct direction relative to the lac promoter was selected as an expression vector for HemAT-Bs and named pCR-HemAT-B.

*E. coli* JM109 was used as a host for the expression of HemAT-Bs. pCR-HemAT/E. coli JM109 was grown in LB medium containing 50 µg/ml ampicillin at 37 °C. The expression of HemAT-Bs was induced by 1 mM isopropyl-β-D-thiogalactopyranoside. The cultivation was continued at 25 °C for 12 h after adding isopropyl-β-D-thiogalactopyranoside.

The cells were harvested by centrifugation and stored at −80 °C until use.

**Purification of the Recombinant HemAT-Bs**—Purification of the recombinant HemAT-Bs was carried out as follows. The cells were resuspended in 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM phenylmethylsulfonyl fluoride and disrupted by sonication. After unbroken cells and other debris were removed by centrifugation, the supernatant was applied to a column (2.6 × 30 cm) of Q-Sepharose (Amersham Biosciences, Inc.) equilibrated with 50 mM Tris-HCl applied to a column (1.6 × 10 cm) of Superdex 200 pg (Amersham Biosciences, Inc.) equilibrated with 50 mM Tris-HCl buffer containing 0.1 mM NaCl, pH 8.5. The column was run at 0.2 ml/min using 50 mM Tris-HCl buffer containing 0.1 mM NaCl, pH 8.5, as an elution buffer. 1–2 mg of purified HemAT-Bs was obtained from approximately 10 g of wet cells of pCR-HemAT/E. coli JM109.

**Characterization of HemAT-Bs**—The purification of HemAT-Bs was carried out by adding a few grains of sodium dithionite into the isolated HemAT-Bs. CO gas was introduced into HemAT-Bs solution by a gas-tight syringe.

The type of the heme in HemAT-Bs was determined by the pyridine ferrohemochrome method. The value of 34 mM$^{-1}$ cm$^{-1}$ at the absorption maximum of the a band for the pyridine ferrohemochrome derived from the protoheme was used to calculate the concentration of the heme in HemAT-Bs. Protein concentration was determined by the Coomassie Protein Assay Reagent (Pierce) or the Advanced Protein Assay Reagent (Cytoskeleton Inc.) using bovine serum albumin as a standard.

The molecular mass of HemAT-Bs was determined by gel filtration, using a Superdex 200-pg gel filtration column (1.6 × 80 cm). The system was calibrated with β-amylase (molecular weight, 200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), carbonic anhydrase (29,000), and cytochrome c (12, 400), using 50 mM Tris-HCl buffer containing 0.1 M NaCl, pH 8.5, as the eluent. These proteins used in the calibration were obtained from Sigma Chemical Co.

**Spectral Measurement**—The electronic absorption spectra were measured on a Hitachi U-3300 UV-visible spectrophotometer. Resonance Raman spectra were measured with laser excitation at 413.1 nm by a Kr$^+$ laser (Spectra Physics, model 2016). The excitation light was focused into the cell, and the laser power was 1 milliwatt (mW) at the cell for the oxygen and deoxy forms of HemAT-Bs but 0.1 mW for CO-bound HemAT-Bs so as to avoid photolysis of coordinated CO. The sample solutions for the Raman measurements were sealed in quartz cells, which were rotated at 500 rpm at room temperature. Typically, 30 µl of the protein solution containing 1.0 mg/ml HemAT-Bs was applied to a column (2.6 cm) of Q-Sepharose (Amersham Biosciences, Inc.) equilibrated with 1 M KCl, pH 8.5. The adsorbed proteins were eluted at a flow rate of 1 ml/min by increasing linearly the concentration of K$_2$HPO$_4$ in the buffer containing 0.1 M NaCl, pH 8.5, as the eluent. These proteins used in the calibration were obtained from Sigma Chemical Co.

Results—HemAT-Bs was expressed as a soluble hemoprotein in the expression system constructed in this study. A nearly homogeneous HemAT-Bs was obtained by column chromatography described under “Experimental Procedures,” as evident from SDS-polyacrylamide gel electrophoresis (Fig. 1a). The pyridine ferrohemochrome derived from HemAT-Bs gave the a band at 556 nm (data not shown), which means that HemAT-Bs contains a protoheme (b-type heme) as a prosthetic group. The molecular mass of the purified HemAT-Bs was estimated to be about 188 kDa by gel filtration (Fig. 1b), suggesting that the purified HemAT-Bs is a homo-tetramer of an identical subunit (48.7 kDa).

