Unusual CO Bonding Geometry in Abnormal Subunits of Hemoglobin M Boston and Hemoglobin M Saskatoon†

Masako Nagai,‡ Yoshimasa Yoneyama,§ and Teizo Kitagawa*,‖

Biological Laboratory, Kanazawa University School of Allied Medical Professions, Kanazawa, Ishikawa 920, Japan, Department of Biochemistry, Kanazawa University School of Medicine, Kanazawa, Ishikawa 920, Japan, and Institute for Molecular Science, Okazaki National Research Institutes, Myodaiji, Okazaki 444, Japan

Received December 28, 1990; Revised Manuscript Received April 2, 1991

ABSTRACT: To clarify the role of the proximal histidine (F8-His), distal His (E7-His), and E11 valine (E11-Val) in ligand binding of hemoglobin (Hb), we have investigated the resonance Raman (RR) spectra of the carbon monoxide adduct of Hbs M (COHb M) in which one of these residues was genetically replaced by another amino acid in either the α or β subunit. In the fully reduced state, all Hbs M gave ν_3 at $\sim$1472 cm^{-1} and $\nu_{Fe\text{-}His}$ at 214–218 cm^{-1}, indicating that they have a pentacoordinate heme and the heme iron is bound to either E7-His or F8-His. The porphyrin skeletal vibrations of the COHb M were essentially unaltered by replacements of E7- or F8-His with tyrosine (Tyr) and of E11-Val by glutamic acid (Glu). The ν_{CO}, $\nu_{Fe\text{-}CO}$, and $\delta_{Fe\text{-}C\text{-}O}$ frequencies of COHb M Iwate (αF8-His → Tyr), COHb M Hyde Park (βF8-His → Tyr), and COHb M Milwaukee (βE11-Val → Glu) were nearly identical with those of COHb A. In contrast, the RR spectra of COHb M Boston (αE7-His → Tyr) and COHb M Saskatoon (βE7-His → Tyr) gave two new Raman bands derived from the abnormal subunits, $\nu_{Fe\text{-}CO}$ at 490 cm^{-1} and ν_{CO} at 1972 cm^{-1}, in addition to those from the normal subunits at 505 cm^{-1} ($\nu_{Fe\text{-}CO}$) and 1952 cm^{-1} (ν_{CO}). The CO adduct of the abnormal subunits exhibited apparently no photodissociation upon illumination of CW laser with a stationary cell under which the normal subunit exhibited complete photodissociation. From the normal coordinate analysis, the Fe–C–O bond in the abnormal subunits of Hb M Boston and Hb M Saskatoon was suggested to be linear perpendicular against the heme plane.

In hemoglobin (Hb),[1] the amino acid residues near the heme iron play important roles for preventing the ferrous iron from oxidation and also for cooperative binding of oxygen. Especially, the highly conserved two histidine (His) residues called the proximal (F8-His) and the distal His (E7-His) are the most substantial (Dickerson & Geis, 1983). The E11 valine in the β subunit (βE11-Val), which is located in the proximity of the ligand binding site and may exert steric hindrance on the bound ligand, is also responsible for the ligand affinity. Substitution of tyrosine (Tyr) for either one of these His or of glutamic acid (Glu) for βE11-Val causes the so-called Hb M disease in which the abnormal subunits are usually stabilized in the oxidized form in vivo and cannot bind oxygen (Bunn & Forget, 1986). However, these abnormal subunits can bind the ligand after chemical reduction in vitro. Generally, for oxygen binding heme proteins, carbon monoxide (CO) is bound to the reduced heme without oxidizing the heme iron. Therefore, CO is expected to serve as a useful probe for exploring modulation of the heme environment in the abnormal subunits of Hbs M.

Resonance Raman (RR) scattering from heme proteins provides detailed structural information on the heme moiety (Asher, 1981; Rousseau et al., 1983; Spiro, 1988; Kitagawa & Ozaki, 1987). Information on the bound CO can be obtained from the C–O stretching (ν_{CO}), Fe–CO stretching ($\nu_{Fe\text{-}CO}$), and Fe–C–O bending ($\delta_{Fe\text{-}C\text{-}O}$) RR bands (Tsubaki et al., 1982; Yu, 1986; Yu & Kerr, 1988; Li & Spiro, 1988). In this paper, we demonstrate that the CO binding is modulated substantially by the replacement of either αE7-His (Hb M Boston) or βE7-His (Hb M Saskatoon) by Tyr but little affected by the substitution of Tyr for either αF8-His (Hb M Iwate) or βF8-His (Hb M Hyde Park) and Glu for βE11-Val (Hb M Milwaukee).

Materials and Methods

Hemoglobins. Purification of five kinds of Hbs M from patients' hemolysate and the reduction of abnormal subunits were carried out as described previously (Nagai, M., et al., 1980, 1987; Nagai, 1985). Purity of the isolated Hbs M was examined by analytical electrofocusing on an ampholine plate gel (pH range 3.5–9), and contamination of Hb A in each Hb M preparation was confirmed to be less than 1%. The Hb concentration was determined after conversion to the pyridine hemochrome by assuming ϵ_{mM}(557 nm) = 34. COHbs M were obtained by flushing with CO gas into the fully reduced Hb solution in 0.05 M Bis-Tris buffer, pH 7.0, containing 0.1 M NaCl. $^{13}C^{16}O$ (99 atom % for ^{13}C), $^{12}C^{18}O$ (98 atom % for ^{18}O), and $^{13}C^{18}O$ (99 atom % for ^{13}C and 95 atom % for ^{18}O) were purchased from Cambridge Isotope Laboratories.

Absorption Spectrum. Absorption spectra of the fully oxidized, fully reduced, and fully CO liganded Hbs M were recorded with a Hitachi U-3210 spectrophotometer.

† This work was supported by the Yamanouchi Foundation for Research on Metabolic Disorders (Y.Y.) and the Joint Program of the Institute for Molecular Science (1989–1990) and also by a Grant-in-Aid for Scientific Research in Priority Areas to T.K. (63635005).

* To whom correspondence should be addressed.
‡ Biological Laboratory.
§ Department of Biochemistry.
‖ Institute for Molecular Science.

[1] Abbreviations: α^A and β^A, normal subunits of Hbs M; α^M and β^M, abnormal subunits of Hbs M; CO, carbon monoxide; COHb, carbon monoxide adduct of Hb; CW, continuous wavelength; Glu, glutamic acid; Hb, hemoglobin; His, histidine; Mb, myoglobin; RR, resonance Raman; Tyr, tyrosine; Val, valine.

0006-2960/91/0430-6495$02.50/0

Table I: Absorption Maxima (nm) of Five Hbs M and Hb A in the Fully Oxidized (Met), Fully Reduced, and Fully CO Forms

hemoglobins	Met			reduced		CO		
	Soret	visible		Soret	visible	Soret	visible	
Hb M Iwate	405.0	486.2	588.5	430.6	557.3	420.2	539.4	569.0
Hb M Boston	405.2	494.6	603.0	430.8	555.7	420.8	538.8	568.7
Hb M Hyde Park	404.6	491.3	576.3	430.2	557.1	419.6	539.2	569.3
Hb M Saskatoon	405.4	487.4	599.0	430.4	555.3	420.2	539.1	568.4
Hb M Milwaukee	405.0	499.5	622.2	430.4	554.6	418.8	538.6	568.8
Hb A	405.7	499.7	630.1	430.4	555.7	419.4	539.1	568.6

