Protein Conformation Changes of HemAT-Bs upon Ligand Binding Probed by Ultraviolet Resonance Raman Spectroscopy

Samir F. EI-Mashtoly,‡ Yuzong Gu, Hideaki Yoshimura, Shiro Yoshioka, Shigetoshi Aono, and Teizo Kitagawa§,*

From Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki, Aichi 444-8787, Japan

‡Present address; Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York, 10461
§Present address; Toyota Physical & Chemical Research Institute, Nagakute, Aichi 480-1192, Japan
*Address correspondence to Teizo Kitagawa: Toyota Physical and Chemical Research Institute, Tel: +81-80-1620-8159; Fax: +81-561-63-6302; E-mail: teizo@ims.ac.jp

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HemAT from *Bacillus subtilis* (HemAT-Bs) is a heme-based O2 sensor protein that acts as a signal transducer responsible for aerotaxis. HemAT-Bs discriminates its physiological effector (O2) from other gas molecules (CO and NO), although all of them bind to a heme. To monitor the conformational changes in the protein moiety upon binding of different ligands we have investigated ultraviolet resonance Raman (UVRR) spectra of the ligand-free, and O2-, CO- and NO-bound forms of full-length HemAT-Bs and several mutants (Tyr70Phe, His86Ala, Thr95Ala, and, Tyr133Phe), and found that Tyr70 in the heme distal side, and Tyr133 and Trp132 from G-helix in the heme proximal side undergo environmental changes upon ligand binding. In addition, the UVRR results confirmed our previous model, which suggested that Thr95 forms a hydrogen bond with heme-bound O2 but Tyr70 does not. It is deduced from this study that hydrogen bonds between Thr95 and heme bound-O2 and between His86 and heme 6-propionate communicate the heme structural changes to the protein moiety upon O2-binding but not upon CO- and NO binding. Accordingly, the present UVRR results suggest that O2 binding to heme causes displacement of G-helix, which would be important for transduction of the conformational changes from the sensor domain to the signaling domain.

Recently, a variety of heme-containing gas sensor proteins have been discovered through gene analysis of various organisms from bacteria to mammals (1-6). The O2-sensing proteins identified so far include FixL (7), phosphodiesterase A1 from *Acetobacter xylulnium* (Ax PDEA1) (8), *E. coli* direct oxygen sensor (Ec DOS) (9), and HemAT (10). FixL, Ax PDEA1, and Ec DOS belong to the PAS family, and containing heme-bound PAS domain as a sensor, but HemAT is globin-like, belonging to the methyl-accepting chemotaxis (MCP) family. The effector domain of FixL serves as a protein kinase, which regulates the expression of nitrogen fixation gene by phosphorylating the FixJ protein (7,11,12). Ax PDEA1 catalyzes the hydrolysis of 3',5'-cyclic di-guanyllic acid (c-di-GMP), which is required for the activation of cellulose synthase in cellulose-producing bacteria (8). Ec DOS also exhibits significant phosphodiesterase activity towards c-di-GMP (13).

HemAT is a heme-based signal transducer protein responsible for bacterial aerotaxis (10,14-17). HemAT consists of two domains, sensor and signaling domains, connected by a linker region. The sensor domain has globin folds and contains a heme that acts as the O2 binding site, while the signaling domain interacts with a histidine kinase protein (CheA), a component of the CheA/CheY two-component signal-transduction system that regulates the rotation direction of the flagellar motor (18-20). The X-ray crystallographic analysis of HemAT has been completed only for the reduced ligand-free and oxidized CN-bound forms of the truncated sensor domain, which stays as a homo-dimer (21).

Figure 1A displays the crystallographic structure of one subunit of the CN-bound form of truncated sensor domain of *Bacillus subtilis* HemAT (HemAT-Bs). The CN ion and His123 are the heme axial ligands in the distal and proximal sides, respectively. The CN ligand
forms a hydrogen bond with Tyr70 (21). The truncated sensor domain of HemAT- Bs contains one Trp and five Tyr residues; Trp132 and Tyr133 are contained in the G-helix in the proximal side of heme, but Tyr70 is present in the distal side, while Tyr13, Tyr49, and Tyr148 residues are located far from the heme (Figure 1A). These aromatic residues will be used as probes to monitor the protein conformational changes upon ligand binding in this ultraviolet resonance Raman (UVRR) study.