**Kinetic Constants for the Reaction of Ferrous HemAT-Bs with Oxygen**—The binding of oxygen to the heme is the first and crucial step of the oxygen sensing and signal transduction by HemAT-Bs. Therefore, elucidation of the oxygen binding properties is essential to understand the functional properties of HemAT-Bs. In this work, the reaction rate constants for oxygen binding and dissociation, $k_{on}$ and $k_{off}$, were determined by laser-flash photolysis and stopped-flow spectroscopy, respectively. The $k_{on}$ and $k_{off}$ values for HemAT-Bs thus determined were 32 µM$^{-1}$ s$^{-1}$ and 23 s$^{-1}$, respectively, which were similar to those of sperm whale Mb (Sw Mb) (Table I). HemAT-Bs exhibits a moderate oxygen affinity, similar to that of Sw Mb but different from another oxygen sensor protein FixL. FixL exhibits an extremely low oxygen affinity due to a very low $k_{on}$ value ($k_{on}$ = 0.14 µM$^{-1}$ s$^{-1}$), although the $k_{off}$ of FixL is comparable to that of HemAT-Bs (Table I).

The dissociation equilibrium constant ($K_d$), given by the ratio $k_{off}/k_{on}$ for HemAT-Bs was 719 nM (Table I), which is comparable to the $K_m$ values of terminal oxidases for oxygen respiration. The $K_m$ value of HemAT-Bs for O$_2$ is comparable to $K_m$ values of terminal oxidases seems reasonable from the functional point of view for HemAT-Bs. Because HemAT-Bs acts as an oxygen-sensing signal transducer in aerophilic re-
In response of *B. subtilis* (9), it should detect the most suitable oxygen concentration for the bacterium, i.e., that for oxygen respiration. If HemAT-Bs has an extremely high oxygen affinity as do *Mt* Hb, *Ascaris* Hb, and *Synechocystis* Hb (Table I), it would be disadvantageous, because a signal transduction would occur to induce chemotaxis toward a too low concentration of oxygen unsuitable for oxygen respiration. The *Kd* value of 719 nM for HemAT-Bs, a moderate oxygen affinity, reveals that HemAT-Bs can detect an oxygen concentration suitable for oxygen respiration.

**Autoxidation of Oxy HemAT-Bs**—HemAT-Bs (O₂) as isolated showed a rate constant for autoxidation of 0.06 h⁻¹ at 37 °C, which is similar to that of *Sw* Mb (Table II). FixL, another oxygen sensor protein, shows a 20- to 40-fold larger autoxidation rate constant compared with that of HemAT-Bs (29, 30). The kinetics parameters for autoxidation and oxygen binding are different between HemAT-Bs and FixL, which suggests some qualitative difference in the heme environmental structure between the two oxygen sensor proteins.

**Electronic Absorption Spectra of HemAT-Bs**—The electronic absorption spectra of HemAT-Bs are shown in Fig. 2. HemAT-Bs as isolated gave the Soret, α, and β peaks at 414, 578, and 543 nm, respectively, as shown in Fig. 2a. This spectrum is typical of six-coordinate, low spin hemoproteins and resembles that of the oxy form of Mb, as described previously (9). When CO was reacted with the isolated HemAT-Bs without any reductant, CO-bound HemAT-Bs was formed (data not shown). These results strongly suggest that HemAT-Bs is purified as the oxy form in which O₂ is bound to the ferrous heme. Resonance Raman spectroscopy revealed that this was the

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**Table I**

| Hemoproteinsa | *k*ₐₙ | *k*ₜₐₜ | *Kd* | References |
|--------------|--------|--------|------|------------|
| HemAT-Bs     | 32²    | 23³    | 719  | This work  |
| *Pc* Hb      | 30.1   | 25.2   | 838  | 23         |
| *Sw* Mb      | 17     | 15     | 882  | 24         |
| human Hb α-chain (R-state) | 23 | 11 | 478 | 25 |
| human Hb β-chain (R-state) | 79 | 28 | 354 | 25 |
| *Mt* Hb      | 25     | 0.199  | 7.96 | 26         |
| *Ascaris* Hb | 1.2    | 0.23   | 192  | 27         |
| *Leg-Hb*     | 130    | 5.6    | 43   | 24         |
| *Synechocystis* Hb | 240 | 0.014 | 0.06 | 28         |
| *Bj* FixL    | 0.14   | 0.20   | 140 × 10³ | 29 |

² Abbreviations: *Sw* Mb, sperm whale myoglobin; Hb, hemoglobin; *Mt* Hb, *M. tuberculosis* hemoglobin; *Ascaris* Hb, *Ascaris lumbricoides* hemoglobin from body-wall; *Leg-Hb*, Soybean leghemoglobin; *Synechocystis* Hb, *Synechocystis* PCC6803 hemoglobin; *Pc* Hb, *P. caudatum* hemoglobin; *Bj* FixL, *Bradyrhizobium japonicum* FixL.

