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Heterogeneity between Two α Subunits of $\alpha_2\beta_2$ Human Hemoglobin and O_2 Binding Properties: Raman, ^{1}H Nuclear Magnetic Resonance, and Terahertz Spectra

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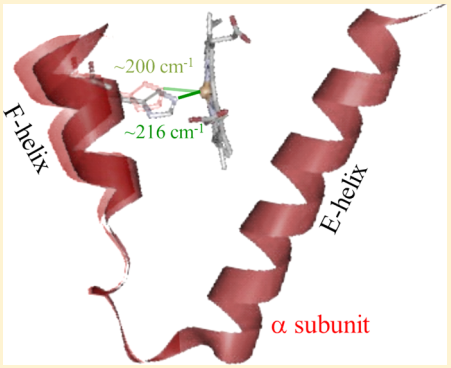
Supporting Information

ABSTRACT: Following a previous detailed investigation of the β subunit of $\alpha_2\beta_2$ human adult hemoglobin (Hb A), this study focuses on the α subunit by using three natural valency hybrid $\alpha(Fe^{2+}\text{-deoxy}/O_2)\beta(Fe^{3+})$ hemoglobin M (Hb M) in which O_2 cannot bind to the β subunit: Hb M Hyde Park (β92His → Tyr), Hb M Saskatoon (β63His → Tyr), and Hb M Milwaukee (β67Val → Glu). In contrast with the β subunit that exhibited a clear correlation between O_2 affinity and Fe^{2+}–His stretching frequencies, the Fe^{2+}–His stretching mode of the α subunit gave two Raman bands only in the T quaternary structure. This means the presence of two tertiary structures in α subunits of the $\alpha_2\beta_2$ tetramer with T structure, and the two structures seemed to be nondynamical as judged from terahertz absorption spectra in the 5–30 cm^{-1} region of Hb M Milwaukee, $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$. This kind of heterogeneity of α subunits was noticed in the reported spectra of a metal hybrid Hb A like $\alpha(Fe^{2+}\text{-deoxy})\beta(Co^{2+})$ and, therefore, seems to be universal among α subunits of Hb A. Unexpectedly, the two Fe–His frequencies were hardly changed with a large alteration of O_2 affinity by pH change, suggesting no correlation of frequency with O_2 affinity for the α subunit. Instead, a new Fe^{2+}–His band corresponding to the R quaternary structure appeared at a higher frequency and was intensified as the O_2 affinity increased. The high-frequency counterpart was also observed for a partially O_2-bound form, $\alpha(Fe^{2+}\text{-deoxy})\alpha(Fe^{2+}\text{-}O_2)\beta(Fe^{3+})\beta(Fe^{3+})$, of the present Hb M, consistent with our previous finding that binding of O_2 to one α subunit of T structure $\alpha_2\beta_2$ tetramer changes the other α subunit to the R structure.



Human adult hemoglobin (Hb A) can effectively transport O_2 from lung to tissue because of its cooperativity in O_2 binding.[1] Hb A is composed of two α subunits (141 residues) and two β subunits (146 residues), forming an $\alpha_2\beta_2$ tetramer.[1] Each subunit has one protoporphyrin IX–Fe complex, called protoheme, which is coordinatively bound to the proximal histidine (HisF8) of the F helix (called the Fe–His bond hereafter) and binds O_2 at its *trans* site. To elucidate cooperative O_2 binding of Hb A, there have been many studies since those of Bohr (Bohr effects, 1904),[2] Hill (Hill plot analysis, 1913),[3] and Adair (Adair equation, 1925).[4] Even recently, some reviews and papers have been published.[5−15]

Cooperative O_2 binding was beautifully explained in terms of transitions between two states, T (tense) and R (relaxed),[16−18] which correspond to the states of low and high O_2 affinity, respectively. Perutz elucidated the X-ray structure of Hb A[16,17] and interpreted that the T and R states practically correspond to structures of deoxy Hb A and oxy Hb A, respectively.[16−18] Accordingly, this Perutz mechanism is based on the change in the quaternary structure during the O_2 binding process. The intermediates involved were directly observed by time-resolved resonance Raman spectroscopy.[7,10,19−21] However, there are other proposals, which point out the importance of the change in tertiary structure between t and r rather than a change in quaternary structure,[6,9,15,22,23] the presence of several quaternary states[1] and structures,[11,24,25] and the changes in protein

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fluctuations, particularly of E and F helices, without a change in quaternary structure.[8,26−29]

Besides the studies that focused on the interplay of both quaternary and tertiary structures, different roles of α and β subunits in the regulation of the O_2 affinity of an $\alpha_2\beta_2$ tetramer are also noted.[10,12,15,30−46] Indeed, when Hb A is separated into subunits, α and β form a homodimer (α_2) and homotetramer (β_4), respectively, but they exhibit no cooperativity in O_2 binding.[1,47] The cooperativity of Hb A appears only in the $\alpha_2\beta_2$ heterotetramer. This implies that some structure changes of one subunit may affect the O_2 affinity of its counterpart ($\alpha \rightarrow \beta$ or $\beta \rightarrow \alpha$) through the Fe−His bond. Indeed, this was demonstrated with cavity mutant Hb A;[12] the Fe−His bond in the β subunit is working to decrease the O_2 affinity of α subunits in Hb A, while a change in the Fe−His bond of one deoxy α subunit is essential to increase the O_2 affinity of the remaining α and β subunits through a quaternary structure change.[12]

It is emphasized that the previous studies, including ours,[5,38,43−45] have assumed the equivalence of two α subunits and two β subunits irrespective of O_2 binding in solution, though as mentioned below some authors noted the observation of two different modes for the α subunits.[10,30,31,33,36]

Unexpectedly, however, it was noticed with deoxy rHb(Hβ92G) of the cavity mutant that the Fe−His stretching mode of the α subunit gave rise to two bands at 201 and 222 cm^{-1}.[12] In rHb(Hβ92G), there is no covalent bond between heme and F helix in the β subunit. This means the presence of two kinds of Fe−His bonds for α subunits, that is, two tertiary structures. Before this finding was published, there had been Raman studies that indicated the presence of two Fe−His bands for only the α subunit in valency hybrid Hb, metal hybrid Hb A such as Fe−Co and Fe−Ni, and protoporphyrin(IX)−mesoporphyrin hybrid Hb A,[10,30,31,33,36] although discussion about an origin of two different modes for the α subunits was not focused. We infer, therefore, that the structural heterogeneity of α subunits of Hb A is inherent and appears depending on its quaternary structure. To elucidate the origins of the heterogeneity of Fe−His bonds of α subunits as well as their relationship with O_2 binding properties, in this study we focus on the α subunit by using three kinds of natural valency hybrid ($\alpha Fe^{2+}\beta Fe^{3+}$) mutant hemoglobins (Hb M): Hb M Hyde Park (β92His → Tyr), Hb M Saskatoon (β63His → Tyr), and Hb M Milwaukee (β67Val → Glu). Hereafter, we call native $\alpha Fe^{2+}\beta Fe^{3+}$ the half-met form. In $\alpha Fe^{2+}\beta Fe^{3+}$, the β heme cannot bind O_2 because of Fe^{3+}. Full-met ($\alpha Fe^{3+}\beta Fe^{3+}$) and fully reduced ($\alpha Fe^{2+}\beta Fe^{2+}$) forms can also be generated.[48−58] The half-met form, $\alpha Fe^{2+}\beta Fe^{3+}$, enables us to observe quaternary structure changes upon binding of a ligand to only the α heme and also the Fe−His frequency of the α subunit as the O_2 affinity increases and/or decreases. Characteristic structural differences among the β subunits of Hb A, Hb M Saskatoon, Hb M Hyde Park, and Hb M Milwaukee are illustrated in Figure 1.

In this paper, the experimental results of resonance Raman (RR), terahertz time-domain, and ^1H nuclear magnetic resonance (NMR) spectroscopy for the Hb Ms mentioned above are described in detail. The heterogeneity of α subunits, which has been observed previously,[10,30,31,33,36] is reconfirmed, and its implications for the dynamical structure of the $\alpha_2\beta_2$ tetramer and the regulation of O_2 affinity are discussed.

