Pathway of Information Transmission from Heme to Protein upon Ligand Binding/Dissociation in Myoglobin Revealed by UV Resonance Raman Spectroscopy*§

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Gas sensory heme proteins respond to their environment by binding a specific gas molecule to heme and transmitting this primary binding signal to the protein. How the binding signal is transmitted from the heme to the protein remains to be clarified. Using UV resonance Raman (UVRR) spectroscopy, we investigated this pathway in sperm whale myoglobin as a model gas sensory heme protein. Based on the UVRR data and the effects of deleting one of three important pathways (His-93, 6-propionate, or 7-propionate), we determined the changes in the conformation of globin that occur upon binding of CO, nitric oxide (NO), or O2 to heme and how they are transmitted from heme to globin. The UVRR results show that heme discriminates different ligands, resulting in different conformations in the globin protein. Specifically, NO induces changes in the spectrum of Trp residues in the A-helix that are significantly different from those induced by O2 or CO binding. On the other hand, binding of O2 to heme produces changes in the Tyr residues of the H-helix that are different from those induced by CO or NO binding. Furthermore, we found that cleavage of the Fe-His-93 covalent bond eliminates communication to the terminal region of the H-helix and that the 7-propionate hydrogen-bonding network is essential for transmitting the CO or NO binding signal to the N and C termini. Finally, the 6-propionate is important only for NO binding. Thus, the hydrogen-bonding network in the protein appears to be critical for intramolecular signal transduction in gas sensory heme proteins.

The structural change in heme that occurs upon ligand binding/dissociation is the main process underlying the cooperativity in oxygen binding by Hb (1) and signal transduction by gas sensory heme proteins (2–4). This process is thought to mediate transmission of the heme ligand-binding signal to the heme-binding domain of the protein. Mb is a small heme-containing protein that can reversibly bind diatomic gaseous molecules (O2, NO,2 and CO). Because Mb is well characterized, it is an ideal model for investigating the interactions between heme and protein moieties of gas sensory heme proteins. The deoxy form of Mb (deoxygenMb) adopts a five-coordinate high spin structure with the iron atom 0.3 Å out of the heme plane, whereas the heme is planar in the ligated form (5–8). The out-of-plane displacement of the iron atom is thought to be responsible for a conformational change between the deoxy and ligated forms of Mb.

Our previous ultraviolet resonance Raman (UVRR) experiments, wherein Mb was excited at 244 nm, suggested that slight structural changes in Trp-7 and Tyr-151 occur in Mb upon CO binding (9). In addition, time-resolved resonance Raman (RR) measurements of the Fe-His stretching mode with visible laser pulses have demonstrated that, for Mb, the structural change takes place rapidly (time constant of ∼120 ps) (10). However, the transmission of the heme structural change to globin via His-93 has become a matter of controversy because a fast structural change has also been observed for the Gly-93(+1Imd) mutant, which lacks the Fe-His covalent bond (11).

Fig. 1 illustrates the hydrogen-bonding networks in the vicinity of heme in sperm whale Mb (swMb) as revealed by x-ray crystallography (6). The proximal His (His-93) forms hydrogen bonds with Ser-92 and Leu-89. The CO2 group of the propionate side chain at position 7 forms hydrogen bonds with His-97 and Ser-92, whereas the CO2 group of the propionate side chain at position 6 participates in a hydrogen-bonding network with Arg-45, H2O, and His-64 (distal His). It is very likely that these hydrogen-bonding systems transmit conformational changes in heme upon ligand binding to the globin protein.

We next examined swMb by UVRR spectroscopy to explore the role of hydrogen bonds in this communication process. We first analyzed the conformational changes in Mb upon binding of various gaseous ligands (CO, NO, and O2). We found that heme discriminates between different ligands by inducing significantly different conformations in the A- and H-helices. Fur-
thermore, we monitored the UVRR spectral changes of Trp and Tyr residues that occur upon CO and NO binding to versions of Mb in which the predicted pathways for transmission of conformational changes (His-93, 6-propionate, or 7-propionate) were eliminated by mutation or chemical modification of the heme. We found that the 7-propionate hydrogen-bonding network in the heme proximal site is essential for communicating the conformational changes to the N and C termini upon binding of CO or NO to heme, whereas the 6-propionate pathway is important only for the communication of NO binding. Furthermore, we found that cleavage of the Fe–His-93 covalent bond eliminates the communication to the C terminus.