³ This value was calculated by the reported value of *t*₁/₂ = 537 h.

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**Table II**

| Hemoproteinsa | *k*ₐₙ | References |
|--------------|--------|------------|
| HemAT-Bs     | 0.06   | This work  |
| *Sw* Mb      | 0.06   | 31         |
| *Mt* Hb      | 0.0013 | 26         |
| *Leg-Hb*     | 0.198  | 26         |
| *Rm* FixL    | 1.3    | 30         |
| *Bj* FixL    | 2.7    | 29         |

² Abbreviations: *Sw* Mb, sperm whale myoglobin; *Mt* Hb, *M. tuberculosis* hemoglobin; *Leg-Hb*, Soybean leghemoglobin; *Rm* FixL, *Rhizobium meliloti* FixL; *Bj* FixL, *Bradyrhizobium japonicum* FixL.

³ These values are determined at 37 °C in air-saturated buffered solution.

⁴ This value was calculated by the reported value of *t*₁/₂ = 537 h.
case, as described below. Upon deoxygenation with sodium dithionite, HemAT-Bs showed a spectrum with the Soret peak at 431 nm and a single peak at 563 nm in the Q-band region, as shown in Fig. 2b. This spectrum is typical of five-coordinate, high spin ferrous hemoproteins, which show the formation of deoxy HemAT-Bs. CO-bound HemAT-Bs was formed upon the reaction of dithionite reduced HemAT-Bs with CO, which showed the Soret, α, and β peaks at 422, 567, and 543 nm, respectively, as shown in Fig. 2c. These electronic absorption spectra of HemAT-Bs were similar to those of Mb as reported previously (9), which is consistent with the fact that the N-terminal region of HemAT-Bs shows an amino acid sequence homology to Mb (9). The values of the absorption maxima observed in this study are slightly different from those reported in Ref. 9. Hou et al. have noted from their absorption spectra the presence of a significant population of deoxy species for both the CO- and O₂-bound forms of HemAT-Bs (9). In our preparation of HemAT-Bs, however, such an incomplete ligand binding was not noticed, which was confirmed by resonance Raman spectroscopy as described below. These differences may cause the difference of the absorption maxima, although the reasons of the difference are not clear at present.

The heme in HemAT-Bs is thought to play an important role for sensing O₂. The ligand conformation, the interaction between the bound O₂ and the amino acid residue(s) in the heme pocket, and/or the heme environmental structure will be responsible for triggering the signal transduction upon the O₂ binding to the heme in HemAT-Bs. To characterize these properties, we measured the resonance Raman spectra of HemAT-Bs in the oxy, deoxy, and CO-bound forms.

**Resonance Raman Spectra of Oxy HemAT-Bs**—The resonance Raman spectrum in the high frequency region (1300–1700 cm⁻¹) of HemAT-Bs(O₂) is shown in Fig. 3a. In Table III, the observed frequencies of the major bands are compared with those of Mb (32, 33), heme-bound heme oxygenase (32, 33), and gas-sensing hemoproteins such as the O₂, CO, and NO sensors of FixL (34–37), CooA (38–40), and sGC (41–43), respectively. It is established that resonance Raman spectra in the high frequency region contain a few marker bands sensitive to the oxidation state (ν₁) and the spin and coordination states (ν₂ and ν₃) of the heme iron (44). HemAT-Bs(O₂) gave two bands at 1471 and 1501 cm⁻¹ in the ν₂ band region, indicative of a mixture of five- and six-coordinate heme species. The relative intensities of these bands depended on the laser power, i.e. the

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**Fig. 2.** Electronic absorption spectra of oxy (a), deoxy (b), and CO-bound HemAT-Bs (c). HemAT-Bs was dissolved in 50 mM Tris-HCl buffer, pH 8.5. HemAT-Bs as isolated was in the oxy form. Deoxy HemAT-Bs was prepared by adding sodium dithionite into oxy HemAT-Bs solution. CO was introduced into deoxy HemAT-Bs solution to prepare CO-bound HemAT-Bs. The molar extinction coefficients for the heme in HemAT-Bs are shown in this figure.
The intensity of the band at 1471 cm$^{-1}$ due to a five-coordinate heme increased as the laser power increased. These results show that the bound O$_2$ in HemAT-Bs(O$_2$) was photodissociated to form the five-coordinate form at the higher laser power. Therefore, the band at 1501 cm$^{-1}$ was assigned to the $v_5$ band of HemAT-Bs(O$_2$). The $v_2$ band of HemAT-Bs(O$_2$) was observed at 1578 cm$^{-1}$.