EXPERIMENTAL PROCEDURES

Preparation and Purification of Hemoglobins. Hb A was purified from human hemolysate by preparative isoelectric focusing.[59] Human hemolysate was prepared from concentrated red cells provided by the Japanese Red Cross Kanto-Koshinetsu Block Blood Center. Hb M Hyde Park and Hb M Saskatoon were purified by preparative isoelectric focusing, and Hb M Milwaukee was purified by using an Amberlite ionic chromatograph from individual Hb M hemolysates according to the reported method.[60]

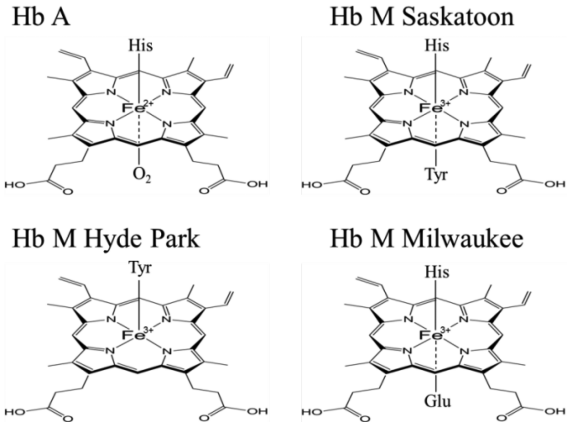

Figure 1. Hemes and axial ligands of the β subunit in Hb A, Hb M Hyde Park (βH92Y), Hb M Saskatoon (βH63Y), and Hb M Milwaukee (βV67E). Hb A is in its oxy form, $\alpha(Fe^{2+}\text{-}O_2)\beta(Fe^{2+}\text{-}O_2)$, and Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee are in half-met forms, $\alpha(Fe^{2+}\text{-deoxy}/O_2)\beta(Fe^{3+})$. Hemes of β subunits of Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee in half-met forms contain ferric irons (Fe^{3+}), which are coordinated with mutated residues, Tyr, Tyr, and Glu, respectively, and cannot bind O_2 or CO.

Visible RR Measurements. Visible RR spectra were excited at 441.6 nm with a He/Cd laser (Kimmon Koha, model CD4805R), dispersed with a 1 m single polychromator (Ritsu Oyo Kogaku, model MC-100DG) using the first-order diffraction of a grating (1200 grooves/nm), and detected with an ultraviolet (UV)-coated, liquid nitrogen-cooled CCD detector (Roper Scientific, LN/CCD-1100-PB/VISAR/1). All the hemoglobin samples were adjusted to a concentration of 200 μM (in heme) in 0.05 M phosphate buffer (from pH 5.7 to 7.6) or 0.05 M borate buffer (from pH 8.0 to 10.3). Deoxy Hb A was prepared by adding a small amount of sodium dithionite (1 mg/mL) to the oxy form after the replacement of the air inside the sample tube with N_2. The deoxy forms of Hb Ms Hyde Park, Saskatoon, and Milwaukee, $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$, were prepared by repeated injection of N_2 gas after degassing the oxy form, $\alpha(Fe^{2+}\text{-}O_2)\beta(Fe^{3+})$, three or four times.

Deoxygenation of $\alpha(Fe^{2+}\text{-}O_2)\beta(Fe^{3+})$ to $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$ was examined by absorption spectroscopy as shown in Figure S1. Absorption bands at 427 and 570 nm and 405 and 600 nm, which indicate the existence of $\alpha(Fe^{2+}\text{-deoxy})$ and $\beta(Fe^{3+})$ in $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$, respectively, were confirmed. It is noted for measurements of visible RR spectra of the high-O_2 affinity species that because an appreciable amount of the oxy form, $\alpha(Fe^{2+}\text{-}O_2)\beta(Fe^{3+})$, was produced in a sample of the deoxy form, $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$, this was used to detect the Fe^{2+}−His RR band of the partially O_2-bound form, $\alpha_1(Fe^{2+}\text{-deoxy})\alpha_2(Fe^{2+}\text{-}O_2)\beta_1(Fe^{3+})\beta_2(Fe^{3+})$.

All measurements were taken at room temperature with a spinning cell (1800 rpm). The laser power at the scattering point was 3.0−4.0 mW. The integrity of samples after visible RR measurements was carefully confirmed with the visible absorption spectra. Visible absorption spectra were recorded

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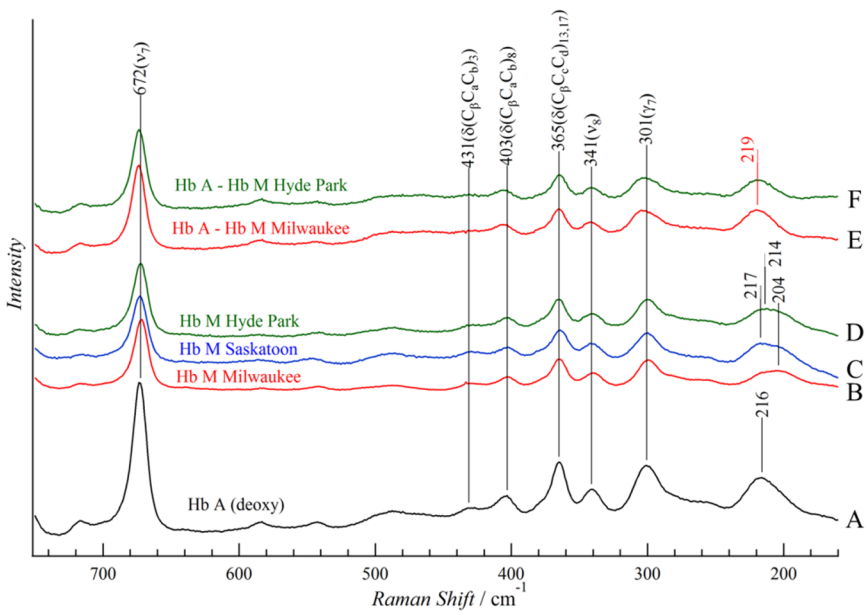

Figure 2. Resonance Raman spectra excited at 441.6 nm of deoxy Hb A (A) at pH 7.0 and the half-met form, $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$, of Hb M Milwaukee at pH 5.7 (B), Hb M Saskatoon at pH 6.9 (C), and Hb M Hyde Park at pH 5.9 (D). Only Hb M Saskatoon contains 2 mM inositol hexaphosphate. Spectra C and D are traces in which the contribution of the fluorescence background is subtracted from the observed spectra. Spectral intensities of three Hb Ms are normalized with the ν_7 band and adjusted to half of that of deoxy Hb A. Spectra E and F show the differences; E = A − B, and F = A − D.

with a Hitachi U-3310 spectrophotometer. When spectral changes were recognized, the Raman spectra were discarded. However, whenever an appreciable change in the visible absorption spectra due to oxygenation was clear, it was adopted and noted in the figure caption.

Measurements of Terahertz (THz) Spectra. To investigate dynamical features of the main chain, THz time-domain spectroscopy (TDS), performed at the Research Center for Development of Far-Infrared Region, University of Fukui, was adopted. Measurements were taken for only Hb M Milwaukee. THz absorption spectra of Hb solutions were measured using a THz time-domain spectrometer (Aispec, IRS-1000/2000) with its transmission geometry. Analysis of THz spectra was performed as described in the previous papers.[61,62] The heme concentration of Hb M Milwaukee was 3 mM. To distinguish the THz TDS spectrum of $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$ from that of $\alpha(Fe^{2+}\text{-}O_2)\beta(Fe^{3+})$, we separately prepared $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$ at pH 5.6 and $\alpha(Fe^{2+}\text{-}O_2)\beta(Fe^{3+})$ at pH 8.5 in 50 mM phosphate buffer. The Hb solution was poured into a sample cell having windows of a silicon plate. The area of the window and the thickness of the cell are 1000 mm^2 (=10 cm^2) and 0.3 mm, respectively. For measurement of $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$, the inside of the cell was filled with N_2 gas before the Hb solution was injected. Measurements were taken 10 times alternately for the reference cell and sample cell. The temperature was kept at 22 °C during measurements. Thus, the observed spectra shown are an average of 10 accumulated spectra.

Measurements of ^{1}H NMR Spectra. The ^{1}H NMR spectra were measured with a Bruker AVANCE 600 FT NMR spectrometer operating at a ^{1}H frequency of 600 MHz at the OPEN FACILITY, the Research Facility Center for Science and Technology, University of Tsukuba. The hemoglobin concentrations of Hb A (deoxy and CO forms), Hb M Saskatoon, $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$, and $\alpha(Fe^{2+}\text{-CO})\beta(Fe^{3+})$ were 1 mM on a heme basis in 0.05 M phosphate buffer (pH 7.0). Deoxy Hb A and CO Hb A were prepared by adding sodium dithionite (1 mg/mL) to the oxy form after replacement of the air inside the sample tube with N_2 and CO, respectively. Hb M Saskatoon, $\alpha(Fe^{2+}\text{-CO})\beta(Fe^{3+})$, at pH 7.0 was prepared by adding CO gas to $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$. Hb M Saskatoon, $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$, at pH 7.0 was prepared by repeatedly removing and adding N_2 gas. To remove oxygen, we used an airtight test tube. The spectra were recorded with a water suppression method by presaturation with approximately 4k−8k scans, a spectral width of 36 kHz (60 ppm), 32k data points, a 90° pulse of 10.2 μs, and recycle times of 0.5 s for the deoxy form and 1−3 s for the CO forms. Chemical shifts are given in parts per million downfield from sodium 2,2′-dimethyl-2-silapentane-5-sulfonate, with the residual H^2HO as an internal reference.

