Heme Structures of Five Variants of Hemoglobin M Probed by Resonance Raman Spectroscopy†

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ABSTRACT: The α-abnormal hemoglobin (Hb) M variants show physiological properties different from the *β*-abnormal Hb M variants, that is, extremely low oxygen affinity of the normal subunit and extraordinary resistance to both enzymatic and chemical reduction of the abnormal met-subunit. To get insight into the contribution of heme structures to these differences among Hb M's, we examined the 406.7-nm excited resonance Raman (RR) spectra of five Hb M's in the frequency region from 1700 to 200 cm^{-1}. In the high-frequency region, profound differences between met-α and met-*β* abnormal subunits were observed for the in-plane skeletal modes (the $\nu_{C=C}$, ν_{37}, ν_2, ν_{11}, and ν_{38} bands), probably reflecting different distortions of heme structure caused by the out-of-plane displacement of the heme iron due to tyrosine coordination. Below 900 cm^{-1}, Hb M Iwate [α(F8)His → Tyr] exhibited a distinct spectral pattern for ν_{15}, γ_{11}, $\delta(C_\beta C_a C_b)_{2,4}$, and $\delta(C_\beta C_c C_d)_{6,7}$ compared to that of Hb M Boston [α(E7)His → Tyr], although both heme irons are coordinated by Tyr. The *β*-abnormal Hb M variants, namely, Hb M Hyde Park [*β*-(F8)His → Tyr], Hb M Saskatoon [*β*(E7)His → Tyr], and Hb M Milwaukee [*β*(E11)Val → Glu], displayed RR band patterns similar to that of metHb A, but with some minor individual differences. The RR bands characteristic of the met-subunits of Hb M's totally disappeared by chemical reduction, and the ferrous heme of abnormal subunits was no longer bonded with Tyr or Glu. They were bonded to the distal (E7) or proximal (F8) His, and this was confirmed by the presence of the ν_{Fe-His} mode at 215 cm^{-1} in the 441.6-nm excited RR spectra. A possible involvement of heme distortion in differences of reducibility of abnormal subunits and oxygen affinity of normal subunits is discussed.

Hemoglobin (Hb)[1] M variants have abnormal ferric heme in either the α or *β* subunit of the $\alpha_2\beta_2$ tetramer, yielding a natural valency-hybrid hemoglobin under physiological conditions. Five variants have so far been known as Hb M (Figure 1). Four of them, namely, Hb M Iwate [α87(F8)His → Tyr], Hb M Boston [α58(E7)His → Tyr], Hb M Hyde Park [*β*92(F8)His → Tyr], and Hb M Saskatoon [*β*63(E7)-His → Tyr], have either the proximal (F8) or the distal (E7) histidine replaced by tyrosine (*1*−*3*). Hb M Milwaukee involves replacement of valine by glutamic acid [*β*67(E11)-Val → Glu] (*2*). These replacements in Hb M's Iwate, Boston, and Hyde Park make the redox potential of the heme iron more negative, so that the oxidized heme becomes toughly resistant to reduction by erythrocyte methemoglobin reductases even under anaerobic conditions and is retained in the ferric state in blood (*4*). Although the abnormal met-subunit of Hb M Saskatoon can be reduced by the met-hemoglobin reductases under anaerobic conditions at the

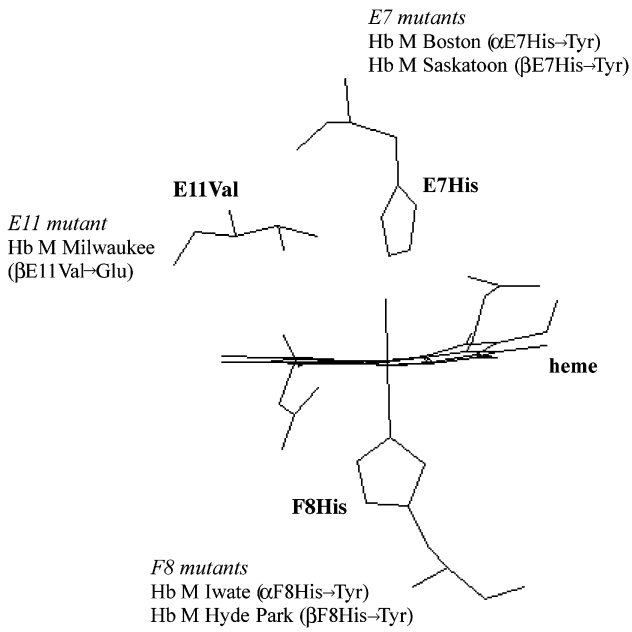

FIGURE 1: Mutation positions of Hb M's in the heme pocket. The coordinate of aquomet-*β* subunit of Hb A was obtained from pdb (1HGB) (*47*).

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[1] Abbreviations: Hb, hemoglobin; Mb, myoglobin; RR, resonance Raman; NiOEP, nickel octaethylporphyrin.

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same rate as metHb A, their autoxidation rates are so fast that some of the abnormal met-subunit still remains in the ferric state in blood (*4*−*6*). In a previous study (*7*), we demonstrated by 488.0-nm excited resonance Raman (RR) spectroscopy that only the heme iron of abnormal β subunit of Hb M Saskatoon adopts the hexacoordinate high-spin structure in contrast to the pentacoordinate high-spin structure in Hb M's Iwate, Boston, and Hyde Park. We have suggested that the unusually facile reducibility of the abnormal met-β subunit of Hb M Saskatoon results from the weak Fe-tyrosinate bond, which allows weak coordination of the proximal histidine, taking the hexacoordinate high-spin structure.

Depending on which subunit is affected, Hb M's also have distinct characteristics in sensitivity to chemical reduction of the abnormal met-subunits and in oxygen affinity of the normal subunits (*8*). The abnormal met-β subunit of Hb M Milwaukee is very slowly reduced by the methemoglobin reductases, while that of Hb M Hyde Park is hardly reduced by the methemoglobin reductases but can be reduced with a moderate rate by dithionite (1 mg/mL) or the ferredoxin and ferredoxin-NADP reductase system (*9*−*11*). On the other hand, abnormal met-α subunits of Hb M's Iwate and Boston are extraordinarily resistant to reduction by the ferredoxin and ferredoxin-NADP reductase system but are very slowly reduced by standing them overnight at room temperature in the presence of dithionite (3 mg/mL) under anaerobic conditions (*10*). Normal α subunits in Hb M's Hyde Park and Saskatoon show nearly normal oxygen affinity and a substantial Bohr effect (*12*, *13*). Although the abnormal subunit of Hb M Milwaukee is very slowly reduced by the methemoglobin reductases, the normal subunit is different in having low oxygen affinity and an increased Bohr effect (*14*). In contrast, normal β subunits in Hb M's Iwate and Boston exhibit extremely low oxygen affinity and nearly no Bohr effect (*15*, *16*).

Through extensive studies of heme proteins and model compounds, marker bands of the porphyrin core size and of electron density in the porphyrin π^* orbital have been discovered among the high-frequency (1700−1200 cm^{-1}) skeletal modes (*17*−*19*). Heme protein RR spectra are also very rich in the low-frequency (900−200 cm^{-1}) region and differ substantially from one protein to another (*20*, *21*). The information contained in these differences has been established through the analysis of the normal mode of nickel octaethylporphyrin (NiOEP) (*22*), cytochrome *c* (*23*), and myoglobin (Mb) (*24*) on the basis of the isotopically labeled hemes.

We examined the 406.7-nm excited RR spectra of abnormal met-α subunits in Hb M's Iwate and Boston and compared them with those of abnormal met-β subunits of Hb M's Hyde Park, Saskatoon, and Milwaukee to investigate the influence of the heme structure both on the reducibility of abnormal met-subunits and on oxygen binding features of normal subunits in Hb M's. In the high-frequency region, several spectral differences were observed between α-abnormal and β-abnormal Hb M's. In the region below 900 cm^{-1}, the F8-substituted Hb M's showed different spectra for modes of the peripheral groups of heme from those of the E7-substituted Hb M's, particularly in α-abnormal Hb M's.