The $v_2$ and $v_3$ modes of HemAT-Bs(O$_2$) observed at 1578 and 1501 cm$^{-1}$, respectively, are slightly lower than the corresponding ones of Mb(O$_2$) ($v_2 = 1584$ cm$^{-1}$ and $v_3 = 1507$ cm$^{-1}$) (33) but coincide with the corresponding ones of FixL*(O$_2$) ($v_2 = 1577$ cm$^{-1}$ and $v_3 = 1502$ cm$^{-1}$) (34). Because the $v_2$ and $v_3$ frequencies are linearly correlated with the Ct-N distance (distance between the center and a nitrogen atom of a porphyrin ring) (45, 46), the lower frequencies of the $v_2$ and $v_3$ modes in HemAT-Bs(O$_2$) may be indicative of an expanded porphyrin core compared with Mb(O$_2$), which is also suggested for FixL*(O$_2$) (34).

The resonance Raman spectra of HemAT-Bs(O$_2$) in the low frequency region are shown in Fig. 4. The spectra of the 16O$_2$- and 18O$_2$-bound forms of HemAT-Bs are shown in the top and middle, respectively, and the 16O$_2$–18O$_2$ difference spectrum is shown in the bottom. A large isotope shift was observed for the band at 560 cm$^{-1}$ where the derivative pattern of the difference spectrum was observed. Therefore, this band is assigned to the Fe–O$_2$ stretching mode ($v_{Fe-O_2}$). However, the peak-to-valley frequency difference in Fig. 4c is as large as 26 cm$^{-1}$, which is slightly larger than the frequency shift expected for an Fe–O$_2$ diatomic oscillator (23 cm$^{-1}$). The 561 cm$^{-1}$ band in Fig. 4a is broader than those of other bands, implying inhomogeneous broadening. Accordingly, a too large isotopic frequency shift might be caused by the existence of more than two bands and the difference of their intensity distributions between 16O$_2$ and 18O$_2$ derivatives. Although this should be clarified in later studies, it is no doubt that the 560 cm$^{-1}$ band primarily involves the $v_{Fe-O_2}$ character.

The ligand modes of various hemoproteins are listed in Table IV. The ligand modes get resonance Raman intensity from the electronic coupling of the ligand orbital to the metal and porphyrin orbitals. In particular, the assignment of a ligand-Fe stretching mode is useful, because it directly re-
The isotope shifts of 3, 19, and 46 cm\(^{-1}\) in the high frequency region is shown in Fig. 3 resonance Raman spectrum of CO-bound HemAT-Bs, HemAT-stretching mode of deoxy HemAT-Bs. This frequency is higher in the region of 200–250 cm\(^{-1}\) for the five-coordinate ferrous species (52). We thus assign the 225 cm\(^{-1}\) band to the Fe-His stretching mode of deoxy HemAT-Bs. This frequency is higher than that of deoxy Mb, and it will be discussed later.

**Resonance Raman Spectra of Deoxy HemAT-Bs**—The high frequency resonance Raman spectrum of deoxy HemAT-Bs is shown in Fig. 3b. Deoxy HemAT-Bs showed the \(v_2\), \(v_3\), and \(v_4\) modes at 1558, 1469, and 1352 cm\(^{-1}\), respectively, at typical frequencies of the deoxy form of five-coordinate heme species in the high spin state.

The resonance Raman spectrum of deoxy HemAT-Bs in the low frequency region is shown in Fig. 5a. The intense line at 225 cm\(^{-1}\), which was undetectable with the O\(_2\)- and CO-bound forms, was observed for deoxy HemAT-Bs. In general, the Fe-His stretching Raman band of hemoproteins is observable in the region of 200–250 cm\(^{-1}\) for the five-coordinate ferrous species (52). We thus assign the 225 cm\(^{-1}\) band to the Fe-His stretching mode of deoxy HemAT-Bs. This frequency is higher than that of deoxy Mb, and it will be discussed later.

**Resonance Raman Spectra of CO-bound HemAT-Bs**—The resonance Raman spectrum of CO-bound HemAT-Bs, HemAT-Bs(CO), in the high frequency region is shown in Fig. 3c. HemAT-Bs(CO) was photolabile and showed two \(v_1\) bands at 1468 and 1495 cm\(^{-1}\), corresponding to a five-coordinate, high spin and a six-coordinate, low spin heme species, respectively. The former species is thought to be formed by photodissociation of CO, whereas the latter species is thought to be formed by photodissociation of CO from the Fe-His orbital of porphyrin to the \(\pi^*\) orbital of the bound ligand through the \(d_{\sigma}\) orbital of Fe, the electron delocalization is larger for O\(_2\) than for CO.