RESULTS

Fe−His Frequencies of Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee. RR spectra of deoxy Hb A and the half-met form, $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$, of Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee excited at 441.6 nm are shown in Figure 2. For the spectra of Hb M Hyde Park and Hb M Saskatoon, some fluorescence background was subtracted to yield a flat baseline. Generally, the absorption maxima (λ_{max}) of met, oxy, and deoxy Hb A are located at 405, 415, and 430 nm, respectively,[63,64] and indeed, the λ_{max} values of $\alpha(Fe^{2+}\text{-deoxy})$ and $\beta(Fe^{3+})$ of the half-met form, $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$, of Hb M Milwaukee were observed at 427 and 405 nm, respectively (Figure S1). The four observed spectra shown in Figure 2A−D are derived from deoxy-Fe^{2+} hemes, because Raman bands of high-spin Fe^{3+} hemes are too weak to be detected upon excitation at 441.6 nm because the resonance effect is much smaller than that for deoxy-Fe^{2+} heme.[15] Although the spectrum of deoxy Hb A reflects both α and β hemes, the spectra of half-met Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee reflect the α heme only.[57] Except for the Fe−His band around 200−220 cm^{-1}, the assignments of spectra are based on the work of Hu et al.[65]

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In Figure 2, the intensities of spectra of Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee, $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$, are normalized with the ν_7 band, and they are adjusted to half of the ν_7 intensity of deoxy Hb A, which also contains the contribution from $\beta(Fe^{2+}\text{-deoxy})$. The width of the Fe−His band of Hb A (A) at 216 cm^{-1} is ∼27 cm^{-1}, while those of Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee are ∼35−40 cm^{-1}, being broader than that of Hb A. This was unexpected, because widths of Fe−His bands of Hb M Iwate and Hb M Boston, $\alpha(Fe^{3+})\beta(Fe^{2+}\text{-deoxy})$, are ∼20 cm^{-1}, because of the lack of the contribution from the α subunit and the approximate homogeneity of the β subunits.[15,30,66,67] Apparently, the Fe−His bands of Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee seem to be composed of two components.

To explore their broadness, difference spectra (E = A − B, and F = A − D) were calculated. The bandwidths of the Fe−His bands in the difference spectra are 22 cm^{-1}, and the bands are symmetrically similar to the Fe−His bands of $\beta(Fe^{2+}\text{-deoxy})$ in Hb M Iwate and Hb M Boston but are higher by ∼2 cm^{-1}.[15] This implies that spectra B and D are close to the spectrum of the α subunit of deoxy Hb A and that the difference spectra (E and F) indicate the spectrum of the β subunit of deoxy Hb A with the T quaternary structure. In other words, the broadness of the Fe−His band of deoxy Hb A arises from the contribution of one of α subunit having a ν_{Fe-His} at ∼200 cm^{-1}.

The pH dependencies of RR spectra of Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee are shown in Figures S2−S4, respectively. The fluorescence backgrounds were subtraced from the observed spectra for Hb M Hyde Park and Hb M Saskatoon. Their expanding spectra of the low-frequency regions are shown in Figures 3−5, respectively. As mentioned above, the spectra shown reflect only the α deoxy heme of $\alpha_2\beta_2$ tetramer Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee, because of resonance effects. At higher pH, the O_2 affinity becomes higher for some of Hb M, and accordingly, another ν_4 band was weakly observed at ∼1375 cm^{-1}. This band is considered to arise from $\alpha(Fe^{2+}\text{-}O_2)$ of oxygenated forms, $\alpha(Fe^{2+}\text{-}O_2)\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})\beta(Fe^{3+})$ or $\alpha(Fe^{2+}\text{-}O_2)\alpha(Fe^{2+}\text{-}O_2)\beta(Fe^{3+})\beta(Fe^{3+})$.[65,68] However, upon Raman excitation at 441.6 nm, intensities of bands derived from the oxy form are considered to be too weak to be effectively observed except for ν_4 and ν_7 bands. Especially in the low-frequency region between 190 and 240 cm^{-1}, bands of oxy Hb A are not observed at all as shown by RR spectra of oxy Hb A at the bottom of Figures 3 and 5. Therefore, we consider that RR bands observed between 190 and 240 cm^{-1} arise from $\alpha(Fe^{2+}\text{-deoxy})$ even though the Hb M samples contain the partially O_2-bound form. This is the reason why we adopted the Raman spectra of high-pH samples for which visible absorption spectra observed before and/or after Raman measurements indicated appreciable progress of oxygenation. If we obtain partially oxygenated forms, we can confirm the presence of the partially oxygenated form by simply interrogating the sample with 413 nm excitation, where the $\nu(Fe-O)$ mode of any oxy form could be detected.

The RR spectra shown in Figures S2−S4 show that pH dependencies of the α subunit of Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee are very weak except for ν_4, ν_7, and ν_{Fe-His} bands. With regard to the ν_{Fe-His} modes, two bands were observed for Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee. Apparent peak tops of Fe−His bands in all the spectra of Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee shifted to high wavenumbers above pH 8, 9, and 10, respectively.

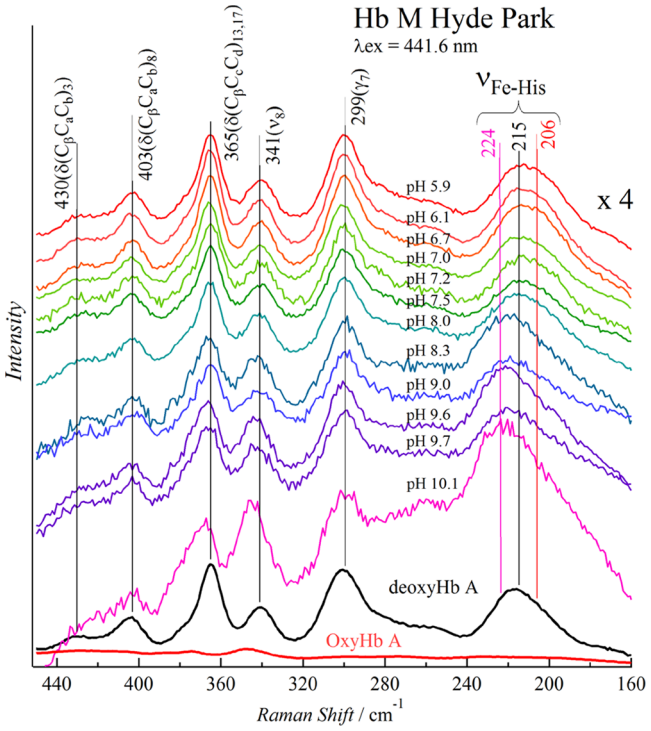

Figure 3. pH dependence of resonance Raman spectra of low-frequency regions of half-met Hb M Hyde Park, $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$, excited at 441.6 nm. Twelve spectra observed from pH 5.9 to 10.1 are displayed. The black and red spectra at the bottom are those of deoxy and oxy Hb A, respectively, at pH 7.0. Spectra from 1520 to 160 cm^{-1} are shown in Figure S2.

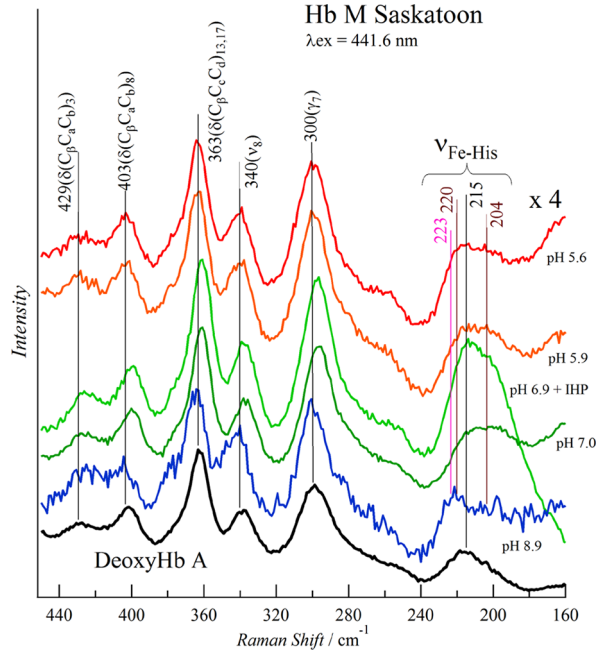

Figure 4. pH dependence of resonance Raman spectra of low-frequency regions of half-met Hb M Saskatoon, $\alpha(Fe^{2+}\text{-deoxy})\beta(Fe^{3+})$, excited at 441.6 nm. Five spectra observed from pH 5.6 to 8.9 are displayed. The black spectrum at the bottom is that of deoxy Hb A at pH 7.0. Spectra from 1420 to 160 cm^{-1} are shown in Figure S3.