One of the important issues concerning the structural biology of gas sensor proteins is to clarify structural changes of the heme and the nearby residues (Tyr70, His86 and Thr95 in Figure 1A) upon binding of the signaling molecule. The structural changes in the active site upon binding of different ligands have been studied with different techniques (15,16,22-24). For instance, by using visible-excited resonance Raman (RR) spectroscopy in combination with site-directed mutagenesis, it is proposed that His86 forms a hydrogen bond with a heme 6-propionate upon binding of O2 to the heme (23). The formation of this hydrogen bond induces conformational changes of the protein by which Thr95 is moved to a position suitable to form a hydrogen bond with the heme-coordinated O2, while Tyr70 does not form a hydrogen bond with the heme-bound O2 (22,23). In contrast, Zhang et al. reported that mutation of Tyr70 (Y70F, Y70L, and Y70W) of the truncated sensor domain of HemAT- Bs brought about larger dissociation constants for O2 than the wild type (WT), and thus proposed that Tyr70 would form a hydrogen bond to the heme-bound O2 in the WT form (16). These contradictory results demand us to probe directly the environmental change of Tyr70 and Tyr133. Furthermore, UVRR spectroscopy will provide some information on the G-helix motion through side-chain vibrations of Trp and Tyr residues, because HemAT- Bs contains Trp132 as well as Tyr133 in the G-helix. We have examined the effect of removal of the hydrogen bond between His86 and heme 6-propionate and that between Thr95 and heme-bound O2 on the protein conformational changes. As discussed below, the UVRR data will reveal specific features regarding the protein structural changes upon binding of different ligands.

Another important issue is concerned with the structural changes in the protein moiety of G-helix. The recent time-resolved resonance Raman (TR3) study suggested that Tyr133 in G-helix (Figure 1A) forms a hydrogen bond with the proximal axial ligand (His123) upon CO-binding (25). This is based on the 2 cm-1 downshift of the Fe-histidine stretching (νFe-His) band in TR3 spectra of WT at hundreds of picoseconds after photodissociation of CO, whereas such a frequency shift was not observed for Y133F mutant. Furthermore, G and H helices of different subunits of homo-dimer form an antiparallel four-helical bundles (Figure 1B), and exhibit appreciable displacement upon CN-binding (21). Although this displacement is small, it is perceptible, and may be related to the signal transduction (21). On the other hand, it is unknown whether or not such conformational changes occur upon binding of the effector molecule, O2.

To monitor the conformational changes in the protein moiety upon binding of O2, CO and NO to a heme, we have applied UVRR spectroscopy to WT and several mutants (Y70F, H86A, T95A, and Y133F) of the full-length HemAT- Bs for the first time. The vibrational spectra of the aromatic side chains such as Tyr and Trp residues can be selectively obtained by choosing an appropriate excitation wavelength in the 220-250 nm region and provide structural information about their conformations, local environments and hydrogen bonding interactions (26,27). Such kinds of UVRR-specific information have been proved to be essential to understand the structural mechanisms of a variety of heme proteins including hemoglobin (28), myoglobin (29), CooA (30), and Ec DOS (31). Thus, UVRR spectra of HemAT- Bs are expected to probe directly the structural and/or environmental changes of Tyr70 and Tyr133.

EXPERIMENTAL PROCEDURE

Sample Preparation—In this study, we used the full-length HemAT- Bs with His6-tag at the C-terminus, which was expressed with E. coli BL21(DE3) cell system under the control of T7 promoter in pET-24(+) vector (Novagen). Site-directed mutagenesis was carried out using QuikChange Site-Directed Mutagenesis Kit (Stratagene). For the expression of HemAT- Bs, the E. coli cells were grown aerobically at 37°C for 4 hours in Terrific Broth containing 30 μg/mL kanamycin. The expression was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final
concentration of 1 mM, and then the cultivation was continued at 22°C for 18 hours. The cells were harvested by centrifugation at 4,000 g and were stored at –80°C until the use.

The cells were thawed and resuspended in the buffer A (50 mM Tris-HCl buffer (pH 8.0) containing 15 mM glycine and 1 M NaCl), and then were broken by sonication. The resulting suspension was centrifuged at 100,000 g for 20 minutes, and the supernatant was loaded on a Ni²⁺-charged HiTrap Chelating column (Amersham Biosciences Corp.). After washing the column with the buffer A, and then with 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl, and then HemAT-Bs was eluted by increasing the concentration of NaCl in the buffer.

For Raman measurements the concentration of protein was adjusted to 80 μM in 50 mM Tris-HCl buffer (pH 7.5). As an internal intensity standard to generate UVRR difference spectra, 400 mM sodium perchlorate (NaClO₄) was contained in the solution for the excitation at 229 nm. In addition, Trp Raman bands were used as a secondary internal standard to normalize the UVRR spectra of different Tyr-mutants.

The oxidized HemAT-Bs was prepared by adding an excess amount of potassium ferricyanide to the purified protein and afterwards potassium ferricyanide was removed by gel filtration with Sephadex G25. Five-times excess of sodium dithionite was added into degassed oxidized HemAT-Bs solution to reduce it. To prepare CO-, NO-, and O₂-bound HemAT-Bs, the reduced HemAT-Bs solution was exposed to CO, NO, and O₂ gas, respectively.