The resonance Raman spectra of HemAT-Bs(CO) in the low frequency region are shown in Fig. 5 (a and b). The 225 cm\(^{-1}\) band was also observed in HemAT-Bs(CO) under increased laser power conditions, as shown in Fig. 5b. This suggests that photodissociation of CO from HemAT-Bs(CO) generates a five-coordinate ferrous HemAT-Bs, whereby the \(v_{Fe-Co}\) band appears at 225 cm\(^{-1}\).

The resonance Raman spectra of the HemAT-Bs(CO) with \(^{13}\text{C}\)O and \(^{12}\text{C}\)O in the low frequency (300–700 cm\(^{-1}\)) and high frequency (1800–2100 cm\(^{-1}\)) regions are shown in Fig. 6. The isotope shifts of 3, 19, and 46 cm\(^{-1}\) were observed for the bands at 494, 570, and 1964 cm\(^{-1}\), respectively, upon substitution of \(^{13}\text{C}\)O for \(^{12}\text{C}\)O. Based on the frequencies of these bands and sizes of the isotope shifts, we assign the 494, 570, and 1964 cm\(^{-1}\) bands to the Fe-CO stretching \((v_{Fe-CO})\), the Fe–O bending, and the C–O stretching \((v_{C-O})\) modes, respectively.

It is established that the \(v_{Fe-CO}\) and \(v_{C-O}\) frequencies display an inverse linear correlation (53, 54) because of the \(\pi\)-electron back donation mentioned above. This \(\pi\)-back donation results in strengthening of the Fe–C bond and concomitant weakening of the C–O bond (or vice versa), leading to the empirical \(v_{Fe-CO}\) versus \(v_{C-O}\) linear inverse line (53, 54). The \(v_{Fe-CO}\) versus \(v_{C-O}\) correlation depends on the nature of the proximal ligand; the CO-bound hemes with an imidazole/histidine as the trans ligand exhibit a correlation line different from those with a thiolate or imidazolate. The \(v_{Fe-CO}\) and \(v_{C-O}\) bands of HemAT-Bs(CO), detected at 494 cm\(^{-1}\) and 1964 cm\(^{-1}\), respectively, fell on the imidazole/histidine correlation curve as illustrated in Fig. 7. This fact means that proximal His is neutral like Mb,
and the high frequency of the Fe-His stretching frequency cannot be ascribed to deprotonation of proximal His. The $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ frequencies suggest that the environment around the oxygen atom of bound CO is less hydrophilic than that of native Mb like that in the open form of Mb. The negligibly weak enhancement of the Fe–C–O bending band observed for HemAT-Bs(CO), as shown in Fig. 6, suggests an undistorted Fe–C–O geometry (54).

**DISCUSSION**

**Structural Characteristics of Heme Pocket of HemAT-Bs**

The $\nu_{\text{Fe-O}_2}$ frequency of HemAT-Bs(O$_2$) in the $^{16}$O$_2$ spectrum
(Fig. 4a, 560 cm⁻¹) is one of the lowest Fe–O₂ stretching frequencies among O₂-bound hemoproteins having a histidine as an axial ligand (33, 34, 47, 55–61). Recently, Yeh et al. (47) have reported a low Fe–O₂ stretching frequency (560 cm⁻¹) for hemoglobin from Mycobacterium tuberculosis (Mt Hb). In conjunction with the mutagenesis studies, they have proposed a unique hydrogen bonding pattern between the bound oxygen and the distal tyrosine at the B10 position (the tenth residue in the B helix) through the sp² orbital of the proximal oxygen atom and have pointed out that this unique hydrogen bonding plays a crucial role in the low frequency of the νₚₐₐ₉⁻O₂ mode for Mt Hb (47). Considering the low frequency of the νₚₐₐ₉⁻O₂ mode of HemAT-Bs(O₂) comparable to that of Mt Hb, such a unique hydrogen bonding as seen for Mt Hb would also be involved between one or more distal amino acid residues and the proximal oxygen atom of the bound O₂ in HemAT-Bs(O₂). Specification of the hydrogen bonding counterpart residue is under progress in this laboratory.

The important determinants of the νₚₐₐ₉⁻His frequency is the hydrogen bonding status of the His N° atom (52), the strain imposed on the axial His by the protein moiety (62), and geometry of bound imidazole (63). In practice, a high frequency shift as large as 25 cm⁻¹ was reported to be caused by deprotonation of bound imidazole in imidazole-coordinated high spin Fe(II) porphyrin (64). In cytochrome c peroxidase, the strong hydrogen bond between the axial His and Asp⁶⁵ enhances the anionic character of the imidazole ring of the axial His, resulting in the higher νₚₐₐ₉⁻His frequency (245 cm⁻¹) (65). When this hydrogen bond is disrupted by the Asp⁶⁵ → Asn mutation, the νₚₐₐ₉⁻His band is shifted to a lower frequency (205 cm⁻¹) (66, 67). Deoxy Mb, which has a weak hydrogen bond on the axial His, gives the νₚₐₐ₉⁻His mode around 220 cm⁻¹ (48). Thus, deoxy HemAT-Bs may possibly have a stronger hydrogen bond compared with deoxy Mb.