EXPERIMENTAL PROCEDURES

Sample Preparation—Native (NT) swMb (Biozyme) and horse Mb (hMb, Sigma) were used without further purification. Mb mutants including W7F-, W14F-, H93G(+Imd), S92A, and R45L Mb were expressed and purified as described previously (12, 13) with some modifications (9). Chemically modified hemes in which the 7- (7M6P) or 6-propionate (6M7P) of protoporphyrin IX was replaced by a methyl group were synthesized as described elsewhere (14). The Fe(III) complexes were reconstituted into the swMb apoprotein (15). The Fe-CO and Fe-His stretching modes of these preparations observed with visible RR spectra were not significantly different from those of the NT Mb, although the heme spectra in the lower frequency region were distinct from that of the NT protein.

For UVRR measurements, the concentration of protein was adjusted to 200 and 300 μM in 50 mM phosphate buffer (pH 7.0) for excitation at 229 and 244 nm, respectively. As an internal intensity standard for UVRR difference spectra, 400 and 100 mM Na2SO4 were included in the samples for excitation at 229 and 244 nm, respectively. The ferric form of NT swMb was directly used from the commercial product. DeoxyMb was prepared by adding a minimal amount of sodium dithionite solution (final concentration 1–2 mM) to the protein solution under a nitrogen atmosphere. The O2-bound form was prepared by passing the dithionite-reduced Mb protein through a small Sephadex G25 column under aerobic conditions and then saturating the sample with oxygen by flushing it with pure oxygen gas in a sealed Raman cell. The CO or NO adducts of Mb were prepared by introducing pure CO or NO gas into the Raman cell with an airtight syringe through a rubber septum. The formation of six-coordinate ligand-bound forms was confirmed by examining the optical absorption spectra.

UV Resonance Raman Measurements—UV resonance Raman measurements were performed as described previously (16). The UV probe light at 229 or 244 nm was generated by an intracavity frequency-doubled argon ion laser (Coherent; Innova 300 FReD). The second harmonic in the laser output was separated from the fundamental with a Pellin-Broca prism and focused into a protein sample solution. An ~100-μl aliquot of the sample solution was placed into a spinning cell with a stirring function (16). Light scattered at a right angle was collected with the objective lens of a UV microscope, dispersed with a 126-cm single monochromator (Spex 1269) equipped with a 3600 groove/mm holographic grating, and detected with an intensified charge-coupled detector (Princeton Instruments; model ICCD-1024MG-E/I). The laser power at the sample point was 0.2–0.3 milliwatt, and the spectral resolutions were set at 6.9 and 7.8 cm−1 for 244 and 229 nm excitations, respectively. The protein sample was replaced with fresh one every 5–10 min, and the total exposure time to obtain each spectrum was ~1 h. The integrity of the sample after exposure to UV laser light was confirmed by comparing the visible absorption spectra obtained before and after the UVRR measurements. If some spectral changes were found, the Raman spectrum was discarded. Raman shifts were calibrated with cyclohexane, trichloroethylene, 1,2-dichloroethane, and toluene.

RESULTS

Changes in the 229-nm-excited UVRR Spectrum of Mb upon Binding of Different Ligands—Fig. 2 shows the raw 229-nm-excited UVRR spectrum of the deoxy (a) form of NT swMb and the deoxy–ferric (b), deoxy–O2 (c), deoxy–CO (d), and deoxy–NO (e) difference spectra for NT swMb. The raw spectrum of NT swMb is dominated by the bands arising from two Trp (Trp-7 and Trp-14) and three Tyr (Tyr-103, Tyr-146, and Tyr-151) residues, which are labeled W and Y, respectively, followed by their mode numbers (17). Because the protein concentrations are the same in all measurements performed at a given excitation wavelength, the intensities of the Raman features were comparable in all of the raw spectra. The difference spectra were calculated so that the band for SO42− (981 cm−1), which was present at the same level in all samples as an internal intensity standard, would be zero.
Difference spectrum b shows strong negative bands at 758 (W18), 877 (W17), 1011 (W16), 1359 (W7), 1558 (W3), and 1616 cm\(^{-1}\) (Y8a/W1), indicating an increase in the intensity of the Trp and Tyr bands in the ferric form. Because of the importance of the physiological ligand, O\(_2\), for Mbs, we examined the UVRR spectral changes of NT Mb upon binding of O\(_2\). It is known that in the O\(_2\)-bound form, the heme iron is rapidly photo-oxidized even at very low laser power. The absorption spectra showed that the sample was partially photo-oxidized to the ferric form (\(~30\%) after exposure to the UV light, independent of solution in the pH range between 7 and 9. Thus, to obtain the spectrum of the O\(_2\)-bound form, we subtracted the contribution of the spectrum of the ferric form from the observed spectrum. The resulting UVRR spectral changes upon O\(_2\) binding are displayed in spectrum c. The low signal-to-noise ratio of spectrum c is due to this double-difference procedure. The deoxy-O\(_2\) spectrum (c) reveals small features near the W18, W16, and W3 bands, implying that the intensity of the Trp Raman bands is slightly enhanced at very low laser power. The absorption spectra showed that the sample was partially photo-oxidized to the ferric form (\(~30\%) after exposure to the UV light, independent of solution in the pH range between 7 and 9. Thus, to obtain the spectrum of the O\(_2\)-bound form, we subtracted the contribution of the spectrum of the ferric form from the observed spectrum. The resulting UVRR spectral changes upon O\(_2\) binding are displayed in spectrum c. The low signal-to-noise ratio of spectrum c is due to this double-difference procedure. The deoxy-O\(_2\) spectrum (c) reveals small features near the W18, W16, and W3 bands, implying that the intensity of the Trp Raman bands is slightly enhanced at very low laser power. The absorption spectra showed that the sample was partially photo-oxidized to the ferric form (\(~30\%) after exposure to the UV light, independent of solution in the pH range between 7 and 9. Thus, to obtain the spectrum of the O\(_2\)-bound form, we subtracted the contribution of the spectrum of the ferric form from the observed spectrum. The resulting UVRR spectral changes upon O\(_2\) binding are displayed in spectrum c. The low signal-to-noise ratio of spectrum c is due to this double-difference procedure. The deoxy-O\(_2\) spectrum (c) reveals small features near the W18, W16, and W3 bands, implying that the intensity of the Trp Raman bands is slightly enhanced at very low laser power.

