Resonance Raman Study on Cytochrome c Peroxidase and Its Intermediate

PRESENCE OF THE Fe(IV)=O BOND IN COMPOUND ES AND HEME-LINKED IONIZATION*

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Resonance Raman spectra of ferrous and ferric cytochrome c peroxidase and Compound ES and their pH dependences were investigated in resonance with Soret band. The Fe(IV)=O stretching Raman line of Compound ES was assigned to a broad band around 767 cm⁻¹, which was shifted to 727 cm⁻¹ upon ¹⁸O substitution. The ¹⁸O-isotopic frequency shift was recognized band. The Fe(IV)=O stretching Raman line of Compound ES was assigned on the basis of the frequency shift observed for ¹⁸Fe isotopic substitution. From the intensity analysis of this band, the pKₐ of the heme-linked ionization of ferrocytochrome c peroxidase was determined to be 7.3. The Raman spectrum of ferri-cytochrome c peroxidase strongly suggested that the heme is placed under an equilibrium between the 5- and 6-coordinate high-spin structures. At neutral pH it is biased to the 5-coordinate structure, but at the acidic side of the transition of pKₐ = 5.5 the 6-coordinate heme becomes dominant. F₇ is bound to the heme iron at pH 6, but Cl⁻ was bound only at acidic pH. Acidification by HNO₃, H₂SO₄, CH₃COOH, HBr, or HI resulted in somewhat different populations of the 5- and 6-coordinate forms when they were compared at pH 4.3. Accordingly, it is inferred that a water molecule which is suggested to occupy the sixth coordination position of the heme iron is not coordinated to the heme iron at pH 6 but that protonation of the pKₐ = 5.5 residue induces an appreciable structural change, allowing the coordination of the water molecule to the heme iron.

Reactivities of the heme iron of peroxidases are distinctly different from those of oxygen carriers despite the fact that both have a histidine-coordinated iron-protoporphyrin IX as a prosthetic group. Spectroscopic investigations for characterizing the iron coordination environments of a peroxidase have been carried out mainly with horseradish peroxidase, a plant peroxidase. Indeed, difference between the Fe(II)–histidine bonds of the two kinds of heme proteins were elucidated from studies on horseradish peroxidase (1–4). The formation of the Fe(IV)=O bond in a reaction intermediate has been confirmed for Compound II of horseradish peroxidase, which has an oxidation state higher by 1 oxidizing equivalent than the ferric state, by means of visible absorption (5), electron nuclear double resonance (6), NMR (7), extended x-ray absorption fine structure (8), and resonance Raman (9, 10) spectroscopies. Unfortunately, however, horseradish peroxidase has not been crystallized yet, and, therefore, precise structural information is not available. It is desirable to examine the spectroscopic features with a peroxidase whose three-dimensional structure has been clarified.

Cytochrome c peroxidase (ferrocytochrome c hydrogen peroxide oxidoreductase, EC 1.11.1.5) catalyzes reduction of hydrogen peroxide like a plant peroxidase (see Ref. 11 for review). The x-ray crystallographic analysis of cytochrome c peroxidase isolated from yeast mitochondria has been accomplished with the resolution of 1.7 Å, and its catalytic mechanism has been discussed from the structural viewpoint (12, 13). Cytochrome c peroxidase reacts rapidly with H₂O₂, donating two reducing equivalents for the cleavage of the HO–OH bond, to give rise to an intermediate called Compound I (11), which corresponds to Compound I of horseradish peroxidase regarding the oxidation state (14). These intermediates return to their native states by 2-equivalent reduction.