EXPERIMENTAL PROCEDURES

Hemoglobins. Hb M's were prepared from patients' hemolysate as follows. Hb M's (Iwate, Boston, and Milwaukee) were separated from Hb A by an Amberlite CG-50 column chromatography (*4*). Hb M's (Hyde Park and Saskatoon) were purified by a preparative isoelectric focusing electrophoresis (*4*). The fully met hemoglobin was prepared by addition of ferricyanide. Half-met Hb M's (abnormal subunit in the met-form and normal subunit in the deoxy-form) were prepared from the half-oxy Hb M's (abnormal subunit in the met-form and normal subunit in the oxy-form) by repeating evacuation and flushing with N_2 gas. Hb M's (Iwate and Boston) were fully reduced by adding sodium dithionite (2 mg/mL) and standing the sample overnight at room temperature under anaerobic conditions (*25*, *26*). Hb M's (Hyde Park, Saskatoon, and Milwaukee) were fully reduced by adding sodium dithionite (1 mg/mL) just before measurement (*10*). Visible absorption spectra were measured with a Hitachi-U3010 spectrophotometer.

Measurements of Resonance Raman Spectra. Raman scattering was excited with the 406.7-nm line of a Kr^+ laser (Spectra-Physics, model 2060) or the 441.6-nm line of a He/Cd laser (Kimmon Electrics, model KR1801C), dispersed with a 100-cm single polychromator (Ritsu Oyo Kogaku, model MC-100DG) and detected with a liquid nitrogen-cooled CCD detector (Princeton Instruments, model LN/CCD-1100-PB) (*26*). The slit width and slit height were set to be 150 μm and 20 mm, respectively. A total of 150 μL of 50 or 200 μM hemoglobin solution (in heme) was used for measurements of the 406.7- or 441.6-nm excited RR spectra, respectively. All measurements were carried out at room temperature with an ordinary spinning cell (1000 rpm). Laser power at the sample point was 2−5 mW. Raman shifts were calibrated with indene (1700−500 cm^{-1}) and CCl_4 (500−200 cm^{-1}), and accuracy of the peak positions of Raman bands was ± 1 cm^{-1}. Integrity of hemoglobin sample was confirmed by recording visible absorption spectra before and after RR measurements.

RESULTS

Absorption and RR Spectra of Hb M Boston in the Fully Met Form and in the Half-Met Form. Figure 2 shows the absorption spectra of the fully met form (both normal and abnormal subunits in the met-form) (A) and the half-met form (abnormal subunit in the met-form and normal subunit in the deoxy-form) (B) of Hb M Boston together with those of metHb A and deoxyHb A, respectively. To extract the spectrum for the abnormal met-α subunit, the difference spectra between the fully met Hb M Boston and metHb A or between the half-met Hb M Boston and deoxyHb A were calculated in which the spectrum of Hb A multiplied by a factor of 0.5 was subtracted. The difference spectra thus calculated are delineated by dotted line. Both difference spectra are nearly identical and have the absorption maxima at 404 nm in the B-band region and at 491 and 603 nm in the Q-band region, respectively. Two peaks at 491 and 603 nm are obviously different from those at 500 and 631 nm for metHb A and that at 555 nm for the normal deoxy-subunits.

Figure 3 shows the 406.7-nm excited RR spectra of the fully met form (A) and the half-met form (B) of Hb M

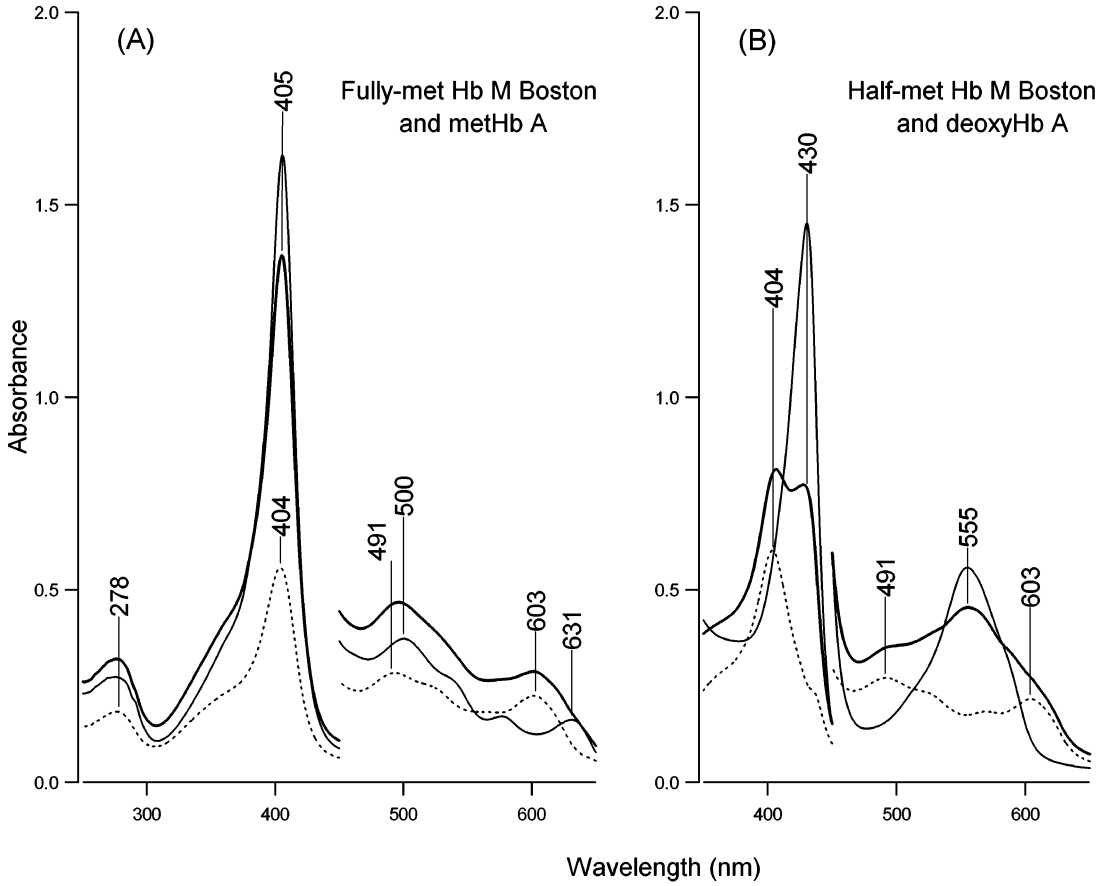

FIGURE 2: Electronic absorption spectra of the fully met (A) and the half-met (B) forms of Hb M Boston in comparison with those of the met- and deoxy-Hb A in 0.1 M phosphate buffer, pH 7.0. Hemoglobin concentrations used were 50 μM (in heme basis) in the region from 250 to 450 nm, and 200 μM in the region from 450 to 650 nm. The path length of an optical cell used was 2 mm. Bold line, Hb M Boston; thin solid line, Hb A; dotted line, the difference spectra (Hb M Boston-minus-1/2 Hb A).