It is noted for Figure S3 that an interesting band appears at 876 cm^{-1} for Hb M Saskatoon only below pH 6. In a previous study

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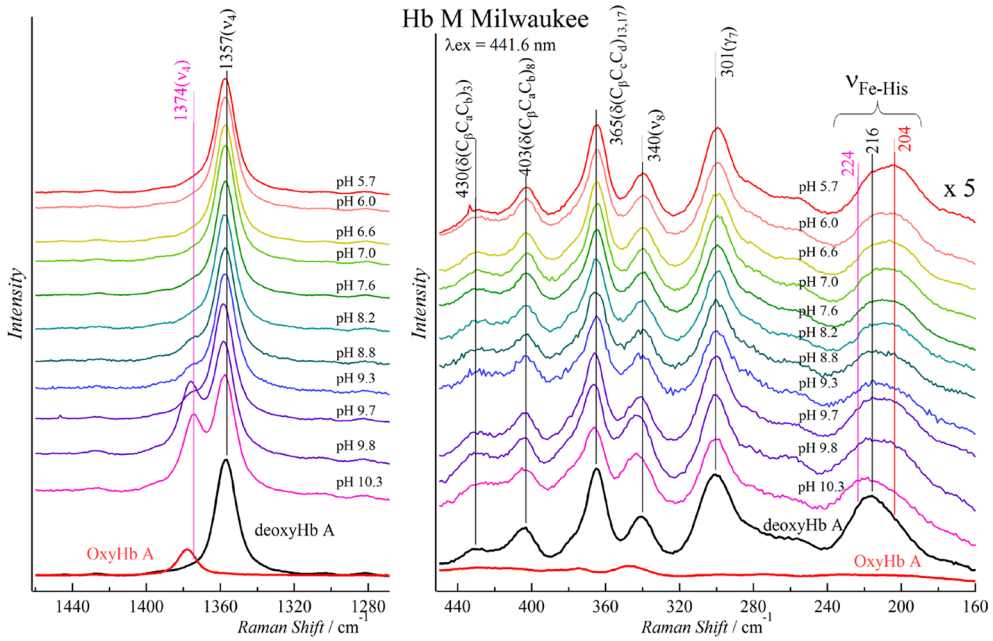

Figure 5. pH dependence of resonance Raman spectra of low-frequency regions and ν_4 of half-met Hb M Milwaukee, $\alpha(Fe^{2+}$-deoxy$)\beta(Fe^{3+})$, excited at 441.6 nm. Eleven spectra observed from pH 5.7 to 10.3 are displayed. The black and red spectra at the bottom are those of deoxy and oxy Hb A, respectively, at pH 7.0. Spectra from 1520 to 160 cm^{-1} are shown in Figure S4.

of fully met Hb M Saskatoon, $\alpha(Fe^{3+})\beta(Fe^{3+})$, its absorption spectrum changed between pH 7.0 and 5.0,[54] and this change was attributed to the abnormal β subunits.[54] The corresponding band is observed for Hb M Boston, $\alpha(Fe^{3+})\beta(Fe^{2+}$-deoxy/CO), upon excitation at 488.0 nm and is tentatively assigned to an internal mode of the coordinated tyrosinate (possibly Y1).[56] Accordingly, the 876 cm^{-1} band of half-met Hb M Saskatoon, $\alpha(Fe^{2+}$-deoxy/$O_2)\beta(Fe^{3+})$, is presumably associated with a heme(Fe^{3+})-bound Tyr(OH/O^-) mode of the β subunit,[54,58] though RR bands of the α subunit are basically intensity-enhanced upon excitation at 441.6 nm. The pH sensitivity seems to arise from the coordinated Tyr.

Deconvolution of Fe−His Bands of the α Subunit. To investigate the pH-dependent spectral change in the Fe−His bands, we deconvoluted the Fe−His band with two or three Gaussian functions. The results for Hb M Hyde Park and Hb M Milwaukee are illustrated in Figure 6, indicating that Fe−His bands of both mutants consist of two or three components. Deconvoluted peak frequencies were set in advance with almost the same values (within peak frequencies of ±1) and half-height bandwidths (20−22 cm^{-1}) in each Hb M within all measured pH regions. The peak frequencies that were used are shown in Figure S5. This calculatons indicated that the half-height bandwidths (20−22 cm^{-1}) of component bands were similar to the Fe−His bands of the β subunit of another valency hybrid Hb M Iwate and Hb M Boston, $\alpha(Fe^{3+})\beta(Fe^{2+}$-deoxy), which were symmetric with ~22 cm^{-1} of half-width.[15]

In Hb M Hyde Park spectra, the Fe−His band consists of two bands, having peaks at 203 and 217 cm^{-1}, in the low-pH region between 5.9 and 7.5, but a third band appears at 225 cm^{-1} at pH >8.0. In Hb M Milwaukee, the Fe−His band consists of two bands, having peaks at 200 and 216 cm^{-1} in below pH 9.3, but a third band appears clearly at 224 cm^{-1} above pH 9.7. In both Hb M Hyde Park and Hb M Milwaukee, the intensity of the third band is greater as the pH becomes higher. In contrast, intensities of the other two bands decrease without a change in frequency. In Hb M Saskatoon (Figure S6), the Fe−His band consists of two

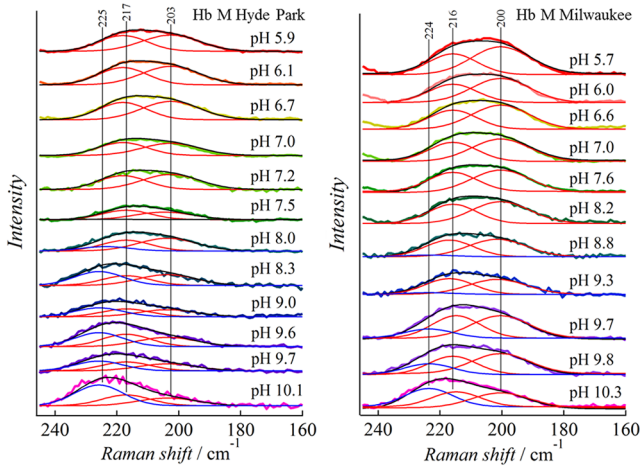

Figure 6. Deconvolutions of pH-dependent Fe−His bands of half-met Hb M Hyde Park (left) and Hb M Milwaukee (right), $\alpha(Fe^{3+})\beta(Fe^{2+}$-deoxy). Observed spectra are the same as those in Figures 3 and 5. Black solid lines show sums of deconvoluted two- or three-component bands (red). Spectra of Hb M Hyde Park above pH 8.0 and those of Hb M Milwaukee above pH 9.7 could not be fitted well with two bands, and a third band is colored blue.

bands, having peaks at 203 and 220 cm^{-1} in a low-pH region between 5.6 and 7.0, but a third band appears at 223 cm^{-1} at pH 8.9. The wavenumbers (223−225 cm^{-1}) of the third band correspond to the R structure.[66] These results suggest that the three Hb Ms contain not only T structure but also R structure in a higher-pH region.