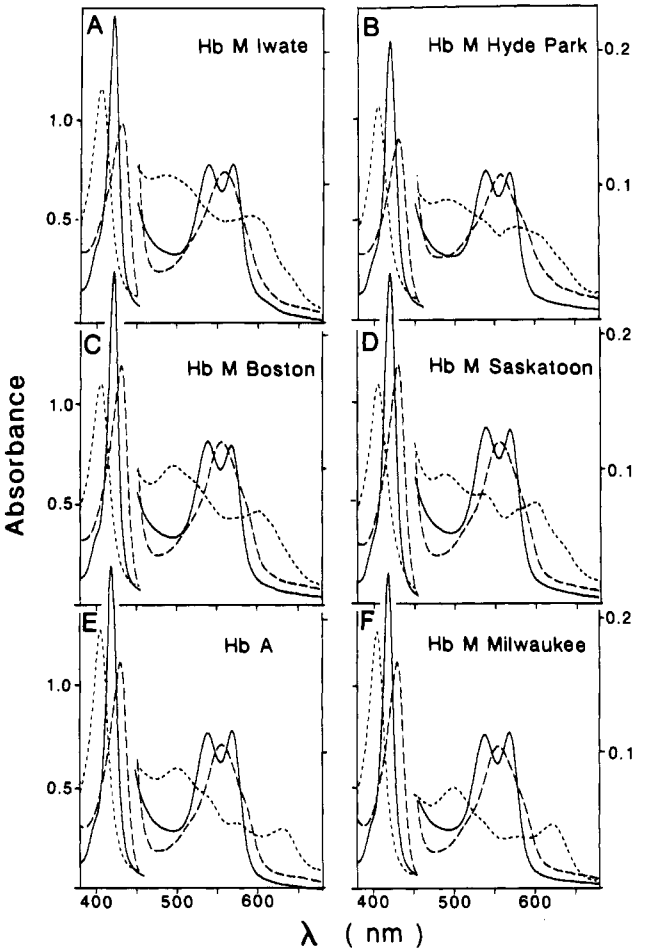


FIGURE 1: Absorption spectra of the fully oxidized (---), fully reduced (--), and fully CO-bound (—) forms of Hb M Iwate (A), Hb M Hyde Park (B), Hb M Boston (C), Hb M Saskatoon (D), Hb A (E), and Hb M Milwaukee (F). Hbs were fully oxidized by ferricyanide (Nagai & Yoneyama, 1983), and after the measurements of their spectra, Hbs M were reduced with dithionite (Nagai, 1985; Nagai, M., et al., 1987). COHbs M were obtained by introducing 100% CO gas into the fully reduced Hb M solutions after measurements of their spectra. Buffer used was 0.05M Bis-Tris buffer, pH 7.0, containing 0.1 M NaCl. Hemoglobin concentrations were ~40 μM (in heme), and a 2-mm light path of Thunberg-type cuvette was used.

Resonance Raman Spectrum. Raman scattering was excited by the 406.7-nm line of a Kr laser (Spectra-Physics Model 164) or the 441.6-nm line of a He/Cd laser (Kinmon Electrics, Model CDR80MGE) and recorded on a JEOL-400D Raman spectrometer equipped with a cooled photomultiplier (RCA-31034a). Raman shifts were calibrated with indene (500–1600 cm^{-1}) (Hendra & Loader, 1968) and CCl_4 (200–500 cm^{-1}). The CO adducts of Hb were measured with an air-tight spinning cell (1800 rpm) by making the laser power as low as possible (less than 10 mW at the sample point), while the fully reduced Hb was measured in a cylindrical cell. The temperature of the sample solution was kept below 10 °C throughout the measurement by flushing with cold N_2 gas against the Raman cell.

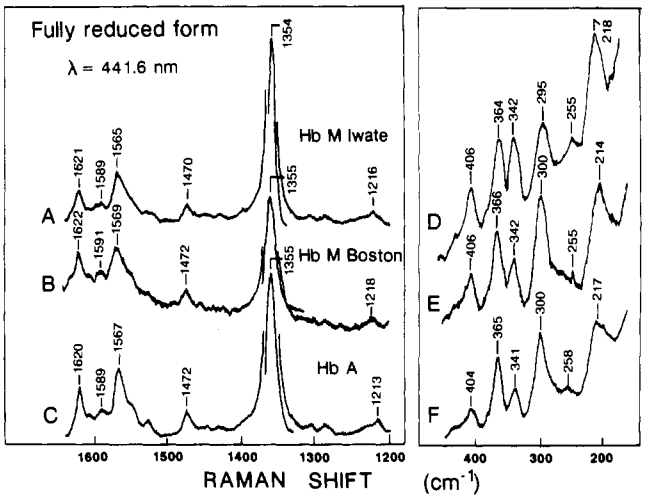


FIGURE 2: RR spectra in the 1200–1650-cm^{-1} region (A–C) and in the 180–450-cm^{-1} region (D–F) of Hb M Iwate (A, D) and Hb M Boston (B, E) in the fully reduced form. RR spectra of deoxyHb A (C, F) are also shown for comparison. Hbs M were reduced with dithionite. Hbs (100 μM in heme) were in 0.05 M Bis-Tris buffer, pH 7.0, containing 0.1 M NaCl. Excitation, 441.6 nm.

RESULTS

Absorption Spectra of Hbs M. Figure 1 shows the absorption spectra of five Hbs M in the fully oxidized, reduced, and CO-bound forms. For comparison, those of Hb A are also included. The wavelengths of the absorption maxima of these spectra are summarized in Table I. Although absorption spectra of four E7- or F8-His substituted Hbs M in the fully reduced and CO-bound forms were nearly identical with those of Hb A, their spectra in fully oxidized form were obviously different from that of Hb A. By contrast, all spectra of Hb M Milwaukee were almost the same as those of Hb A except for a shift of the charge-transfer band of the oxidized form from 630 nm of Hb A to 622 nm of Hb M Milwaukee.

RR Spectra of Fully Reduced Hbs M. The ferric hemes of the abnormal subunits of four Hbs M are coordinated by a phenolate group of the replaced F8- or E7-Tyr (Nagai et al., 1989). However, it is unlikely that the reduced iron (Fe^{2+}) is bonded to the Tyr because of relatively weak affinity of a phenolate group of Tyr for it (Bunn & Forget, 1986; Peisach & Gersonde, 1977). RR spectroscopy has been successfully used for determination of the coordination number of iron ions in heme proteins and model compounds (Spiro & Burke, 1976; Kitagawa et al., 1976; Kitagawa & Teraoka, 1979; Andersson et al., 1989). In order to get information on the coordination structure of these Hbs M in the fully reduced state, we examined their RR spectra. The RR spectra in the higher (1200–1650-cm^{-1}) and lower (180–450-cm^{-1}) frequency regions of the fully reduced Hb M Iwate and Hb M Boston are compared with that of the deoxygenated Hb A in Figure 2. The fully reduced Hb M Iwate (A) and Hb M Boston (B) bear close resemblance to the deoxyHb A (C) in their RR spectra.

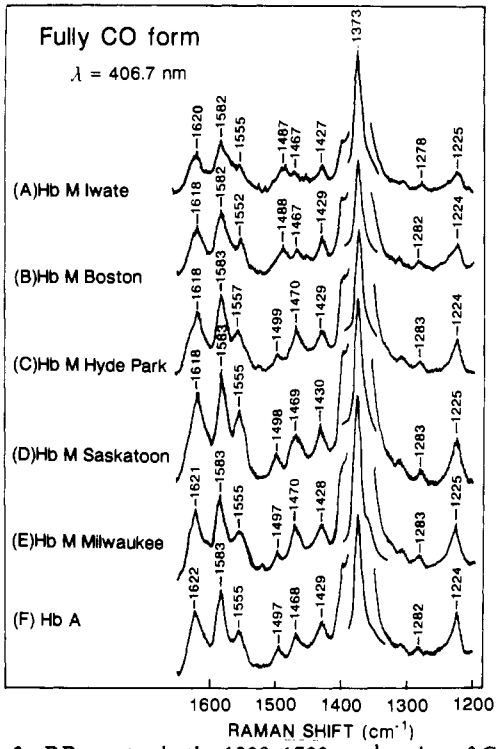


FIGURE 3: RR spectra in the 1200–1700-cm^{-1} region of COHb M Iwate (A), COHb M Boston (B), COHb M Hyde Park (C), COHb M Saskatoon (D), COHb M Milwaukee (E), and COHb A (F). All Hbs (100 μM in heme) were in 0.05 M Bis-Tris buffer, pH 7.0, containing 0.1 M NaCl and dithionite. Raman cell (2 cm in diameter) was spinning (1800 rpm) and kept below 10 °C by flushing with cold N_2 gas. Excitation, 406.7 nm.

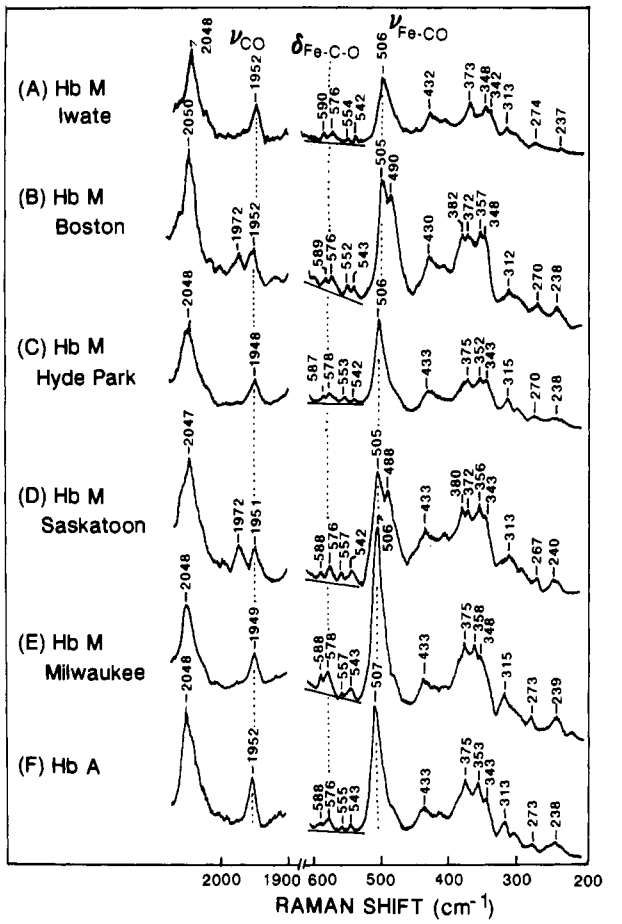


FIGURE 4: RR spectra of COHb M Iwate (A), COHb M Boston (B), COHb M Hyde Park (C), COHb M Saskatoon (D), COHb M Milwaukee (E), and COHb A (F) in the higher (1900–2100-cm^{-1}) and lower (200–600-cm^{-1}) frequency regions. Experimental conditions are the same as those for Figure 3.