There are some differences between cytochrome c peroxidase and horseradish peroxidase. 1) Specificity of reductants is extremely narrow for cytochrome c peroxidase whereas it is very broad for horseradish peroxidase. 2) Compound II is more stable than Compound I for horseradish peroxidase, but the situation is reversed with cytochrome c peroxidase. 3) The extra oxidizing equivalents of Compound ES are preserved in the form of an oxyferri heme and a protein cation radical (15–18), but those of Compound I are in the form of the oxyferri heme and a porphyrin cation radical (6, 19–21). 4) The x-ray crystallographic analysis on the resting cytochrome c peroxidase points out that the 6th coordination position of the heme iron is occupied by a water molecule (13), whereas various spectroscopic data on the resting horseradish peroxidase suggest that the heme iron adopts the 5-coordinate structure (1, 22–24).

The resonance Raman (RR) spectra of heme proteins have

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provided detailed structural information on the heme vicinity. This technique succeeded in identifying a structural change caused by the heme-linked ionization (25) for various plant peroxidases (4) and more recently in revealing the occurrence of oxygen exchange between the Fe(IV)=O heme of Compound II of horseradish peroxidase and bulk water (26). These features seem to be specific to a peroxidase. Accordingly, in this study, we investigated the RR spectra of yeast cytochrome c peroxidase which is the same as that used for the x-ray crystallographic analysis. This paper will not only give the first experimental evidence for the presence of the Fe(IV)=O bond in Compound ES of cytochrome c peroxidase but also bring some structural information on the heme-linked ionization of the resting and reduced cytochrome c peroxidase.

EXPERIMENTAL PROCEDURES

Cytochrome c peroxidase was purified from bakers' yeast and recrystallized with the method of Yonetani et al. (27). The crystal was dissolved in potassium phosphate buffer, pH 6.0, unless otherwise stated. When the enzyme was relatively fluorescent, it was dialyzed against 5 mM phosphate, pH 6.0, for a night, adsorbed on a DEAE-cellulose column, eluted with 0.5 M phosphate, pH 6.0, and then dialyzed against an appropriate buffer again. The concentration of the enzyme was determined spectrophotometrically on the basis of 280 nm at 408 nm. Reconstitution of cytochrome c peroxidase with the 56Fe- or 54Fe-incorporated protoporphyrin IX was performed according to Yonetani (28). Preparation of the [56Fe]heme was described previously (1), and a bovine hemin (Sigma, Type I) was used as the [56Fe]heme.

Compound ES was obtained by adding an equimolar amount of H4O2 to the resting cytochrome c peroxidase at pH 7.0 at room temperature, and its RR spectra were observed with a spinning cell within 3 min after its formation. Since Compound ES was photolabile, the laser power used was made as low as possible.

The enzyme was reduced with a minimal amount of solid dithionite. After reduction of the sample, the cell was evacuated down to 0.01 mm Hg and closed with a cock. Acidification of the sample was carried out with several kinds of acids specified in the figure captions, because there may occur the coordination of an anion to the heme iron besides a simple pH change. The pH values were determined after the Raman measurements.

Raman scattering was excited with a helium/cadmium laser (Kinetic, 5000). The frequencies of Raman lines were determined from 10 independent measurements. The frequencies of a band were designated at left specify the proportion of the acidic form in the mixed. For these simulated curves, frequencies of a band were used as the RR spectra of pure acidic and alkaline forms, respectively, and adopted the following procedures to determine pHK for this change.

RESULTS

Fig. 1 compares the RR spectra of ferrocyanochrome c peroxidase reconstituted with 56Fe- and 54Fe-incorporated protoporphyrin IX. The frequencies of Raman lines were determined from 10 independent scans, and their uncertainties were inferred from the standard deviations. Only the Raman line of the 56Fe cytochrome c peroxidase at 246.9 cm⁻¹ is shifted to higher frequency over the uncertainty upon 56Fe substitution, while the frequencies of other Raman lines are practically the same between the two samples. The 247 cm⁻¹ line is polarized and hence should arise from a totally symmetric mode. Accordingly, this line is assigned to the Fe(II)-histidine stretching vibration.

