Site-specific Protein Dynamics in Communication Pathway from Sensor to Signaling Domain of Oxygen Sensor Protein, HemAT-Bs

**TIME-RESOLVED ULTRAVIOLET RESONANCE RAMAN STUDY**

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**Background:** HemAT is an O₂ sensor and functions as a signal transducer for aerotaxis. Time-resolved ultraviolet resonance Raman (UVRR) studies of wild-type and Y70F mutant of the full-length HemAT-Bs and the truncated sensor domain were performed to determine the site-specific protein dynamics following carbon monoxide (CO) photodissociation. The UVRR spectra indicated two phases of intensity changes for Trp, Tyr, and Phe bands of both full-length and sensor domain proteins. The W16 and W3 Raman bands of Trp, the F8a band of Phe, and the Y8a band of Tyr increased in intensity at hundreds of nanoseconds after CO photolysis, which was followed by recovery in hundreds of nanoseconds after photolysis, thus stimulating downstream chemotactic signaling in *Bacillus subtilis*. Besides, the signaling domain of HemAT has an amino acid sequence homologous to that of the signaling domain of Tsr, a typical methyl-accepting chemotactic protein (MCP) of *Escherichia coli*. Thus, the binding of O₂ to the heme induces a conformational change in the protein in the heme pocket and ultimately in the signaling domain of HemAT, thus stimulating downstream chemotactic signaling in *Bacillus subtilis*.

**Significance:** Understanding the communication pathway from the sensor to signaling domain of HemAT-Bs.

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HemAT-Bs is a heme-based signal transducer protein responsible for aerotaxis. Time-resolved ultraviolet resonance Raman (UVRR) studies of wild-type and Y70F mutant of the full-length HemAT-Bs and the truncated sensor domain were performed to determine the site-specific protein dynamics following carbon monoxide (CO) photodissociation. The UVRR spectra indicated two phases of intensity changes for Trp, Tyr, and Phe bands of both full-length and sensor domain proteins. The W16 and W3 Raman bands of Trp, the F8a band of Phe, and the Y8a band of Tyr increased in intensity at hundreds of nanoseconds after CO photodissociation, and this was followed by recovery in ~50 μs. These changes were assigned to Trp-132 (G-helix), Tyr-70 (B-helix), and Phe-69 (B-helix) and/or Phe-137 (G-helix), suggesting that the change in the heme structure drives the displacement of B- and G-helices. The UVRR difference spectra of the sensor domain displayed a positive peak for amide I in hundreds of nanoseconds after photolysis, which was followed by recovery in ~50 μs. This difference band was absent in the spectra of the full-length protein, suggesting that the isolated sensor domain undergoes conformational changes of the protein backbone upon CO photolysis and that the changes are restrained by the signaling domain. The time-resolved difference spectrum at 200 μs exhibited a pattern similar to that of the static (reduced − CO) difference spectrum, although the peak intensities were much weaker. Thus, the rearrangements of the protein moiety toward the equilibrium ligand-free structure occur in a time range of hundreds of microseconds.

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The absorption spectra of the ligand-bound forms of the full-length HemAT-Bs and its truncated sensor domain are identical irrespective of the ligand species (3, 4), suggesting that the proteins have a similar heme pocket. The structural changes that occur in the heme pocket upon the binding of different ligands have been investigated with different techniques (3, 4, 13–15). Static visible-excited resonance Raman studies have shown that the O₂-bound form of the wild-type (WT) HemAT-Bs has three conformers, closed, open-α, and open-β forms, with different hydrogen bond interactions between the heme-bound O₂ and surrounding amino acid residues. The closed, open-α, and open-β forms give the Fe-O₂ stretching (ν_{Fe-O₂}) band at 554, 566, and 572 cm⁻¹, respectively (13). The closed and open-α forms disappear when Thr-95 is mutated to Ala, in which the ν_{Fe-O₂} band is observed only at 569 cm⁻¹. However, the three conformers are all present in the Y70F mutant. These results suggest that Thr-95 forms hydrogen bonds with the heme-coordinated O₂, whereas Tyr-70 is not involved in the hydrogen bonding interaction with the heme-bound O₂ (13). In addition, it is proposed that His-86 forms a hydrogen bond with the heme 6-propionate upon the binding of O₂ to the heme (14). The formation of hydrogen bonds between His-86 and the heme 6-propionate and between Thr-95 and heme-bound O₂ would result in considerable conformational change in the E- and B-helices (13, 14). However, Zhang et al. proposed that Tyr-70 would form a hydrogen bond to the heme-bound O₂ based on the observation that mutation of Tyr-70 made the dissociation constant of the heme-bound O₂ larger than that of WT (4).