The effect of strain from the protein on the νₚₐₐ₉⁻His frequency is most clearly seen for deoxy Hb, which gives different νₚₐₐ₉⁻His frequencies between the T νₚₐₐ₉⁻His = 215 cm⁻¹) and R νₚₐₐ₉⁻His = 221 cm⁻¹) structures (62). The low νₚₐₐ₉⁻His frequency is a consequence of the strain in the T quaternary structure, and the magnitude of strain is directly related to oxygen affinity (68). In the absence of strain, the νₚₐₐ₉⁻His frequency is close to that of deoxy Mb. The high νₚₐₐ₉⁻His frequency is also observed for a cavity mutant of Mb (226 cm⁻¹), in which the axial His is replaced with Gly in the presence of exogenous imidazole (69). In this case, an exogenous imidazole acts as the proximal ligand, and the strain on the coordinated imidazole should be absent. Such a cavity mutant was also prepared for sGC (70), heme-bound hemoxygenase (71), and CoOAS (39), which show similar phenomena. Accordingly, the higher vibrational frequency of the νₚₐₐ₉⁻His mode in deoxy HemAT-Bs is indicative of less strain being imposed on the Fe–His bond compared with other hemoproteins listed in Table IV except for Mt Hb.

Desbois and coworkers (63) investigated the geometry dependence of νₚₐₐ₉⁻His frequencies and found that νₚₐₐ₉⁻His frequencies fall on a straight line with the inclination of −0.5 cm⁻¹/deg when they are plotted against dihedral angles (ϕ) formed by the imidazole plane and the nearest N(pyrrole)–Fe–N(pyrrole) axis. This correlation is different between the imidazolate and imidazole groups, although both give higher frequency for smaller ϕ values. On the basis of this correlation, Desbois and co-workers (63) interpreted the high νₚₐₐ₉⁻His frequency (228–231 cm⁻¹) of cytochrome c’ in terms of a large ϕ angle for strongly hydrogen-bonded imidazole coordination.

Recently, Andrew et al. (72) observed a relatively high νₚₐₐ₉⁻His frequency (231 cm⁻¹) for Alcaligenes xylosoxidans cytochrome c’. According to the x-ray structure of this protein in the oxidized form, the ϕ angle is 33°, and accordingly, the high νₚₐₐ₉⁻His frequency seemed to be due to deprotonation of proximal His. However, the x-ray structure for the oxidized form did not exhibit a hydrogen bonding counterpart of proximal His. Furthermore, the νₚₐₐ₉⁻CO versus νₚₐ₉⁻CO correlation of this protein indicated the coordination of neutral imidazole at the trans position of CO. Therefore, Andrew et al. assumed that the structure in the proximity of heme changes with the oxidation state of iron as well as upon ligand binding. Although the x-ray crystallographic structure is not available for HemAT, its νₚₐₐ₉⁻His frequency is too high to interpret it in terms of a neutral imidazole coordination even for ϕ = 0°, but its νₚₐₐ₉⁻CO versus νₚₐ₉⁻CO correlation suggests the coordination of a neutral imidazole. Thus, the same kind of dilemma as seen for A. xylosoxidans cytochrome c’ is present. Also in the case of HemAT-Bs, a structural change would take place in the proximal side of the heme upon the ligand binding, as is suggested for A. xylosoxidans cytochrome c’. Such a conformational change in the proximal heme pocket might be concerned with signal transduction in HemAT-Bs.

### TABLE V

| Proteins          | υₚₐ₉⁻CO | υₚₐ₉⁻Cₕ⁻Cₕ,₁₂ | υₚₐ₉⁻Cₕ⁻Cₕ,₁₄ | υₚ₉⁻Cₕ⁻Cₙₙ,₂ | References |
|-------------------|---------|---------------|---------------|--------------|------------|
| HemAT-Bs(O₂)      | 344     | 375           | 407           | 428          | This work  |
| HemAT-Bs(CO)      | 355     | 375           | 413           | 428          | This work  |
| Δυₚₐ₉⁻Hₙₙ       | 11      | 0             | 6             | 0            |            |
| Mb(O₂)            | 344     | 377           | 410           | 438          | 59         |
| Mb(CO)            | 346     | 378           | 410           | 436          | 76         |
| Δυₚ₉⁻Hₙₙ       | 2       | 2             | 0             | -2           |            |
| Δυₚₐ₉⁻Hₙₙ (Mb)   |         |               |               |              |            |

a. δ(Cₕ,Cₕ,₁₂), porphyrin-propionate bending mode; δ(Cₕ,Cₕ,₁₄) and δ(Cₕ,Cₙₙ,₂), the bending mode of 4-vinyl and 2-vinyl group, respectively.

b. Δυₚₐ₉⁻Hₙₙ = 1(HemAT-Bs(CO)–HemAT-Bs(O₂)).

c. Horse myoglobin.

d. Δυₚ₉⁻Hₙₙ = 1(Mb(CO)–Mb(O₂)).