The ν_3 bands of Hb M Iwate and Hb M Boston [mode numbers are based on Abe et al. (1978) and Li et al. (1990)] were observed at 1470 and 1472 cm^{-1}, respectively, like that of Hb A (1472 cm^{-1}). The ν_4 band is seen at 1354–1355 cm^{-1}. If they have a tetracoordinate ferrous heme, ν_3 and ν_4 bands are expected to appear at around 1500 and 1375 cm^{-1}, respectively (Kitagawa & Teraoka, 1979; Andersson et al., 1989). Therefore, this observation indicates that reduced heme irons of the abnormal subunits as well as those of the normal subunits adopt a pentacoordinate structure like deoxyHb A. The $\nu_{Fe\text{-}His}$ band is clearly observed in the spectra of Hb M Iwate (at 218 cm^{-1}) and Hb M Boston (at 214 cm^{-1}), and their relative intensities to the band around 300 cm^{-1} are stronger in the mutants than in deoxyHb A, indicating that the abnormal subunits of Hb M Iwate and Hb M Boston have the histidine-coordinated ferrous hemes similar to the normal subunits. Although the relative intensities of Raman bands are subject to alteration by higher order structures of the protein moiety, all these features suggest close similarity among the fully reduced forms of Hb M Iwate, Hb M Boston, and Hb A regarding the structure of the porphyrin skeleton and the iron coordination. RR spectra of the fully reduced Hb M Hyde Park, Hb M Saskatoon, and Hb M Milwaukee were also nearly identical with that of deoxyHb A (data not shown).

RR Spectra of COHbs M in the 1200–1700-cm^{-1} Region. The above results seem to imply that CO is unusually bound to the proximal side of the heme in the abnormal subunits of Hb M Iwate and Hb M Hyde Park, since the distal His must be bound to the heme iron. Figure 3 shows the RR spectra of COHb M Iwate (A), COHb M Boston (B), COHb M Hyde Park (C), COHb M Saskatoon (D), COHb M Milwaukee (E), and COHb A (F) in the 1200–1700-cm^{-1} region. The ν_4 bands of the CO form of Hbs M and Hb A are nearly the same (1373 cm^{-1}) and distinctly different from that of the deoxyHbs (1355–1357 cm^{-1}). The ν_4 band at ~1357 cm^{-1} was sometimes weakly observed due to partial photolysis of COHb by laser irradiation, but it was at most 10% throughout the present measurements.

The ν_3 bands of COHb M Iwate (1487 cm^{-1}) and COHb M Boston (1488 cm^{-1}) were shifted to lower frequency by 10 cm^{-1} than those of other COHbs M and COHb A (1497 cm^{-1}). Probably, the ν_3 bands of the abnormal subunits of the two Hbs M appear at lower frequency with higher intensity and obscure the ν_3 band of the normal subunit. The ν_3 band is known to serve as a core-size marker of porphyrin (Spaulding et al., 1975). This suggests that the porphyrin cores of abnormal α subunits of COHb M Iwate and COHb M Boston are more expanded than that of normal Hb, although its direct origin could not be clarified. The vinyl side-chain bands (Choi et al., 1982a,b) are observed at 1618–1622 cm^{-1}, and they show no systematic differences. Other Raman bands associated with the porphyrin ring vibrations were nearly the same. This means that the main configuration of heme moiety is not modified by the amino acid substitutions in all Hbs M.

C–O Stretching, Fe–CO Stretching, and Fe–C–O Bending Frequencies of COHbs M. Figure 4 shows RR spectra of five COHbs M and COHb A in the ν_{CO}, $\nu_{Fe\text{-}CO}$, and $\delta_{Fe\text{-}C\text{-}O}$ regions. The RR spectrum of COHb A (Figure 4F) exhibited $\nu_{Fe\text{-}CO}$ at 507 cm^{-1}, $\delta_{Fe\text{-}C\text{-}O}$ at 576 cm^{-1}, and ν_{CO} at 1952 cm^{-1}. These values are in agreement with those reported previously (Tsubaki et al., 1982). Substitution of Tyr for the proximal His in either subunit, or Glu for Val (βE11), did little to affect these three vibrations (Figure 4A,C,E). The result from

COHb M Milwaukee is consistent with the results from the site-directed mutagenesis experiment to the β chain of COHb A (Nagai, K., et al., 1987), which resulted in no change of these frequencies after replacement of βE11-Val with other bulky groups. In contrast, substitution of Tyr for the distal His in either subunit induced definite changes in the RR spectra (Figure 4B,D). In the spectra of COHb M Boston and COHb M Saskatoon, two new bands were observed at 490 and 1972 cm^{-1} in addition to the ordinary bands at 505 cm^{-1} (ν_{Fe-CO}) and 1952 cm^{-1} (ν_{CO}) due to the normal subunits. These new bands were assigned to the ν_{Fe-CO} and ν_{CO} modes of their abnormal subunits, respectively, from the isotope substitution experiments described later. The frequencies and intensities of all other RR bands were found to be practically identical with those of COHb A, indicating that the general porphyrin structure was also unaltered by the E7-His substitution. Concerning the ν_{CO} frequency of COHb, infrared (IR) spectroscopy is also useful for detailed component analysis (Choc & Caughey, 1981; Potter et al., 1983; Satterlee et al., 1978), and in fact we have obtained additional supports for altered CO binding structure in abnormal subunits of Hb M Boston and Hb M Saskatoon by IR and ^{13}C NMR spectroscopy.[2]

In the low-frequency region, RR bands of heme are mainly associated with the in-plane deformation modes of the skeletal and peripheral groups (Abe et al., 1978; Lee et al., 1986; Li et al., 1990). Around the bending frequency of Fe–C–O (576 cm^{-1}), there are three other Raman bands (543, 555, and 588 cm^{-1}) which are tentatively assigned to ν_{49}, ν_{25}, and ν_{24}, respectively (Li et al., 1990). The δ_{Fe-C-O} frequencies of all Hbs M were almost identical with that of COHb A. When their intensities are compared with that at 543 cm^{-1} (ν_{49}), it is noticed that the intensity of the δ_{Fe-C-O} bands of Hb M Boston and Hb M Saskatoon, the E7 mutant Hbs M, are nearly half those of other Hbs M and Hb A, suggesting a possibility that the δ_{Fe-C-O} RR band of the abnormal subunits of E7 mutant Hbs M is noticeably weak.

In the lower frequency region of the spectra of Hb M Boston and Hb M Saskatoon (E7 mutants), an additional band was observed at 380 cm^{-1}, and in the spectra of Hb M Iwate and Hb M Hyde Park (F8 mutants) the 238-cm^{-1} RR band was greatly decreased in intensity. These features may suggest that the porphyrin peripheral groups are affected differently by the substitution of Tyr for either the proximal His (F8) or distal His (E7). The frequency and relative intensity of the RR band at 2048 cm^{-1}, which would be a combination band of ν_4 and ν_7, were almost unaltered through all Hbs M and Hb A.

Carbon Monoxide Isotope Effects. Figure 5 shows the effects of CO isotope substitution on RR spectra of COHb M Boston (A) and COHb M Hyde Park (B). It is quite obvious that only the three CO-related Raman bands, ν_{CO}, ν_{Fe-CO}, and δ_{Fe-C-O}, are sensitive to isotope substitution. The ν_{CO} band of COHb M Hyde Park was shifted from 1948 ($^{12}C^{16}O$) to 1906 ($^{13}C^{16}O$), 1907, ($^{12}C^{18}O$), and 1860 cm^{-1} ($^{13}C^{18}O$), respectively. The same extents of frequency shifts were also observed for Hb M Iwate, Hb M Milwaukee, and normal subunits of Hb M Boston and Hb M Saskatoon (Table IIA). By contrast, CO isotope substitution in the abnormal subunits of Hb M Boston and Hb M Saskatoon induced greater frequency shifts than those of the other Hbs M and Hb A, although the difference is small: from 1972 ($^{12}C^{16}O$) to 1924 ($^{13}C^{16}O$), 1924 ($^{12}C^{18}O$), and 1880 cm^{-1} ($^{13}C^{18}O$).