Assignment of Trp Spectral Changes Induced by CO Binding in the 229-nm-excited Spectra—Fig. 3 displays the raw UVRR spectrum of the deoxy form (a) of NT swMb excited at 229 nm and the deoxy – CO difference spectra for NT (b), W14F- (c), and W7F-Mb (d). The frequency of the W17 band (~875 cm\(^{-1}\)) serves as a marker of hydrogen bonding of the Trp indole ring (18). This band of NT Mb (a) appears at 877 cm\(^{-1}\), indicating moderate strength of hydrogen bonding, consistent with the crystal structure, wherein both Trp-7 and Trp-14 form hydrogen bonds with water molecules (6).

The W7 band appears as a doublet at 1360/1340 cm\(^{-1}\) in the raw spectrum of NT Mb (a). The expanded spectra W7 bands of the W7F- and W14F-Mb are displayed in Fig. 3, inset A. This
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doublet arises from Fermi resonance between the \( N_1 - C_8 \) stretching fundamental (W7 mode) and the combination of out-of-plane bending vibrations (17). The intensity ratio of the W7 doublet serves as a marker of the hydrophobicity of the environment around the indole ring (17, 18). The \( I_{1358}/I_{1340} \) ratio for Trp-14 is larger than that for Trp-7, which is consistent with the fact that Trp-14 is buried in a more hydrophobic environment than Trp-7 (5, 6).

The frequency of the Trp W3 mode is sensitive to the absolute value of the torsion angle \( (\chi^{2-1}) \) about the bond \( (C_\text{H}-C_\text{C}) \) connecting the indole ring to the peptide main chain (18). It appears at 1558 cm\(^{-1}\) in spectrum \( a \), and expanded spectra for NT and the W14F- and W7F-Mb (Fig. 3, inset \( B \)) indicate that the more precise frequencies are 1557 cm\(^{-1}\) for Trp-7 and 1560 cm\(^{-1}\) for Trp-14. The 1557 cm\(^{-1}\) band for Trp-7 suggests a \( \chi^{2-1} \) of 114°, which is comparable with the value of 118° determined from the crystal structure (6); however, the frequency of 1560 cm\(^{-1}\) for Trp-14 is unexpectedly high and does not fit well with the empirical correlation curve (18).

As shown in Fig. 3b, the W18, W17, W16, W7, and W3 bands of NT Mb exhibit prominent negative features, implying an increase in intensity of the Trp bands in the CO-bound form. Similar features have been observed for the W14F-Mb (Fig. 3c). The difference spectrum of W7F-Mb (Fig. 3d), however, displays no such negative features. These results indicate that the spectral changes that occur in NT Mb upon CO binding mainly arise from Trp-7 rather than from Trp-14.