### RESULTS

**UVRR Spectral Changes of the Sensor Domain and Full-length Proteins of HemAT-Bs upon O₂ Binding**— Figure 2 depicts the 229-nm-excited raw UVRR spectrum of the O₂-bound form of full-length WT HemAT-Bs (a) and the difference spectra, O₂ – ligand-free, for full-length (b) and truncated sensor domain proteins (c). The raw spectrum of full-length WT (a) is dominated by the bands arising from one Trp (Trp132) and six Tyr residues (Tyr13, Tyr49, Tyr70, Tyr133, Tyr148, and Tyr184), which are labeled by W and Y, respectively, followed by their mode numbers (26). The frequency of W₁₇ band (~875 cm⁻¹) is known to serve as a marker of hydrogen bonding of Trp indole ring (33,34). The W₁₇ frequency of WT (a) is observed at 876 cm⁻¹. This frequency corresponds to a moderate strength of hydrogen bonding (33,34). The crystal structure of the CN-bound form of the truncated sensor solution was incorporated into a spinning cell having a stirring function (32), the inside of which was replaced with the corresponding gas. Raman scattered light at right angle was collected with a UV microscope objective lens, dispersed with a 126 cm single monochromator (Spex 1269) equipped with a 3600 groove/mm holographic grating, and detected by an intensified charge-coupled detector (ICCD, Princeton Instruments, model ICCD-1024MG-E/1). We adopted spectral resolutions of 7.8 cm⁻¹ for spectra. The laser power at the sample point was very low (0.3 mW) to avoid the photodissociation of the ligand from the heme. The protein sample was replaced with fresh one every 10 minutes and the total exposure time to get one spectrum was about one hour. The integrity of the sample after exposure to UV laser light was carefully confirmed by comparing the visible absorption spectra obtained before and after the UVRR measurements. If some spectral changes were recognized, the Raman spectrum was discarded. Raman shifts were calibrated with cyclohexane, trichloroethylene, 1,2-dichloroethane, and toluene.

**Ultraviolet Resonance Raman Measurements**—UVRR measurements were performed by using instrumentation described in details previously (32). The 229-nm excitation light was generated by an intracavity frequency-doubling of the 457.9 nm line of an Ar ion laser (Coherent, Innova 300 FReD). The second harmonic in the laser output was separated with a Pellin-Broca prism and focused into a sample solution. About 100 μL aliquot of the protein solution was incorporated into a spinning cell having a stirring function (32), the inside of which was replaced with the corresponding gas. Raman scattered light at right angle was collected with a UV microscope objective lens, dispersed with a 126 cm single monochromator (Spex 1269) equipped with a 3600 groove/mm holographic grating, and detected by an intensified charge-coupled detector (ICCD, Princeton Instruments, model ICCD-1024MG-E/1). We adopted spectral resolutions of 7.8 cm⁻¹ for spectra. The laser power at the sample point was very low (0.3 mW) to avoid the photodissociation of the ligand from the heme. The protein sample was replaced with fresh one every 10 minutes and the total exposure time to get one spectrum was about one hour. The integrity of the sample after exposure to UV laser light was carefully confirmed by comparing the visible absorption spectra obtained before and after the UVRR measurements. If some spectral changes were recognized, the Raman spectrum was discarded. Raman shifts were calibrated with cyclohexane, trichloroethylene, 1,2-dichloroethane, and toluene.
domain in the homo-dimer, suggests that Trp132 does not form a hydrogen bond with any nearby residues (21). The W3 mode of Trp, which is known to be sensitive to the absolute value of torsion angle, \(|\chi^2\angle 1\) about the C9-C3 bond connecting the indole ring to the peptide main chain (35), is observed at 1554 cm\(^{-1}\) for the full-length WT, which corresponds to a \(|\chi^2\angle 1\) angle of 102°. The X-ray structure of the truncated sensor domain indicated that Trp132 in each monomer of the cyanide bound form has \(|\chi^2\angle 1\) values of 111 and 127° (21). These values are larger than that predicted from the W3 frequency. The W17 and W3 vibrations of truncated sensor domain are also observed at frequencies similar to those of the full-length protein (not shown). These observations suggest that the hydrogen bonding interaction and side chain conformation of Trp132 might be slightly different between the O2- and CN-bound forms.

Because of the importance of an effector molecule, O2, for HemAT-Bs, next we focus on the UVRR spectral changes upon binding of O2 to the heme. We note that the absorption spectrum of the O2-bound form showed no significant change after exposure to UV light, indicating that the O2-bound form was not oxidized under our experimental conditions. The O2 – ligand-free difference spectra (Figure 2b,c) yield negative peaks at 758 (W18), 876 (W17), 1011 (W16), 1174 (Y9a), 1358 (W7), 1554 (W3), and 1617 cm\(^{-1}\) (Y8a), indicating the decrease in the intensities of the Trp and Tyr bands in the O2-bound form. These results suggest that Trp132 and some Tyr residues experience environmental changes upon O2-binding.