[2] M. Nagai, A. Dong, Y. Yoneyama, and W. S. Caughey, manuscript in preparation.

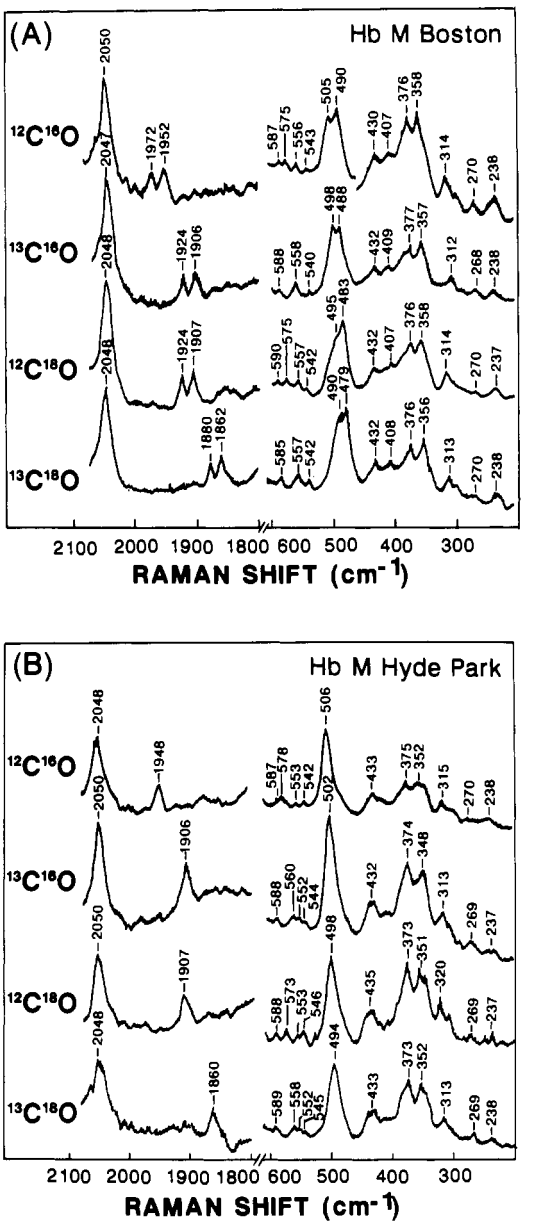


FIGURE 5: Carbon monoxide isotope effects on the higher frequency (1800–2100-cm^{-1}) and lower frequency (200–600-cm^{-1}) RR spectra of COHb M Boston (A) and COHb M Hyde Park (B). Experimental conditions are the same as those for Figure 3.

Two other isotope-sensitive bands were also noticed in the lower frequency spectrum of COHb M Hyde Park (Figure 5B). The band at 506 cm^{-1} (ν_{Fe-CO}) exhibited a montonous shift toward lower frequency in the order $^{12}C^{16}O \rightarrow {}^{13}C^{16}O \rightarrow {}^{12}C^{18}O \rightarrow {}^{13}C^{18}O$, representing a simple dependence on the sum of masses of both atoms. On the other hand, the other band at 578 cm^{-1} (δ_{Fe-C-O}) showed the zigzag frequency shift upon increase of the sum of masses of the two atoms as demonstrated for COHb A (Tsubaki et al., 1982; Yu & Kerr, 1988; Li & Spiro, 1988). Usually, isotope substitution of terminal oxygen has little influence on the δ_{Fe-C-O} frequency (Tsubaki et al., 1982; Yu & Kerr, 1988). The similar isotope shifts of ν_{Fe-C-O} and δ_{Fe-C-O} were also obtained with Hb M Iwate, Hb M Milwaukee, and the normal subunits of both Hb M Boston and Hb M Saskatoon (Table IIB,C).

On the other hand, the isotope shifts of the corresponding RR bands of the abnormal subunits of COHb M Boston and COHb M Saskatoon were different from those of the other COHbs M and COHb A. As shown in Figure 5A, the shift

Table II: Carbon Monoxide Isotope Effects on RR Band Frequencies of COHbs M and COHb A

	(A) ν_{CO} (cm^{-1})	$\Delta^{13}C$	$\Delta^{18}O$	$\Delta^{13}C^{18}O$
Hb A	1952	−45	−46	−90
Hb M Boston-β^A	1952	−46	−45	−90
Hb M Saskatoon-α^A	1951	−45	−46	−91
Hb M Iwate	1952	−46	−45	−90
Hb M Hyde Park	1948	−42	−41	−88
Hb M Milwaukee	1949	−44	−43	−89
Hb M Boston-α^M	1972	−48	−48	−92
Hb M Saskatoon-β^M	1972	−47	−48	−95
	(B) ν_{Fe-CO} (cm^{-1})	$\Delta^{13}C$	$\Delta^{18}O$	$\Delta^{13}C^{18}O$
Hb A	507	−6	−11	−13
Hb M Boston-β^A	505	−7	−10	−15
Hb M Saskatoon-α^A	505	−6	−8	−13
Hb M Iwate	506	−8	−12	−14
Hb M Hyde Park	506	−4	−8	−12
Hb M Milwaukee	506	−4	−9	−12
Hb M Boston-α^M	490	−2	−7	−11
Hb M Saskatoon-β^M	488	−2	−5	−10
	(C) δ_{Fe-C-O} (cm^{-1})	$\Delta^{13}C$	$\Delta^{18}O$	$\Delta^{13}C^{18}O$
Hb A	576	−14	−1	−19
Hb M Milwaukee	578	−17	+1	−19
Hb M Iwate	576	−18	−1	−18
Hb M Hyde Park	578	−18	−5	−20
Hb M Boston	575	−17	0	−18
Hb M Saskatoon	576	−18	−1	−18

of ν_{Fe-CO} in the abnormal subunit of Hb M Boston was very small in the case of $^{13}C^{16}O$ ligation: from 490 ($^{12}C^{16}O$) to 488 cm^{-1} ($^{13}C^{16}O$) in COα^M of Hb M Boston compared to a shift from 505 ($^{12}C^{16}O$) to 498 cm^{-1} ($^{13}C^{16}O$) in COβ^A of Hb M Boston.

The similar zigzag frequency shifts of δ_{Fe-C-O} upon isotope substitution of CO were observed in the spectra of COHb M Boston and COHb M Saskatoon, but its counterpart due to the abnormal subunit could not be established; a clear δ_{Fe-C-O} band was undetectable in the spectra of these E7 mutant Hbs M except for ligation with $^{13}C^{16}O$ or $^{13}C^{18}O$, because the shifted δ_{Fe-C-O} band overlapped with the 556-cm^{-1} band (ν_{25}). In addition to the presence of three other RR bands around the bending mode, the low intensity of this band sometimes made the detection of δ_{Fe-C-O} in these E7 mutant Hbs M very difficult. Since the band around 580 cm^{-1} was suggested to possibly be an overtone of the Fe–C–O bending vibration (Tsuboi, 1988), we carefully searched for an isotope-sensitive band in the 280–300-cm^{-1} region. As shown in Figure 5, there is no indication for the presence of an isotope-sensitive band in that frequency region. However, it does not mean that the proposed assignment is wrong at all, because the fundamental of δ_{Fe-C-O} is Raman inactive when the Fe–C–O is linear perpendicular to the porphyrin plane.

Photodissociation. The bound CO was completely photodissociated from COHb A by stopping the spinning of the Raman cell as shown in Figure 6A, and the resultant RR spectrum (dotted line) was the same as that of the deoxyHb. For Hb M Saskatoon (B), on the other hand, one set of the CO-related RR bands disappeared upon the stop of spinning of the Raman cell while the other set remained as shown by the dotted line. The abnormal subunit of COHb M Saskatoon did scarcely dissociate the bound CO in the stationary cell and gave the ν_{CO} and ν_{Fe-CO} RR bands at 1972 and 489 cm^{-1}, respectively. It was estimated from the changes of ν_4 and RR band at around 2048 cm^{-1} that about half of the CO was released from COHb M Saskatoon. The same phenomenon was observed for COHb M Boston.