Ultraviolet resonance Raman (UVRR) spectroscopy is a powerful tool for studying protein structure because it allows the observation of the Raman bands of the polypeptide backbone and aromatic side chains with high selectivity (16–27). It is well established that the vibrational bands of Trp, Tyr, and Phe function as structural markers of proteins (16). The static UVRR studies of HemAT-Bs did not indicate any change in the hydrogen bonding interaction of any of the Tyr residues upon the binding of different ligands (28), consistent with the model of hydrogen bonding interaction between bound O₂ and Thr-95 (28).

Determination of the structural dynamics of the protein moiety associated with the ligand binding/dissociation of heme is of great interest in elucidating the initial events of the signaling mechanism. Yoshimura et al. (29) suggested that Tyr-133 forms a hydrogen bond with the heme-proximal ligand (His-123) in the CO-bound form. The transient absorption difference spectrum obtained at ~4 μs following CO photolysis typically displays a bathochromic shift relative to the equilibrium absorption difference spectrum near 435 nm, suggesting an unrelaxed heme pocket geometry immediately after CO photolysis (30). Moreover, the CO rebinding to the heme after laser photolysis is monophasic and occurs on a millisecond time scale (4). These studies have directly probed the dynamics of heme after CO photolysis, but the dynamics of the protein matrix remain to be investigated.

Accordingly, in this study, nanosecond to microsecond time-resolved UVRR spectroscopy was used to monitor the structural changes in the full-length WT and Y70F mutant as well as the truncated sensor domain of HemAT-Bs following CO photodissociation, thus enabling site-specific observation of protein dynamics. Because the apparent quantum yield of photodissociation on the nanosecond time scale for heme-bound O₂ is too low to perform this measurement, we used CO as the ligand. Although O₂ is physiologically a signaling ligand, the much greater photosensitivity of the CO complex warrants its use as O₂ mimic, especially because this strategy of using CO as a ligation surrogate is a commonly accepted practice (24–27).

**Experimental Procedures**

**Sample Preparation**—The full-length and the sensor domain of HemAT-Bs with a C-terminal His₆ tag were expressed in an *E. coli* BL21 cell system under the control of the T7 promoter in the pET-24(+) vector (Novagen). Site-directed mutagenesis was carried out using a QuikChange site-directed mutagenesis kit (Stratagene). For the expression of HemAT-Bs, the *E. coli* cells were grown aerobically at 37 °C for 4 h in Terrific Broth containing 30 μg/ml kanamycin. The expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM, and then the cultivation was continued at 22 °C for 18 h. The cells were harvested by centrifugation at 4,000 × g and were stored at ~80 °C until use. HemAT was purified as reported previously, and the heme remained bound to the protein throughout purification (14).

For both the static and time-resolved UVRR experiments, the CO-bound and CO-free forms were prepared as follows. 80 μM oxidized protein (50 mM Tris-HCl buffer, pH 7.5), was transferred to an airtight spinning cell, into which CO gas was incorporated to a pressure of 1 atmosphere after evacuation of the internal pressure down to 0.01 mmHg. The pump/fill procedure was repeated at least three times, and finally a small amount of anaerobic dithionite solution was added (final concentration, 0.5 mM) for the complete reduction of the protein.
The CO-free form was prepared by adding a small amount of anaerobic dithionite solution (final concentration, 0.5 mM) to the oxidized form under a nitrogen atmosphere. For both the static and time-resolved UVR-R spectra measurements, 400 nm sodium perchlorate (NaClO₄) was included in the solution as an internal intensity standard to calculate the UVR-R difference spectra.

**Time-resolved UVR-R Measurements**—Time-resolved UVR-R experiments were performed as described previously (31, 32). In brief, the probe beam was generated with a subnanosecond Ti:Sapphire oscillator (Tsunami pumped by a Millennia 105; Spectra Physics) and an amplifier system (Spitfire pumped by an Evolution X; Spectra Physics) operating at 1 kHz. The laser output at 880 nm was quadrupled by two BBO crystals, and the output pulse energy of the fourth harmonic at 220 nm was >10 μJ. The pump beam (25 ns) was the 532-nm SHG output of a Nd:YAG laser (Navigator I; Spectra Physics), which was Q-switched at 1 kHz by triggering it with a pulse generator (DG535; Stanford Research Systems). The pulse generator was also used to send an appropriately delayed trigger to the Q-switched Nd:YLF laser (Evolution X) in the regenerative amplifier (Spitfire) at 1 kHz to synchronize the pump and probe pulses with a timing jitter of ±25 ns. The pump and probe beams were made coaxial using a dichroic mirror and focused onto the sample with a cylindrical lens with a 135° back-scattering collection geometry. The energy of the probe beam at the sample position was adjusted to 0.25 μJ, whereas the energy of the pump beam in the probe area was 1 mJ.