Boston in comparison with those of metHb A (C) and deoxyHb A (D). Assignments of RR bands are based on the results of Mb (*24*). The 406.7-nm excitation mainly enhances the Raman bands of the ferric heme but only weakly those from the deoxy heme because of different absorption maxima. Therefore, both normal and abnormal subunits contribute to the RR spectrum of the fully met Hb M Boston, while the RR spectrum of the half-met form reflects mostly that from the heme of abnormal met-α subunit. Although the spectrum of the fully met Hb M Boston is different from that of metHb A for the ν_2 ($C_\beta-C_\beta$ stretch), ν_3 ($C_\alpha-C_m$ stretch), and several other RR bands, it is difficult to distinguish between the RR bands of abnormal met-α subunit and those of normal met-β subunit. In the RR spectrum of the half-met Hb M Boston, the ν_2, ν_3, and ν_4 (pyrrole half-ring)$_{sym}$ bands of the abnormal met-α subunit are separated from those of the normal deoxy-β subunit (*marked in Figure 3), but are still partly overlapped with the bands for the normal deoxy-subunit. The ν_2 and ν_3 bands, marker bands for spin and coordination states, are located at 1571 and 1487 cm^{-1} for the abnormal met-α subunit and at 1561 and 1479 cm^{-1} for the normal met-subunit, respectively. This indicates that the heme iron of abnormal met-α subunit takes the pentacoordinate high-spin structure, while that of the normal subunits of metHb A adopts the hexacoordinate high-spin structure (*27*−*30*). Hb M Boston gives three extra bands (underlined in Figure 3) at 1502, 1277, and 828 cm^{-1}, that are ascribed to Y19a, ν_{CO}, and Y1 modes of the tyrosine residue attached to the heme (*7*, *31*, *32*). We note that the ν_{15} band (pyrrole breathing) at 753 cm^{-1} is greatly intensified in the spectrum of Hb M Boston, implying that the porphyrin skeleton is much disordered in the abnormal subunit (*23*).

The 406.7-nm Excited RR Spectra of the Half-Met Hb M's. As seen in Figure 3, Raman bands for the abnormal subunit of Hb M Boston are more clearly observed in the half-met form than in the fully met form. Therefore, we compared the RR spectra of five Hb M's in the half-met form in Figures 4, 6, and 7. In these figures, the spectra of metHb A and deoxyHb A are also included at the bottom. Figure 4 shows the RR spectra of five Hb M's of the half-met form (A−E), metHb A (F), and deoxyHb A (G) in the high-frequency region (1680−1280 cm^{-1}). The oxidation state marker band, ν_4, of all Hb M's has two peaks at 1369 cm^{-1} from the abnormal met-subunit and at 1358 cm^{-1} from the normal deoxy-subunit. The ν_3 band is observed at 1487 cm^{-1} in the spectra of Hb M's Iwate, Boston, and Hyde Park and at 1473 cm^{-1} in the Hb M's Saskatoon and Milwaukee. The ν_2 and ν_{37} bands are seen at 1571 and 1589 cm^{-1} in the spectra of Hb M's Iwate and Boston and at 1564−1565 cm^{-1} and 1580−1582 cm^{-1} in the spectra of Hb M's Hyde Park, Saskatoon, and Milwaukee, respectively. The $\nu_{C=C}$ (vinyl C=C stretching mode) is observed at the higher frequencies in the spectra of α-abnormal Hb M's (Iwate and Boston) than in those for β-abnormal Hb M's (Hyde Park, Saskatoon, and Milwaukee). As several RR bands for the abnormal met-subunit including the $\nu_{C=C}$, ν_{37}, ν_3, and ν_4 overlap partly with

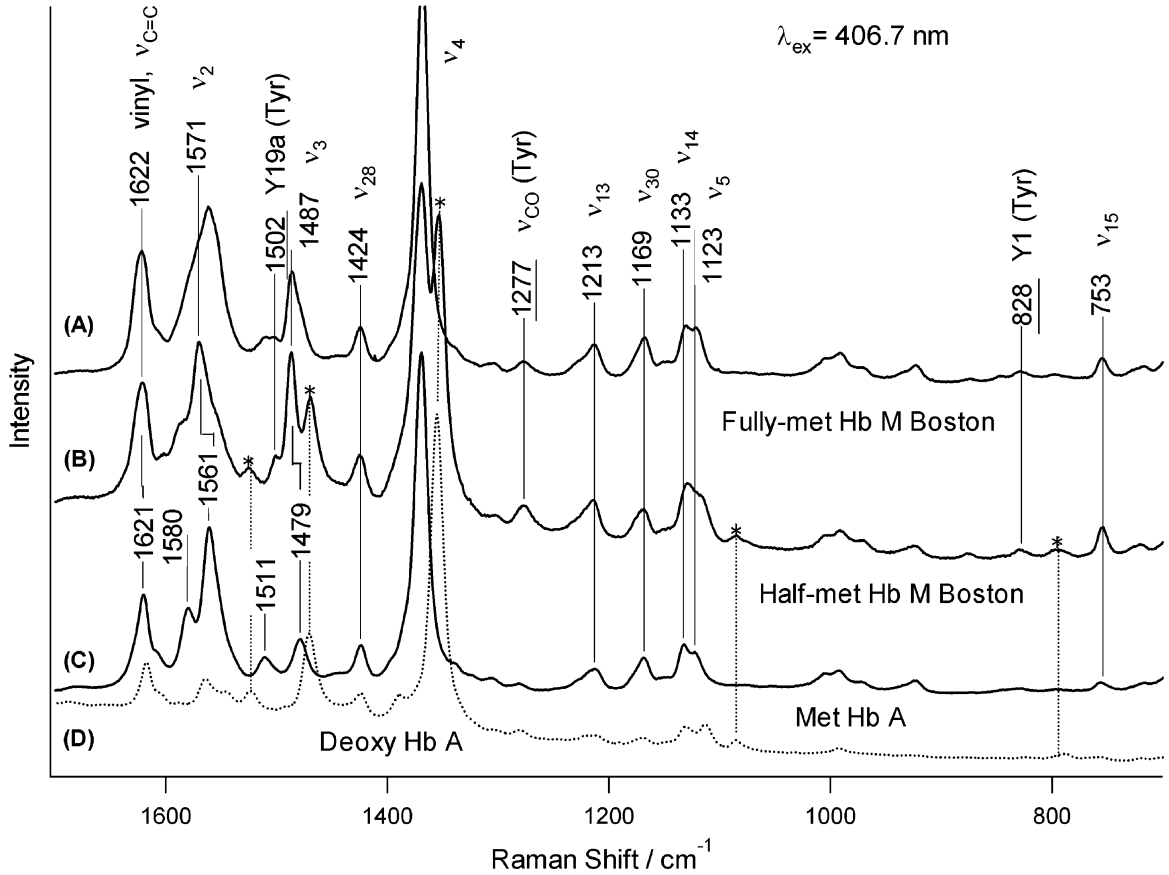

FIGURE 3: 406.7-nm excited RR spectra of the fully met Hb M Boston (A), the half-met Hb M Boston (B), metHb A (C), and deoxyHb A (D). Hemes of both normal and abnormal subunits are oxidized in the fully met Hb M Boston. In the half-met Hb M Boston, the abnormal α subunit is in the met-form, but the normal β subunit is in the deoxy-form. Peak assignments for porphyrin bands are according to Hu et al. (*24*), and these for the coordinated tyrosine related bands (underlined) are due to Que (*31*) and Nagatomo et al. (*32*). RR bands from the deoxy-form are marked by an asterisk in the spectrum of the half-met Hb M Boston (B). Hemoglobin concentration was 50 μM (in heme) in 0.1 M phosphate buffer, pH 7.0.

those for the normal deoxy-subunit as in Hb M Boston, peak frequencies in Figure 4 represent apparent values. Because contributions of the normal subunits of Hb M's would be similar to those of Hb A, the difference spectrum either between the fully met Hb M's and the metHb A or between the half-met Hb M's and the deoxyHb A was calculated to determine the exact peak frequency. Peak frequencies obtained from the two difference spectra were in good agreement (data not shown).