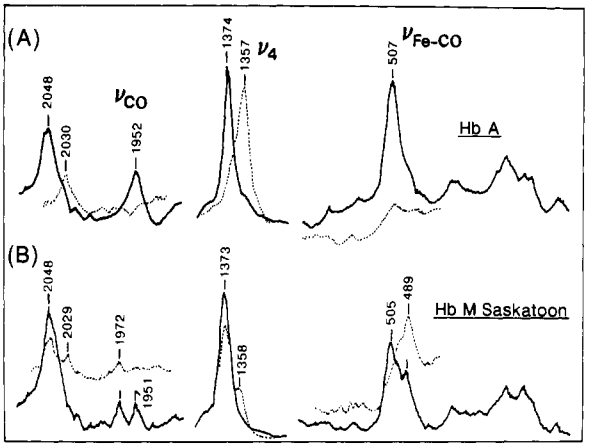


FIGURE 6: RR spectra of COHb A (A) and COHb M Saskatoon (B) observed with (—) or without (···) spinning the Raman cell. Other experimental conditions are the same as those for Figure 3.

Discussion

Inverse Relation between ν_{Fe-CO} and ν_{CO} of COHbs M. An inverse relationship between the ν_{Fe-CO} and ν_{CO} frequencies of CO-bound heme proteins and their model compounds was first demonstrated by Yu et al. (1983). The ν_{Fe-CO} of the normal and abnormal subunits of Hbs M and Hb A are plotted against ν_{CO} in Figure 7, where data from other heme proteins are also included. The present data hold the same inverse relation as was observed for other heme proteins. As also superimposed in the same figure, such a negative linear correlation has been noted with a wide variety of CO adducts of heme proteins and their model compounds and explained in terms of the π back-donation characteristic of the Fe–CO linkage (Tsubaki et al., 1982; Yu & Kerr, 1988). These compounds can be categorized into three groups: weak trans ligand group (compounds 1 and 2 in Figure 7), imidazole as the trans ligand (compounds 3–39), and strong trans ligand group (compounds 40–47). The plots for normal and abnormal subunits of Hbs M all fall on the line of the imidazole trans ligand group. This serves as additional support for the distal His ligation to the heme in abnormal subunits of COHb M Iwate and COHb M Hyde Park.

CO Ligation to the Proximal Site in F8-His-Substituted Abnormal Subunits of Hb M Iwate and Hb M Hyde Park. Our previous RR studies on Hbs M have revealed that the heme irons in the abnormal subunits of four Hbs M are coordinated by the substituted Tyr in the oxidized form (Nagai et al., 1989). On the other hand, Egeberg et al. (1990) recently characterized Hb M like mutants of sperm whale Mb obtained from the site-directed mutagenesis and identified the RR bands due to the Fe^{3+}–O stretching and the phenolate internal modes for the oxidized form of both E7-Tyr and F8-Tyr mutants, in agreement with our observation. The fully reduced Hb M Iwate and Hb M Boston display RR spectra similar to that of deoxyHb A in 1200–1700-cm^{-1} region, implying that their abnormal subunits also have a pentacoordinate heme structure like normal hemoglobin (Andersson et al., 1989; Abe et al., 1978; Li et al., 1990). This is also consistent with the results from the mutant Mbs (Egeberg et al., 1990).

When the abnormal subunits were reduced, the Fe–O-(phenolate) bonding was deduced to be broken from the changes in the absorption spectra (Peisach & Gersonde, 1977). The present study suggested the coordination of His to the abnormal subunits of all the deoxyHb M mutants treated here. However, according to Egeberg et al. (1990), the F8-Tyr mutant of Mb does not give the ν_{Fe-His} RR band while the

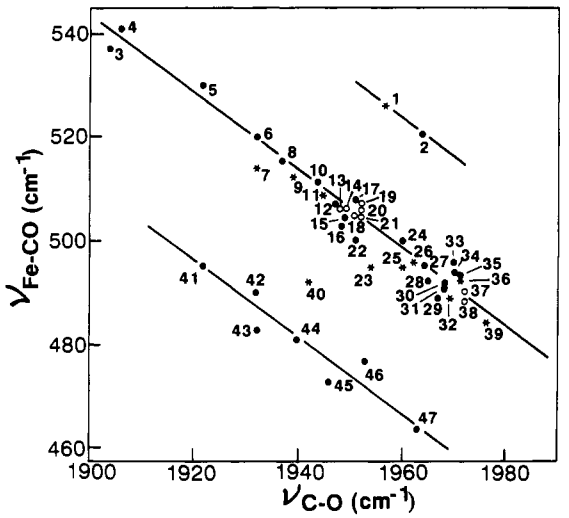


FIGURE 7: Correlation between the ν_{Fe-CO} and ν_{CO} Raman frequencies of heme proteins (●), heme model compounds (*), and normal and abnormal subunits of COHbs M (○). Slopes of the three solid lines are all −0.75. Numbers in the figure stand for the following compounds: 1, α,α,α,α-*meso*-tetrakis(*O*-pivalamidophenyl)porphyrin iron(II) (tetrahydrofuran), $Fe^{2+}(Tp_{iv}PP)(THF)$ (Kerr, 1984; Kerr et al., 1983); 2, cytochrome oxidase (Argade et al., 1984; Volpe et al., 1975); 3, horseradish peroxidase (HRP) at low pH (Evangelista-Kirkup et al., 1986); 4, HRP at pH 7 (Uno et al., 1987); 5, cytochrome *c* peroxidase (CCP) at low pH (Smulevich et al., 1986); 6, *Glycera* polymeric Hb (Carson et al., 1985); 7, heme-5 strapped with 13 hydrocarbon (FeSP-13), (Yu et al., 1983); 8, elephant Mb (Kerr et al., 1985); 9, FeSP-14 (*N*-methylimidazole, *N*-MeIm) (Yu et al., 1983); 10, sperm whale Mb at pH 8.4 (Tsubaki et al., 1982); 11, FeSP-15(*N*-MeIm) (Yu et al., 1983); 12, sperm whale Mb at pH 7 (Ramsden & Spiro, 1989); 13, Hb M Hyde Park (this work); 14, Hb M Milwaukee (this work); 15, leghemoglobin (Rousseau et al., 1983; Fuchsman & Appleby, 1979); 16, CCP at high pH (Smulevich et al., 1986); 17, carp Hb (Tsubaki et al., 1982; Onwubiko et al., 1982); 18, Hb M Saskatoon-α^A (this work); 19, Hb A (this work); 20, Hb M Iwate (this work); 21, Hb M Boston-β^A (this work); 22, mutant CCP (Arg-48 → Leu) at pH 8.5 (Smulevich et al., 1988); 23, heme-5(*N*-MeIm) (Yu et al., 1983); 24, insect (*Chironomus*) Hb (Yu et al., 1984); 25, Fe^{2+}(protoporphyrin IX dimethyl ester)(imidazole), Fe^{2+}(PPDME)(ImH) (Evangelista-Kirkup et al., 1986); 26, $Fe^{2+}(T_{iv}PP)(1,2\text{-}Me_2Im)$ (Kerr, 1984; Kerr et al., 1983); 27, mutant Mb (E7-Met) (pH 7) (Morikis et al., 1989); 28, mutant Mb (E7-Gly) (pH 7) (Morikis et al., 1989); 29, sperm whale Mb at pH 4 (Ramsden & Spiro, 1989); 30, mutant Hb (βE7-Phe) (Lin et al., 1990); 31, carp Hb + inositol hexaphosphate (IHP) (Rousseau et al., 1984); 32, $Fe^{2+}(T_{iv}PP)$(*N*-MeIm) (Kerr, 1984; Collman et al., 1976); 33, *Glycera* monomeric Hb (Carson et al., 1985); 34, mutant Hb (αE7-Gly) (Lin et al., 1990); 35, mutant Hb (βE7-Gly) (Lin et al., 1990); 36, Fe^{2+}(octaethylporphyrin)(4-NH_2-pyridine), Fe^{2+}(OHP)(4-NH_2py) (Kerr, 1984; Alben & Caughey, 1968); 37, Hb M Boston-α^M (this work); 38, Hb M Saskatoon-β^M (this work); 39, Fe^{2+}(tetraphenylporphyrin, TPP)(py) (Kerr et al., 1985); 40, Fe^{2+}(PPDME)(Im^-) (Evangelista-Kirkup et al., 1986); 41, aged CCP (Smulevich et al., 1986); 42, HRP at pH 11 (Smulevich et al., 1986); 43, $P\text{-}450_{camphor(cam)}$ (putidaredoxin from *Pseudomonas putida*) (Makino et al., 1984); 44, $P\text{-}450_{cam}$ from *P. putida* (+camphor) (Uno et al., 1985); 45, $P\text{-}450_{cam}$ (+norcamphor) (Uno et al., 1987); 46, $P\text{-}450_{scc}$ (scc = cholesterol side-chain cleavage) from bovine (Tsubaki & Ichikawa, 1985); 47, $P\text{-}450_{cam}$ from *P. putida* (−camphor) (Uno et al., 1985).