Intensity comparison between spectra b and c indicates that the intensity change of Y8a is larger for the full length protein than for the truncated sensor domain (see also Figure S1 in Supplemental Data). Since, this difference spectrum (Figure 2b) reflects the spectral changes of Tyr residues upon O2-binding and the truncated sensor domain (residues 10-178) does not contain Tyr184, which is located in the linker region (residues 176-195) between the sensor and signaling domains, the change in Y8a band (spectrum b) is partially assigned to Tyr184. This suggests that an environmental change of Tyr184, in fact, takes place upon binding of O2 to the heme. However, the overall similarity between the spectra (b) and (c) means that the structural changes of Trp and Tyr residues in the sensor domain upon O2-binding are hardly affected by the presence of the linker region.

UVRR Spectral Changes of HemAT-Bs upon Binding of Different Ligands – Figure 3 compares the UVRR spectral differences among the O2- (b), CO- (c) and NO-bound forms (d) of the full-length WT, for which the 229-nm-excited raw UVRR spectrum of the O2-bound form is presented again by spectrum (a) for reference. For clarification, spectra (b)-(d) are represented by the differences, ligand-bound – ligand-free reduced forms. Although, CO and NO are not effector molecules for HemAT-Bs, we have monitored the spectral changes for Trp and Tyr residues of WT upon CO- (c) or NO-binding (d) to elucidate whether or not the protein moiety can discriminate between different ligands. The difference spectrum c (CO – ligand-free) displays negative features for Trp and Tyr bands similar to those observed upon O2 binding (b), but the features of W18 and W16 modes are slightly different between CO- and O2-binding. In addition, spectrum c reveals stronger negative peaks for Y9a (1174 cm\(^{-1}\)), Y8b (1598 cm\(^{-1}\)) and Y8a (1617 cm\(^{-1}\)) bands than those in spectrum b, implying that the Raman intensity of some Tyr residues are more reduced in the CO- than O2-form.

Furthermore, the difference spectrum d (NO – ligand-free) yields negative features for Trp and Tyr bands similar to those observed upon O2-binding (b), but the features for the W3 and Y8a modes appear different between NO and O2. For instance, the W3 feature is stronger than Y8a in spectrum b, but it is reversed in spectrum d. Thus, the UVRR difference spectra indicate that the Raman bands of Trp and Tyr residues change in different ways upon binding of different ligand species (O2, CO, or NO). This suggests that the protein structural changes including those of Trp132 and some Tyr residues are specific to a ligand species.

Spectral Changes of Tyr Residues Induced by O2 Binding – The 229-nm excited UVRR spectra of Tyr mutants are shown in Figure 4, where the raw spectra of the O2-form of Y133F (a) and Y70F (b) of full-length HemAT-Bs are displayed, while the difference spectra, O2 – ligand-free are also represented for Y133F (c), and Y70F (d). The Trp and Tyr Raman bands in spectra a and b are located at frequencies similar to those of WT (Figure 3a). As shown in Figure 4c, the W18, W16, W7, and W3 bands of Y133F mutant exhibit prominent negative features, implying the intensity decreases of the Trp bands in the

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O$_2$-bound form are similar between WT (Figure 3b) and Y133F (Figure 4c). However, the intensities of difference peaks of Y9a and Y8a are weaker for Y133F (Figure 4c) than for WT (Figure 3b). On the other hand, all Tyr negative peaks are abolished by mutation of Tyr70 (Figure 4d). These results are clearly seen in the double difference spectra (spectra e and f in Figure S2 in Supplemental Data), demonstrating similarity between the spectra of WT and Y133F and some dissimilarity between spectra of WT and Y70F with regard to Tyr bands. This would mean that binding of O$_2$ to heme induces conformational changes of the protein in both the distal (Tyr70) and proximal (Tyr133) sides of heme, but the former is much larger than the latter.

Spectral Changes of Tyr Residues Induced by CO Binding – Figure 5 depicts the raw UVRR spectrum of the CO-form of full-length WT excited at 229 nm (a) and the difference spectra, CO – ligand-free, for WT (b), Y70F (c), and Y133F (d). Spectrum b reveals negative peaks for Y9a, Y7a, Y8b and Y8a bands, indicating that some Tyr residues undergo spectral changes upon CO binding. These negative peaks in WT spectrum (b) became weaker in Y70F (c) and Y133F spectra (d), indicating the contribution from both Tyr70 and Tyr133 to the difference spectrum of WT.