Changes in the 229-nm-excited UVRR Spectrum upon CO Binding to Mb with a Modified Heme and Mutant Mbs—Fig. 4 shows the raw UVRR spectrum of NT deoxyMb \( (a) \) excited at 229 nm and the deoxy – CO difference spectra for NT \( (b), H93G(+Imd) \) \( (c) \), 6M7P- \( (d) \), 7M6P- \( (e) \), and S92A Mb \( (f) \). Unexpectedly, the difference spectrum for H93G(+Imd) \( (c) \), in which exogenous imidazole is bound as a ligand of iron instead of His-93, is very similar to that of NT \( (b) \) despite the absence of a covalent bond between the heme and globin. Because this strongly suggests that the Fe-His-93 bond is not directly responsible for the spectral change of Trp-7, we examined other possibilities by obtaining spectra \( d-f \). As described by Hayashi et al. (14), we synthesized the modified iron-protoporphyrin in which the 6- or 7-propionate is selectively replaced by a methyl group and used them to reconstitute Mb apoprotein (6M7P- and 7M6P-Mb, respectively). The deoxy – CO difference UVRR spectra were obtained in a similar way as for NT Mb. The difference spectrum of 6M7P-Mb \( (d) \) exhibits negative features for Trp bands similar to those observed for NT Mb \( (b) \). In contrast, the difference spectrum of 7M6P-Mb \( (e) \) shows small positive/large negative peaks at 750/760 and 1006/1014 cm\(^{-1}\). In addition, a strong negative peak appeared at 1562 cm\(^{-1}\) \( (W3) \) in contrast to the negative peak at 1557 cm\(^{-1}\) in spectra \( b-d \) (Fig. 4, inset). This frequency shift is probably caused by an upshift of the Trp-7 W3 band coupled with a large increase in intensity upon CO binding and may indicate a slight increase \( (10°-20°) \) in the \( \chi^{2-1} \) angle of Trp-7 \( (9, 18) \); however, we cannot rule out the possibility that Trp-14 undergoes spectral changes in 7M6P-Mb because W3 of Trp-14 is located at 1560 cm\(^{-1}\). Regardless, the spectrum of 7M6P-Mb \( (e) \) differs significantly from the spectra of NT and H93G(+Imd) Mb, indicating that the structural change in Trp-7 was perturbed by removal of the 7-propionate group or its hydrogen-bonding network.

To confirm the effect of hydrogen bonding by 7-propionate, its hydrogen-bonding partner, Ser-92 (Fig. 1), was replaced with Ala (Fig. 4, spectrum \( f \)). Its spectrum is similar to spectrum \( e \) but different from spectrum \( b \). Furthermore, the difference spectra \( (e \text{ and } f) \) reveal a derivative pattern near 1616 cm\(^{-1}\), indicating an upshift of the Y8a/W1 frequency in the CO-bound form, whereas this frequency is not up shifted in spectra \( b-d \). Consequently, we concluded that the structural change of heme upon CO binding is communicated to Trp-7 via the hydrogen-bonding network through the 7-propionate and Ser-92.
Assignments of Spectral Changes upon NO Binding in the 229-nm-excited Spectra—Fig. 5 displays the raw UVRR spectrum of the deoxy form of NT Mb (a) and the difference spectra (deoxy-NO) of NT Mb (b) and the W14F- (c) and W7F-Mb (d). The inset shows the enlarged difference spectra of W18 mode. The sample concentration was 200 μM in 50 mM phosphate buffer, pH 7.0. 400 mM Na2SO4 was used as an internal intensity standard. a.u., arbitrary units.

FIGURE 5. The 229-nm-excited UVRR spectra of NT and Trp mutants of swMb. Shown are the spectrum for the deoxy form of NT Mb (a) and the difference spectra (deoxy-NO) of NT Mb (b) and the W14F- (c) and W7F-Mb (d). The inset shows the enlarged difference spectra of W18 mode. The sample concentration was 200 μM in 50 mM phosphate buffer, pH 7.0. 400 mM Na2SO4 was used as an internal intensity standard. a.u., arbitrary units.

UVR Spectral Changes of Mb Mutants upon NO Binding in the 229-nm-excited Spectra—Fig. 6 shows the raw UVRR spectrum of the deoxy form of NT swMb (a) and the deoxy-NO difference spectra for NT Mb (b) and the H93G (c), S92A (d), and R45L (e) forms of Mb. The sample concentration was 200 μM in 50 mM phosphate buffer, pH 7.0. 400 mM Na2SO4 was used as an internal intensity standard. a.u., arbitrary units.