Figure 5 shows the difference spectra of five Hb M's calculated between the fully met Hb M's and the metHb A. The band frequencies of ν_3 mode for the abnormal subunits of three Hb M's Iwate, Boston, and Hyde Park appear at 1486−1487 cm^{-1}. On the other hand, ν_3 modes of Hb M Saskatoon and Hb M Milwaukee are seen at 1476 and 1479 cm^{-1}, respectively. This indicates that the hemes of abnormal met-subunits in the former three Hb M's take the penta-coordinate high-spin structure, while those of the latter two Hb M's adopt the hexacoordinate high-spin structure as reported previously (*7*). The α-abnormal Hb M's Iwate and Boston display distinct ν_{11} bands ($C_\beta-C_\beta$ stretch, B_{1g} symmetry) at 1554 cm^{-1}. The β-abnormal Hb M's Saskatoon and Milwaukee exhibit a weak band of ν_{38} ($C_\beta-C_\beta$ stretch, E_u symmetry) at 1513 cm^{-1} similar to 1511 cm^{-1} band for metHb A (Figure 4F). Small but distinct bands due to Y19a (ring vibration) of the attached Tyr are observed at 1501−1503 cm^{-1} for all Tyr-substituted Hb M's but not for Hb M Milwaukee. The $\nu_{C=C}$ band at 1621 cm^{-1} of metHb A (Figure 4F) is shifted up to 1625 cm^{-1} in the spectra of α-abnormal Hb M's (Iwate and Boston), but appears at 1620 cm^{-1} in those of β-abnormal Hb M's (Hyde Park, Saskatoon, and Milwaukee).

Figure 6 (A−E) shows the RR spectra of the half-met forms of five Hb M's in the frequency region between 850 and 600 cm^{-1} with those of metHb A (F) and deoxyHb A (G). A small but distinct band is observed around 825 cm^{-1} in the spectra of all four Hb M's with E7-Tyr or F8-Tyr, that was ascribed to the Y1 mode of tyrosine residue attached to the heme (*32*). Interestingly, Hb M's with the F8-Tyr (Iwate and Hyde Park) yield the peak of Y1 modes at frequencies different from those of Hb M's with the E7-Tyr (Boston and Saskatoon) irrespective of the subunit class, that is, the former at 823−824 cm^{-1} and the latter at 826−828 cm^{-1}. The ν_7 mode is observed at 674 cm^{-1} in metHb A. Its peak positions for Hb M's were obtained from the difference spectra between the half-met Hb M's and deoxyHb A. They are variable among Hb M's: Iwate at 672 cm^{-1}, Boston at 674 cm^{-1}, Hyde Park at 675 cm^{-1}, Saskatoon at 671 cm^{-1}, and Milwaukee at 672 cm^{-1} (Table 1).

MetHb A displays two pyrrole related RR bands at 753 cm^{-1} (ν_{15}, pyrrole breathing) and at 715 cm^{-1} (γ_{11}, pyrrole folding) (F). The frequencies and intensities of these bands are variable among Hb M's. Regarding the ν_{15} band for the abnormal met-subunit, the abnormal met-α subunit shows a

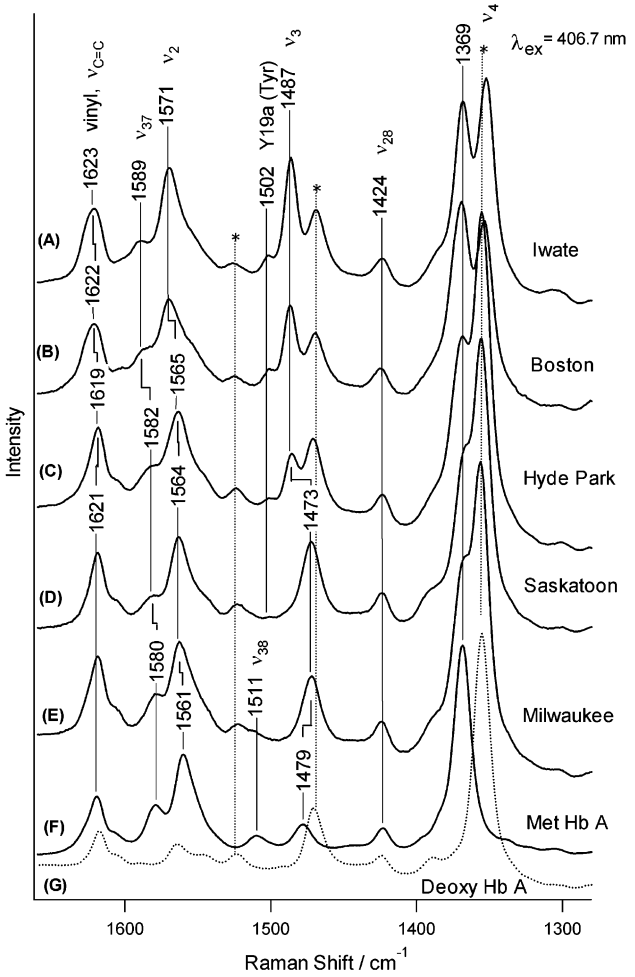

FIGURE 4: High-frequency RR spectra of the half-met Hb M Iwate (A), Hb M Boston (B), Hb M Hyde Park (C), Hb M Saskatoon (D), Hb M Milwaukee (E), and Hb A in the met-form (F) and in the deoxy-form (G). In the half-met Hb M's, the abnormal subunit is in the met-form and the normal subunit is in the deoxy-form. Other conditions are the same as those in Figure 3.

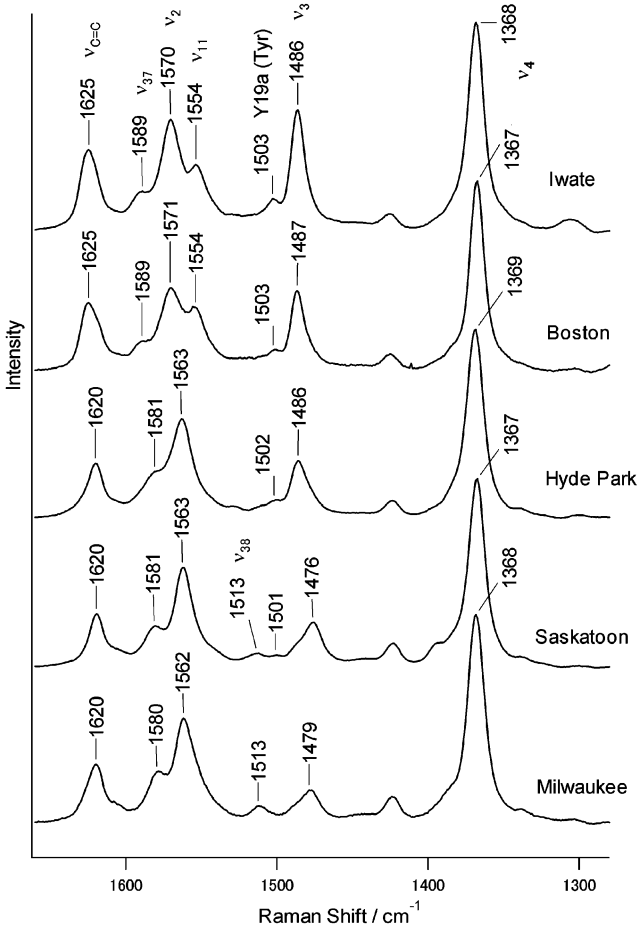

FIGURE 5: RR spectra for abnormal met-subunits in five Hb M's extracted as the difference spectrum between the fully met Hb M and metHb A.

more prominent band than the abnormal met-β subunit. The ν_{15} band of Hb M Boston (B) at 753 cm^{-1} is shifted down to 750 cm^{-1} in Hb M Iwate (A), indicative of different influences between the F8-His and E7-His substitutions. The ν_{15} modes for the abnormal met-β subunits of Hb M's Hyde Park (C) and Saskatoon (D) are less intensified than those for the abnormal met-α subunits and not shifted. The ν_{15} band is not so much intensified in the spectra of Hb M Milwaukee (E).

The abnormal met-subunits of Hb M's also show variations of the γ_{11} band in both intensity and peak frequency. We note that considerable differences in the intensity of γ_{11} band are observed between the F8-His substituted Hb M's (Iwate and Hyde Park) and the E7-His substituted Hb M's (Boston and Saskatoon), that is, the former two display a more prominent band than the latter two. The γ_{11} band is strongly intensified with an upshift by 3 cm^{-1} in the αF8-His substituted Hb M Iwate spectrum but weakened with a further upshift in the αE7-His substituted Hb M Boston spectrum. Although the γ_{11} band is also intensified with an upshift by 3 cm^{-1} in the βF8-His substituted Hb M Hyde Park spectrum, it is slightly intensified and not shifted for the βE7-His substituted Hb M Saskatoon. Hb M Milwaukee shows γ_{11} band similar to the metHb A. This result indicates that F8-His substitution in the α subunit has potent influence on the out-of-plane distortion of the heme in addition to the in-plane skeletal modes.