Relation between Structure and Kinetic Characteristics—Resonance Raman spectroscopy of HemAT-Bs(O₂) has revealed that a distal amino acid residue is hydrogen bonding to the...
proximal atom of bound oxygen molecule as described above. Although MtHb (46) and Ascaris Hb (77) have a hydrogen bonding network similar to that of HemAT-Bs, they have an oxygen affinity very different from that of HemAT-Bs. The difference in oxygen affinity is mainly due to very small $k_{\text{off}}$ values for MtHb and Ascaris Hb compared with that for HemAT-Bs (Table I), whereas all have similar $k_{\text{on}}$ values except for Ascaris Hb. Indeed, the $k_{\text{off}}$ value for HemAT-Bs (O$_2$) is greater than those for MtHb and Ascaris Hb by 100- and 5000-fold, respectively. We note here that $k_{\text{off}}$ depends on the free energy of the whole protein in the oxygenated form relative to that of the transient state. When the strong hydrogen bond between the bound oxygen and distal residues causes some strain in other parts of the protein, the stabilization of the oxygenated form by the strong hydrogen bonds would be partially canceled. Such a feature is really seen for deoxy Hb with T structure, in which the inter-subunit hydrogen bonds are stronger and free energy is lower in the T than R state, but the Fe–His bond is weaker in the T than R state due to the strain imposed on the proximal His by the protein. In other words, the globin moiety is stabilized whereas the heme group is destabilized by the hydrogen bonds between subunits. The binding of a ligand to one heme of Hb is conveyed to another heme via changes in the inter-subunit hydrogen bonds. Accordingly, it is highly likely that HemAT-Bs utilizes a similar character of protein for signal transduction and thus to work as an O$_2$ sensor.

It is interesting to note that Paramecium caudatum (Pc) Hb exhibits similar kinetics and equilibrium parameters for oxygen binding to those of HemAT-Bs (23). Although Pc Hb possesses a distal E7 glutamine and a B10 tyrosine that form a hydrogen bond to the proximal and terminal oxygen atoms, respectively (23), it gives a $k_{\text{off}}$ value (25.2 s$^{-1}$) larger than those for Mt Hb and Ascaris Hb but close to that of HemAT-Bs ($k_{\text{off}} = 23$ s$^{-1}$). The $\nu_{\text{Fe-O}_2}$ mode of Pc Hb appears at 563 cm$^{-1}$.
which is similar to those of HemAT-Bs and Mt Hb (Table IV). Das et al. (23) have proposed that the oxy complex of Pc Hb is an equilibrium mixture of a hydrogen-bonded closed structure and non-hydrogen bonded open structure and that oxygen will dissociate preferentially from the open structure. Because the Fe–O₂ stretching Raman band of HemAT-Bs(O₂) is broad and likely to be composed of multiple bands as mentioned above, we cannot rule out the possibility that there are two oxygenated forms in HemAT-Bs(O₂), that is, hydrogen-bonded and non-hydrogen-bonded forms, and that oxygen is dissociated from the latter.

HemAT-Bs(O₂) shows an autoxidation rate significantly slower than those of LegHb and FixL as shown in Table II. The autoxidation rate of HemAT-BS(O₂) is close to that of Mt Hb. It is well known that the bound O₂ of Mt Hb is stabilized by a hydrogen bond from the distal His, and accordingly, the bound O₂ of HemAT-Bs would be stabilized to a similar extent. An extremely small autoxidation rate constant for Mt Hb is probably caused by hydrogen bonding to the proximal oxygen atom within a closed distal heme pocket (26). The increased autoxidation rate of HemAT-Bs compared with Mt Hb, despite the strong hydrogen bonding to the proximal oxygen atom similar to that in Mt Hb, might be due to the presence of the second conformer with a relatively open distal heme pocket.