Of course, for Fe−His bands at higher pH values, we can fit the bands with two components, which need broadening of the bandwidth compared with that at lower pH values. However, we do not have any ideas for interpretation of why wider bandwidths are needed at high pH. As we did not change the temperatures of Hb samples in this study, it is difficult for us to interpret a

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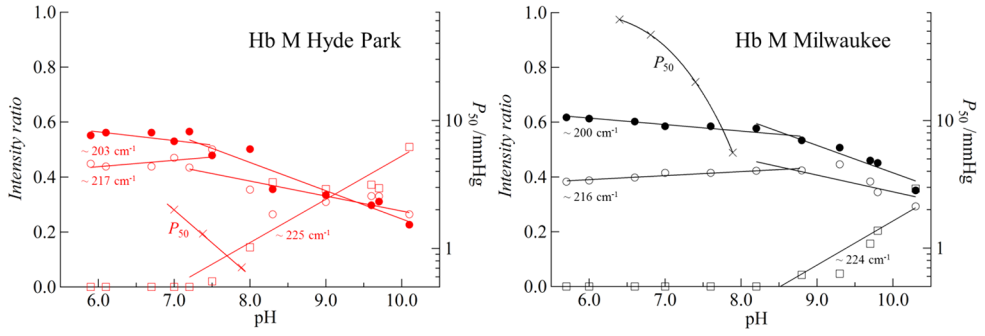

Figure 7. pH dependence of intensities of the deconvoluted individual Fe−His bands shown in Figure 6. By normalization of total intensities of the Fe−His band in each spectrum to unity, relative intensities of the deconvoluted Fe−His band were calculated and are plotted against pH for Hb M Hyde Park (left) and Hb M Milwaukee (right). Filled circles, empty circles, and empty squares represent intensities of bands at ∼200, ∼216, and ∼224 cm^{-1}, respectively. Fitted lines are regression curves as determined by the linear least-squares method. Times signs indicate values of O_2 affinity, P_{50}, plotted against the right axis. The values were taken from refs 69−71. Lines of P_{50} are smooth free hand lines.

broadening of bandwidth by increases in collision frequencies of Hb molecules in solution. Therefore, we maintained the bandwidth and peak frequencies during the simulation. Instead, we considered that the interpretation based on the occurrence of another Fe−His band corresponding to R structure is appropriate; i.e., two Fe−His bands of T structure and one Fe−His band of R structure are overlapped.

pH Dependence of Intensities of Deconvoluted Fe−His Bands of the α Subunit. We calculated intensities of the deconvoluted Fe−His band by integration of individual Gaussian functions. Here, to determine the distributions of deconvoluted Fe−His bands, we calculated them under the assumption that the total intensities of deconvoluted Fe−His bands are unity. Relative intensities of the deconvoluted component bands are plotted versus pH in Figure 7. This demonstrates that relative intensities of two component bands of Hb M Hyde Park and Hb M Milwaukee scarcely change until pH values of 7.5 and 9.3, respectively, are reached and that the third band with a higher frequency (∼224 cm^{-1}) appears at pH 8.0 and 9.7, respectively. The third Fe−His band probably is derived from $\alpha(Fe^{2+}$-deoxy) having R structure in the partially O_2-bound form, $\alpha(Fe^{2+}$-$O_2)\alpha(Fe^{2+}$-deoxy)$\beta(Fe^{3+})\beta(Fe^{3+})$.[30,66,67] For the sake of convenience, the pH dependencies of oxygen affinities (P_{50}) of both Hb Ms, $\alpha(Fe^{2+}$-deoxy)$\beta(Fe^{3+})$, are also plotted in Figure 7.[71] These curves of P_{50} are distinctly different from the curves of Fe−His RR bands.

Terahertz Spectra of Hb M Milwaukee. Figure 8 shows absorption spectra in the THz region (<35 cm^{-1}) of 3 mM solutions of half-met Hb M Milwaukee, $\alpha(Fe^{2+}$-deoxy)$\beta(Fe^{3+})$, at pH 5.6 (low affinity) and $\alpha(Fe^{2+}$-$O_2)\beta(Fe^{3+})$ at pH 8.5 (high affinity). Blue and red solid lines are observed THz spectra of $\alpha(Fe^{2+}$-deoxy)$\beta(Fe^{3+})$ and $\alpha(Fe^{2+}$-$O_2)\beta(Fe^{3+})$ solutions, respectively. Apparently, both spectra are almost the same. To examine the detailed difference, we calculated their differences. The black solid line represents a difference spectrum, $\alpha(Fe^{2+}$-deoxy)$\beta(Fe^{3+})$ − $\alpha(Fe^{2+}$-$O_2)\beta(Fe^{3+})$. This suggests that there is no evidence of significant differences. Although the concentrations of Hb M Milwaukee, $\alpha(Fe^{2+}$-deoxy)$\beta(Fe^{3+})$, and $\alpha(Fe^{2+}$-$O_2)\beta(Fe^{3+})$ are much higher (3 mM) than those used for Raman measurements (0.2 mM), absorption of water might be still too strong to detect their difference, even if present.

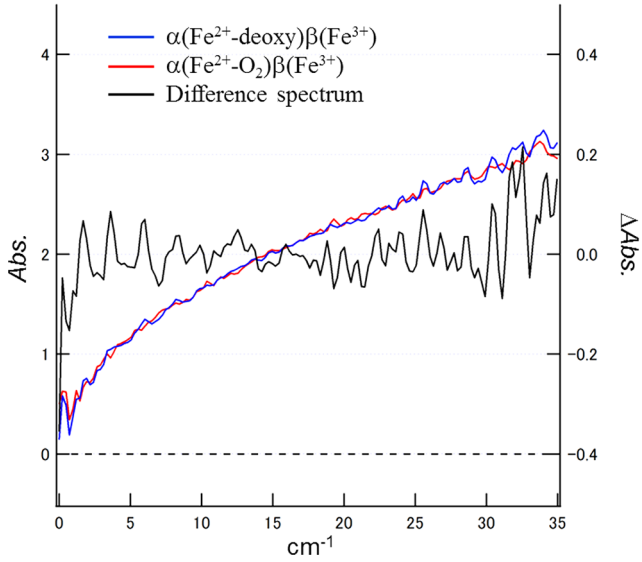

Figure 8. Absorption spectra in the terahertz region (<35 cm^{-1}) of 3 mM solutions of half-met Hb M Milwaukee, $\alpha(Fe^{2+}$-deoxy)$\beta(Fe^{3+})$, at pH 5.6 (low-affinity form) and $\alpha(Fe^{2+}$-$O_2)\beta(Fe^{3+})$ at pH 8.5 (high-affinity form), added with 50 mM phosphate buffer. Blue and red solid lines are observed spectra of $\alpha(Fe^{2+}$-deoxy)$\beta(Fe^{3+})$ and $\alpha(Fe^{2+}$-$O_2)\beta(Fe^{3+})$ with reference to the left ordinate, respectively. The black solid line indicates an $\alpha(Fe^{2+}$-deoxy)$\beta(Fe^{3+})$ − $\alpha(Fe^{2+}$-$O_2)\beta(Fe^{3+})$ difference spectrum with reference to the right ordinate.

DISCUSSION

Two Distinct Fe−His Frequencies Found in the α Subunit. Figure 6 and Figure S6 show that Fe−His bands of three Hb Ms consist of two components in a lower-pH region. We ascribe the doublet properties of the Fe−His band to "heterogeneity" of α subunits, because the Fe−His frequency reflects the tertiary structure of globin. The similar heterogeneity of the Fe−His bands in the α subunit has been reported previously.[10,12,30,31,33,36] Ondrias et al.[31] demonstrated that the RR spectrum of $\alpha(Fe^{2+}$-deoxy)$\beta(Co^{2+}$-deoxy) hybrid Hb excited at 441.6 nm gave two Fe−His bands at 201 and 212 cm^{-1}, and these two bands are observed only for the T structure. Even recently, Jones et al.[10] reported the protoheme-selective RR spectra of α(meso)β(proto) and α(proto)β(meso) heme-hybrid Hb A under sol−gel conditions. The α(proto)β(meso) Hb in sol−gels showed two kinds of Fe−His frequencies upon excitation at 441.6 nm.[10] In our recent study of a cavity mutant Hb, rHb(βH92G), which has properties similar to those of valency hybrid Hbs with regard to the absence of the Fe−His bond in the β subunit, we were able to observe the Fe−His

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frequencies of the α subunit at 201 and 222 cm^{-1} separately from the Fe−Im(imidazole) mode of the β subunit.[12] Surprisingly, a quaternary structure of rHb(βH92G) in the deoxy form exhibited characteristics of T quaternary structure,[12] though the O_2 affinity of its α subunit was high (~2 mmHg) compared with that of wild-type deoxy Hb A. A half-met Hb M Milwaukee, $\alpha(Fe^{2+}$-deoxy$)\beta(Fe^{3+})$, at pH 7 also showed T structure as determined by ^{1}H NMR[50] and UVRR spectra,[5] and a half-met Hb M Saskatoon, $\alpha(Fe^{2+}$-deoxy$)\beta(Fe^{3+})$, at pH 7.0 also showed T structure as determined by ^{1}H NMR spectra as shown in Figure S7.

On the other hand, there have been a few reports that Fe−His band is deconvoluted without distinction between the α and β subunits.[72−74] These analyses resulted in the presence of four or five bands in the frequency region from 190 to 230 cm^{-1}, pointing out the presence of heterogeneity not only among α subunits but also among β subunits.[72−74] However, we did not find appreciable heterogeneity of the Fe−His band for β subunits.