Figure 7 shows the RR spectra in the frequency region between 480 and 200 cm^{-1} for the half-met forms of five Hb M's (A−E), metHb A (F), and deoxyHb A (G). As the deoxyHb A does not display any discernible RR band in this region, all bands can be ascribed to the met-heme. The RR bands in this region are known to be sensitive to the conformation of the peripheral substituents (vinyl or propionate) of the heme (*22*−*24*). The two peaks of metHb A at 382 and 368 cm^{-1} and those at 432 and 407 cm^{-1} can be ascribed to the $\delta(C_\beta C_c C_d)_{6,7}$ (a bending mode of the propionate side chains) and to the $\delta(C_\beta C_a C_b)_{2,4}$ (a bending mode of the vinyl substituents), although the separation of two bands is not so clear for the latter. The separation of the $\delta(C_\beta C_a C_b)_{2,4}$ modes is clearer for all Hb M's. In the spectrum of Hb M Iwate (A), the higher frequency band at 425 cm^{-1} is stronger than the lower frequency band at 403 cm^{-1}, and both bands are shifted toward low frequencies by 4−7 cm^{-1}. The bands for other Hb M's appear at 405−410 and 430−435 cm^{-1}, and are also intensified.

As to the $\delta(C_\beta C_c C_d)_{6,7}$ mode, the abnormal met-subunits of Hb M's display the frequency and intensity specific to each species. A single prominent band at 367 cm^{-1} is seen in Hb M Iwate (A), while the band in Hb M Boston (B) is split into two components at 384 and 367 cm^{-1}. Hb M Saskatoon exhibits a band pattern similar to that of Hb M

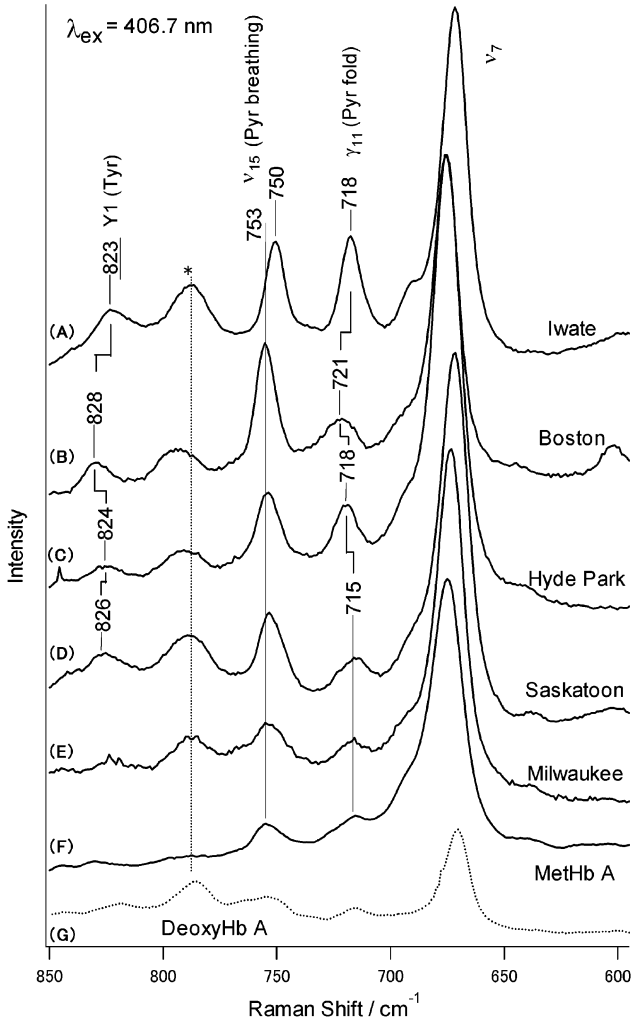

FIGURE 6: RR spectra of the half-met Hb M Iwate (A), Hb M Boston (B), Hb M Hyde Park (C), Hb M Saskatoon (D), Hb M Milwaukee (E), and Hb A in the met- (F) and in deoxy-forms (G) in the 850 and 600 cm^{-1} region. Experimental conditions are the same as those in Figure 3.

Boston, in which the higher frequency component is more intense than the lower frequency one. It is interesting that these E7-substituted Hb M's show a similar band pattern irrespective of the subunit class and also that the band pattern for Hb M Saskatoon resembles that of metHb A. In contrast, a reversed intensity pattern of the two bands is observed for the F8-substituted Hb M Hyde Park. Hb M Milwaukee shows also an intensity pattern similar to that of Hb M Hyde Park.

In the RR spectrum of metHb A (F), three peaks are observed at 345, 307, and 259 cm^{-1}. On the basis of the results of NiOEP (*22*) and Mb (*24*), these bands can be assigned to ν_8 (a pyrrole stretch and substituent bending mode), γ_7 (an out-of-plane methine wag mode involving C_α and C_m), and ν_9 (a pyrrole substituent bending mode) of the ferric heme, respectively. The ν_8 and ν_9 bands, which are mixed modes of Fe-N(pyrrole) stretching and pyrrole-substituent bending vibrations, have been shown to be sensitive to the substituent conformations in the case of NiOEP (*22*). The abnormal met-subunits show the intensified ν_8 band without a frequency shift except ν_8 of Hb M Boston with an upshift by 5 cm^{-1}. The γ_7 mode for the abnormal met-α subunit of Hb M Iwate exhibits an upshift to 312 cm^{-1}, while that of Hb M Boston is split into two bands at 306 and 296 cm^{-1}, indicative of appreciable difference in the out-of-plane distortion of hemes in two α subunits. Although the ν_9 mode for the abnormal met-α subunit of Hb M Boston is shifted up to 264 cm^{-1}, the abnormal met-subunits of other Hb M's give a broad band at 259−261 cm^{-1}. In this case, the E7-His substituted subunit shows a more intensified band than the F8-His substituted subunit. These results suggest that the bonding between the heme iron and the substituted Tyr or Glu residue in the abnormal subunits affect differently the structural distortion of the heme.

The 441.6-nm Excited RR Spectra of the Fully Reduced Hb M's. Figure 8 shows the 441.6-nm excited RR spectra of the fully reduced Hb M's and deoxyHb A in the high (1650−950 cm^{-1}) and the low (900−200 cm^{-1}) frequency regions. The spectra of Hb M's are almost the same as that of deoxyHb A in the high-frequency region. However, only Hb M Iwate (A) exhibits a different spectrum from other Hb M's and deoxyHb A in the low-frequency region (*26*). For the fully reduced Hb M Iwate, the ν_9 band appears as a distinct peak at 252 cm^{-1}, and several bands are shifted to lower frequencies by 1−6 cm^{-1}, ν_6 (788 → 786 cm^{-1}), ν_{15} (754 → 753 cm^{-1}), $\delta(C_\beta C_c C_d)_{6,7}$ (364 → 362 cm^{-1}), γ_7 (299 → 293 cm^{-1}), and ν_9 (253 → 252 cm^{-1}). In the fully reduced state, the ν_3 band emerges at 1470 cm^{-1} in the spectra of all five Hb M's as in that of deoxyHb A, indicating that the heme iron for the abnormal subunits in all Hb M's adopts the pentacoordinate high-spin structure. Upon reduction, the ferrous heme loses its axial ligand (either Tyr or Glu) but gets new axial coordination of the E7- or F8-His. This is confirmed by appearance of the Fe-His stretching mode, ν_{Fe-His}, at 215 cm^{-1} in all fully reduced Hb M's.