Primary Structure of HemAT-Bs—It has been reported that the N-terminal region of HemAT-Bs and HemAT-Hs exhibit an amino acid sequence homology to Mb (9). Alignment of the heme binding domain of HemAT-Bs (10) with some globin sequences is shown in Fig. 8. Among these proteins, the proximal histidine at the F8 position is conserved, but amino acid residues at the B10 and E7 positions are not. It has been recently confirmed that His²¹² corresponding to a histidine at the F8 position does indeed act as the proximal ligand of the heme in HemAT-Bs (10). A tyrosine at the B10 position in Mt Hb and Pc Hb hydrogen bonds to the bound oxygen (23, 47). In HemAT-Bs, however, the residue at the B10 position would be a leucine, an amino acid not capable of hydrogen bonding, as is the case for Sw Mt Hb. The distal residue at the E7 position in Sw Mt Hb and Pc Hb, His⁶⁴ and Gin⁴², respectively, also hydrogen bonds to the bound oxygen (23, 78). In HemAT-Bs, the residue at the E7 position would be a serine, Ser⁸⁷. Although a serine is a possible candidate for an amino acid residue hydrogen bonding to the bound oxygen, there is not any direct evidence at present that the Ser⁸⁷ is involved in the hydrogen bonding network in HemAT-Bs(O₂).

In a preliminary resonance Raman study of S87A HemAT-Bs, in which Ser⁸⁷ is replaced by Ala, the ν₁₆₆₉–₁₆₆₈ mode was observed at 563 cm⁻¹. In the case of Chlamydomonas eugametos Hb, the mutation of the B10 Tyr or E7 Gln, both of which hydrogen bond to the bound oxygen, results in the up-shift of the ν₁₆₆₉–₁₆₆₈ mode by 7 or 15 cm⁻¹, respectively (79). Compared with the case for C. eugametos Hb, the ν₁₆₆₉–₁₆₆₈ mode is not perturbed as much by the mutation of Ser⁸⁷ to Ala in the case of HemAT-Bs. Thus we need additional experiments to find out if Ser⁸⁷ hydrogen bonds to the bound oxygen and to determine the distal amino acid residue that hydrogen bonds to the bound oxygen.

Oxygen Sensing and Signal Transduction Mechanisms for HemAT-Bs—A conformational change induced by the ligand binding to the heme is a crucial step for the effector sensing and signal transduction in the heme-based sensor proteins. For the CO sensor protein CooA, CO replaces one of the endogenous axial ligands, Pro², upon the binding to the six-coordinated ferrous heme, which results in a conformational change in the N-terminal region and/or in a relocation of the heme with a conformational change of the proximal heme pocket (39, 80, 81). The ligand exchange between Pro² and CO is the first step of the CO sensing in the case of CooA. This sensing mechanism would be specific for CooA among CooA, FixL, and HemAT-Bs, because only the heme in CooA is six-coordinate in the resting state (20, 80). FixL and HemAT-Bs contain a five-coordinate heme in their resting state, and oxygen binds to a vacant distal site of the heme.

In the case of FixL, the sensing mechanism is completely different from that for CooA. Gong et al. (82) have reported that oxygen binding to the heme in FixL induces rearrangements of the hydrogen bonding network between the heme 6- and 7-protoporphyrin and amino acid residues (Arg²⁰⁶ and His²¹⁴) in the F/G loop region that results in the flattening of the heme plane upon the ligand binding. They propose that the signal transduction might be mainly driven by the motion of the heme plane induced by oxygen binding (82). On the other hand, Mukai et al. (83) have proposed that the interaction between IL₃⁹⁰ (and/or IL₂¹⁰), which are located on the F/G loop region, and the iron-bound oxygen is essential for oxygen sensing by FixL.

Although both of FixL and HemAT-Bs are oxygen sensor proteins, they show no structural homology. HemAT-Bs is a member of globin-coupled sensor proteins (10), whereas FixL is a member of the PAS domain superfamily (84). Furthermore, oxygen binding and autoxidation kinetics are completely different between FixL and HemAT-Bs, as described in this work. These results suggest that the oxygen-sensing mechanism for HemAT-Bs will be different from that for FixL.

Oxygen binding to the heme iron in Hb results in a movement of the iron ion into the heme plane along with the movement of the proximal histidine and F helix, which is a trigger of the allosteric control for Hb. As described in this work, HemAT-Bs shows amino acid sequence homology and similar spectroscopic and ligand binding properties to those of Mb and Hbs. These results suggest that a similar conformational change, i.e., a movement of the heme iron with the proximal ligand and F helix, upon the oxygen binding could be a trigger of the signal transduction for HemAT-Bs. Given that this is the case, however, CO should have the same effect as O₂. Because CO is not a physiological effector of HemAT-Bs, HemAT-Bs should discriminate between O₂ and CO. Hydrogen bonding to the bound oxygen is considered to play a crucial role for the ligand discrimination as discussed above, but further studies are required to determine if this is the case.

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