An approximate 1.0-ml sample was placed in an airtight quartz NMR tube attached directly to a motor as a spinning cell and was maintained at ~15 °C by flushing with cooled N₂ gas to avoid sample degradation. The Raman scattered light was collected and focused onto the entrance slit of a prism prefilter (Bunkoh-Keiki) coupled to a 1-m single spectrograph (Spex, 1000 m; Jobin Yvon) by two achromatic double lenses. The dispersed light was detected with a UV-coated, liquid-nitrogen-cooled CCD detector (Spec10:400B/LN; Roper Scientific). Raman shifts were calibrated with cyclohexane to an accuracy of ±1 cm⁻¹ for the well defined Raman bands. Time-resolved UVR-R spectra were measured with delay times (Δt) of between 0.1 and 200 μs. Sample integrity after exposure to laser irradiation was confirmed by visible absorption spectra (U-3310; Hitachi). The data recording processes as well as synchronization of the laser pulses and mechanical shutters were controlled by a computer program designed to perform quasi-simultaneous measurements of a series of pump-probe, probe-only, and dark spectra under the same conditions and at nearly the same time.

**Fitting Analysis**—Suppose that a molecule, M₀, is changed to M₂ through an intermediate, M₁, whose concentrations are denoted by [M₀], [M₁], and [M₂], respectively, and that the time constants of the formation and decay of M₁ are τ₁ and τ₂, respectively. Their transient concentrations at Δt after the start of the reaction, [Mᵢ(Δt)] (i = 0, 1, 2), are explicitly represented by Equation 1,

\[
[Mᵢ(Δt)] = [Mᵢ(0)][1 - K(τᵢ/τ₂)exp(−Δt/τ₁) + Kexp(−Δt/τ₂)]
\]

(Eq. 1)

where [Mᵢ(0)] is the initial concentration of the sample and \( K = 1/(τᵢ/τ₂ - 1) \). The Raman intensity of the \( i \)th species observed at a certain wave number (ν) is proportional to the concentration of the species and its intrinsic intensity. Then, the total transient Raman intensity at ν and Δt, \( I(ν,Δt) \), can be represented by Equation 2.

\[
I(ν,Δt) = I₀(ν)exp(−Δt/τ₁) + I₁(ν)(1 - K(τᵢ/τ₂)exp(−Δt/τ₂)) + Kexp(−Δt/τ₂)
\]

(Eq. 2)

Here, \( I₁(ν) \) is the intrinsic spectrum of the \( i \)th species. For clarity, the temporal changes in the observed spectrum are represented as \( ΔI(ν,Δt) \), which is a difference spectrum, \( I(ν,Δt) - I(ν,0) \). All of the observed time-resolved UVR-R difference spectra, \( ΔI(ν,Δt) \), were loaded into MATLAB 7.11 software (R2010b; MathWorks), and the global fitting analysis (33) using a custom algorithm was performed with two exponential functions represented by Equation 3 in the Δt time range between 0.1 and 200 μs,

\[
ΔI(ν,Δt) = a₁(ν)exp(−Δt/τ₁) + a₂(ν)exp(−Δt/τ₂) + a₀
\]

(Eq. 3)

where \( a₁(ν) \) and \( a₂(ν) \) mean the τᵢ values (i = 1, 2) preexponential factors, which can be related to \( I₁(ν) \) through Equation 2, and \( a₀ \) is an offset that practically mimics the static difference spectrum, \( I₁(ν) - I₀(ν) \). This procedure will be referred to as analysis I.

Furthermore, to identify the peak positions in the time-resolved spectra, band deconvolution using Gaussian functions was performed for the probe-only and time-resolved difference spectra with Igor Pro 6.2 Software (WaveMetrics). The intensities of the \( i \)th band, \( ΔI₁(ν,Δt) \), were calculated, and the intensities of each band were fitted with two exponential functions of the Equation 2 type by using the ExpDec 2 function with OriginPro 8 (OriginLab Corporation). This procedure will be referred to as analysis II.

**RESULTS**

**Time-resolved UVR-R Spectra of Sensor Domain of HemAT-Bs**—Raman bands arising from the Trp, Tyr, and Phe residues are selectively enhanced upon excitation at ~220–250 nm and can be used as structural probes of their surroundings (16, 17). The probe-only raw UVR-R spectra of the CO-bound form of the sensor domain of WT HemAT-Bs excited at 220 nm is shown in Fig. 2a. This spectrum is dominated by the bands arising from one Trp (Trp-132), five Tyr (Tyr-13, Tyr-49, Tyr-70, Tyr-133, and Tyr-148), and six Phe (Phe-3, Phe-14, Phe-69, Phe-104, Phe-112, and Phe-137) residues, which are labeled W, Y, and F, respectively, followed by their mode numbers (16). The spectra b–j in Fig. 2 represent time-resolved UVR-R difference spectra, \( ΔI(ν,Δt) \), obtained by subtracting the CO-bound probe-only spectrum (a), \( I(ν,0) \), from the observed spectra, \( I(ν,Δt) \), mea-
measured at individual delay times from -0.1 to 200 μs (pump/probe - probe-only). These difference spectra were calculated so that the ClO₄⁻ band (933 cm⁻¹), which was present in all of the raw spectra as an internal intensity standard, was completely canceled. The static (reduced - CO) difference spectrum is also shown by trace k. The raw UVRR spectrum of HemAT-Bs excited at 220 nm is appreciably different from the reported spectrum excited at 229 nm (28), due to the variation in resonance enhancement of the Raman intensities. This reflects the fact that Trp, Tyr, and Phe residues have electronic transitions at different wavelengths near this excitation wavelength (16, 17).