DISCUSSION

Coordination Structures of the Heme of Abnormal Subunits in Hb M's. It is well-known that the ν_3 and ν_{10} frequencies are sensitive to coordination number of the heme iron. The peak frequencies of ν_3 and ν_{10} modes appear at 1480−1483 cm^{-1} and at 1610−1620 cm^{-1} for the hexacoordinate ferric high-spin state, and at 1486−1490 cm^{-1} and 1628−1630 cm^{-1} for the pentacoordinate ferric high-spin state, respectively (*28*−*30*, *33*). However, in the B-band excited RR spectra, the ν_{10} mode is not observed for the metHb because of its overlap with the strong vinyl $C_a{=}C_b$ stretching band at 1621 cm^{-1} (*24*). In the present study, ν_3 modes for abnormal met-subunits of three Hb M's (Iwate, Boston, and Hyde Park) afforded peaks at 1486−1487 cm^{-1} while Hb M Saskatoon was at 1476 cm^{-1}. These results confirm the findings of our previous Q-band excited RR study that the heme iron for the abnormal subunit of the former three Hb M's adopts the pentacoordinate high-spin structure while that of Hb M Saskatoon takes the hexacoordinate high-spin structure (*7*). The ν_3 mode of the abnormal met-β subunit of Hb M Milwaukee is found at 1479 cm^{-1} (Figure 5), implying that the heme iron of the abnormal subunit adopts the hexacoordinate high-spin structure. On the basis of the Q-band excited Raman spectrum, Nagai et al. (*34*) suggested that in the abnormal subunit of Hb M Milwaukee, the γ-carboxyl group of βE11-Glu occupied the sixth coordination position of the ferric heme in place of a water molecule of aquomet-Hb A. The X-ray crystallographic study has indicated that the γ-carboxyl group of βE11-Glu is bonded to the ferric heme iron in Hb M Milwaukee (*35*). Taking

Table 1: Frequencies and Mode Assignments of Resonance Raman Bands Observed for Abnormal Met-α and Met-β Subunits in Hb M's[a]

	abnormal met-α subunits		abnormal met-β subunits			
ν_i description	Iwate	Boston	Hyde Park	Saskatoon	Milwaukee	metHb A
$\nu_{C=C}\,\nu(C_a{=}C_b)$	**1625**[b]	**1625**	1620	1620	1620	1621
$\nu_{37}\,\nu(C_\alpha{-}C_m)$asym	**1589**	**1589**	1581	1581	1580	1580
$\nu_2\,\nu(C_\beta{-}C_\beta)$	**1570**	**1571**	1563	1563	1562	1561
$\nu_{11}\,\nu(C_\beta{-}C_\beta)$	**1554**	**1554**	nd[c]	nd	nd	nd
$\nu_{38}\,\nu(C_\beta{-}C_\beta)$	nd	nd	nd	1513	1513	1511
$\nu_3\,\nu(C_\alpha{-}C_m)$sym	1486	1487	1486	1476	1479	1479
Y1 ν(Tyr)	823	828	824	826	nd	nd
$\nu_{15}\,\nu$(Pyr breathing)	**750(i)**[d]	**753(i)**	753	753	753	753
$\gamma_{11}\,\gamma$(Pyr-fold)asym	718(i)	721	718	715	715	715
$\nu_7\,\delta$(Pyr-def)sym	672[e]	674[e]	675[e]	671[e]	672[e]	674
$\delta(C_\beta C_a C_b)_{2,4}$	**425/403**	**435/410**	432/405	430/405	432/407	432/407
$\delta(C_\beta C_c C_d)_{6,7}$	**367**	**384/367**	382/369	381/366	384/368	382/368
$\nu_8\,\nu$(Fe−N)	344	349	345	344	344	345
$\gamma_7\,\gamma(C_\alpha{-}C_m)$	**312**	**306/296**	308	304	305	307
$\nu_9\,\delta(C_\beta{-}C_1)$sym	**261**	**264**	259	259	259	259

[a] Assignments are based on NiOEP (*22*) and metMb (*24*). [b] Bold letter, distinct RR bands in abnormal met-α subunits. [c] nd, not detected. [d] (i) means a greatly intensified band. [e] Peak positions were obtained from the difference spectrum between the half-met Hb M and deoxyHb A.

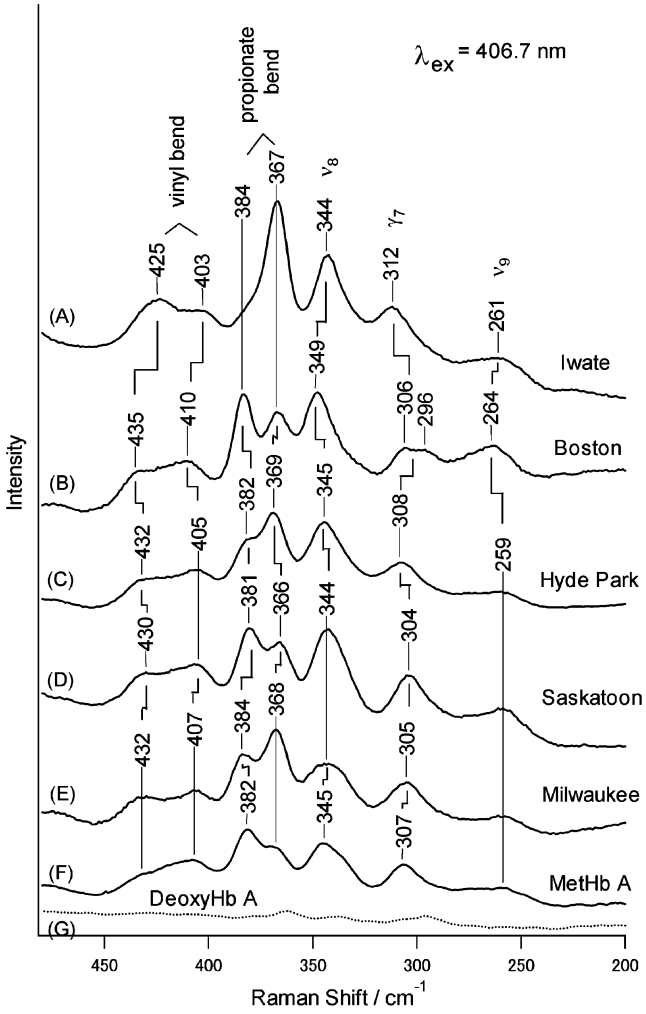

FIGURE 7: Low-frequency RR spectra of the half-met Hb M Iwate (A), Hb M Boston (B), Hb M Hyde Park (C), Hb M Saskatoon (D), Hb M Milwaukee (E), and Hb A in the met- (F) and in deoxy-forms (G). Experimental conditions are the same as those in Figure 3.

these results together, the heme irons of Hb M's Iwate, Boston, and Hyde Park are bonded only with either F8-Tyr or E7-Tyr, while those of Hb M's Saskatoon and Milwaukee are bound to both F8-His and E7-Tyr or E11-Glu.

RR Bands for the Coordinated Tyrosine. In the Q-band excited RR study, Nagai et al. (*7*) pointed out that the internal vibrations of the coordinated tyrosine yielded extra RR bands at 1607, 1505, 1278, and 603 cm^{-1} for Hb M Boston besides the usual RR bands for the iron porphyrin. These bands have been regarded as the characteristic fingerprint bands for tyrosine coordination to the metal ion (*31*). In the Q-band excited RR study on Hb M Boston, Nagatomo et al. (*32*) ascribed the RR band at 829 cm^{-1} to Y1 mode of the coordinated tyrosine. We note that F8-His substituted Hb M's (Iwate and Hyde Park) give the Y1 mode at lower frequencies (823−824 cm^{-1}) than E7-His substituted Hb M's (Boston and Saskatoon) (826−828 cm^{-1}) (Figure 6). According to 2.1 Å resolution X-ray crystallography of oxyHb A (*36*), the proximal His (F8) is located closer to the heme iron than the distal His (E7) in both α and β subunits. The distance from the axial His to the heme iron may have influence on the frequency of Y1 mode and thus cause the frequency difference between the F8-His substituted Hb M and the E7-His substituted Hb M described above.