FIGURE 6. The 229-nm-excited UVRR spectra of NT and mutant swMbs. Shown are the spectrum for the deoxy form of NT Mb (a) and the deoxy-NO difference spectra for NT Mb (b) and the H93G (c), S92A (d), and R45L (e) forms of Mb. The sample concentration was 200 μM in 50 mM phosphate buffer, pH 7.0. 400 mM Na2SO4 was used as an internal intensity standard. a.u., arbitrary units.
Changes in the 244-nm-excited UVRR Spectrum of Mb upon Binding of Different Ligands—The raw UVRR spectrum of the deoxy form of NT swMb is shown in Fig. 7, trace a. The Tyr bands are significantly enhanced when compared with Fig. 2a. Trace b shows the deoxy – CO difference spectrum of NT swMb, revealing a derivative pattern near 1177 cm\(^{-1}\) (Y9a), thus indicating an upshift of the Y9a frequency in the CO-bound form. This change was not observed for hMb, which lacks Tyr-151 (replaced by Phe) and therefore is attributed to Tyr-151 in swMb (9). The most prominent feature in spectrum b is a positive peak at 1619 cm\(^{-1}\), which is due to a decrease in intensity of the Y8a band in the CO-bound form. It may also contain contributions from C=\(C\) stretching of heme vinyl groups. In addition, it shows a positive feature at 1560 cm\(^{-1}\), indicating an increased intensity of W3 in the deoxy form. These changes in spectrum b are identical to those observed previously by Haruta et al. (9).

Fig. 7, traces c and d, show the difference spectra of the deoxy – NO forms of swMb and hMb, respectively, and traces e and f show the difference spectra of deoxy – O\(_2\) and deoxy – ferric forms of swMb, respectively. The intensity-normalized raw spectra of the Y9a band are displayed in the inset for the ligand-bound (dotted line) and -free forms (solid line) as references for the difference spectra shown in traces b–f. Trace c reveals a derivative feature at 1177 cm\(^{-1}\) and a positive band at 1619 cm\(^{-1}\), similar to those observed upon CO binding.

To assign the spectral change of the Y9a band observed upon NO binding by swMb, we examined the UVRR spectra of hMb, which lacks Tyr-151. Trace d shows the deoxy – NO difference spectrum of NT hMb, which has a small negative peak instead of a derivative. This indicates a small increase in intensity without a frequency shift for Tyr-103 and Tyr-146 upon NO binding. Accordingly, the upshift in Y9a upon NO binding in swMb can be attributed to Tyr-151.

We additionally examined the UVRR spectral changes of NT Mb upon binding of O\(_2\) and subtracted the ferric contribution to the O\(_2\)-bound form in a way similar to that carried out for the 229 nm excitation (Fig. 2c). The UVRR spectral changes upon O\(_2\) binding, displayed in spectrum e, reveal a positive feature near Y9a, implying a decrease in intensity for the O\(_2\)-bound form. We noted that the reduction in intensity of Y9a upon O\(_2\) binding is coupled with a frequency upshift (Fig. 7, inset). In addition, the Y8a band (1619 cm\(^{-1}\)) of the O\(_2\)-bound form (e) appears less intense than the positive peaks in spectra b and c. We further noted that trace f displays features at W3 and Y8a distinct from those observed upon O\(_2\) binding. Therefore, we concluded that the spectral changes of Tyr caused by O\(_2\) binding, especially those near the Y9a band (traces b–e), differ from those observed caused by NO and CO binding.

Changes in the 244-nm-excited UVRR Spectrum upon CO Binding to Mb with a Modified Heme and Mutant Mbs—Fig. 8 shows the raw UVRR spectrum of the deoxy form of NT swMb excited at 244 nm and the deoxy – CO difference spectra of NT swMb (b), H93G(+Imd) (c), 6M7P-(d), R45L (e), 7M6P-(f), and S92A Mb (g). H93G(+Imd) (c) exhibits a negative Y9a peak, which appears to be different from other spectra. Arg-45 is a hydrogen-bonding partner of 6-propionate (Fig. 1), and, therefore, its mutation to Leu disrupts the hydrogen-bonding network through 6-propionate. The difference spectra for 6M7P-(d) and R45L Mb (e) are very similar and have features like those observed for NT Mb (b). On the contrary, the features at 1558 and 1619 cm\(^{-1}\) in 7M6P-Mb (f) are distinct from those in spectrum b.