We first determined mathematically how many independent patterns are contained in the spectra b-j (Fig. 2) using analysis I. It was found that two independent patterns are contained, and these are depicted in Fig. 3. This means that all of the observed time-resolved spectra can be represented mathematically as a linear combination of the two spectra and thus the presence of two observable intermediate species in this process. Accordingly, we analyzed the observed spectra on the basis of a reaction model shown in Scheme 1.

When the starting CO adduct of HemAT-Bs is denoted by A, the first intermediate, B, is generated in a very short time, but this photodissociation process is beyond the time resolution of the present measurements. Provided that the two species observed in the present measurements are C and D intermediates, the two independent spectra in Fig. 3 correspond to a - b and c - d. Accordingly, spectra Ic and Id in Equation 2 correspond to Ic and Id, respectively. The basic assumption is that the B intermediate is too short lived to be detected. Then, the intermediate C arises and decays with time constants of τc and τd, respectively. The spectrum of C gives positive peaks for W16, W3, F8a, Y8a, and amide I. In contrast, the spectrum of D displays negative peaks for W3, F8a, and Y8a, similar to the static (reduced - CO) difference spectrum, suggesting that Ic is close to the spectrum of the ligand-free product, although the intensities of the negative peaks in spectrum D are weaker than those observed for the static difference spectrum.

The spectral regions between 980 and 1040 cm⁻¹ and between 1510 and 1640 cm⁻¹ in Fig. 2 are enlarged, and representative spectra are depicted in Fig. 4. The observed bands were deconvoluted into component bands under the assumption of a Gaussian band shape using analysis II. The deconvolution successfully reproduces the probe-only spectrum (top).
Time-resolved UV Resonance Raman Study of HemAT-Bs

The component bands, \( \Delta I(\nu, \Delta t) \), are represented by blue solid lines, and the sum of the component bands are represented by red broken lines, which reproduce the observed spectra shown by the black solid lines very well. Thus, strong bands were observed at 1000 (F12, ring-breathing), 1010 (W16, ring-breathing), 1554 (W3, C-C stretching of the pyrrole ring), 1605 (F8a, ring C-C-stretching), and 1617 (Y8a, ring C-C-stretching) cm\(^{-1}\).

The difference spectra were also fitted and represented in the same way as those of the probe-only spectrum. The static (reduced – CO) difference spectrum (Fig. 2c or Fig. 4b) displays negative peaks for the W3, F8a, and Y8a bands, indicating the stronger intensities of the Trp, Phe, and Tyr bands in the CO-bound than CO-free form. At the earliest time point following CO photodissociation, positive difference peaks appeared, reaching a maximum at approximately \( \Delta t = 0.5 \) \( \mu \)s (Fig. 2d) and then diminishing (Fig. 2h). It is noted that W16 band appeared at 1017 cm\(^{-1}\) (Fig. 2d or 4c), whereas it is located at 1010 cm\(^{-1}\) in the probe-only spectrum (Fig. 2a or 4a). When a frequency shift takes place, usually a derivative type pattern appears in a difference spectrum when the shifted band has an intensity similar to that of the original band. However, when the shifted band has significantly larger intensity compared with that of the original band, only a positive band may apparently appear. The 1017 cm\(^{-1}\) band corresponds to this case, that is, the W16 band of Trp-132 is up-shifted by \( \sim 7 \) cm\(^{-1}\) and intensity enhanced upon CO photolysis. This frequency shift is also observed for the C intermediate (Fig. 3). At 100 \( \mu \)s after CO photolysis, negative peaks appeared for W3, F8a, and Y8a (Fig. 2a), and their intensities increased at 200 \( \mu \)s (Fig. 2). This spectrum is similar to the static (reduced – CO) difference spectrum (Fig. 2k), although the intensities of the negative peaks in Fig. 2j are weaker than those observed for the static difference spectrum. The observed two phases of intensity changes for the Trp, Tyr, and Phe of the sensor domain mentioned above reflect a structural change of protein upon CO photolysis and its relaxation which induce significant environmental changes of these aromatic residues.