E7-Tyr mutant does. In the RR spectra of deoxy Hb M Iwate with αF8-Tyr (Figure 2D), the ν_{Fe-His} RR band is relatively intense. Since the α and β subunits of deoxy Hb A have different spectra in the low-affinity state (Nagai & Kitagawa, 1980) and the ν_{Fe-His} band intensity becomes stronger and its frequency becomes higher as the oxygen affinity becomes higher (Matsukawa et al., 1985), it is practically difficult to deduce the spectrum of abnormal subunit by subtracting Figure 2F from Figure 2D. So, we cannot rule out the possibility that the 218-cm^{-1} band in Figure 2D solely arises from the normal β chain. However, when the RR spectrum of

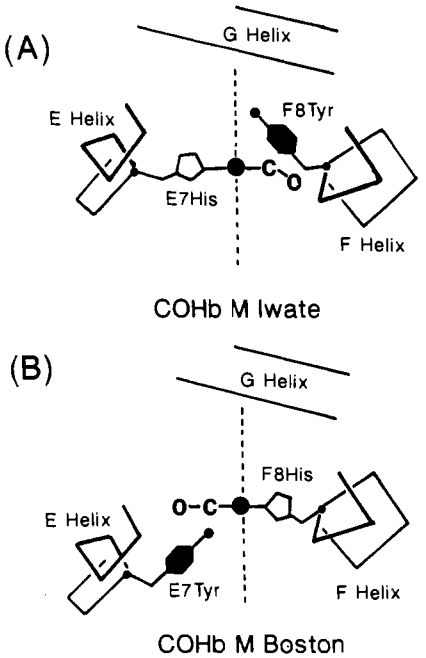


FIGURE 8: Plausible orientations of CO in the abnormal subunits of Hb M Iwate (A) and Hb M Boston (B). The original schematic representation of heme structure for Hb M Boston (Dickerson & Geis, 1983) was modified. Heme plane is shown by a dotted line, and the iron ion is denoted by a black circle on it.

half-reduced Hb M Iwate, in which the normal β and abnormal α subunits stay in the reduced and oxidized states, respectively, was subtracted from Figure 2D, there remained a band around 220 cm^{-1} in any reasonable difference calculations. Judging from this fact and the overall similarity among the RR spectra of Hb M Iwate, Hb M Boston, and Hb A shown in Figure 2 in addition to the plots in Figure 7, it is more natural to assume that the abnormal subunit of deoxy Hb M Iwate has the Fe–His bond which gives rise to its stretching RR band around 220 cm^{-1}. This means that the "distal" His binds to the heme iron in the abnormal subunits of Hb M Iwate and Hb M Hyde Park in the fully reduced state. In this regard, the deoxy form of the F8-Tyr Mb mutant might be different from abnormal subunits of deoxy Hb M Iwate and deoxy Hb M Hyde Park.

Examining the absorption spectrum of COHb M Iwate in the fully reduced state, Peisach and Gersonde (1977) found that the CO-liganded abnormal α subunits bore a close resemblance to the CO-liganded normal β subunits. They suggested that the proximal site of the heme iron was occupied by CO whereas the E7-His was bound to the distal site as illustrated in Figure 8A. In the RR spectrum of the fully reduced COHb M Iwate, we revealed that ν_{CO}, ν_{Fe-CO}, and δ_{Fe-C-O} frequencies were almost identical with those of COHb A, although a few changes in porphyrin vibrational modes were present probably due to the ligation of imidazole from the distal side. This confirms that the proximal side of abnormal subunits in Hb M Iwate can provide circumstances capable of binding CO as the distal side usually does. The same results were also obtained with the RR spectrum of COHb M Hyde Park in the fully reduced state. These observations suggest that the Fe–C–O linkage adopts a slightly bent structure as in the distal side of the normal subunits, and presumably the interaction of the substituted Tyr with CO in the proximal side is responsible for it.

These results are not always compatible with the results from the Mb mutants; contrary to the results of Hb M Iwate, the F8-Tyr Mb gave the ν_{Fe-CO} at 499 cm^{-1}, which is distinctly

Table III: Observed and Calculated Frequencies of COHb A (A) and CO-Bound Abnormal Subunit of Hb M Boston (B)

	$^{12}C^{16}O$		$^{13}C^{16}O$		$^{12}C^{18}O$		$^{13}C^{18}O$	
	obsd	calcd	obsd	calcd	obsd	calcd	obsd	calcd
				(A) Hb A[a]				
ν_{CO}	1952	1952	1907	1906	1906	1909	1862	1862
$\delta_{Fe\text{-}C\text{-}O}$[b]	576	577	562	563	575	567	557	553
$\nu_{Fe\text{-}CO}$	507	507	501	500	496	498	494	491
				(B) Abnormal Subunit of Hb M Boston[c]				
ν_{CO}	1972	1970	1924	1923	1924	1927	1880	1880
$\delta_{Fe\text{-}C\text{-}O}$[b]	575	579	558	563	575	571	557	555
$\nu_{Fe\text{-}CO}$	490	491	488	486	483	480	479	475

[a] Assumptions in calculations are as follows: r(Fe–C), 1.8 Å; r(C–O), 1.2 Å; K_1(Fe–C), 2.43 mdyn/Å; K_2(C–O), 14.62 mdyn/Å; H(Fe–C–O), 0.22 mdyn·Å; F(Fe···O), 0.2 mdyn/Å; θ(Fe–C–O), 160°. [b] $\delta_{Fe\text{-}C\text{-}O}$ was calculated as an overtone whose fundamental should be at 288 cm^{-1} (Tsuboi, 1988). Obsd, observed; calcd, calculated. [c] Assumptions in calculations are as follows: K_1(Fe–C), 2.49 mdyn/Å; K_2(C–O), 14.82 mdyn/Å; θ(Fe–C–O), 170°; other conditions are the same as in footnote *a*.

lower than that of the wild type (508 cm^{-1}; Egeberg et al., 1990). In contrast, the low frequency of the $\nu_{Fe\text{-}CO}$ mode of the abnormal subunits of Hb M Boston and Hb M Saskatoon (490 cm^{-1}) is consistent with the results from the E7-Tyr mutant of Mb which gives rise to the $\nu_{Fe\text{-}His}$ RR band at 494 cm^{-1}.

Bonding Geometry of CO in Abnormal Subunits of Hb M Boston and Hb M Saskatoon. In order to deduce a type of distortion in the CO heme of the abnormal subunits of Hb M Boston and Hb M Saskatoon, we carried out simple normal-coordinate calculations for an isolated three-body oscillator, Fe–C–O, by using Wilson's GF matrix method (Wilson, 1939). Since the vibrational coupling between the FeCO group and the porphyrin macrocycle is not so large (Champion et al., 1982), this treatment would be allowed as the first approximation. The potential function (V) used is

$$2V = K_1\Delta r_{Fe\text{-}CO}^2 + K_2\Delta r_{CO}^2 + H\Delta\vartheta_{FeCO}^2 + F\Delta r_{FeO}^2$$

where $\Delta r_{Fe\text{-}CO}$, Δr_{CO}, $\Delta\vartheta_{FeCO}$ and Δr_{FeO} represent the displacement coordinates for the Fe–CO stretching, C–O stretching, Fe–C–O bending, and Fe–O nonbonding interaction, respectively. In this treatment we assumed that the 576-cm^{-1} RR band was associated with the overtone of the Fe–C–O bending vibration (Tsuboi, 1988). The force constants were adjusted with the isotopic shift data observed for COHb A while the structural data were taken from the X-ray crystallographic analysis (Baldwin, 1980) and the IR study (ϑ = 160°) (Moore et al., 1988).