The RR spectrum of ferrocytochrome c peroxidase in the 1200-1700 cm⁻¹ region (not shown) exhibited a typical pattern of 5-coordinate high-spin form with the marker lines at 1355 (v2) and 1470 cm⁻¹ (v3) (the mode number is based on Ref. 29). These frequencies are almost coincident with those of ferrohorseradish peroxidase (v2 = 1356 and v3 = 1472 cm⁻¹ (30)). The vinyl stretching mode (31, 32) of ferrocyanochrome c peroxidase was identified at 1619 cm⁻¹ which is distinctly lower than that of ferrohorseradish peroxidase (1626 cm⁻¹), but the same frequency as that of deoxy-Mb (1618 cm⁻¹), 30). Therefore, the conformation of the peripheral vinyl groups has presumably no relevance to the catalytic activity of the heme iron. This seems consistent with the previous results which demonstrated that cytochrome c peroxidase reconstituted with 2,4-substituted heme exhibited the activity similar to that of the native enzyme (33).

Fig. 2A illustrates the pH-dependent change of the RR spectra of ferrocyanochrome c peroxidase in the low frequency region. Only the Fe(II)-histidine stretching Raman line exhibits a downward shift as pH is raised while the frequencies of other Raman lines around 300-450 cm⁻¹ as well as those in the higher frequency region are little shifted. The RR spectral change occurred in the pH region between 6 and 8. Therefore, we regarded the spectra observed for pH 5.6 and 8.6 as the RR spectra of pure acidic and alkaline forms, respectively, and adopted the following procedures to determine pHK for this change.

Base line-subtracted spectra of the pure acidic and pure alkaline forms were derived first, and then the two spectra were mixed digitally in an arbitrary ratio. Examples of the synthesized spectra are depicted in Fig. 3, where the numbers designated at left specify the proportion of the acidic form mixed. For these simulated curves, frequencies of a band center at v, %, and ½ height of the maximum of the Fe(II)-histidine stretching RR bands and also the relative peak heights at the frequencies "a" and "b" were determined and are plotted against the proportion of the acidic form in the inset of Fig. 3. It is apparent that the shift of the frequencies of the band center at half-peak height are not linear to the mixing ratio. This may be due to the presence of overlapped side bands at higher frequencies. The relative peak height is
FIG. 2. A, the pH-dependent RR spectral change of ferrocytochrome c peroxidase (ferroCcP) upon excitation at 441.6 nm. B, plots the proportion of the acid form present against pH (see text about the calculation method). Solid line and broken line denote theoretical curves expected for dissociation of 1 and 2 protons with pK_a = 7.3, respectively.

FIG. 3. Computer-simulated RR spectra. I and 0 correspond to the base line-subtracted RR spectra observed for pH 5.6 and 8.6, respectively. The numbers specified at the left side of individual curves denote the proportion of curve I in taking the digital sum of curves I and 0. The inset plots the center frequencies of the Fe(II)-histidine stretching Raman lines against the proportion of the acid form (curve I). The center frequencies were determined at ½ (Δ), ⅓ (O), and ⅔ (■) peak height. The ratios of peak heights at position b and a (I_b/I_a) (□) are also plotted against the proportion of the acid form mixed.

The frequencies of the band center at ½ peak height of the Fe(II)-histidine stretching Raman bands were determined with the RR spectra observed for various pH values. The amounts of proportion of the acidic form present at a given pH, which were determined from the frequencies of the observed band centers and the calibration curve shown in the inset of Fig. 3, are plotted against pH in Fig. 2B. The solid and broken lines are theoretical curves calculated for dissociation of one and two protons with pK_a = 7.3, respectively. The agreement between the observed and either of the calculated values appears satisfactory. Thus, the pK_a value of the heme-linked ionization of ferrocytochrome c peroxidase was determined to be 7.3, which is in agreement with the value (= 7.6) determined from the measurements of the redox potential (34), although the number of dissociating protons could not be exclusively determined from the present experiment. The RR spectra of ferrocytochrome c peroxidase at pH 5.6 and 8.6 in the higher frequency region (1200-1700 cm⁻¹) were identical (not shown), indicating no change in a spin state or a coordination number upon the heme-linked ionization, although Conroy et al. (34) suggested the appearance of the low spin form above pH 7.6 from the observation of visible absorption spectra.