Structure Changes Because of Binding of O_2 to the α Subunit: Appearance of a Third Band. In Figure 5, the bands at 1357 and 1374 cm^{-1} arise from $\alpha(Fe^{2+}$-deoxy) and $\alpha(Fe^{2+}$-$O_2)$ subunits of partially O_2-bound Hb M Milwaukee. Thus, it is plausible that the third band at 224 cm^{-1} arises from the partially O_2-bound molecules. Here, we try to show that this is really the case. First, we define a fraction (f_i) of molecular species bound with i molecules of O_2 in half-met Hb M: f_0 for $\alpha_1(Fe^{2+}$-deoxy$)\alpha_2(Fe^{2+}$-deoxy$)\beta_1(Fe^{3+})\beta_2(Fe^{3+})$, f_1 for $\alpha_1(Fe^{2+}$-deoxy$)\alpha_2(Fe^{2+}$-$O_2)\beta_1(Fe^{3+})\beta_2(Fe^{3+})$ and $\alpha_1(Fe^{2+}$-$O_2)\alpha_2(Fe^{2+}$-deoxy$)\beta_1(Fe^{3+})\beta_2(Fe^{3+})$, and f_2 for $\alpha_1(Fe^{2+}$-$O_2)\alpha_2(Fe^{2+}$-$O_2)\beta_1(Fe^{3+})\beta_2(Fe^{3+})$. Here, $f_0 + f_1 + f_2 = 1$. The spectra at the bottom of Figure 5 were obtained for Hb A under the same experimental conditions that were used for Hb M Milwaukee. Assuming that the intensity (I) ratio of ν_4 of Hb A, $I(\nu_{1357})/I(\nu_{1374})$, is a and indicates the intrinsic Raman intensity ratio of deoxy and oxy hemes under the present experimental conditions, and that the corresponding intensity ratio for Hb M Milwaukee at pH 10.3 is equal to x, their ratio is related to the fraction of O_2-bound molecules, Y, as $x/a = (1 - Y)/Y$.

The Raman intensities of ν_4 bands of Hb A and Hb Milwaukee at pH 10.3 were estimated by simulating their ν_4 bands with Gaussian functions, yielding a and x with values of 5.0 and 1.5, respectively. Thus, we obtain the estimate $Y = 0.77$.

This Y and the magnitude of the Hill coefficient are sufficient to estimate all f_i values as follows. The fraction (f_i) can be expressed using Adair constants (K_i) and the partial pressure (p) of O_2 as $f_i = g_i(p)/(1 + 2K_1p + K_1K_2p^2)$, where $g_0(p) = 1$, $g_1(p) = 2K_1p$, and $g_2(p) = K_1K_2p^2$. These equations are different from those for Hb A, which has four O_2 binding sites.[4,75] Y can be calculated from the Adair constant and partial pressure of O_2 by using the following equation:

$$Y = \frac{K_1p + K_1K_2p^2}{1 + 2K_1p + K_1K_2p^2}$$

According to the expression, the Hill coefficient is writen as a function of the ratio of K_2 and K_1 as

$$\left[\frac{d}{d\log_{10}p}\log_{10}\left(\frac{Y}{1-Y}\right)\right]_{Y=1/2} = \frac{2\sqrt{K_2/K_1}}{1+\sqrt{K_2/K_1}}$$

Indeed, variation of Hill coefficient n depending on the ratio between K_2 and K_1 can be seen in Figure S8. For Hb M Milwaukee with a Hill coefficient (n) of 1.4,[70,71] K_2/K_1 is estimated to be 5.4. The f_i values thus calculated are plotted against Y in Figure S9. At the O_2 saturation value of $Y = 77\%$, f_0, f_1, and f_2 are calculated to be 10, 23, and 67%, respectively. Thus, the population of the partially O_2-bound molecules, f_1, is certainly appreciable. Therefore, the Fe−His band of f_1 is expected to contribute to an observed spectrum. The $(2f_0 + f_1)/(f_1 + 2f_2)$ ratio of populations of deoxy and oxy hemes calculated from these fractions is 0.27. This value is consistent with that (0.30) obtained from $(1 - Y)/Y$.

The n values previously reported[69−71] for Hb M Hyde Park and Hb M Milwaukee, $\alpha Fe^{2+}\beta Fe^{3+}$, are almost the same as that of Hb M Milwaukee. Similar behaviors are rationalized, accordingly. Then, the third Fe−His band observed at 224 cm^{-1} is considered to arise from the changed α heme in f_1 species. The Fe−His frequencies of 225 and 223 cm^{-1} observed at pH 8.3 and 8.9 for Hb M Hyde Park and Hb M Saskatoon (Figures 3 and 4), respectively, may also be derived from the changed α heme in f_1 species, because of the appearance of the ν_4 band at 1374 and 1357 cm^{-1}. In conclusion, the change in quaternary structure to R structure takes place during binding of O_2 to the α subunit, yielding a higher oxygen affinity and appreciable cooperativity (1.2−1.6) in Hb Ms Hyde Park, Saskatoon, and Milwaukee at high pH.[69−71]

Previously, Shibayama et al.[33] studied Ni−Fe hybrid Hb, $\alpha(Fe^{2+}$-deoxy/oxy$)\beta(Ni^{2+})$, and pointed out that the Fe−His band of $\alpha(Fe^{2+}$-deoxy$)\beta(Ni^{2+})$ was present around 201−203 cm^{-1} in a low-O_2 affinity state ($K_1 > 5$ mmHg) and around 220−221 cm^{-1} in a high-O_2 affinity state ($K_1 < 2$ mmHg) and that the Fe−His band was broader and apparently flattened in the intermediate state. Here, a shoulder seems to be present near 220 cm^{-1} in the Fe−His band when $K_1 > 5$ mmHg. Although the situation described above is not caused by O_2 binding, the results of the Ni−Fe hybrid Hb are consistent with our results. Namely, quaternary structures of Ni−Fe hybrid Hb, $\alpha(Fe^{2+}$-deoxy/oxy$)\beta(Ni^{2+})$, when $K_1 > 5$ mmHg, 5 mmHg $\geq K_1 \geq 2$ mmHg, and $K_1 < 2$ mmHg are probably T, a mixture of T and R, and R, respectively.

Dependence of the Oxygen Affinity of the α Subunit on Tension in the F Helix of the β Subunit. In rHb(Hβ92G), there is no covalent bond between the heme and F helix in the β subunit (an Fe−imidazole bond is present). This is equivalent to the lack of an Fe−His bond. The $\alpha(Fe^{2+}$-deoxy) of rHb(Hβ92G) has a high oxygen affinity that is the same as that of sperm whale Mb,[12] while $\alpha(Fe^{2+}$-deoxy) of deoxy Hb A has a low oxygen affinity. Therefore, it is considered that the difference in oxygen affinity of the α subunit arises from the presence or absence of the Fe−His bond in the β subunit of the $\alpha_2\beta_2$ tetramer.

If the presence of the Fe−His bond of the β subunit generates some tension on the F helix of the α subunit through intersubunit interactions and lowers the Fe−His frequency of the α subunit, the relaxation of the F helix of the β subunit due to a lack or movement of an Fe−His bond like in Hb M Hyde Park is expected to affect the F helix of the α subunit and thus its Fe−His bond and/or spin state and coordination number of the heme iron of the α subunit, even if the α subunit is in the oxidized state. In fact, the absence of the Fe−His bond in the β subunits, like rHb (Hisβ92 → Gly), gave a higher O_2 affinity and caused a high-frequency shift of the Fe−His mode for its α subunit.[12] Also, the binding of a ligand (CO) to its β subunit caused an increase in the coordination number of the heme iron of the α subunit, like in the case of Hb M Iwate and Hb M Boston,[15,56] meaning reduction of the tension of the F helix in the α subunit. Such an

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observation is reported with Co−Fe hybrid Hb, $\alpha(Co^{2+})\beta(Fe^{2+}$-deoxy),[76] and Ni−Fe hybrid Hb, $\alpha(Ni^{2+})\beta(Fe^{2+}$-deoxy).[32]

In the fully met form of Hb M Saskatoon, $\alpha Fe^{3+}\beta Fe^{3+}$, Fe^{3+}−O (Tyr) stretching frequencies of an abnormal β subunit are observed at 598 cm^{-1} at pH 10 and at 581 at pH 7. This Fe^{3+}−O bond is cleaved at pH 5.[54,58] Assuming that the strength of the Fe^{3+}−His bond at its *trans* site is inversely proportional to the Fe^{3+}−O bond strength, the Fe^{3+}−His bond of the β subunit is expected to become stronger at pH 7 than that at pH 10. If the same change occurs in half-met Hb M Saskatoon, $\alpha Fe^{2+}\beta Fe^{3+}$, the tension of the F helix in the β subunit would be stronger at pH 7 than at pH 10, and thus, it is reasonably explained that the oxygen affinity of the α subunit is lower at lower pHs.[71]