The contribution from Tyr70 appears larger than that from Tyr133 as shown by the double difference spectra (spectra e and f in Figure S3 in Supplemental Data). The larger contribution from Tyr70 than from Tyr133 in addition to the absence of Trp negative peaks except for W3 mode distinguish between the CO- and O$_2$-bound forms. These results also mean that Tyr70 and Tyr133 besides Trp132 in the sensor region undergo spectral changes upon CO binding, which are different from those seen for O$_2$-binding.

Effects of Mutations of Thr95 and His86 on the O$_2$-Induced Spectral Changes – Yoshimura et al. proposed that a hydrogen bond is formed between His86 and heme 6-propionate only when O$_2$ is bound to the heme in HemAT-Bs (23). The formation of this hydrogen bond induces a conformational change of the CE loop and E-helix, by which Thr95 is moved to a position suitable to form a hydrogen bond with the heme bound-O$_2$. The conformational changes around the heme upon O$_2$ binding would be propagated to the protein moiety within the sensor domain and afterwards to the signaling domain. If this is the case, these hydrogen bonds must control the conformational changes in the protein moiety. To examine this idea, we performed UVRR measurements of H86A and T95A mutants, in which a hydrogen bond between His86 and 6-propionate or that between Thr95 and heme bound-O$_2$ would be absent, respectively.

Figure 6 displays the raw UVRR spectra of O$_2$-bound form of T95A (a) and H86A (b), and the difference spectra, O$_2$ – ligand-free for the T95A (c) and H86A (d). The raw spectra of T95A and H86A are quite similar to that of WT (Figure 3a) implying that the mutations of the Thr95 and His86 incorporate no significant effect on the Raman frequencies of Trp and Tyr residues. The difference spectrum for T95A (Figure 6c) is different from that for WT (Figure 3b) regarding the negative features at W18, W17, W16, W7, W3, and Y8a; the difference peak intensities are much weaker in T95A spectrum than in WT (see also Figure S4 in Supplemental Data).

We previously reported for the O$_2$-bound WT HemAT-Bs that there are three O$_2$-isotope sensitive bands ($\nu$Fe-O$_2$) at 554, 566, and 572 cm$^{-1}$ (22). In T95A mutant, the bands at 554 cm$^{-1}$, which is deduced to arise from the species with a hydrogen bond between the proximal oxygen atom of heme-bound O$_2$ and a protein residue, and 566 cm$^{-1}$ disappeared, and only the band at 572 cm$^{-1}$ was observed (22). The frequency of 566 cm$^{-1}$ implies the presence of moderately strong hydrogen bond between the distal oxygen atom of heme-bound O$_2$ and a protein residue similar to that in MbO$_2$. The results suggested that T95A mutant has a single conformation of the distal heme pocket, which would correspond to the open form with $\nu$Fe-O$_2$ at 572 cm$^{-1}$ (22). Thus, the small negative features in spectrum c (Figure 6) are produced as a result of intensity reduction of the open form (572 cm$^{-1}$) compared with that of ligand-free form. Therefore, the removal of a hydrogen bond between Thr95 and heme bound-O$_2$ significantly perturbs the conformation of Trp132, Tyr133, and Tyr70 residues and thus may make the information transduction impossible.

On the other hand, H86A spectrum (Figure 6d) displays negative peaks at W18, Y1, W17, W16, W7, and W3 bands similar to those for WT (Figure 3b). However, the negative peak of Y8a in the WT spectrum (Figure 3b) is absent in the H86A spectrum (Figure 6d). In addition, Y9a mode appears as a small derivative near 1174
cm\(^{-1}\), implying that \(Y9a\) band is shifted to higher a frequency in the \(O_2\)-bound form.

In \(H86A\) mutant, only the \(\nu_{Fe-O2}\) band at 566 cm\(^{-1}\) disappeared (23), implying that \(H86A\) mutant yields two conformations corresponding to the open form with \(\nu_{Fe-O2}\) at 572 cm\(^{-1}\) and the closed form with \(\nu_{Fe-O2}\) at 554 cm\(^{-1}\). Thus, the negative features in spectrum d (Figure 6) are probably due to the existence of two conformations with \(\nu_{Fe-O2}\) of 572 and 557 cm\(^{-1}\).

Therefore, the removal of a hydrogen bond between His86 and 6-propionate perturbs only the conformation of Tyr70 and Tyr133 residues, resulting in the disappearance of the 566 cm\(^{-1}\) species. Taken this result together with that of T95A, we conclude that the hydrogen bonds between His86 and 6-propionate and between Thr95 and heme-bound \(O_2\) significantly influence the conformational states of Tyr70, Trp132, and Tyr133.