Fig. 4 shows the 441.6-nm excited RR spectra of ferricytochrome c peroxidase at pH 9.3 (A), pH 6.0 (B), and in the presence of fluoride at pH 6.0 (C). The Raman lines of ferricytochrome c peroxidase at pH 6.0 (B) at 1629(dp), 1572(p), 1495(p), and 1374(p) cm⁻¹ are assigned to the ν₁₀, ν₂, ν₃, and ν₄ modes, respectively (p and dp denote polarized and depolarized, respectively). These frequencies are close to those of ferrithorseradish peroxidase with the 5-coordinate high-spin heme (ν₁₀ = 1630, ν₂ = 1573, ν₃ = 1492, and ν₄ = 1375 cm⁻¹), but distinct from those of aquo-metMb with the 6-coordinate not always sensitive enough to determine uniquely the proportion of the acid form. However, the center frequencies of the band center at ½ peak height appeared to give the proportion of the acidic form reasonably, and hence this was adopted as the calibration curve.
Excitation, 441.6 nm. pH 6.0, exhibiting the typical pattern of 6-coordinate high-spin heme proteins. This confirmed the reported RR data.

When fluoride was added to the solution at pH 6.0 (Fig. 4C), the Raman lines were shifted to lower frequency, characteristic frequencies of the 6-coordinate ferric low-spin heme proteins. This confirmed the reported RR data obtained from the 514.5-nm excitation (36).

On the other hand, when pH was raised to 9.3 (Fig. 4A), the marker lines were replaced by those at 1635 cm⁻¹, 1619, 1564, 1564, 1479, and 1372 cm⁻¹, exhibiting the typical pattern of 6-coordinate high-spin heme proteins. This confirmed the occurrence of a spin state transition as pointed out by Shelnutt et al. (38) who presumed its pKₐ to be 7.3. We stress here that the RR spectra (A), (B), and (C) of Fig. 4 represent three distinctive states of the ferric heme of cytochrome c peroxidase, that is 6-coordinate low-spin, 5-coordinate high-spin, and 6-coordinate high-spin states, respectively, and that ferricytochrome c peroxidase at neutral pH gives spectrum of the 5-coordinate high-spin type.

The kinetic study on the reaction of ferricytochrome c peroxidase with hydrogen peroxide suggested the presence of an ionizable group with pKₐ = 5.5. Deprotonation of this residue is considered to be essential for cytochrome c peroxidase to react rapidly with hydrogen peroxide (39). More recently, Shelnutt et al. (38) found a frequency shift of the ν₁ line by 0.8 cm⁻¹ upon transition at pH 5.5. We observed much larger changes in the RR spectra upon acidification. Detailed examination, however, revealed that the spectral changes are not merely a pH effect but a pH- and anion-dependent phenomenon.

The pH-dependent RR spectral changes upon excitation at 406.7 nm are illustrated in Fig. 5A, where the visible difference spectra are also depicted in Fig. 5B. The acidic versus neutral difference spectra gave a positive peak at 405 nm and negative troughs at 376 and 421 nm, suggesting that there are two molecular species in equilibrium. They are conveniently called the 405- and 421-nm species. The 421-nm species does not always mean to have the absorption maximum precisely at 421 nm, but it would have absorption near 421 and 376 nm. The RR spectrum of ferricytochrome c peroxidase excited at 441.6 nm preferentially reflects the 421-nm species which is dominant at neutral pH. Excitation of Raman scattering at 406.7 nm is expected to probe most sensitively the 405-nm species.