Furthermore, a weak positive feature was observed near 1660 cm\(^{-1}\) at 0.1 \( \mu \)s after photolysis (Fig. 2c and see also supplemental Fig. S1). The intensity of this band reaches a maximum at 0.5 \( \mu \)s (Fig. 2d), then decreases, and finally disappears at \( \sim 50 \) \( \mu \)s (Fig. 2h), as the other positive peaks described above had done. This band may be assigned to amide I vibration. To confirm this assignment, we performed static UVRR measurements with excitation at 206 nm because amide bands are expected to be in closer resonance with a shorter excitation wavelength (16, 17). Indeed, the amide I band was observed more clearly at 1660 cm\(^{-1}\) than CO-free form. At the latest time point following CO photolysis, the positive features appeared for W3, F8a, and Y8a (Fig. 2). The results suggest that an appreciable change occurs in the protein skeleton at \( \sim 0.5 \) \( \mu \)s after CO photolysis.

To discuss the behavior of the Trp, Tyr, and Phe residues individually, the ratio of the area intensities of the individual \( \Delta I(\nu, \Delta t) \) to \( I(\nu, 0) \) for the W16, W3, F8a, and Y8a bands were obtained and are plotted against \( \Delta t \) in Fig. 5. The plots were fitted with two exponential functions of the Equation 2 type by analysis II, as shown by the smooth curves which were drawn, and the time constants and amplitudes were determined as shown in Table I. Their averages are \( \tau_1 = 0.20 \pm 0.04 \) and \( \tau_2 = 45 \pm 17 \mu \)s, which are in reasonable agreement with those obtained from analysis I (\( \tau_1 = 0.20 \) and \( \tau_2 = 47 \mu \)s).

Time-resolved UVRR Spectra of Full-length HemAT-Bs—To investigate the effect of the HemAT-Bs signaling domain on the protein dynamics of the sensor domain, we performed time-resolved UVRR experiments for WT full-length HemAT-Bs, and the results are displayed in Fig. 6A. The raw UVRR spectrum (a) is dominated by the bands arising from one Trp, six Tyr, and twelve Phe residues. Because the full-length HemAT-Bs contains twice the number of Phe residues compared with the sensor domain, the intensity of the Phe modes is larger than that of the Tyr modes (see supplemental Fig. S3). At 0.5 \( \mu \)s after CO photolysis (d), small positive features start to appear for W3, F8a, and Y8a. The positive band of W3 almost disappeared at 10 \( \mu \)s (g), whereas small positive features near F8a and Y8a remained. These results are different from those observed for the sensor domain, for which the positive features of W3, F8a, and Y8a disappeared at 50 \( \mu \)s after CO photolysis (Fig. 2). For the full-length protein, on the other hand, negative peaks appeared for W3, F8a, and Y8a at 50 \( \mu \)s (Fig. 6h), and they become stronger in intensity until 200 \( \mu \)s. For the isolated sen-
The Raman bands of W3, F8a, and Y8a were extracted from Fig. 6A in the same manner as for the sensor domain (Fig. 4) and the ratios of $\Delta I(\nu,\Delta t)$ to $I(\nu,0)$ are plotted against the delay time as indicated in Fig. 6B. The plots were subjected to the fitting analysis II. The time constants and amplitudes thus determined are also listed in Table 1. The average values are $\tau_1 = 0.44 \pm 0.02$ and $\tau_2 = 47 \pm 7 \mu s$, which are in reasonably good agreement with those obtained from analysis I ($\tau_1 = 0.67$ and $\tau_2 = 42 \mu s$). The results indicate that the $\tau_1$ process is approximately three times slower for the full-length protein, whereas the $\tau_2$ process is little influenced, meaning that the rate of the initial structure change of the sensor domain is significantly affected by the presence of the signaling domain.

**Time-resolved UVRR Spectra of Full-length Y70F Mutant**—The Fourier Transform Infrared (FTIR) study by Pinakoulaki et al. (15) suggested that Tyr-70 is crucial for ligand recognition and discrimination. To investigate the role of Tyr-70, we performed time-resolved UVRR experiments on the Y70F mutant of full-length HemAT-Bs, and the results are shown in Fig. 7A. Although the raw UVRR spectrum ($a$) looks similar to Fig. 6A, the spectral evolution pattern ($b$–$j$) is notably different. The independent patterns contained in Fig. 7A were obtained mathematically by analysis I and the two independent spectra are depicted as spectra C and D in supplemental Fig. S5. Although the quality of these spectra is not so good, the absence of Y8a in both spectra C and D (supplemental Fig. S5) seems plausible compared with the corresponding spectra of the WT proteins (supplemental Fig. S4). Furthermore, the Y8a band is definitely absent in the static difference spectrum (reduced $- CO$) for the Y70F mutant (Fig. 7A$k$). This is presumably due to the absence of Tyr-70, indicating that Tyr-70 is the origin of the spectral changes observed for Y8a upon CO photolysis in the WT.

