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RESONANCE RAMAN STUDY OF THE HEME-LINKED IONIZATION IN REDUCED HORSERADISH PEROXIDASE

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SUMMARY The resonance Raman spectra of reduced horseradish peroxidase (oxidoreductase, EC 1.11.1.7) and its cyanide complex in the 200-600 cm^{-1} region were measured. Among many Raman lines observed, only the line at 244 cm^{-1} (pH 6.5) exhibited the pH dependent frequency shift. This line disappeared in the cyanide complex. The 244-cm^{-1} line was intense upon excitation at 441.6 nm but unrecognizable at 488.0 nm. Consequently this line is assignable to the Fe-N_ε(His, proximal) stretching mode in accord with the 220-cm^{-1} line of the Fe-N_ε(His F8) stretching line of deoxy Mb. It is concluded that the ionization of an amino acid residue with pK_a = 7.17 is transmitted to heme via Fe-N_ε(His) bond in the proximal side.

INTRODUCTION

The presence of heme-linked ionization at pH~7 in reduced horseradish peroxidase (HRP) was first suggested by Theorell (1) and its importance has been noticed in relation with the proton-balance between individual reaction intermediates of this enzyme (2). Detailed comparative analysis of the acid-base properties of HRP and Mb were worked out by Yamazaki et al. (3), who concluded that the pK~7 transition of reduced HRP is associated with protonation of distal histidine. The change of pK values depending upon the sixth ligand is ascribed to hydrogen bond between the distal histidine and the sixth ligand (4). However, in the case of absence of the sixth ligand coordinated to the heme iron in reduced HRP as generally accepted, the mechanism how the protonation of distal histidine is transmitted to the heme would be one of the basic problems to be solved.

Resonance Raman scattering from hemeproteins has revealed the molecular vibrations of hemes (5). The Raman spectra of HRP in the higher frequency region (>1200 cm^{-1}) have been investigated by several groups (6-10) but the

HRP, horseradish peroxidase

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lower frequency region is left unexamined. Recently Kitagawa et al. (11) demonstrated that the Fe-N_ε(His F8) stretching Raman line is observable at 220 cm^{-1} for deoxy Mb upon excitation at 441.6 nm. Furthermore, Nagai et al. (12) observed the corresponding Raman line of deoxy Hb at 216 cm^{-1}, pointing out that only this mode could serve as an sensitive indicator of the higher order structures of deoxy Hb. The corresponding Raman line of reduced HRP may reflect the pK~7 transition and reveal structural details involved. Accordingly we examined the resonance Raman spectra of reduced HRP in the 200-600 cm^{-1} region.

MATERIALS AND METHODS

Isoenzyme C of HRP with RZ = 3.2 (Toyobo, grade I-C) was dissolved in 0.3 M phosphate or Tris-HCl buffer to give a 0.5 mM HRP solution. The pH of the solution was adjusted with concentrated HCl or NaOH solution. The enzyme was reduced by small amount of solid dithionite under argon atmosphere (99.999 %). Sperm whale myoglobin was kindly given by Dr. K. Nagai (Nara Medical College). ^{13}C and ^{15}N enriched potassium cyanide was purchased from Boxy Brown Co. Raman scattering was excited at 441.6 nm with a He-Cd laser (Kinmon Electrics, model CDR30MGH) or at 457.9 nm with an Ar laser (Spectra Physics, model 164) and was recorded on a JEOL-400D Raman spectrometer. Frequency calibration of Raman spectrometer was performed with indene for each experiment.

RESULTS

Figure 1 compares the resonance Raman spectra of reduced HRP (pH 6.5) and deoxy Mb (pH 6.5) excited at 457.9 nm. All Raman lines are polarized except for the line at 1605 cm^{-1}, which is depolarized and therefore assigned to ν_{10} in accord with Abe et al. (14). Other marker lines (15) of reduced HRP and deoxy Mb are alike; 1473 and 1472 cm^{-1} for ν_3 and 1358 and 1355 cm^{-1} for ν_4 of HRP and Mb, respectively. The close similarity strongly suggests that ferro heme of HRP assumes the penta-coordinated high-spin structure as that of deoxy Mb. In the lower frequency region, however, the two spectra distinctly differ. The 220- and 239-cm^{-1} lines of deoxy Mb were previously assigned to the modes involving primarily the Fe-N_ε(His F8) stretching (11) and peripheral vinyl bending vibrations (16), respectively. To inquire into a vibrational property of the 244-cm^{-1} line of reduced HRP, we treated the cyanide complex.