Differences of the Heme Structure between Abnormal Met-α and Met-β Subunits of Hb M's. Table 1 summarizes the frequencies of RR bands for the abnormal met-α and met-β subunits in five Hb M's together with those of metHb A. As shown in the bold letter, several RR bands of the abnormal met-α subunit appear at different frequencies from those of the abnormal met-β subunit, while the latter are nearly equal to those for the normal met-subunits of metHb A.

Vinyl $C_a{=}C_b$ stretching modes for the abnormal met-α subunit are shifted up by 5 cm^{-1}. Vinyl groups are known to mix with some of the skeletal modes such as ν_2 and ν_{11} (*24*). The ν_2 mode for the abnormal met-α subunit is also shifted up by 7−9 cm^{-1}. The ν_{11} mode for the abnormal met-α subunit is observed at 1554 cm^{-1}, but that for the abnormal met-β subunit is absent. Vinyl groups are also known to break the symmetry classification and thus to induce Raman activity for the E_u mode (*37*, *38*). In the high-frequency region (Figure 4), the ν_{37} and ν_{38} bands (E_u mode) of the normal met-subunits of metHb A are obviously seen at 1580 and 1511 cm^{-1}, respectively. Because of overlap with the prominent ν_2 band, the ν_{37} bands for the abnormal

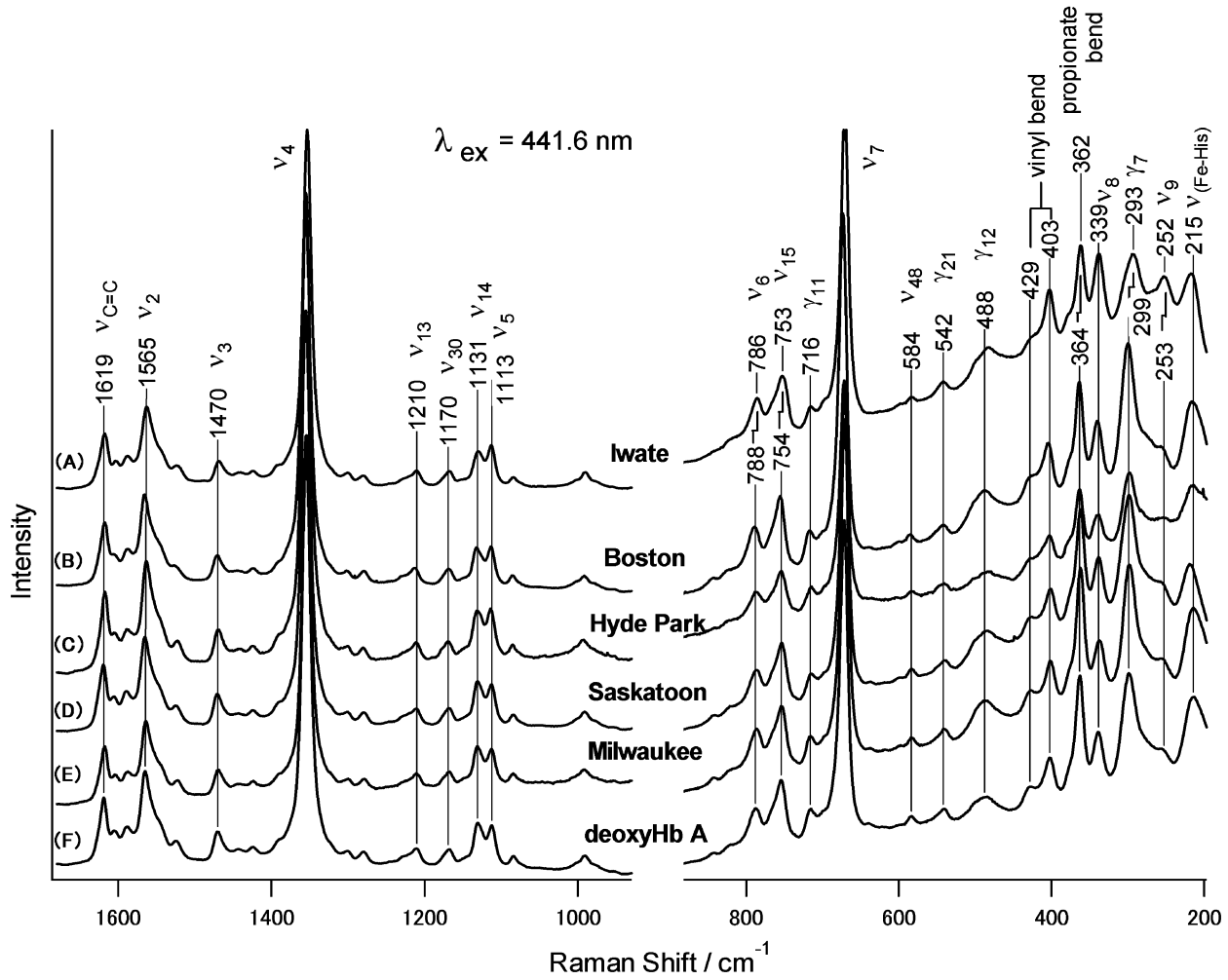

FIGURE 8: The 441.6-nm excited RR spectra of the fully reduced Hb M's and deoxyHb A. The concentration of hemoglobin was 200 μM (in heme) in 0.1 M phosphate buffer, pH 7.0.

subunit of Hb M's do not show distinct peaks such as the normal met-subunits of metHb A. The difference spectra between fully metHb M's and metHb A revealed the ν_{37} band with weak but significant intensity at 1589 cm^{-1} for the abnormal met-α subunit and at 1581 cm^{-1} for the abnormal met-β subunit (Figure 5, Table 1). This indicates that the ν_{37} mode for the abnormal met-α subunit is shifted up by 9 cm^{-1}, while that for the abnormal met-β subunit remains unaltered. The ν_{37} mode for the abnormal met-α subunit exhibits weaker intensity than that for the abnormal met-β subunit. The ν_{38} bands with weak intensities are seen at 1513 cm^{-1} only for the abnormal met-β subunits of two Hb M's with the hexacoordinate high-spin structure (Hb M Saskatoon and Hb M Milwaukee). On the other hand, this mode for the abnormal met-α subunit is absent. The bonding of either phenolate or carboxylate to the heme iron is expected to induce some specific local alteration in the protein structure surrounding the heme pocket, which probably affects conformation of the vinyl substituents. Accordingly, the observed differences in the in-plane skeletal modes between the abnormal met-α subunit and the abnormal met-β subunit seem also to reflect local differences induced in a protein structure surrounding the heme.

Below 500 cm^{-1}, striking differences in the band pattern of vinyl and propionate substituents are observed among the abnormal subunits of Hb M's. For the vinyl bending modes, $\delta(C_\beta C_a C_b)_{2,4}$, obvious differences are seen between Hb M Iwate and Hb M Boston. The 2-vinyl and 4-vinyl bending modes for Hb M Iwate are located at 425 and 403 cm^{-1}, respectively, and the former is more intensified than the latter (Figure 7). In contrast, in the spectrum of Hb M Boston, these modes are shifted up to 435 and 410 cm^{-1}, and the latter exhibits higher intensity than the former. The β abnormal Hb M's display a similar band pattern for these modes with peak frequencies at 430−432 and 405−407 cm^{-1}, respectively. For the propionate bending modes, δ-$(C_\beta C_c C_d)_{6,7}$, a marked difference is observed among Hb M's. The propionate bending mode for the abnormal met-α subunit appears as a prominent band at 367 cm^{-1} in the Hb M Iwate spectrum but is split into two bands at 384 and 367 cm^{-1} in Hb M Boston, the intensities of which are higher at 384 cm^{-1} than at 367 cm^{-1}. The abnormal met-β subunits give also two bands. Interestingly, the F8-His substituted Hb M's exhibit a band pattern different from that of E7-His substituted Hb M's in the intensity of the two peaks at 381−384 cm^{-1} and at 366−369 cm^{-1}. The abnormal subunits of the E7-His substituted Hb M's give the propionate bending bands similar to those of metHb A. The abnormal met-β subunit of Hb M Milwaukee exhibits a band pattern similar to that of the F8-His substituted Hb M's. Sperm whale myoglobin gave the propionate bending mode at 371 cm^{-1}. The mutations that cause weakening of the hydrogen bonding