To confirm that the spectral change in 7M6P-Mb is due to changes in the hydrogen-bonding network, we changed the hydrogen-bonding partner of 7-propionate from Ser to Ala by site-directed mutagenesis. The results are shown in spectrum g. As expected, the overall features of spectra f and g are similar, but the effects of 7M6P and S92A on Y9a at 1177 cm\(^{-1}\) (spectra f and g, respectively) are slightly different; the S92A mutation (g) gives rise to a derivative pattern at Y9a similar to that of NT Mb (b), whereas a small negative feature is observed for 7M6P-Mb (f).
Conformational Changes in the N Terminus upon Ligand Binding—There are two Trp residues in the A-helix of swMb. These residues can be used in UVRR spectroscopy to probe the conformational changes in the N terminus upon ligand binding because they provide information about the protein tertiary structure in solution. We found that the intensity of the 229-nm-excited Trp-7 signal is enhanced in the CO-bound form. On the other hand, binding of NO to the heme reduces the intensity of both Trp-7 and Trp-14 Raman bands. The intensities of the Trp bands are known to be sensitive to environmental hydrophobicity and/or hydrogen-bonding interactions of the Trp side chain (18–20). Because a frequency shift is not observed for the hydrogen bond marker band (877 cm⁻¹), we inferred that the hydrogen bonds between Trp-7 or Trp-14 and water molecules hardly change during the transition from the deoxy to the CO- or NO-bound forms. Thus, it is reasonable to conclude that the hydrophobicity around Trp-7 only changes upon CO binding, whereas the hydrophobicity around both Trp-7 and Trp-14 changes upon NO binding.

W7 mode is known as a marker for environmental hydrophobicity around Trp (18). The ratio of the intensity of the W7 doublet (I₁₃₅₉/I₁₃₄₀) confirmed that the hydrophobicity around Trp-7 only changes upon CO binding, whereas the hydrophobicity around both Trp-7 and Trp-14 changes upon NO binding. The higher frequency component (1359 cm⁻¹) of the W7 doublet is more intense in the CO-bound form and less intense in the NO-bound form. On the other hand, the intensity of the lower frequency component of W7 mode (1340 cm⁻¹) is unchanged in the CO- and NO-bound forms. Thus, the order of I₁₃₅₉/I₁₃₄₀ is CO > deoxy > NO. Therefore, it is clear that Trp-7 shifts to a more hydrophobic environment upon CO binding, whereas Trp-14 and Trp-7 shift to a more hydrophilic environment upon NO binding. In addition, the Trp residues in the O₂-bound form show spectral changes close to those observed upon CO binding but significantly different from those of the NO-bound form, suggesting that the Trp environments become more hydrophobic upon O₂ binding.

The superimposition of three reported crystal structures for O₂-, CO-, and NO-bound Mbs with the deoxy form (6–8) reveals that there is noticeable movement of the A-helix upon NO binding (Fig. 9), whereas there is a smaller movement upon CO or O₂ binding. The root-mean-square deviations (r.m.s.d.) of Cα atoms in A-helix (residues 4–18) with respect to deoxy form are 0.63, 0.08 and 0.29 Å for NO-, CO-, and O₂-bound forms, respectively (the resolutions of crystal structures are 1.15 Å in deoxy (6), 1.7 Å in NO- (7), 1.15 Å in CO- (6), and 1.0 Å in O₂-bound forms (8 Mb). The r.m.s.d. was calculated with Swiss-PdbViewer (21). Considering the low resolution for the NO-bound form, its r.m.s.d. value could be fluctuated. Nevertheless, the significant increment of r.m.s.d. in the NO-bound form may suggest rather larger displacement from the deoxy form. These observations are compatible with our results in that Trp residues of the A-helix experience spectral changes four times larger upon NO binding than upon CO or O₂ binding. This implies a large tertiary structural change in the A-helix toward a more hydrophilic environment upon binding of NO and a smaller structural change toward a more hydrophobic environment upon binding of CO or O₂.

C-terminal Conformational Changes upon Ligand Binding—The C-terminal region of the H-helix in swMb includes two Tyr residues, Tyr-146 and Tyr-151. A third tyrosine, Tyr-103, is located in the G-helix. The UVRR results show that the Y9a
band is upshifted in all the ligand-bound forms of Mb; however, the UVRR spectra of hMb, which lacks Tyr-151, does not show a shift in the frequency of Y9a for either the CO-bound (9) or the NO-bound form. Thus, the frequency of the Y9a band of Tyr-151 in swMb shifts upon ligand binding. In addition, the Y9a frequency shift is coupled with an intensity of decrease upon binding of O₂, as indicated by a positive peak at 1177 cm⁻¹. These results suggest that Tyr-151 undergoes environmental changes upon binding of O₂, becoming a form that differs from the CO- and NO-bound forms, although the other Tyr residues (Tyr-103 and Tyr-146) may also make small contributions to the change in the intensity of the Y9a and Y8a bands in swMb.