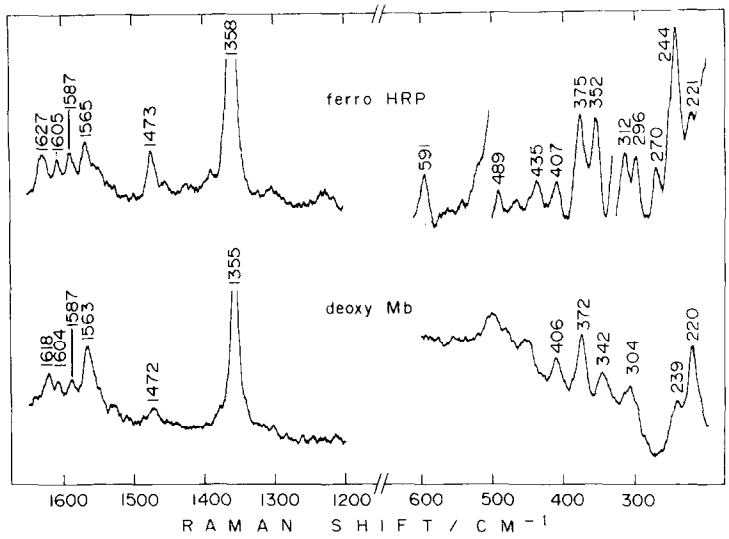
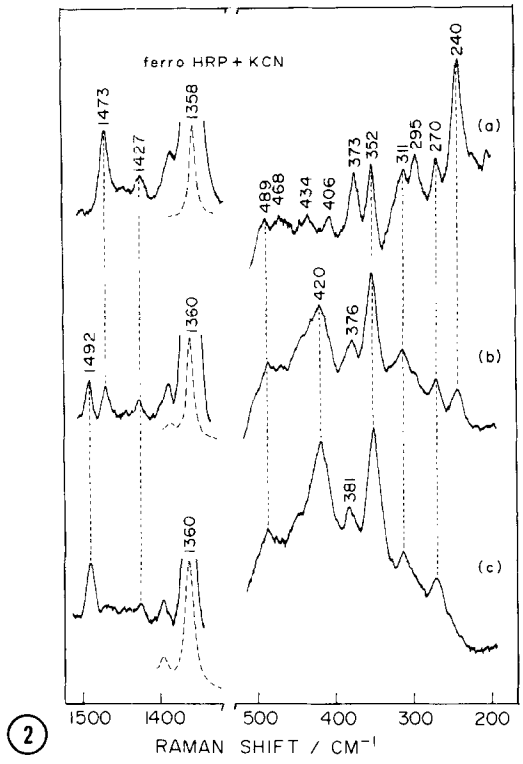


Fig. 1 Resonance Raman spectra of reduced horseradish peroxidase (pH 6.5) and deoxy Mb (pH 6.5) excited at 457.9 nm (power 18 mW). Instrumental conditions: (200-600 cm^{-1}) slit width, 5 cm^{-1}; sensitivity, 500 counts/s; scan speed, 10 cm^{-1}/min; time constant, 16 sec. (1200-1700 cm^{-1}) slit width, 4 cm^{-1}; sensitivity, 1000 counts/s, scan speed 25 cm^{-1}/min; time constant, 3.2 sec.

Figure 2 displays the resonance Raman spectra of reduced HRP in the presence of excess KCN at pH 7.5 (a), 9.3 (b) and 10.6 (c). Coordination of CN^- to the heme iron can be monitored by the ν_3 band which is known to be located around 1470 or 1490 cm^{-1} for the ferrous high- or low-spin state, respectively (15). The 1470-cm^{-1} line of trace (a) disappears completely at pH 10.6 and instead a new line appears at 1492 cm^{-1} as seen in trace (c), indicating the formation of hexa-coordinated low-spin cyanide complex at alkaline pH. This agrees with the results of Loehr and Loehr (6). Note that the ν_4 line of the cyanide complex appears at 1360 cm^{-1}. The frequency of this line reflects a nature of chemical bond between Fe and the sixth ligand (15), and 1360 cm^{-1} of reduced HRP·CN indicates that the Fe-CN bond is of the σ type (15). It is one of great differences between HRP and Mb that deoxy Mb can not form a stable complex with the σ-type ferrous low-spin character.

In the lower frequency region, the Raman lines of ferrous high-spin HRP at 240 and 295 cm^{-1} disappear and a new intense line appears at 420 cm^{-1} upon formation of the cyanide complex. We failed to detect a $^{13}C^{15}N$ isotopic frequency shift for the 420-cm^{-1} line as well as for other lines of reduced



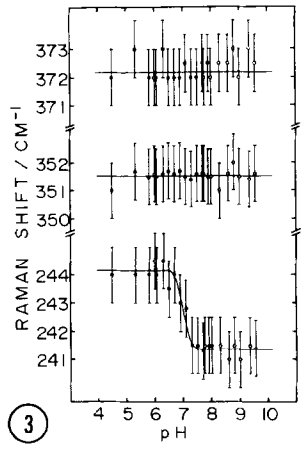


Fig. 2 Resonance Raman spectra of reduced horseradish peroxidase in the presence of 50-fold excess KCN to 0.4 mM horseradish peroxidase solution. (a) pH 7.5 (b) pH 9.3 (c) pH 10.6. excitation, 441.6 nm (15 mW). Instrumental conditions are same as those in Fig. 1.