This relation between Fe−His frequencies of the α subunit and tensions in the F helix of the β subunit is summarized in Figure 9, where F helices of α and β subunits are colored red and blue, respectively, and names of proteins are specified in the upper parts of helices. Two Fe−His bands of the α subunit are classified into the lower-frequency (200−203 cm^{-1}) and higher-frequency (212−222 cm^{-1}) groups. Only the latter frequencies and oxygen affinity decrease as the tension imposed on the F helix in the β subunit becomes larger. The cavity mutant rHb(βH92G) with the smallest tension in the F helix of the β subunit gives rise to the higher-frequency α Fe−His band at 222 cm^{-1}, while $\alpha(Fe^{2+}$-deoxy)$\beta(Co^{2+})$ with the largest tension in the β subunit yields this band at 212 cm^{-1}. However, this Fe−His frequency dependence on oxygen affinity for the α subunit is much more obscure than that for the β subunit.[15] The corresponding frequencies of the α subunit of three Hb Ms are much less changed among them (216, 217, and 220 cm^{-1}) in spite of appreciable differences in oxygen affinity (40, 2, and 10 mmHg in P_{50}).[69−71,77]

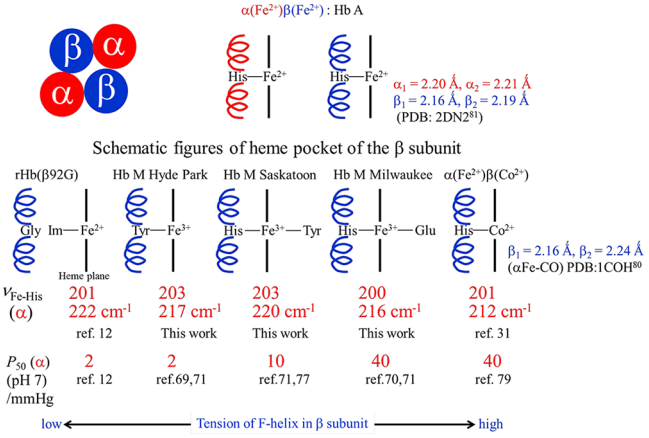

Figure 9. Schematic illustration of heme environments in the β subunit and two Fe−His frequencies of the α subunit of several valency and metal hybrid Hbs and their P_{50} values.

Therefore, though the decreases in the tension of the F helix in the β subunit increase the oxygen affinity of the α subunit and its Fe−His frequency slightly only for one component, they hardly affect the heterogeneity of α subunits, i.e., the presence of two groups of Fe−His frequencies (∼202 and ∼218 cm^{-1}). However, upon partial binding of O_2 to one of two α subunits of Hb M, the change in the quaternary structure to R increases the oxygen affinity of the remaining α subunit[12] and simultaneously causes the appearance of the third Fe−His band at ∼224 cm^{-1}. This explains the occurrence of positive cooperativity (1.2−1.6) at higher pH values for present Hb Ms. Pathways of structure change concomitant with binding of a ligand to the α subunit include cleavage of hydrogen bonds, Tyrα42−Aspβ99 and Aspα94−Trpβ37, as previously proposed.[78]

In summary, high O_2 affinities (low P_{50}) of the α subunit at high pH would be due to both the decreases in the tension of the F helix in the β subunit and the change in quaternary structure to R by partial binding of O_2 to one of two α subunits.

Differences in the Bohr Effect between Two Types of Hb Ms. Previously, we examined the O_2 affinity, ν_{Fe-His}, and intersubunit H bonding changes upon binding of O_2 to α-abnormal Hb Ms: Hb M Iwate (αH87Y) and Hb M Boston (αH58Y).[15] In this study, we examined it with the other type of Hb M, i.e., β-abnormal Hb Ms: Hb M Hyde Park (βH92Y), Hb M Saskatoon (βH63Y), and Hb M Milwaukee (βV67E). Characteristic differences in O_2 binding properties, Bohr effects, ν_{Fe-His}, and quaternary structure among five Hb Ms are summarized in Table 1. The properties of cavity mutant Hbs, rHb (αH87G) and rHb (βH92G), are also included in this table for comparison.

All these half-met Hb Ms have shown little cooperativity but could take the quaternary T structure in the deoxy state, $\alpha Fe^{3+}\beta Fe^{2+}$-deoxy or αFe^{2+}-deoxyβFe^{3+}. This means that the H-bonds at the $\alpha_1\beta_2$ subunit interface were formed in all Hb Ms, in which one of subunit is oxidized and the other subunit is in the deoxy form. However, cooperativity could not appear in the absence of the Fe^{2+}−His F8 bond. The substantial difference between α-abnormal Hb Ms and β-abnormal Hb Ms is the alkaline Bohr effect. Hb M Iwate and Hb M Boston (α-abnormal Hb M) do not show an alkaline Bohr effect, but Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee (β-abnormal Hb M) exhibit almost the full extent of the alkaline Bohr effect. The major residues for the alkaline Bohr effect are considered to be

Table 1. Characteristic Differences among α-Abnormal Hb Ms, β-Abnormal Hb Ms, and Cavity Mutants

Hb	P_{50} (mmHg) (pH 7)	Hill coefficient (n)	Bohr effect (%)	quaternary structure[a] ($\alpha Fe^{3+}\beta Fe^{2+}$-CO, αFe^{2+}-COβFe^{3+})	ν_{Fe-His}[b]	refs
rHb (αH87G)	5.6/60	0.45	7	T	217	12
Hb M Iwate (αH87Y)	50	1.0, 1.1	10	T	217	15, 71, 82
Hb M Boston (αH58Y)	27.5	1.2	0	T	217	15, 56, 71
rHb (βH92G)	2	1.2	46	∼R	201, 222	12
Hb M Hyde Park (βH92Y)	2	1.0, 1.3−1.4	80	no data	203, 217	69, 71
Hb M Saskatoon (βH63Y)	10	1.2	110	∼R	203, 220	71, 77
Hb M Milwaukee (βV67E)	40	1.2, 1.4−1.6	155	∼R	200, 216	5, 70, 71

[a]Quaternary structure is estimated by ultraviolet resonance Raman spectroscopy (UVRR) or ^{1}H NMR. "∼R" shows the existence of low-wavenumber shifts of Tyr bands caused by a change in quaternary structure from T to R by UVRR or disappearance of the ^{1}H signal of the Tyrα42···Aspβ99 hydrogen bond by ^{1}H NMR. [b]Observed ν_{Fe-His} wavenumbers are those of the $\alpha Fe^{3+}\beta Fe^{2+}$-deoxy or αFe^{2+}-deoxyβFe^{3+} form.

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Hisβ146 and Hisα89.[83,84] The results presented here indicate that the release of a proton from Hisβ146 is absolutely dependent on binding of the ligand to the α subunit. In deoxy Hb A, the carboxyl group of Hisβ146 is hydrogen-bonded with Lysα40.[81] When the ligand binds to the α subunit, the quaternary structure changes to R and separates these contacts of β_1 and α_2 by ~6 Å.[81] Therefore, there are no intersubunit contacts around Hisβ146 in oxy Hb A.[81] In the oxy form, the imidazole of Hisα89 interacts with $N_\epsilon H_3^+$ of Lysα139.[81] The imidazole is probably deprotonated. In the deoxy form, the imidazole of Hisα89 does not interact with $N_\epsilon H_3^+$ of Lysα139. The Hisα89 is probably protonated. As shown in Table 1, binding of oxygen to the α subunit causes a change in the quaternary structure from T to R in many cases except at lower pH in the presence of IHP.[85] This is why amino acid residues such as Hisβ146 and Hisα89, in which protonation states, i.e., pK_as, are different between deoxy and oxy forms, are responsible for large Bohr effect upon binding of O_2 to the α subunit.

Thus, in β-abnormal Hb Ms, binding of the ligand to the α subunit exhibits almost the full extent of the alkaline Bohr effect by a change in quaternary structure. On the other hand, for Hb M Iwate and Hb M Boston, binding of the ligand to the β subunit maintains T structure and thus hardly changes the Aspβ94···Hisβ146 H-bond until a pH of 8 is reached,[15] yielding no Bohr effect. These intersubunit changes around Hisβ146 could not be induced by binding of the ligand to the β subunit as observed in Hb M Iwate and Hb M Boston. This is the origin of the difference in the Bohr effect between two types of Hb Ms.