**DISCUSSION**

**Protein Conformational Changes upon Binding of Different Ligands**

Full-length HemAT-Bs protein contains a single Trp (Trp132) and six Tyr (Tyr13, Tyr49, Tyr70, Tyr133, Tyr148, and Tyr184) residues. Trp132 and Tyr133 are located in the proximal side of heme, but Tyr70 in the distal side, while Tyr49, Tyr148, and Tyr184 residues are far from the heme (Figure 1A). Only Tyr184 stays in the linker region between the sensor and the signaling domains. These aromatic residues are used as probes to monitor the protein structure in the present UVRR spectroscopy. The UVRR results demonstrated that Raman intensities of Trp132 decrease for the \(O_2\)-, \(CO\)-, and \(NO\)-bound forms in different ways. Generally, the Raman intensity of Trp is sensitive to environmental hydrophobicity and/or hydrogen-bonding interactions of the indole side chain (28,36,37). Since a frequency shift is not observed for the hydrogen bond marker band (876 cm\(^{-1}\)), a moderate strength hydrogen bonding between Trp132 and nearby residues or a water molecule is hardly altered by ligand binding. Thus, it is reasonable to conclude that the environment around Trp132 shifts to more hydrophilic in the \(O_2\)-bound form.

The UVRR bands of Tyr residues also exhibited an intensity decrease upon binding of ligands in a manner specific to a ligand species, and these changes were assigned to Tyr70, Tyr133 (Figures 4-5). These spectral changes are not compatible with deprotonation or formation of strong hydrogen bond of the phenol side chain. Our previous RR study with visible excitation (22,23) suggested that Tyr70 does not form a hydrogen bond with heme-coordinated \(O_2\). However, Zhang et al. proposed that Tyr70 would form a hydrogen bond to the heme-bound \(O_2\) on the basis of the observation that the mutation of Tyr70 gave a larger dissociation constant for the heme-bound \(O_2\) than that for WT (16). On the other hand, Yoshimura et al. recently suggested that Tyr133 forms a hydrogen bond with the proximal ligand (His123) in the \(CO\)-bound form (25). This proposal is based on 2 cm\(^{-1}\) downshift of the \(\nu_{Fe-His}\) band in TR\(^3\) spectra of WT in hundreds of picoseconds after the photodissociation of \(CO\). This frequency shift did not occur in that of \(Y133F\) mutant. However, the size of downshift of \(\nu_{Fe-His}\) suggests a very weak hydrogen bond (38), if present.

It was clarified from the model compound studies that the intensity of Tyr bands is influenced by the hydrophobicity of its surroundings (37). On the other hand, the formation or breaking of a hydrogen bond between the hydroxy side chain of Tyr and any surrounding residue is expected to influence significantly both the intensity and frequency of Tyr bands (28,31,39,40). Since UVRR results of WT, Y70F, and Y133F showed no significant frequency shift for any of Tyr Raman bands, all Tyr residues including Tyr70 and Tyr133 do not undergo a change of hydrogen bonds. Only intensities of Tyr70 and Tyr133 are altered upon
ligand binding, we conclude that the environment around Tyr70 and Tyr133 shifts to more hydrophilic in the ligand-bound forms without a change in their hydrogen bonding interactions. These results confirmed our previous model (22,23), which suggested that Tyr70 does not form a hydrogen bond but Thr95 does it with heme-coordinated O2. Presumably, the change of hydrophobicity around Tyr133 would be associated with the observed νFe–His shift.

Both the full-length and truncated sensor domain proteins of HemAT-Bs revealed similar spectral changes for Trp and Tyr residues upon O2 binding (Figure 2). Although the main contributors to Tyr difference peaks arise from Tyr70 and Tyr133, Tyr184 in the linker region exhibits small spectral change (Y8a band) upon ligand binding, and the magnitude of its change seems to depend on a ligand species (Figure 3). This implies that conformation change of the sensor domain upon ligand binding, specific to a ligand species, is communicated to the signaling domain through the linker region. One of the possible explanations for the small spectral change of Tyr184 is that this residue may be exposed to solvent and binding of O2 will cause only small changes in its environment. However, the main changes in UVRR spectra are attributed to environmental changes of Trp132, Tyr70 and Tyr133 in the heme neighborhood.

The previous RR results showed that Thr95 forms a hydrogen bond to the heme-bound O2 of HemAT-Bs (22,23). In contrast, the RR and FTIR results indicated the absence of the hydrogen bonding interaction between the heme coordinated-CO and Thr95 (23,24). Therefore, it is proposed that the hydrogen bonding interactions of the heme-bound O2 with the surrounding residues such as Thr95 is crucial for ligand discrimination in HemAT-Bs (23). Such a specific hydrogen bonding between a heme-bound O2 and a protein residue has been also observed for other O2-sensor heme proteins such as FixL and Ec DOS (4,5). The present results in Figure 3 are compatible with the idea that heme of HemAT-Bs discriminates between O2 and CO, resulting in different conformational changes in the protein moiety of HemAT-Bs. In addition, binding of O2, CO, or NO to a five-coordinate high-spin ligand-free (reduced) form changes the spin-state of the heme iron of HemAT-Bs (15). Thus, the observed structural changes upon O2-binding are mainly due to conversion of the high–spin to low-spin state and the formation of hydrogen bonds between O2 and Thr95, while those observed upon CO- or NO-binding are probably due to only the change in the spin-state of heme iron.