Fig. 3 The pH dependence of frequencies of a few selected Raman lines of reduced horseradish peroxidase. Open circles and closed circles show the data for Tris buffer and phosphate buffer, respectively. The size of circles represents uncertainty of pH and the error bars indicate the maximum errors in frequencies.

HRP·CN shown in Fig. 2(c) and thus to observe the Fe-CN stretching mode. Therefore, the Raman lines exhibited by trace (c) and the corresponding ones of reduced HRP (trace a) are assignable to the porphyrin in-plane modes.

Figure 3 illustrates pH-dependence of selected Raman lines of reduced HRP. Neither the 352- and 375-cm^{-1} lines nor other lines not plotted in Fig. 3 exhibited a pH dependent frequency shift. However, only the 244-cm^{-1} line showed an appreciable frequency shift with the middle point at pH~7. As the porphyrin skeletal vibrations are strongly coupled with one another, it is unlikely that only one porphyrin in-plane mode shows the frequency shift. This implies that the 244-cm^{-1} line possesses a dissimilar property from other

Raman lines and is therefore inferred to arise primarily from the Fe-N(axial) stretching vibration. This assignment is consistent with its selective intensity-enhancement at 441.6 nm as in deoxy Mb and also with its disappearance upon formation of the cyanide complex shown in Fig. 2, because the Fe-N(axial) stretching Raman line displayed remarkable intensity-reduction upon conversion from ferrous high- to low-spin state for iron picket-fence porphyrin complexes (17).

The same pH-titration experiment was carried out for deoxy Mb. No Raman lines showed a pH dependent frequency shift in the pH range, 4.8-8.4. Thus the frequency shift shown in Fig. 3 is peculiar to reduced HRP and its middle point agrees closely with the reported pK value of 7.17 obtained from spectrophotometric and pH-stat titration experiments for isoenzymes B+C (2). Consequently it is most reasonable to assume that the protonation of distal histidine is transmitted to heme through the Fe-N(His, proximal) bond, causing some change of visible absorption spectrum of the heme.

DISCUSSION

Coordination of proximal histidine to the heme iron was demonstrated with EPR (18) and ultraviolet difference spectroscopies (19). The coordinated histidine was shown to be inaccessible to solvent H_2O for oxidized HRP in contrast with for aquo metMb (20). Therefore, it is not surprising that the Fe-N_ε(His) stretching modes of the two proteins have distinctly different frequencies. If the Raman lines were both due to fairly pure Fe-N_ε(His) stretching mode, the observed frequency difference may suggest that the Fe-N_ε stretching force constant might be ca. 1.2 times larger in reduced HRP than in deoxy Mb. With the Morse potential and harmonic approximation, the Fe-N_ε bond energy in reduced HRP is deduced to be ca. 1.2 of that in deoxy Mb.

Mb is known to show no Bohr-effect in contrast with HRP (3), although an appreciable spectrophotometric change was detected at pH 5.57 for deoxy Mb (21) Thus the lack of the pH dependent frequency shift in deoxy Mb seems reasonable. The pK value for the heme-linked protonation of reduced HRP complexes increased

in order of HCN < CO < NO < O_2 and the change of pK was ascribed to the altered ionization of distal histidine hydrogen-bonded to the sixth ligand. As the pK values increase in an increasing order of back donation of electrons from iron, the hydrogen bond between the sixth ligand and distal histidine might be essential for these complexes, in which the heme could sense the ionization of distal histidine via the sixth ligand, that is, change of electron delocalization. If there were no sixth ligand coordinated, how does the heme sense the ionization of distal histidine?

The present Raman spectrum in the higher frequency region indicated lack of the sixth ligand in reduced HRP. Furthermore all the porphyrin modes were unaffected by variation of pH. Only the Fe-N_ε(His) stretching frequency reflected the pK~7 transition. This observation might be interpreted by assuming that the ionization of distal histidine triggered a change of tertiary structure resulting in distortion of Fe-N_ε(His) bond in the proximal side.

An alternative interpretation may assume a hydrogen bond between proximal histidine and an appropriate amino acid residue. If the situation of hydrogen bond were altered with pH, the basicity of N_ε and thus the Fe-N_ε bond strength would be altered. Coordination of the sixth ligand might affect the Fe-N_ε bond at the trans position and in turn the hydrogen bond between protein and proximal histidine. In this model the molecular structure in the proximal side is essential in difference between HRP and Mb. At the present stage we refrain from settling our conclusion, until more detailed study under progress is completed.

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