Possible Origins of the Heterogeneity of the α Subunit. Because Fe−His bond reflects the situation of the F helix, the existence of two Fe−His frequencies implies the presence of two structures as illustrated in Figure 10A. Recent high-resolution X-ray crystallographic analysis revealed that the Fe−His bond lengths in the α and β subunits of deoxy Hb A (T structure) are 220 and 221 pm and 216 and 219 pm, respectively.[81] The static heterogeneity of Fe−His bonds is greater in β than in α subunits in the Hb A crystal, but the heterogeneity of Fe−His frequencies in the β subunit is not observed in solution. Crystal packing forces may lead to a different static structure compared to the most stable structure in solution. Therefore, the heterogeneity demonstrated in this study is likely to arise from a dynamical one within the T quaternary structure as illustrated in panels B and C of Figure 10.

Because the relative intensities of two Fe−His bands around 200−203 and 216−220 cm^{-1} remain the same with changes in pH, model C is unlikely. In other words, tetramer molecules are homogeneous but two α subunits in a tetramer have different structures as illustrated in Figure 10A. Then, two structures shown in Figure 10 (B-a and B-b) are in equilibrium. The conversion between the two equilibrium structures involves large-amplitude motions of the F helix and is likely to be accompanied by a change in the electric dipole moment of the subunit and, thus, of a tetramer molecule. If the structural exchange occurs in several subpicoseconds or picoseconds, this dynamical feature results in THz absorption, which originates in relaxation of electric dipole moments. Therefore, we tried to detect it with terahertz absorption spectroscopy. However, Figure 8 shows that there is no difference in absorbance between deoxy Hb M Milwaukee, $\alpha(Fe^{2+}$-deoxy$)\beta(Fe^{3+})$, at pH 5.6 with a low-O_2 affinity structure and oxy Hb M Milwaukee, $\alpha(Fe^{2+}$-$O_2)\beta(Fe^{3+})$, at pH 8.5 with a high-O_2 affinity structure in the 0.15−1.0 THz region (5.0−33 cm^{-1}).

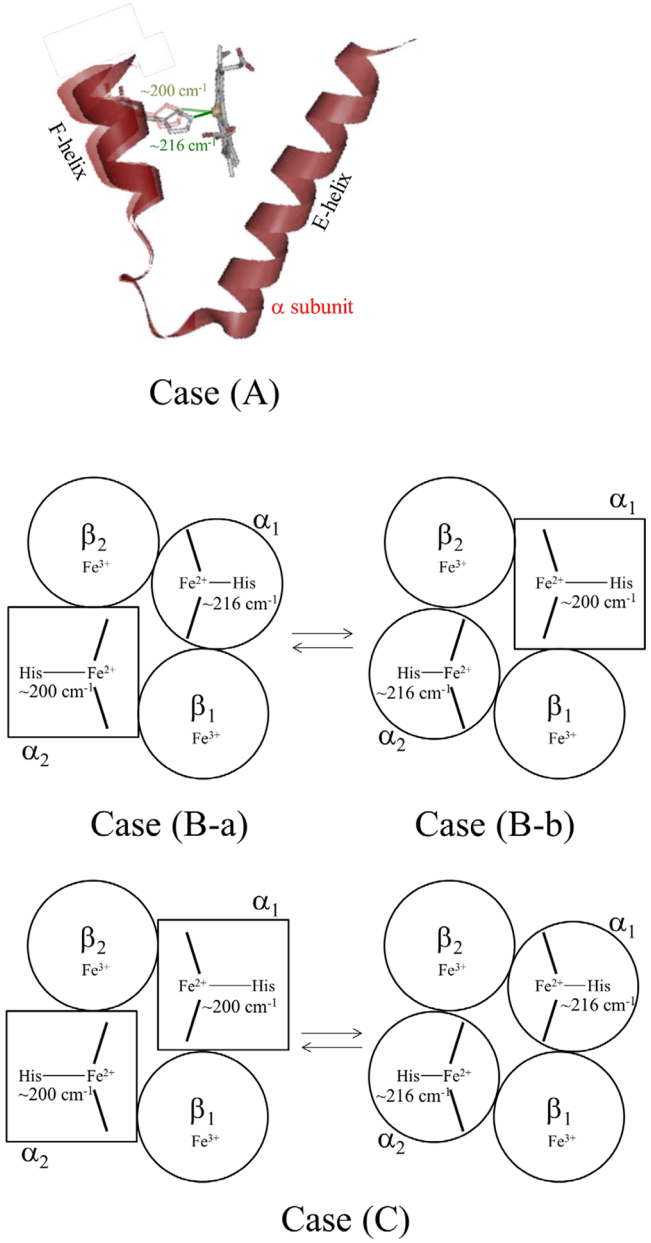

Figure 10. Origin of heterogeneity of the Fe−His band in the α subunit of Hb M Milwaukee. (A) Illustration of two likely structures of α subunits that yield different Fe−His frequencies. (B) Model for the coexistence of two different α subunits in a single tetramer molecule. (C) Model for the coexistence of two kinds of tetramers that contain different α subunits. Both arrows (⇄) in panels B and C would mean slow dynamics between two static disorders if they exist. Panel A was drawn by using the X-ray structure of the deoxy form (Protein Data Bank entry 2DN2) of human adult hemoglobin.[81]

Yonetani and Kanaori[8] proposed in their global allostery mechanism that the O_2 affinity of Hb A is regulated by amplitudes of fluctuations of E and F helices.[28] Their analysis requires that frequencies of fluctuations be between 1 GHz (0.033 cm^{-1}) and 10 THz (330 cm^{-1}) on the basis of molecular dynamics calculation.[8,28] Though our THz experiments cover only a part of the proposed frequency region, we were not able to confirm the fluctuations of the F helix in this region. Therefore, the fluctuations of the F helix might have frequencies lower than 0.15 THz (5.0 cm^{-1}). Otherwise, the absorption of water was too strong to detect a transition dipole due to motions of the F helix

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in proteins $\alpha(Fe^{2+}$-deoxy$)\beta(Fe^{3+})$ and $\alpha(Fe^{2+}$-$O_2)\beta(Fe^{3+})$ of Hb M Milwaukee.

CONCLUSION

Heterogeneity in the Fe−His bonds of the α subunits in human adult hemoglobin has not been investigated in detail so far, although its existence has been known.[10,30,31,33,36] The study presented here demonstrates that a series of $\alpha(Fe^{2+})\beta(Fe^{3+})$, half-met Hb Ms (Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee) gave rise to two Fe−His frequencies of the α (deoxy) subunit in the T structure in the lower-pH region and additionally yielded another band corresponding to the R structure in the higher-pH region. This explains a change to a high-affinity state at high pH and the existence of weak coperativity for O_2 binding. The decreases in tension in the F helix in the β subunit due to cleavage or elongation of its connection to heme little affect the two Fe−His frequencies of α subunit, but do affect O_2 affinities of the α subunit. The heterogeneity of Fe−His frequencies in the α subunit that disappears in the R quaternary structure[30] might be the origin of weak cooperativity in O_2 binding of half-met Hb M. In contrast to those of the β subunit, the Fe−His frequencies of the α subunit did not exhibit a clear correlation with O_2 affinities.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00733.

Absorption spectra of Hb M Milwaukee, $\alpha(Fe^{2+}$-deoxy/$O_2)\beta(Fe^{3+})$ (Figure S1); pH dependence of resonance Raman spectra of half-met Hb M Hyde Park, $\alpha(Fe^{2+}$-deoxy$)\beta(Fe^{3+})$, excited at 441.6 nm in the range from 1520 to 160 cm^{-1} (Figure S2); pH dependence of resonance Raman spectra of half-met Hb M Saskatoon, $\alpha(Fe^{2+}$-deoxy$)\beta(Fe^{3+})$, excited at 441.6 nm in the range from 1420 to 160 cm^{-1} (Figure S3); pH dependence of resonance Raman spectra of half-met Hb M Milwaukee, $\alpha(Fe^{2+}$-deoxy$)\beta(Fe^{3+})$, excited at 441.6 nm in the range from 1520 to 160 cm^{-1} (Figure S4); peak frequencies of the deconvoluted Fe−His band by Gauss functions of Hb M Hyde Park and Hb M Milwaukee (Figure S5); deconvolutions of pH-dependent Fe−His bands of half-met Hb M Saskatoon, $\alpha(Fe^{3+})\beta(Fe^{2+}$-deoxy) (Figure S6), ^{1}H NMR spectra of Hb A and half-met Hb M Saskatoon at pH 7.0 (Figure S7); calculation of the cooperativity of two ligand (O_2) binding Hbs (Figure S8); and fractions of Hb M Milwaukee having different numbers of bound O_2 calculated from parameters for binding equilibrium constants (Hill coefficient of 1.4) (Figure S9) (PDF)

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Notes

The authors declare no competing financial interest.

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