Insights into Signal Transduction Mechanism of HemAT-Bs – A conformational change specific to O2 binding would occur in HemAT in the heme neighborhood, and then intramolecular signal transduction would take place from the sensor domain to the signaling domain through the linker region. As a result, the self-kinase activity of CheA is regulated through the HemAT-CheA interaction by ligand binding to heme. This signaling event takes place only with O2, but not with other gas molecules which bind to the heme. Yoshimura et al. proposed that a hydrogen bond is formed between His86 and heme 6-propionate only when O2 is bound to the heme (23). This proposal seems likely because the formation of a hydrogen bond induces conformational change in the heme distal side, by which Thr95 is displaced to a proper position to form a hydrogen bond with the bound-O2. In the present study, we have investigated the role of these hydrogen bonds and found that the removal of these hydrogen bonds in H86A and T95A mutants strongly perturb the conformational changes of Tyr70 (B-helix), Tyr133, and Trp132 (G-helix) in both the distal and proximal sides of heme. Recently, we have shown that the heme structural changes upon ligand binding in myoglobin are communicated to the globin through heme propionates (29). This is based on the observation that the mutations of the near-by residues or chemical modification of the propionate side chain of heme significantly changes the conformation of Trp residues in the A helix of the globin, which are far from the heme in myoglobin. Similarly, the present UVRR results suggest that hydrogen bonds between Thr95 and heme-bound O2 and between His86 and heme 6-propionate in HemAT-Bs communicate the structural changes of heme to the protein moiety (B- and G-helices) upon O2-binding.

Furthermore, the present UVRR results showed for the first time that Trp132 and Tyr133 on the G-helix in the proximal side of heme undergo a change in hydrophobicity upon O2- or CO-binding. Recently, Pinakoulaki et al. pointed out the presence of a ligand cavity in the protein from the FTIR experiments for the heme-bound CO of HemAT-Bs and suggested that the docked CO is interacting with Tyr133 (41). This observation is compatible with different
conformational changes of G-helix residues (Trp132 and Tyr133) upon binding of different ligands. The conformational changes of G-helix residues are quite important for the signal transduction mechanism of HemAT- Bs, as discussed below. The signal transduction mechanism in MCPs that are homodimeric membrane-bound proteins has been proposed (42,43). A pair of two antiparallel helices in a MCP dimer forms a transmembrane four-helix bundle that connects the periplasmic sensor domain and the cytoplasmic signaling domain. The binding of the effector molecule to the sensor domain induces a slide and/or a rotational movement of this helix bundle, which is a key step in the signal transduction mechanism of MCPs (42,43).

HemAT- Bs is a member of MCPs. Although, HemAT- Bs lacks the transmembrane region, the G and H helices of the two subunits of homo-dimer form an antiparallel four-helical bundles (Figure 1B), exist in the C-terminal region of the sensor domain, and the H helices are apparently continuous to the extended helical structure of the signaling domain. Given that HemAT- Bs adopts the same mechanism for intramolecular signal transduction as membrane-bound MCPs, the helix bundle consisting of the G and H helices will correspond to the transmembrane helix bundle in MCPs. In fact, X-ray analysis of the truncated HemAT- Bs has demonstrated that both G and H helices experience displacement upon CN-binding (21), although CN is not bound to the reduced form. If similar displacements of the four-helical bundle occur upon binding of O2, it would trigger the transduction of the conformational changes from the sensor domain to the signaling domain.

Furthermore, the truncated sensor domain of HemAT- Bs maintains classic globin folds (21). In myoglobin, binding of an external ligand to the heme induces significant displacement of F-, and E-helices (44). In addition, the protein structural changes are propagated from the heme to C-terminal region of the H-helix upon ligand binding through the structural change in the proximal side of the heme (29). X-ray structure of the truncated HemAT- Bs has showed that F-helix suffer displacement upon CN-binding (21). In addition, Yoshimura et al. proposed that E-helix experiences considerable conformational changes upon O2-binding (23). On the other hand, further study is necessary to monitor the structural changes in H-helix of HemAT- Bs upon O2-binding.

In conclusion, the present UVRR study of the ligand-bound (O2, CO, and NO) forms of WT and several mutants of HemAT- Bs has brought new insights into the communication pathway between the heme and the protein moiety. More concretely, the hydrogen bonds between heme bound-O2 and Thr95 and between His86 and heme 6-propionate are considered to communicate the heme structural changes upon O2-binding to the protein moiety. Furthermore, the UVRR results suggest that G-helix experiences different amounts of displacement upon ligand binding, depending on the ligand species, which would be important for communication of the ligand specific conformational changes from the sensor domain to the signaling domain.