The individual band-fitting analysis was carried out through analysis II in the same way as done for the WT full-length protein, and the results are summarized in Fig. 7B and Table 1. Two time constants were obtained, $\tau_1 = 0.62 \pm 0.02$ and $\tau_2 = 51 \pm 1 \mu s$, which are similar to those obtained for the WT full-length protein, although the first phase was slightly slower. This means that a conformational change around Tyr-70 is not tightly cou-

**TABLE 1**

<table>
<thead>
<tr>
<th>Mode</th>
<th>$\tau_1$ ($\mu s$)</th>
<th>$\tau_2$</th>
<th>Full-length</th>
<th>Y70F</th>
</tr>
</thead>
<tbody>
<tr>
<td>W16</td>
<td>0.24 (5.5)</td>
<td>27 (7.9)</td>
<td>0.46 (7.2)</td>
<td>0.63 (6.8)</td>
</tr>
<tr>
<td>W3</td>
<td>0.17 (3.8)</td>
<td>53 (9.2)</td>
<td>0.44 (8.6)</td>
<td>0.60 (6.0)</td>
</tr>
<tr>
<td>F8a</td>
<td>0.16 (4.8)</td>
<td>43 (13)</td>
<td>0.42 (12.3)</td>
<td>0.62</td>
</tr>
<tr>
<td>Y8a</td>
<td>0.21 (5.2)</td>
<td>55 (14)</td>
<td>0.44</td>
<td>47</td>
</tr>
<tr>
<td>Average</td>
<td>0.20</td>
<td>45</td>
<td></td>
<td>51</td>
</tr>
</tbody>
</table>
pled with conformational changes associated with the W3 and F8a signals, particularly in the slower phase.

**DISCUSSION**

Ligand binding to (or dissociation from) the heme iron in the sensor domain induces a structural change in heme, which is communicated to the surrounding protein, causing its own structural change in turn, including an alteration in the hydrogen-bonding interactions and/or environmental changes related to the side chains of the Trp, Tyr, and Phe residues as well as conformational changes in the polypeptide skeleton. This study detected the UVRR spectral changes in these aromatic residues as a function of time in the range of 0.1–200 s following CO photolysis. The temporal changes in the spectra indicated the presence of two observable intermediates, named C and D species, which yielded different spectral patterns, characterized by changes in the intensity of the marker bands. Consequently, the UVRR spectral changes sensitively reflect shifts in the absorption maximum of specified aromatic residues caused by environmental changes, allowing an examination of communication pathway (34, 35).

**Structural Changes of Protein around Trp-132 Residue**—The full-length HemAT-Bs contains a single Trp residue, Trp-132, in the sensor domain. This residue is located in the G-helix in the heme proximal pocket. The crystal structure of the CN-bound form of the truncated sensor domain suggested that Trp-132 does not form a hydrogen bond with the surrounding residues (12). However, the previous static UVRR results had suggested that Trp-132 forms a moderately strong hydrogen bond with nearby residues or water in the CO-free form, and this hydrogen bond is hardly altered upon the binding of different ligands to a reduced heme (28). Trp residues have been often used as a practical probe for helix displacement in heme proteins. In the case of Mb, for instance, the Trp-7 and Trp-14 residues were used to probe the displacement of A-helix upon the binding of different ligands to heme (20, 27). For CooA, a CO sensor protein, Trp-110 was used to probe the alteration of the C-helix upon CO binding (23). Similarly, in this study, Trp-132 of HemAT-Bs was used as a probe to monitor the displacement of the G-helix.

For the C intermediate of the sensor domain, positive peaks were observed for W16 and W3. The W16 band is intensity enhanced and up-shifted to 1017 cm\(^{-1}\) at \(\Delta t \approx -0.2\) μs, whereas the W3 band is reduced in intensity around \(\Delta t \approx -45\) μs in the D intermediate (Fig. 3). Similar results were observed for the full-length protein (supplemental Fig. S4). The intensity of the Trp bands is known to be sensitive to environmental
hydrophobicity and/or hydrogen bonding interactions (18, 34, 35). Because the hydrogen bond of Trp-132 with a nearby residue or water molecule is hardly altered by ligand binding (28), it is reasonable to conclude that the hydrophobicity around Trp-132 is changed upon CO photolysis. According to a recent time-resolved absorption and photoacoustic study of HemAT (30), volume changes occur at 50 ns after CO photolysis, suggesting an increase of the solvent-accessible area of the protein upon CO dissociation. Chi and Asher (34) suggested that a Trp residue in a less hydrophobic environment yields a blue-shifted Bb absorption maximum. It is also known that W16 vibration gains intensity in resonance with the Bb transition (36, 37). As the wavelength of the 220 nm probe pulse lies on the blue side of the Bb absorption band, an increase in the intensity of the Trp bands observed at the early delay times implies an appreciable approach of the absorption maximum toward the excitation wavelength and thus a decrease in the local hydrophobicity. This is compatible with an increase of the solvent accessible area of the protein near Trp-132 at the early stage.