We calculated the dependence of the isotopic frequency shifts as well as the absolute frequencies on the Fe–C–O bond angle (Table III). The calculated isotopic frequency shifts for $^{13}C^{16}O$ with regard to $^{12}C^{16}O$ were plotted against the bond angle in Figure 9. Since the results might depend on the values of force constants used, we also calculated these frequencies for 10% increased values of individual force constants, and the results were plotted in the same figure with different symbols. Accordingly, distribution of different symbols give a size of errors which arise from a change of any force constants by 10%. Solid lines indicate the highest and lowest possible values for each mode under a set of force constants within 10% change from the best-fit values for COHb A. Although the absolute frequencies change greatly with the force constants assumed, the isotopic frequency shifts change with reasonable sensitivity, depending only on the vibrational displacement of the carbon atom. It is apparent that the $\nu_{Fe\text{-}CO}$ and $\delta_{Fe\text{-}C\text{-}O}$ frequencies depend sensitively on ϑ; as does the increase of the bond angle (decrease of bent), the bending frequency increases whereas the Fe–CO stretching frequency decreases. The ν_{CO} frequency also exhibits slight dependence on ϑ. The differences in $\Delta\nu_{Fe\text{-}CO}(^{13}C)$ and $\Delta\nu_{CO}(^{13}C)$ between

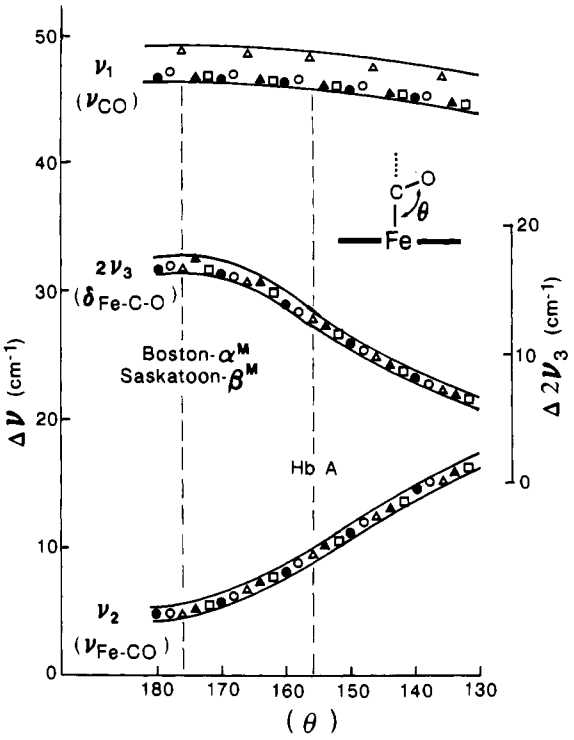


FIGURE 9: Calculated ^{13}CO isotopic frequency shifts of COHb as a function of the Fe–C–O bond angle. The isotope shifts were calculated with four different sets of force constants in which one of the force constants was increased by 10% from the best-fit values and the calculated values for different angles are plotted for clarification. The solid lines denote the highest and lowest values from the present calculations. Difference in the observed ^{13}C isotope shift of ν_{CO} between the abnormal subunit of Hb M Boston and Hb A is about 2 cm^{-1}, and that of $\nu_{Fe\text{-}CO}$ is 4 cm^{-1}. When the bond angle of Hb A is assumed to be 156° (right vertical broken line in the figure), the differences of the isotopic frequency shifts suggest that the bond angle of the abnormal subunit would be 176° (left vertical broken line). Assumed molecular parameters are as follows: Fe, 55.847; C, 12.011; O, 16; r(Fe–C), 1.8 Å; r(C–O), 1.2 Å; K_1(Fe–C), 2.43 mdyn/Å; K_2(C–O), 14.62 mdyn/Å; H(Fe–C–O), 0.22 mdyn·Å; F(Fe···O), 0.2 mdyn/Å. (●) Calculated by the best-fit set of force constants; (○) calculated by using 10% increment of K_1(Fe–C); (△) 10% increment of K_2(C–O); (▲) 10% increment of H(Fe–C–O); (□) 10% increment of F(Fe···O).

Hb A and the abnormal subunits of Hb M Boston and Hb M Saskatoon can be reasonably explained in terms of the difference in ϑ: 176° for Hb M Boston and Hb M Saskatoon and 156° for Hb A.

According to Morikis et al. (1989), the heme pocket of Mb can assume two alternative conformations, that is, "open" and "closed" forms, whose relative population is a function of pH. At acidic pH below 4.5, protonation of distal His causes a conformation change to "open" form, yielding the ν_{CO} and

ν_{Fe-CO} RR bands at 1966 and 491 cm^{-1} (Ramsden & Spiro, 1989; Morikis et al., 1989), respectively, while at neutral pH the deprotonated distal His sterically interferes against the perpendicular configuration of CO to the heme plane. Accordingly, the usual ν_{CO} (=1946 cm^{-1}) and ν_{Fe-CO} (=508 cm^{-1}) frequencies of COMb at neutral pH reflect the bent configuration of CO, which is referred as the "closed" form. The two conformations of COMb are supported by the X-ray crystallographic (Kuriyan et al., 1986) and ^1H NMR studies (Lecomte & La Mar, 1985). Presumably, the heme pocket conformation in the abnormal subunits of Hb M Boston and Hb M Saskatoon is similar to the "open" conformation of COMb, and thus CO is close to the heme normal is illustrated in Figure 8B. The ligand affinity of these abnormal subunits is expected to be very high, and the apparent resistance against photodissociation of Hb M Saskatoon and Hb M Boston would be ascribed to the extremely fast recombination.

Acknowledgments

We thank Drs. R. Jagenburg (Hb M Boston), H. Sakai (Hb M Iwate), S. Ryo and S. Horita (Hb M Saskatoon), K. Karita (Hb M Hyde Park), and A. V. Pisciotta (Hb M Milwaukee) for their courtesy in providing us with blood containing abnormal hemoglobins. We also thank Drs. R. Fukuda and Y. Nagai for helpful discussions.

Registry No. HbM Iwate, 9035-03-4; HbM Boston, 39340-61-9; HbM Hyde Park, 9088-23-7; HbM Saskatoon, 9035-07-8; HbM Milwaukee, 9035-05-6; HbA, 9034-51-9; His, 71-00-1; Val, 72-18-4; ^{13}C, 14762-74-4; ^{18}O, 14797-71-8; Fe, 7439-89-6; C, 7440-44-0; O, 7782-44-7.

References

Abe, M., Kitagawa, T., & Kyogoku, Y. (1978) *J. Chem. Phys. 69*, 4526–4534.

Alben, J. O., & Caughey, W. S. (1968) *Biochemistry 7*, 175–183.

Andersson, L. A., Mylrajan, M., Sullivan, E. P., & Strauss, S. H. (1989) *J. Biol. Chem. 264*, 19099–19102.

Argade, P. V., Ching, Y. C., & Rousseau, D. L. (1984) *Science 225*, 329–331.

Asher, S. A. (1981) *Methods Enzymol. 76*, 371–413.

Baldwin, J. M. (1980) *J. Mol. Biol. 136*, 103–128.

Bunn, H. F., & Forget, B. G. (1986) in *Hemoglobin: Molecular, Genetic and Clinical Aspects*, pp 623–633, Saunders Co., Philadelphia.

Carson, S. D., Constantinidis, I., Satterlee, J. D., & Ondrias, M. R. (1985) *J. Biol. Chem. 260*, 8741–8745.

Champion, P. M., Stallard, B. R., Wagner, G. C., & Gunsalus, I. C. (1982) *J. Am. Chem. Soc. 104*, 5469–5472.

Choc, M. G., & Caughey, W. S. (1981) *J. Biol. Chem. 256*, 1831–1838.

Choi, S., Spiro, T. G., Langry, K. C., & Smith, K. M. (1982a) *J. Am. Chem. Soc. 104*, 4337–4344.

Choi, S., Spiro, T. G., Langry, K. C., Smith, K. M., Budd, D. L., & La Mar, G. N. (1982b) *J. Am. Chem. Soc. 104*, 4345–4351.

Collman, J. P., Brauman, J. I., Halbert, T. R., & Suslick, K. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3333–3337.

Dickerson, R. E., & Geis, I. (1983) in *Hemoglobin: Structure, Function, Evolution, and Pathology*, pp 19–64 and 107–168, Benjamin/Cummings, Menlo Park, CA.

Egeberg, K. D., Springer, B. A., Martinis, S. A., Sligar, S. G., Morikis, D., & Champion, P. M. (1990) *Biochemistry 29*, 9783–9791.

Evangelista-Kirkup, R., Smulevich, G., & Spiro, T. G. (1986) *Biochemistry 25*, 4420–4425.

Fuchsman, W. H., & Appleby, C. A. (1979) *Biochemistry 18*, 1309–1321.

Hendra, P. J., & Loader, E. J. (1968) *Chem. Ind. (London)*, 718–719.

Kerr, E. A. (1984) Ph.D. Dissertation, Geogia Institute of Technology, Atlanta, GA.

Kerr, E. A., Mackin, H. C., & Yu, N.-T. (1983) *Biochemistry 22*, 4373–4379.

Kerr, E. A., Yu, N.-T., Bartnicki, D. E., & Mizukami, H. (1985) *J. Biol. Chem. 260*, 8360–8365.

Kitagawa, T., & Teraoka, J. (1979) *Chem. Phys. Lett. 63*, 443–446.

Kitagawa, T., & Ozaki, Y. (1987) *Struct. Bonding (Berlin) 64*, 71–114.