to the 7-propionate side chain resulted in a downshift to 364 cm^{-1} (*39*). Monomeric HbI from a clam, *Lucina pectinata*, gave a weak peak at 370 cm^{-1} that was suggested to arise from the 7-propionate side chain in the presence of a moderate hydrogen bonding with Arg99 (*40*). According to the X-ray crystallographic study, hydrophilic side chains of propionate extend toward the outside of the hemoglobin molecule and are accessible to the surrounding medium (*41*). The propionate groups of the α subunit assume a different conformation from those of the β subunit. Namely, in the heme of β_1 subunit, the 6-propionate side chain forms an intersubunit hydrogen bond with α_1E1-Ser but that of 7-propionate does not. In the heme of β_2 subunit, the 7-propionate side chain forms an intersubunit hydrogen bond with α_2A6-Thr, but that of 6-propionate is free. On the other hand, the propionate side chains of hemes in the α_1 and α_2 subunits are not involved in the intersubunit contacts and are quite symmetric (*41*). Different from Mb and HbI, the 7-propionate side chain is free in the α subunit of Hb A but the 6-propionate side chain of α subunit forms an intrasubunit hydrogen bond with N_δ of CD3-His. The 6-propionate of α subunit of Hb A is also linked to a water molecule that is hydrogen-bonded to N_ξ of E10-Lys and to the carbonyl of E7-His (*41*). The shift of the propionate bending mode to a lower frequency in the abnormal α subunit of Hb M Iwate might be due to weakening of these hydrogen bonds by binding of F8-Tyr with the heme iron.

The Heme Structure and Physiological Properties in Hb M's. The abnormal subunits of axial His substituted Hb M's are stabilized in the ferric form and no longer bind with oxygen. The phenolate ion has a much stronger affinity to the ferric iron compared with the affinity of imidazole to the ferric iron. This explains why the phenolate ion is bonded to the heme iron in place of the imidazole in their abnormal subunits and the redox potential becomes more negative. The carboxylate (a strong anion) of βE11-Glu is also bonded to the iron in Hb M Milwaukee that takes the hexacoordinate high-spin structure. Among the axial-His substituted Hb M's, only Hb M Saskatoon can be reduced by erythrocyte methemoglobin reductases at the same rate as metHb A, whereas Hb M Milwaukee is reduced very slowly. In contrast, Hb M's Iwate, Boston, and Hyde Park are not reduced at all by the enzymes. However, the abnormal met-β subunit of Hb M Hyde Park can be reduced at a moderate rate by the ferredoxin and ferredoxin-NADP reductase system, while Hb M's Iwate and Boston are not be reduced (*10*). In metHb A, the iron is considered to stay in the plane of the heme as in oxyHb A adopting the R conformation in the quaternary structure. The present study has shown that the abnormal met-β subunits of Hb M's Saskatoon and Milwaukee give the peak frequencies of the in-plane skeletal modes similar to those in the normal met-subunits of metHb A. Accordingly, these two Hb M's assume the R conformation in the quaternary structure. The abnormal met-β subunit of Hb M Hyde Park exhibits similar band pattern to the normal met-subunits of metHb A, but the ν_{38} mode is not detected as in the abnormal met-α subunits of Hb M's Iwate and Boston. As described above, only Hb M Hyde Park can be reduced by the ferredoxin and ferredoxin-NADP reductase system among Hb M's with the pentacoordinate high-spin structure. However, the differences in the reducibility among the pentacoordinate Hb M's cannot be explained only by the characteristic RR band pattern of the abnormal met-β subunit of Hb M Hyde Park. The X-ray crystallographic study, although low resolution, is available on Hb M Hyde Park (*42*). The electron-density map at high resolution may help to answer this problem.

In the present study, distinct differences in both the in-plane skeletal modes and the modes for peripheral substituents were found between the abnormal met-α and met-β subunits (Table 1). Compared with the abnormal met-β subunit, the abnormal met-α subunit showed changes in intensities and peak frequencies for the in-plane skeletal modes and the out-of-plane modes (especially γ_7), indicative of larger distortion of the heme structure induced by the out-of-plane displacement of the heme iron due to the coordinated tyrosine. According to the X-ray diffraction analysis, the distance between the proximal histidine and the heme plane changes from 2.7 Å in deoxy (T)- to 2.1 Å in oxy (R)-Hb A (*36*). A difference electron-density map of Hb M Boston at 3.5 Å resolution showed that in the abnormal met-α subunit, the iron atom was further shifted by about 1 Å to the distal side (*43*). The X-ray crystallographic study also demonstrated that Hb M Boston was locked in the T conformation of the quaternary structure irrespective of the oxy- and deoxy-forms of the normal subunit. The similar shift of the iron position was observed for the electron-density map of Hb M Iwate (*42*), in which the heme iron of the abnormal met-α subunit was about 2 Å closer to helix E than that in deoxyHb A, probably because the tyrosine side chain was much larger than the histidine side chain.

The crystal structure at low resolution (*42*) and nuclear magnetic resonance studies of Hb M Iwate (*44*) suggested that Hb M Iwate was frozen in the T conformation as in Hb M Boston and did not undergo the T → R transition upon ligand binding to the normal subunit. The displacement of the heme iron toward the E helix due to the tyrosine coordination presumably gives considerable distortion of the heme structure in the abnormal met-α subunits, which stabilizes the quaternary structure of the abnormal met-α subunit in the T conformation. Thus, the redox potentials of Hb M's Iwate and Boston (−1.13 V) are extremely lowered from that of metHb A (+ 0.144 V) (*10*). The normal β subunits in the α abnormal Hb M's (Iwate and Boston) have extremely low oxygen affinity. Accordingly, these Hb M's have no cooperativity and almost no Bohr effect. On the other hand, in the axial-His substituted β abnormal Hb M's (Hyde Park and Saskatoon), normal α subunits show relatively normal oxygen affinity. This was explained by disturbances of the F helix and the FG corner due to accommodation of Tyr, which probably enables these Hb M's to undergo the T−R transition that results in a normal Bohr effect and normal oxygen affinity (*45*).

The present study has shown that the abnormal met-β subunits in the axial-His substituted Hb M's display RR spectra almost similar to that of the normal met-subunits in Hb A. This implies that the heme structure in the abnormal met-β subunit is not as structurally distorted as that in the abnormal met-α subunit. On the other hand, the normal α subunit of Hb M Milwaukee has low oxygen affinity. The X-ray crystallographic study (*35*) has suggested that the met-β subunit takes up the deoxy tertiary conformation and forces the quaternary structure to favor the T structure that results in low oxygen affinity. However, it is considered that

the met-β subunit of Hb M Milwaukee undergoes a change of tertiary structure upon oxygenation to the normal α subunit, allowing a change to the quaternary R structure, which attains a substantial Bohr effect. In the same way as the axial-His substituted β abnormal Hb M's, the abnormal β subunit of Hb M Milwaukee exhibited similar RR spectra to that of the normal met-subunits in Hb A except the bending modes of the peripheral substituents. It is likely that bonding of the carboxylate of βE11-Glu to the heme iron brings about the tertiary structure of the subunit different from that of the tyrosine coordinated one. It was reported that nitric oxide could reduce the abnormal β subunit of Hb M Saskatoon but not that of Hb M Milwaukee due to the strong intramolecular bonding between glutamic acid (βE11) and the heme iron (*46*). This suggests a possibility that the abnormal β subunit assumes an intrinsic tertiary structure in Hb M Milwaukee. This structural alteration of the abnormal β subunit in Hb M Milwaukee might constrain the normal α subunit to reduce the oxygen affinity.

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