ASSIGNMENT OF THE Fe–N_ϵ (His F8) STRETCHING BAND IN THE RESONANCE RAMAN SPECTRA OF DEOXY MYOGLOBIN

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1. Introduction

In our course of a project for elucidating structural aspects involved in the *T–R* transition of hemoglobin (Hb), we have observed a systematic change in frequency of a Raman line of deoxy Hb around 220 cm^{-1} upon the *T–R* transition [1]. Since the translocation of heme iron from the out-of-plane to in-plane position is considered to trigger the change of quaternary structure of Hb [2], the vibrational frequency associated with the Fe–N_ϵ (His F8) bond, which links heme to globin, might sense most sensitively the quaternary structures. On the other hand, when histidine is bound to an axial position of heme iron as seen for some heme enzymes, the Fe–N_ϵ (His) stretching frequency would provide an important insight into molecular mechanism of biological reactions catalyzed by the enzyme. Therefore the assignment of the Fe–N_ϵ (His) stretching band is fundamentally important in application of Raman spectroscopy to hemeproteins. Thus we investigated, in the present study, resonance Raman spectrum (RRS) of deoxy myoglobin (Mb) in the lowest frequency region. A Raman line of deoxy Mb at 220 cm^{-1} exhibited a frequency shift upon replacement of ^{56}Fe by ^{54}Fe and was assigned to the Fe–N_ϵ (His F8) stretching mode.

2. Materials and methods

Sperm whale Mb was prepared according to Yamazaki et al. [3]. Rabbit cytochrome b_5 (cyt b_5) was a generous gift of Dr C. Hashimoto-Yutsudo of this institute. Both Mb and cyt b_5 were reduced by adding dithionite solution anaerobically to the sample solution equilibrated with Ar gas (99.999%) in a Raman cell. Protoporphyrin (PP) dimethylester, obtained according to Drago et al. [4], was converted to free acid into which ^{54}Fe (95%, Rhostoff-Einfuhr) was incorporated by the method of Yonetani and Asakura [5]. The labeled heme was purified on a column of Kieselgel 80 (Merck, No. 7734) with a lower layer of the pyridine/chloroform/water/isooctane mixture (20:10:10:1, v/v). Preparation of globin and incorporation of the labeled and unlabeled hemes into globin were performed according to Yonetani and Asakura [5]. Raman scattering was

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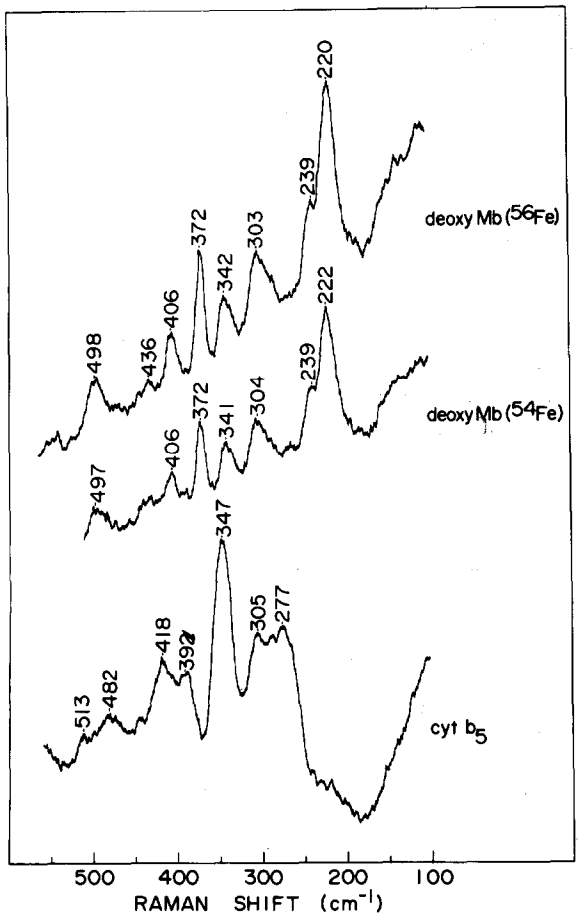

Fig.1. Resonance Raman spectra of deoxy Mb (^{56}Fe) (top), deoxy Mb (^{54}Fe) (middle) and reduced cytochrome b_5 (bottom). Deoxy Mbs were in 0.1 M phosphate buffer (pH 6.5) and cytochrome b_5 was in 0.1 M Tris–HCl buffer (pH 7.5). Heme concentration was approx. 0.4 mM. Instrumental conditions: laser, 441.6 nm; power, 12 mW (sample point); slit width, 5 cm^{-1}, sensitivity, 250 counts/s; scan speed, 10 cm^{-1}/min; time constant, 16 sec.

excited at 441.6 nm with a He-Cd laser (Kinmon Electrics) and recorded on a Jeol-400D Raman spectrometer. The Raman spectrometer was calibrated with indene [6].