The RR spectrum of ferricytochrome c peroxidase at pH 6 (a) is indeed appreciably different from trace B of Fig. 4, although the sample is identical. This result suggests that even at pH 6.0 an appreciable amount of the 405-nm species is present, but it could not be detected upon excitation at 441.6 nm or longer wavelengths. Frequencies of each Raman line were determined from the parallel and perpendicular-polarization components (dp) of the spectrum. The parallel peak at 1622 cm⁻¹, which is presumably due to the peripheral vinyl stretching mode (31), became more prominent upon excitation at 406.7 nm and thus hides the depolarized ν₁₀ line at 1629 cm⁻¹ in the unpolarized spectrum.

As pH is lowered by H₂SO₄, the 1629 (ν₁₀) and 1495 cm⁻¹ (ν₆) lines, the marker lines of the 5-coordinate ferric high-spin heme, became weaker, but the Raman line at 1482 cm⁻¹, which is indicative of the 6-coordinate high-spin heme (22), was more intensified. At pH 4.3 (c) the 1481 cm⁻¹ line is more intense than the 1493 cm⁻¹ line upon excitation at 406.7 nm, but their relative intensity is reversed upon excitation at 441.6 nm as shown by trace (c'). It is, therefore, likely that the 405-nm species corresponds to the 6-coordinate high-spin heme, and its RR spectrum is emphasized upon excitation at 406.7 nm.

When it was acidified by HCl to the same pH, there appeared two ν₃ lines with almost the same intensity as shown at the bottom (d). In this case, however, the excitation at...
441.6 nm gave the RR spectrum similar to (d) in contrast to difference between (c) and (c'). Since neutralization of the acidified enzyme after measurements of spectrum (d) restored the original spectrum, irreversible denaturation does not occur in this experiment. The acidification to pH 4.3 by CH₃COOH brought almost the same spectrum as trace (c), but that by HNO₃ brought a spectrum similar to trace (b). When the enzyme was acidified to pH 4.3 by HBr or HI, the spectral change was similar to the case acidified by HNO₃.

Fig. 6 shows the RR spectra of ferricytochrome c peroxidase in the lower frequency region. The RR spectrum at alkaline pH (A) is clearly different from that at pH 7.7 (B) due to spin state change. When the enzyme was acidified to pH 4.5 (C) with HNO₃, the intensities of Raman lines at 346 and 336 cm⁻¹ are reversed and the Raman line at 278 cm⁻¹ is shifted to 285 cm⁻¹. When it was neutralized again, spectrum (B) was restored. The spectral change due to the pH change was essentially the same as the case acidified with CH₃COOH or H₂SO₄. The resultant RR spectrum at pH 4.5 resembled the spectrum obtained in the presence of fluoride at pH 6.0 (D). In the case of chloride, the RR spectrum remained unaltered at pH 6.0, but when pH was brought to 4.3 without a change of chloride concentration, it gave spectrum (E), which is of the 6-coordinate type but is slightly different from spectra (C) and (D).

These observations suggest that the acidification by HCl has some unique feature. To clarify the phenomenon, difference absorption spectra were examined for the enzyme in the presence of 50 mM KCl. As shown in Fig. 7, the spectral changes upon pH changes above pH 5.1 appeared different from those below pH 5.1. When the size of difference peak heights near 408 and 425 nm is plotted against pH, the changes are represented with two straight lines as depicted in Fig. 7b. The similar plots for the acidification by HNO₃ are also included in Fig. 7b. They are represented by a single nonlinear curve and are distinct from those for the presence of KCl. Therefore, acidification in the presence of chloride brings another feature besides simple acidification, and the other feature is likely to be binding of chloride to the heme iron like the fluoride ion.

When the binding of an anion to the heme iron is involved during acidification, the apparent pKₐ value may be altered, and thus the variant Raman spectra at pH 4.3 are understandable. The Raman spectrum in the lower frequency region may be somewhat altered by the anion species bound. If this interpretation is accepted, then it leads to the conclusion that the protonation of the pKₐ = 5.5 residue allows the coordination of an anion or a water molecule to the 6th coordination position, but its pKₐ value and relative population of the 5- and 6-coordinate heme depend on the anion species present.