The previous static UVRR results indicated that Trp-132 and Tyr-133 in the G-helix undergo a change in hydrophobicity upon the binding of O2 and CO to the heme (28). In addition, the superimposition of the crystal structures of the CN-free and CN-bound forms of the sensor domain reveals that there is a noticeable alteration of the G-helix upon CN binding. The root mean square deviations of the Cn atoms in the G-helix (131–151) with respect to the ligand-free reduced form are ~0.41 and 0.43 Å for the A and B subunits, respectively. Taken together with the present results, it is most plausible that change in hydrophobicity around Trp-132 is achieved by displacement of the G-helix at 0.2 μs after CO photolysis. However, the negative W3 peak at 45 μs is similar to those obtained for the static (reduced - CO) difference spectrum and indicates that the environment around Trp-132 is altered so as to be more hydrophobic through structural rearrangements of the G-helix during relaxation of the protein conformation.

**Structural Changes of Protein in Proximity of Tyr-70 Residue**

The full-length HemAT-Bs contains six Tyr residues, five of which are located in the sensor domain (Tyr-13, Tyr-49, Tyr-70, Tyr-133, and Tyr-148), whereas Tyr-184 stays in the linker region between the sensor and signaling domains. The present study using the WT protein revealed the appearance of a positive peak for Y8a in the C intermediate, whereas there was a negative peak in the D intermediate (Fig. 3 and supplemental Fig. S4). These peaks were absent in the Y70F spectra (supplemental Fig. S5), which allows the clear assignment that it is Tyr-70 that gives rise to the spectral changes. It is known from studies of model compounds that the intensity of the Tyr bands
is influenced by the hydrophobicity of its surroundings (34) and that the formation or breaking of a hydrogen bond between its phenoxyl side chain and a nearby residue significantly influences both the intensity and frequency of the Tyr bands (18, 19, 21, 38). Because no frequency shift was observed for any of the Tyr bands in the time-resolved spectra, it is reasonable to conclude that the Tyr-70 residue in the B-helix only undergoes a hydrophobicity change upon CO photolysis. This is consistent with a previous static UVRR study indicating that there is no change in the hydrogen bonding interactions of any of the Tyr residues upon the binding of CO and O₂ to the heme (28). The present conclusion is also apparently consistent with the previous visible RR study, where the heme-bound O₂ was found to be hydrogen-bonded to Thr-95, but not to Tyr-70 (13), although the residue interacting with the heme-bound CO is different from the O₂ case. This is because the Fe-O-O and Fe-C-O bond angles are different. In fact, Pinakulaki et al. (15) recently reported FTIR data which imply that the hydrogen-bonding counterresidue is not Thr-95 in the CO-bound form. Their results using the Y70F mutant suggested that Tyr-70 should have a role in the hydrogen bond formation in the distal heme pocket upon CO binding, even though Tyr-70 itself does not form a hydrogen bond.

The superimposition of the crystal structures of the CN-free and CN-bound forms revealed appreciable movement of the B-helix upon CN-binding, especially of the B-subunit. The root mean square deviations of the Cα atoms in the B-helix (63–72) with respect to the CN-free form were fairly large (0.16 and 0.32 Å for the A and B subunits, respectively). Thus, taken together with the present results, we suggest that the observed Y8a positive peak of Tyr-70 at 0.2 μs after CO photolysis can be attributed to the displacement of the B-helix and that the negative peak of Y8a at ∼45 μs, which is similar to that obtained for the static (reduced – CO) difference spectrum, indicates that the hydrophobicity near Tyr-70 changes through the structural relaxation of the B-helix.

Finally, it should be mentioned that Yoshimura et al. (29) suggested from the time-resolved visible RR results that Tyr-133 forms a hydrogen bond with the heme-proximal ligand (His-123) in the CO-bound form on the basis of a 2 cm⁻¹ downshift of the Fe-His stretching (νFe-His) band approximately 100 ps after CO photodissociation. The present instrument cannot probe such a rapid event, but UVRR investigation of the corresponding dynamics of Tyr-133 may be of interest in relation to the B intermediate (defined in Scheme 1).