Because the crystal structures show that Tyr-103 is exposed to the solvent in all states (6–8), it is not expected that it contributes to the observed spectral changes. In contrast, Tyr-146, which is embedded in a hydrophobic environment, is involved in the helix-loop interaction between the F-G loop and the H-helix by forming a hydrogen bond with the backbone of Ile-99. Therefore, it is likely that Tyr-146 also contributes to the spectral changes in Y9a for the NO- and O₂-bound forms and in Y8a for the CO-, NO-, and O₂-bound forms. This would confer changes in the hydrophobicity around C-terminal residues Tyr-151 and Tyr-146 or cause substantial alterations in their hydrogen bonding upon CO and NO binding.

Studies of Hb A have suggested that the change in the position of the C-terminal residue is physiologically important. Specifically, removal of the C-terminal residue in the α (Arg-141) or β (His-146) subunit locks the tetramer in the R state and eliminates cooperativity (22, 23). This means that ligand binding to one subunit is communicated to other subunits via the C-terminal residue. Given this information, it is reasonable that Tyr-151 exhibits structural changes upon ligand binding. It is interesting that this structural change varies according to the ligand because the cooperativity of Hb also depends on the ligand.

Transmission of Structural Changes from Heme to the N terminus upon CO or NO Binding—To understand how the conformational changes are transmitted from heme to the N and C termini upon CO and NO binding, we monitored the changes in the UVRR spectra of the aromatic residues in forms of Mb in which one of the predicted pathways (His-93, 6-propionate, or 7-propionate) was eliminated by mutation or chemical modification of heme. The H93G (+1Imd) mutant serves as a mimic of NT Mb in which the covalent bond between heme and globin is cleaved and an exogenous imidazole binds to iron freely in the proximal cavity (13). The sub-picosecond time-resolved RR spectra showed ultra-rapid heme domino following ligand dissociation in this cavity mutant, suggesting that the out-of-plane movement of iron is independent of its chemical linkage to His-93 in NT Mb (11). This left the question of whether this covalent bond is responsible for communication between the heme and the protein moiety. Unexpectedly, the UVRR difference spectra of H93G(+1Imd) Mb are very similar to those of NT Mb upon CO and NO binding, despite the absence of a covalent bond between the heme and globin. This strongly suggests that the Fe-His-93 bond is not directly responsible for transferring the structural changes from the heme to the A-helix upon CO or NO binding.

As shown in Fig. 1, the 6-propionate side chain of heme is directed to the distal side of heme and forms a hydrogen bond with Arg-45. In the case of CO binding, Trp-7 of 6M7P-Mb, which lacks the 6-propionate, shows environmental changes similar to those of NT Mb, suggesting that the hydrophobic environments around the A-helix in NT and 6M7P Mbs are similar. On the other hand, binding of NO to the R45L mutant, in which the hydrogen bond between Arg-45 and 6-propionate is removed, displays negative bands, whereas NT Mb shows positive peaks. These results suggest that, as in the S92A mutant, the environment of the A-helix in the R45L mutant changes from hydrophilic to hydrophobic upon NO binding.

The crystal structures show that the Fe-C-O binding geometry is nearly linear and that its oxygen atom is 3.21 Å away from the Ne of His-64, indicating a very weak hydrogen bond (6). In contrast, Fe-N-O shows a bent geometry with an angle of 112°, and its nitrogen atom is 2.78 Å away from the Ne of His-64 (7), indicating a stronger hydrogen bond. Fig. 1 shows that the distal His-64 is involved in the hydrogen-bonding network with Arg-45 and 6-propionate through a water molecule. Regarding the strength of the electrostatic field, which is modulated by NO or CO, the elimination of the hydrogen bond between the 6-propionate and Arg-45 could disrupt the distal pocket in the NO-bound form more significantly than in the CO-bound form. This structural feature is compatible with our present results showing that the 6-propionate is important for transmitting the conformational changes from heme to the N terminus upon NO binding.

Fig. 1 shows that the 7-propionate side chains of heme form hydrogen bonds with Ser-92 and His-97. The spectral changes upon binding of CO to 7M6P- or S92A Mb are significantly different from those for NT. In addition, for the S92A mutant, NO binding causes mainly negative peaks in the difference spectrum, whereas that for NT shows positive peaks. Thus, the disruptions of the 7-propionate hydrogen-bonding network in the proximal side affect the conformational change in the N-terminal region upon CO or NO binding. The presence of a Ser or Thr at the F7 position in mammalian Mbs is highly conserved, and they form stable hydrogen bonds with nitrogen of the proximal His-93 and the 7-propionate hydroxyl group, although such hydrogen bonds are not present at the F7 position of the α- and β-chains of Hb A (24). Replacement of Ser-92 with Ala, Val, or Leu causes disorder in the heme pocket (25–27). Despite the disorder in the geometry, the kinetic data show only a slight increase in the association rate for CO and an essentially unaltered dissociation rate (25, 26). Our finding is consistent with these results in that the proximal hydrogen bonds are not only essential for maintaining the protein structure but also for transmitting the heme conformational changes to the globin moiety.