We have demonstrated that UVRR spectroscopy is a powerful tool to elucidate fine structural changes of protein associated with the binding of ligand to the heme. The method can be applied in a time-resolved mode to monitor dynamical changes in the protein moiety upon photodissociation of the gaseous ligand using the pump-probe (nanoseconds to microsecond) technique. Time-resolved UVRR study of HemAT- Bs is our next project.

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REFERENCES

FOOTNOTES

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1The Abbreviation used are: Ax PDEA1, phosphodiesterase A1 protein from Acetobactoer xylinum; Ec DOS, direct oxygen sensor from Escherichia coli; MCP, methyl accepting chemotaxis protein; c-di-GMP, 3',5'-cyclic diguanylic acid; HemAT-Bs, HemAT from Bacillus subtilis; UVRR, ultraviolet resonance Raman; WT, wild-type; PAS, an acronym formed from the names of proteins in which imperfect repeat sequences were initially recognized: the Drosophila period clock protein (PER), vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT), and Drosophila single-minded protein (SIM); FTIR, Fourier transform infra-red.

Supplemental Data. Figures S1-4 (PDF). This material is available free of charge via the Internet at ????

FIGURE LEGENDS

Figure 1. X-ray structure of the CN-bound form of truncated HemAT-Bs protein (PDB ID: 1OR4; ref. 21). The heme, Trp, Tyr, His86, Thr95, and His123 residues are explicitly represented in Panel A, where a hydrogen bond between Tyr70 and heme-bound-CN is depicted by a black broken line. Panel B shows the top view of the same protein, which exhibits the flanking of the core helices, G, and H. The helices are labeled in accord with the nomenclature of the globin fold.

Figure 2. The 229-nm excited UVRR spectra of HemAT-Bs. Shown are the raw spectrum for the O2-bound form of WT (a) and the O2 – ligand-free difference spectra for full-length WT (b) and truncated sensor domain (c).

Figure 3. The 229-nm excited UVRR spectra of full-length WT HemAT-Bs. Shown are the raw spectrum for the O2-bound form of WT (a) and the following difference spectra for WT: (b) O2 – ligand-free, (c) CO – ligand-free, and, (d) NO – ligand-free.
Figure 4. The 229-nm excited UVRR spectra of Tyr mutants of full-length HemAT-Bs. Shown are the raw spectra for the O$_2$-bound form of Y133F (a) and Y70F (b), and the O$_2$ – ligand-free difference spectra for Y133F (c) and Y70F (d) of full-length proteins.

Figure 5. The 229-nm excited UVRR spectra of WT and Tyr mutants of full-length HemAT-Bs. Shown are the raw spectrum for the CO-bound form of WT (a), and the CO – ligand-free difference spectra for WT (b), Y70F (c), and Y133F (d) of full-length proteins.

Figure 6. The 229-nm excited UVRR spectra of HemAT-Bs mutants. Shown are the raw spectra for the O$_2$-bound form of T95A (a) and H86A (b), and the difference spectra O$_2$ – ligand-free for T95A (c), and H86A (d) full-length HemAT-Bs proteins.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure S1. The enlarged $\text{O}_2$ ligand-free difference spectra for the full-length (solid line) and the sensor domain (dotted line) of HemAT- Bs are overlaid.
Figure S2. The 229-nm excited UVRR difference spectra of WT and Tyr mutants of HemAT-Bs. Shown are the raw spectrum for the O$_2$-bound form of WT (a) and the O$_2$ – ligand-free difference spectra for WT (b), Y133F (c), and Y70F (d) of full-length proteins, and the double difference spectra of WT – Y133F (e = b – c) and WT – Y70F (f = b – d). The double difference spectra (e-f) show the contribution of Tyr133 and Tyr70 residues to the WT difference spectrum (b).
Figure S3. The 229-nm excited UVRR difference spectra of WT and Tyr mutants of HemAT-Bs. Shown are the raw spectrum for the CO-bound form of WT (a) and the CO−ligand-free difference spectra for WT (b), Y70F (c), and Y133F (d) of full-length proteins, and the double difference spectra of WT−Y70F (e = b − c) and WT−Y133F (f = b − d). The double difference spectra (e-f) show the contribution of Tyr70 and Tyr133 residues to the WT difference spectrum (b).
Figure S4. Shown are the O$_2$-ligand-free difference spectra for WT (a), T95A (b), and H86A (c) of full-length HemAT-Bs proteins.
Protein conformation changes of HemAT-Bs upon ligand binding probed by ultraviolet resonance raman spectroscopy
Samir F. El-Mashtoly, Yuzong Gu, Hideaki Yoshimura, Shiro Yoshioka, Shigetoshi Aono and Teizo Kitagawa

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