The RR spectrum of Compound ES excited at 406.7 nm is compared with that of the native enzyme in Fig. 8. This spectrum of Compound ES (B) is apparently different from that reported previously (36) due to different excitation wavelengths. However, the marker lines are observed at 1641 (ν₁), 1509 (ν₂), and 1378 cm⁻¹ (ν₃), which are in agreement with the reported frequencies of Compound ES of yeast cytochrome c peroxidase (36) and also of Compound II of horseradish peroxidase (40, 41). These are close to but still higher than those of the ferric low-spin cytochrome c peroxidase (Fig. 4A). This trend is consistent with reduction of the number of d electrons and thus decreased delocalization of electrons to the porphyrin π* (ε₂̃) orbital in the Fe(IV) than Fe(III) low-spin state (42, 43). In the lower frequency region, a doublet at 347 and 334 cm⁻¹ is combined to a single line at 341 cm⁻¹, and the intensity of the 375-cm⁻¹ line decreased. This change is characteristic of the spectral changes due to a change of the spin state shown by spectra (B) and (A) in Fig. 6. It was rather unexpected that the RR spectrum below 300 cm⁻¹ remained unaltered upon formation of Compound ES.

Fig. 9 shows the RR spectra of Compound ES with isotopic substitution. The RR spectrum of Compound ES formed in D₂O (B) has an additional line at 772 cm⁻¹ compared with the spectrum of native enzyme (A). Curiously the corresponding line was not definite in H₂O as shown by spectrum (C), but a weak broad feature seems to exist at the higher frequency side of the 753-cm⁻¹ line. Even when H₂¹⁸O and H₂¹⁴O were used to produce Compound ES, the observed spectrum was unaltered (D). However, when H₂¹⁸O and H₂¹⁴O were used, a weak shoulder was recognized around 730 cm⁻¹ as shown by spectrum (E). It is emphasized that the difference between spectra (C) and (E), as shown by trace (F), exhibited clear positive and negative peaks at 767 and 727 cm⁻¹, respectively. This exclusively indicates that the 767-cm⁻¹ feature in spectrum (C) arises from the Fe(IV)=¹⁸O stretching vibration and is shifted to 727 cm⁻¹ in spectrum (E) due to the substitution to the Fe(IV)=¹⁴O heme.

The difference between spectra (C) and (D) is represented by trace (G). There is no clear peak. The absence of spectral difference between the products derived from H₂¹⁸O and H₂¹⁴O in H₂¹⁸O was previously noticed for horseradish peroxidase Compound II at neutral pH (28). This strongly sug-

![Fig. 6. The RR spectra of ferricytochrome c peroxidase in the lower frequency region.](image-url)
Resonance Raman Study of Cytochrome c Peroxidase

FIG. 7. a, The acid versus neutral difference spectra of ferricytochrome c peroxidase in the presence of 50 mM KCl. The enzyme is acidified by HCl. Standard is pH 6.5. A: 1, pH 6.0; 2, pH 5.6; 3, pH 5.2. B: 1, pH 5.1; 2, pH 4.7; 3, pH 4.0. b, plots the difference peak heights against pH. The closed symbols indicate the corresponding peak heights from Fig. 5B obtained upon acidification by HNO₃.

The exchange phenomenon of bound oxygen with bulk water was previously noticed for Compound II of horseradish...
peroxidase (26) and confirmed again in this experiment for Compound ES of cytochrome c peroxidase. The similar oxygen exchange between the Fe(IV)=O heme and water is reported for an intermediate of cytochrome P-450 (49) and its model compounds (50, 51). Accordingly, this may be a characteristic feature of the Fe(IV)=O heme.