Transmission of Structural Changes of Heme to the C terminus upon CO Binding—As mentioned above, the UVRR results showed that the environment around Tyr-151 in the C terminus changes upon ligand binding. In contrast, the spectral changes for 6M7P- and R45L mutants are similar to those in NT Mb, indicating that the distal hydrogen bonds...
are not responsible for transmitting structural changes to the C terminus. On the contrary, the spectra for 7M6P- and S92A Mb exhibit significant changes in the Y8a band (1619 cm⁻¹), suggesting a difference in the communication through the 7-propionate. In particular, the dissimilar patterns at Y9a between 7M6P- and S92A Mb could be due to the absence of the hydrogen bond between the 7-propionate and His-97 in 7M6P-Mb (Fig. 1). Consequently, the absence of one of the hydrogen bonds between the 7-propionate and both Ser-92 and His-97 significantly perturbs the transmission of the structural change from heme to the C terminus. Thus, the hydrogen bonds between the 7-propionate and the F-helix regulate the changes in protein conformation that take place upon CO binding.

Role of Heme-propionate Side Chains in Heme Sensor Proteins—In heme-based sensor proteins, the conformational changes induced by binding of a gas molecule to the heme in the sensor domain modulate the activity of the functional domain. A central issue is how the heme structural changes initiated by binding of a gas molecule are propagated to the environment around the heme and, further, to the functional domain. The FixL proteins, which contain a histidine kinase domain that is regulated by oxygen binding, discriminate between O₂, NO, and CO and switch to the inactive state only upon binding of O₂ (28, 29). X-ray data for *Bradyrhizobium japonicum* FixL heme-binding domain reveal that the binding of O₂ forces Arg-220 to rupture its salt bridge to the 7-propionate in the unligated (ferri/deoxy) state and then to move to the distal heme pocket and form a hydrogen bond with O₂. Subsequently, Arg-206 forms a hydrogen bond with the released 7-propionate. In contrast, the binding of NO and CO does not cause such an alteration in the salt bridge (30). Similarly, the 7-propionate plays an important role in the intramolecular signaling of the direct oxygen sensor protein, EcDOS. Specifically, the hydrogen-bonding network between 7-propionate, Arg-97, and heme-coordinated O₂ in EcDOS is replaced by a hydrogen bond between the 7-propionate and the heme axial ligand (Met-95) upon reduction (31).

Furthermore, the RR spectra reveal that the frequency of the propionate bending mode (δ(C=C_7C_8C_9)), which is indicative of hydrogen bonding of the heme propionate (32), is located at 383 cm⁻¹ in the O₂-bound form but at 379 cm⁻¹ in the CO-bound form (33), implying that the 7-propionate hydrogen-bonding network is different for the O₂ and CO forms.

The ability of Mb to discriminate ligands is related to the ligand affinities. Practically, the electrostatic interactions between the bound ligand and the distal His along with the steric hindrance within the heme pocket (34, 35) regulate the ligand affinity. Our work demonstrates that such interactions in the heme site could influence the conformational changes in the terminal regions of the protein. In addition, some changes in the hydrogen bonds between two propionate side chains and ambient residues are involved in the process of ligand binding and play distinct roles for different ligands. This kind of interaction seems to be significant for ligand discrimination by *Bradyrhizobium japonicum* FixL heme-binding domain (28–30) and EcDOS heme-binding domain (33, 36). Based on this common viewpoint, transmission of the structural changes from heme to protein in Mb could reveal some essential aspects of the intramolecular signal transduction by heme-based sensor proteins.

In conclusion, the UVRR difference spectra selectively detected that there are changes in the environments around the Tyr residues in C-terminal and Trp residues in N-terminal of swMb upon binding of O₂, CO, and NO to the heme iron. The conformational changes in these two regions are parts of conformational changes that occur in the whole protein of swMb. Our results show that the heme discriminates between different ligands by inducing significantly different protein conformational changes in the A- and H-helices. Furthermore, we showed the roles of the Fe-His and heme propionates in communicating the conformational changes to the N and C termini.

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