3. Results and discussion

Figure 1 shows the RRS of the unlabeled (top) and labeled (middle) deoxy Mbs. The two spectra were measured successively under the same instrumental conditions three times for each, and moreover two independent preparations were examined in the same way. The Raman spectrum of the unlabeled but reconstituted sample coincided with that of natural deoxy Mb. The repeated measurements confirmed that the Raman line of deoxy Mb at 220 cm^{-1} was appreciably shifted toward higher frequency upon replacement of ^{56}Fe by ^{54}Fe, although Desbois et al. failed to observe it [7]. The corresponding Raman line was observed at 207 cm^{-1} for Fe^{2+} (PP) (2-MeIM) (2-MeIm: 2-methyl imidazole) and this line was shifted to 211 cm^{-1} upon ^{54}Fe substitution [1]. The frequency shift expected for an isolated Fe–N (2-MeIm) stretching vibration upon the isotopic replacement of iron is as large as 2 cm^{-1} and this would also be applicable to deoxy Mb. Accordingly we assigned the 220-cm^{-1} line of deoxy Mb to the Fe–N_ϵ (His F8) stretching mode.

Cyt b_5 is known to contain Fe (PP) with two histidine residues as axial ligands [8]. In its spectrum, shown at the bottom of fig.1, the 220 cm^{-1} line of deoxy Mb was absent and instead a new line appeared at 277 cm^{-1}. When there is another N-ligand at the position *trans* to the other, the two Fe–N (ligand) stretching vibrations are split into the symmetric and antisymmetric modes, the former of which is expected to appear in the Raman spectrum. Provided that the Fe–N stretching vibrations were nearly free from coupling with any porphyrin modes, the Fe–N stretching frequency for the case of a single N-ligand coordinated as for deoxy Mb, would be represented as $\nu_I = [K(1 + m_N/m_{Fe})/m_N]^{1/2}/2\pi c$, where K is the Fe–N stretching force constant and m_{Fe} and m_N are masses of Fe and the ligand, respectively. On the other hand, for the N–Fe–N system, the symmetric stretching frequency is represented as $\nu_{II} = [(K + k)/m_N]^{1/2}/2\pi c$, where k is a stretching-stretching interaction constant. Since practical values of k and m_N are not available, it is difficult to predict how ν_{II} differs from ν_I. However, in the model system such as Fe^{2+} (PP) (2-MeIm) and Fe^{2+} (PP) $(Im)_2$ (Im: imidazole), ν_{II} was higher than ν_I [1]. This is consistent with the observed difference between deoxy Mb and cyt b_5.

An argument against the present assignment may arise from the observations by Kincaid et al. [9] who assigned the 372-cm^{-1} line of deoxy Mb to the

Fe–N_ϵ (His F8) stretching mode and also by Desbois et al. [7] who assigned the 406-cm^{-1} line to it. However, it must be emphasized here that both lines never exhibited the isotopic frequency shift upon replacement of ^{56}Fe by ^{54}Fe for deoxy Mb, whereas the 220-cm^{-1} line of Fe^{2+} (PP) (2-MeIm) in the spectrum of Kincaid et al. indeed showed a frequency shift upon the change of mass of axial ligand [9]. Furthermore, we confirmed that the 220-cm^{-1} line in the spectrum of Kincaid et al. was shifted to 207 cm^{-1} upon addition of 2.5% cetyltrimethylammonium bromide (CTAB) as shown in the previous paper [1] but other frequencies were almost the same between the two measurements. Addition of CTAB might result in monomerization of the porphyrin complexes or alternatively provide hydrophobic environment around the porphyrin complex by forming micelles. Whichever the reason may be, the Fe-N (2-MeIm) stretching would be most likely to be affected by it compared with the porphyrin modes, and accordingly this observation also supports our assignment.

Kincaid et al. [9] pointed out that the 372- and 220-cm^{-1} modes are vibrationally coupled with each other and therefore both lines show a frequency shift upon change of mass of axial ligand. If some porphyrin modes were coupled with the Fe–axial ligand stretching vibration, the coupling would occur via vibrational displacement of the Fe ion. When a change of mass of axial ligand caused a frequency shift of a few Raman lines and it was due to the vibrational coupling, then one of them which predominantly involves the Fe–axial ligand stretching, should be most sensitive to the mass of iron. Therefore, the 372-cm^{-1} line of deoxy Mb might be coupled with the Fe–N_ϵ (His F8) stretching but it would be in less significant way than the 220-cm^{-1} line.

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