The oxygen exchange in Compound II of horseradish peroxidase took place only at lower pH than pH of the heme-linked ionization (26). Therefore, a dissociable proton of the heme vicinity should be involved in the exchange reaction, although the corresponding pK value of Compound ES of cytochrome c peroxidase has not been determined yet. The Fe(IV)=O stretching frequency of horseradish peroxidase Compound II is shifted to higher frequency by 10 cm\(^{-1}\) upon protonation of the pK = 8.6 residue. This was confirmed recently by Sitter et al. (52) in an independent study. Nevertheless, the Fe(IV)=O stretching mode of cytochrome c peroxidase Compound ES exhibited no frequency shift between pH 4 and 11. The Fe(IV)=O stretching frequency of horseradish peroxidase Compound II is slightly higher in D\(_2\)O than in H\(_2\)O only in the protonated form, and that of cytochrome c peroxidase Compound ES also appears at slightly higher frequency in D\(_2\)O than in H\(_2\)O. Furthermore, this Raman line was always weak despite the fact that the corresponding line of horseradish peroxidase Compound II became stronger above pH 8.6. These facts lead us to assume that the residue involved in the heme-linked ionization of cytochrome c peroxidase Compound ES is protonated at those pH values, and the oxygen is vigorously exchanged.

The Fe(IV)=O stretching Raman line was definitely more intense in D\(_2\)O than in H\(_2\)O for Compound II of horseradish peroxidase (28, 52), and the same phenomenon is observed for Compound ES of cytochrome c peroxidase (compare Fig. 9, B with C). If the rate of photoreduction of Compound ES is slower in D\(_2\)O than in H\(_2\)O as in the enzymatic reaction of horseradish peroxidase (5), the accumulated intensity of Compound ES during the measurements (128 s) would become more intense for the D\(_2\)O solution. This is one of plausible interpretations of the present results. On the other hand, the low Fe(IV)=O stretching frequency of cytochrome c peroxidase compared with that of horseradish peroxidase may imply the stronger hydrogen bond between the bound oxygen atom and the protonated residue. If there were some heterogeneity of a protein conformation in the heme vicinity, there would be Fe(IV)=O bonds having different strengths of the hydrogen bond. This would cause heterogeneous band broadening, and its effect would be more significant when the hydrogen bond is stronger. The higher Fe(IV)=O stretching frequency in D\(_2\)O would mean weaker hydrogen bonding and thus might imply less heterogeneous broadening. However, this interpretation seems less likely because the Raman difference spectrum shown in Fig. 9F indicates appreciable difference between the bandwidths of the Fe(IV)=18O and Fe(IV)=1H O stretching modes.

If the apparent weak intensity of the Fe(IV)=O stretching band was ascribed to band broadening, there would be another plausible mechanism to cause the difference between the H\(_2\)O and D\(_2\)O solutions. According to the x-ray crystallographic analysis (12,13), several water molecules are located in the heme vicinity. Among them, H\(_2\)O-595 stays at 2.4 Å from the Fe(III) ion. Since a rotational vibration of H\(_2\)O in water is reported at 710 cm\(^{-1}\) at room temperature, it is expected to shift to a higher frequency at a lower temperature (35). If a water molecule were accessible close to the Fe(IV)=O bond of Compound ES and its rotational vibration were located around 770 cm\(^{-1}\), the vibrational energy transfer might take place from the Fe(IV)=O stretching mode to it. Since the same mode of D\(_2\)O is located at a lower frequency (530 cm\(^{-1}\)) (53), the energy transfer would no more be important in D\(_2\)O. Then it is understandable that the Fe(IV)=O stretching band is very broad in H\(_2\)O but not in D\(_2\)O. A similar idea was previously proposed to interpret band broadening of the C=NH stretching Raman line of bacteriorhodopsin in H\(_2\)O, although in this case the energy transfer to the bending mode was considered (54). Whatever the mechanism is, it is fairly likely that a dissociable proton is located near the 6th coordination position of the heme iron and plays an essential role in the oxygen exchange reaction. Since the residue which offers this proton is probably involved in the heme-linked ionization, its study is important.