**Structural Changes in Protein around Phe Residues**—In the spectra of the C and D intermediates of both the sensor and full-length proteins, the F8a Raman band only undergoes intensity changes upon CO photolysis. In the C intermediate of the sensor domain, a positive peak is observed for F8a at ∼0.2 μs, whereas a negative peak is seen at ∼45 μs. These changes are most likely to arise from changes that take place in the environment around Phe-69 in the B-helix and/or Phe-137 in the G-helix (Fig. 1), which are only ∼3.5 Å apart from the heme (12). The environmental changes in the vicinity of Phe-69 and/or Phe-137 are presumably attributed to the displacement of the B- and G-helices, as explained above.

**Insights into Signal Transduction Mechanism of HemAT-Bs**—In the present study, we monitored the protein dynamics that occurred upon CO photolysis and found that both the B- and G-helices are displaced at 0.45 μs (0.20 μs in the case of the isolated sensor domain) and at 45 μs (for both the truncated and full-length proteins). These results suggest that both the B- and G-helices are involved in the signaling pathway. We also used the Y70F mutant to demonstrate that the conformational changes that took place around Tyr-70 in the B helix on the distal side of heme and around Trp-132 in the G-helix on the proximal side of heme are not tightly coupled with each other.

A signal transduction mechanism has been proposed for general MCPs as follows. The binding of an effector molecule to the sensor domain induces sliding and/or rotational movement of the transmembrane four-helix bundle that connects the periplasmic sensor domain with the cytoplasmic signaling domain. This movement is considered to be a key step in the signal transduction mechanism of MCP (39, 40). Although HemAT-Bs lacks the transmembrane region, the G- and H-helices of the two subunits form an antiparallel four-helix bundle, as illustrated in Fig. 8A, and they are present in the C-terminal region of the sensor domain. In addition, the H-helix is continu-
uous with the extended helical structure of the signaling domain (Fig. 8B). X-ray analysis of the truncated HemAT-Bs demonstrated that both the G- and H-helices (as well as B-helix) undergo significant displacement upon CN binding which may trigger conformational changes from the sensor domain to the signaling domain (12). Our present observation of the Trp-132 signal in the G-helix provides further experimental support for this proposed signaling mechanism.

The importance of the G-helix was previously suggested by Pinakoulaki et al. (41) who pointed out from the FTIR spectra that HemAT-Bs has a ligand accommodation cavity in the proximal heme pocket near Tyr-133 of the G-helix and that the long-lived docking of CO causes protein fluctuations around the cavity. These protein fluctuations may create an exit channel by which a ligand can escape from the protein. Such dynamics near Tyr-133 at the early stage, however, cannot be probed in the present study due to the limitation of the (instrumental) time resolution, as explained above. This will be taken up in future investigations.

On the other hand, Yoshimura et al. (14) proposed that certain initial events are evoked upon O2 binding. They found the formation of a hydrogen bond between His-86 from the CE loop and the heme propionate. In this scheme, when a ligand binds to the heme, the conformation of the propionate is altered, and then this hydrogen bond triggers a conformational change in the CE loop. This conformational change is propagated to the E-helix and finally shifts Thr-95 to the proper position so as to form a hydrogen bond with the heme-bound O2 (14). We have previously shown that these hydrogen bonds communicate the structural changes in the heme to the B- and G-helices of the protein and presumably also to the signaling domain upon the binding of O2 (28). It is important to note here that such a hydrogen bond network is absent in the CO-bound form, and this is a key point in distinguishing O2 from CO. The signaling mechanism deduced from the present Raman experiments is illustrated schematically in Fig. 9. The displacement of the B-helix (on the distal side of heme) may be associated with this molecular recognition between O2 and CO.

It is highlighted in Fig. 9 that, although Tyr-70 in the B-helix and Trp-132 in the G-helix change in hydrophobicity upon the binding of CO to the heme, the structural changes of the sensor domain are not conveyed to the signaling domain for CO, based on the absence of the amide I peak for the full-length protein and the absence of the peak from Tyr-184 located in the linker region. To understand the signal transduction mechanism fully, the dynamics of the H-helix and the interaction between the sensor and signaling domains should be investigated by making mutant proteins with UVRR probes (Trp or Tyr) at a greater number of sites.

**CONCLUSIONS**

To the best of our knowledge, this is the first site-specific observation of the protein dynamics in the communication pathway from the sensor to signaling domain of HemAT-Bs and was performed using time-resolved UVRR spectroscopy. The temporal behavior of the Raman intensities of the Trp, Tyr, and Phe residues suggests the presence of at least two phases of conformational changes in a time range of nanoseconds to hundreds of microseconds following CO photodissociation. The increase in the intensity of Trp, Tyr, and Phe bands within hun-
dreds of nanoseconds indicates a displacement of the B- and G-helices, suggesting that the heme structural changes are communicated to specific sites in the sensor domain. The recovery in the intensity at around 45 μs is followed by a further decrease in the intensity at hundreds of microseconds, suggesting that a continuous conformational relaxation occurs in the ligand-free reduced form.

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