Kitagawa, T., Kyogoku, Y., Iizuka, T., & Saito, M. I. (1976) *J. Am. Chem. Soc. 98*, 5169–5173.

Kuriyan, J., Wilz, S., Karplus, M., & Petsko, G. A. (1986) *J. Mol. Biol. 192*, 133–154.

Lecomte, J. T. J., & La Mar, G. N. (1985) *Biochemistry 24*, 7388–7395.

Lee, H., Kitagawa, T., Abe, M., Pandey, R. K., Leung, H.-K., & Smith, K. M. (1986) *J. Mol. Struct. 146*, 329–347.

Li, X.-Y., & Spiro, T. G. (1988) *J. Am. Chem. Soc. 110*, 6024–6033.

Li, X.-Y., Czernuszewicz, R. S., Kincaid, J. R., Stein, P., & Spiro, T. G. (1990) *J. Phys. Chem. 94*, 47–61.

Lin, S.-H., Yu, N.-T., Tame, J., Shih, D., Renaud, J.-P., Pagnier, J., & Nagai, K. (1990) *Biochemistry 29*, 5562–5566.

Makino, R., Iizuka, T., Ishimura, Y., Uno, T., Nishimura, Y., & Tsuboi, M. (1984) in *Ninth International Conference on Raman Spectroscopy*, pp 492–493, Tokyo.

Matsukawa, S., Mawatari, K., Yoneyama, Y., & Kitagawa, T. (1985) *J. Am. Chem. Soc. 107*, 1108–1113.

Moore, J. N., Hansen, P. A., & Hochstrasser, R. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5062–5066.

Morikis, D., Champion, P. M., Springer, B. A., & Sligar, S. G. (1989) *Biochemistry 28*, 4791–4800.

Nagai, K., & Kitagawa, T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2033–2037.

Nagai, K., Luisi, B., Shih, D., Miyazaki, G., Imai, K., Poyart, C., Young, A. D., Kwiatkowsky, L., Noble, R. W., Lin, S.-H., & Yu, N.-T. (1987) *Nature 329*, 858–860.

Nagai, M. (1985) *Acta Haematol. Jpn. 48*, 2015–2022.

Nagai, M., & Yoneyama, Y. (1983) *J. Biol. Chem. 258*, 14379–14384.

Nagai, M., Yubisui, T., & Yoneyama, Y. (1980) *J. Biol. Chem. 255*, 4599–4602.

Nagai, M., Takama, S., & Yoneyama, Y. (1987) *Acta Haematol. 78*, 95–98.

Nagai, M., Yoneyama, Y., & Kitagawa, T. (1989) *Biochemistry 28*, 2418–2422.

Onwubiko, H. A., Hazzard, J. H., Noble, R. W., & Caughey, W. S. (1982) *Biochem. Biophys. Res. Commun. 106*, 223–228.

Peisach, J., & Gersonde, K. (1977) *Biochemistry 16*, 2539–2545.

Potter, W. T., Hazzard, J. H., Kawanishi, S., & Caughey, W. S. (1983) *Biochem. Biophys. Res. Commun. 116*, 719–725.

Ramsden J., & Spiro, T. G. (1989) *Biochemistry 28*, 3125–3128.

Rousseau, D. L., Ondrias, M. R., La Mar, G. N., Kong, S. B., & Smith, K. M. (1983) *J. Biol. Chem. 258*, 1740–1746.
Rousseau, D. L., Tan, S. L., Ondrias, M. R., Ogawa, S., & Noble, R. W. (1984) *Biochemistry 23*, 2857–2865.
Satterlee, J. D., Teintze, M., & Richards, J. H. (1978) *Biochemsitry 17*, 1456–1462.
Smulevich, G., Evangelista-Kirkup, R., English, A., & Spiro, T. G. (1986) *Biochemistry 25*, 4426–4430.
Smulevich, G., Mauro, J. M., Fishel, L. A., English, A. M., Kraut, J., & Spiro, T. G. (1988) *Biochemistry 27*, 5486–5492.
Spaulding, L. D., Chang, C. C., Yu, N.-T., & Felton, R. H. (1975) *J. Am. Chem. Soc. 97*, 2517–2525.
Spiro, T. G. (1988) in *Biological Applications of Raman Spectroscopy*, Vol. 3, Wiley-Interscience, New York.
Spiro, T. G., & Burke, J. M. (1976) *J. Am. Chem. Soc. 98*, 5482–5489.
Tsubaki, M., & Ichikawa, Y. (1985) *Biochim. Biophys. Acta 827*, 268–274.
Tsubaki, M., Srivastava, R. B., & Yu, N.-T. (1982) *Biochemistry 21*, 1132–1140.
Tsuboi, M. (1988) *Indian J. Pure Appl. Phys. 26*, 188–191.
Uno, T., Nishimura, Y., Makino, R., Iizuka, T., Ishimura, Y., & Tsuboi, M. (1985) *J. Biol. Chem. 260*, 2023–2026.
Uno, T., Nishimura, Y., Tsuboi, M., Makino, R., Iizuka, T., & Ishimura, Y. (1987) *J. Biol. Chem. 262*, 4549–4556.
Volpe, J. A., O'Toole, M. C., & Caughey, W. S. (1975) *Biochem. Biophys. Res. Commun. 62*, 48–53.
Wilson, E. B. (1939) *J. Chem. Phys. 7*, 1047–1052.
Yu, N.-T. (1986) *Methods Enzymol. 130*, 350–409.
Yu, N.-T., & Kerr, E. A. (1988) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) Vol. 3, pp 40–95, Wiley-Interscience, New York.
Yu, N.-T., Kerr, E. A., Ward, B., & Chang, C. K. (1983) *Biochemistry 22*, 4534–4540.
Yu, N.-T., Benko, B., Kerr, E. A., & Gersonde, K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5106–5110.

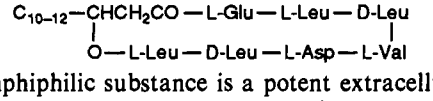
Cell-Free Biosynthesis of Surfactin, a Cyclic Lipopeptide Produced by *Bacillus subtilis*†

Christian Ullrich, Britta Kluge, Zbigniew Palacz, and Joachim Vater*
Institut für Biochemie und Molekulare Biologie der Technischen Universität Berlin, Franklinstrasse 29, D-1000 Berlin 10, Federal Republic of Germany

Received November 14, 1990; Revised Manuscript Received March 22, 1991

ABSTRACT: The lipopeptide antibiotic surfactin is a potent extracellular biosurfactant produced by various *Bacillus subtilis* strains. Biosynthesis of surfactin was studied in a cell-free system prepared from *B. subtilis* ATCC 21332 and OKB 105, which is a transformant producing surfactin in high yield [Nakano, M. M., Marahiel, M. A., & Zuber, P. (1988) *J. Bacteriol. 170*, 5662–5668]. Cell material was disintegrated by treatment with lysozyme and French press. A cell-free extract was prepared by ammonium sulfate fractionation, which catalyzed the formation of surfactin at the expense of ATP. Lipopeptide biosynthesis required the L-amino acid components of surfactin and D-3-hydroxytetradecanoyl-coenzyme A thioester. D-Leucine which is present in surfactin was not utilized but inhibited the biosynthetic process. The structure of surfactin, synthesized enzymatically in vitro, was confirmed by chromatographic comparison with the authentic compound and by amino acid analyses. An enzyme fraction was prepared by gel permeation chromatography which catalyzed ATP/pyrophosphate exchange reactions dependent on the component amino acids of surfactin. This enzyme fraction was capable of binding substrate amino acids covalently, probably via thioester linkages. The formation of these intermediates was inhibited by various thiol blocking reagents and phenylmethanesulfonyl fluoride. De novo synthesis of the lipopeptide was not observed with this partially purified enzyme fraction most likely due to the lack of an acyltransferase activity required for linking the β-hydroxy fatty acid to the peptide moiety.

Surfactin belongs to a class of cyclic lipopeptide antibiotics produced by various strains of *Bacillus subtilis*. It consists of a peptide moiety containing seven amino acids in combination with a β-hydroxy fatty acid which occurs as a mixture of closely related variants depending on the composition of the fermentation medium (Kakinuma et al., 1969a,b,c,d):



This amphiphilic substance is a potent extracellular biosurfactant with antifungal properties and antitumor activity against Ehrlich ascites carcinoma cells (Kameda et al., 1974). Surfactin inhibits fibrin clot formation and lyses erythrocytes and several bacterial spheroplasts and protoplasts (Arima et

† This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 9, Teilprojekt A5).
* To whom correspondence should be addressed.

0006-2960/91/0430-6503$02.50/0