Iron-Histidine Stretching Mode and the Heme-linked Ionization—It was argued that the Fe(II) isotope-sensitive Raman line of hemoproteins may not arise from the Fe(II)-histidine stretching mode but may arise more preferably from the Fe(II)-pyrrole stretching mode (55). This argument brought some confusion in interpretation of RR spectra. Recently, however, a complete set of the isotope substitution experiments was carried out in Ref. 56, and the results supported exclusively the previous assignment. Therefore, the Fe(II) isotope-sensitive Raman line of horseradish peroxidase at 247 cm\(^{-1}\) is assigned to the Fe(II)-histidine stretching mode. As for ferricytochrome c peroxidase, we were confident that the Raman lines at 404, 375, and 345 exhibited no shift upon \(^{54}\)Fe substitution. However, the weak intensity and broad bandwidth of the 278 cm\(^{-1}\) line did not allow us to determine whether it exhibited an isotope shift or not. We also failed to find an isotope-sensitive band for the fluoride complex with relatively sharp lines.

Previously the higher Fe(II)-histidine stretching frequency of ferrophorseradish peroxidase (244 cm\(^{-1}\)) compared with that of deoxy-Mb (220 cm\(^{-1}\)) was attributed to strong hydrogen bonding of proximal histidine (1, 2). The Fe(II)-histidine stretching frequency of ferricytochrome c peroxidase (247 cm\(^{-1}\)) is close to that of ferrophorseradish peroxidase. Indeed, x-ray crystallographic analysis of cytochrome c peroxidase revealed the presence of a hydrogen bonding chain through proximal histidine (His-1751, Asp-285, and Trp-191 (15)). Therefore, the presumption derived from Raman study has now been justified by the present observation.

Table I summarizes the Fe(II)-histidine stretching frequencies of various ferrous hemoproteins examined so far. It is apparent that the Fe(II)-histidine stretching frequencies of all the peroxidases including isozymes of Japanese radish peroxidase, turnip peroxidase, lactoperoxidase, intestine peroxidase, horseradish peroxidase, and cytochrome c peroxidase are located around 244–258 cm\(^{-1}\), which are distinctly higher than those of oxygen carriers and cytochrome oxidases. Therefore, it is established that the strong hydrogen bonding of proximal histidine is a unique characteristic of all peroxidases.

The Fe(II)-histidine stretching Raman line of ferrocytochrome c peroxidase exhibited a pH-dependent frequency shift. The intensity analysis enabled us to determine the pK value to be 7.3, in good agreement with the reported value (7.5) determined from the redox potential measurements (34). Here we stress that the Fe(II)-histidine stretching mode is a good probe of the heme-linked ionization. Indeed this mode of all the plant peroxidases exhibited a pH-dependent frequency shift while all other RR lines remained unshifted (4). The midpoint pH for the frequency shift can practically be regarded as the pK, value of the heme-linked ionization.
However, the Fe(II)–histidine stretching Raman line of animal peroxidases does not show a reversible frequency shift when pH is lowered (57).

*Heme Structure and Heme-linked Ionization*—There is no doubt that the heme of ferrocytochrome c peroxidase adopts the 5-coordinate high-spin structure. The present conclusion for ferricytochrome c peroxidase may be argued, because the x-ray crystallographic analysis claimed occupation of a water ligand (H$_2$O-595) in the sixth coordination position of the heme iron. It is emphasized that the RR spectrum is quite sensitive to the coordination number of heme (22, 58) and that spectra (B) and (C) in Fig. 4 correspond to the typical spectra of the 5- and 6-coordinate high-spin hemes. In neutral solution of ferricytochrome c peroxidase there may be a small amount of the 6-coordinate high-spin heme in equilibrium, but it is very unlikely that the RR spectrum of ferricytochrome c peroxidase would be interpreted in terms of the 6-coordinate high-spin heme. On the other hand, the Fe–(H$_2$O) distance in ferricytochrome c peroxidase is reported to be 2.4 Å (13). This is considerably longer than 2.0 Å of the Fe–(H$_2$O) distance in aquo-Mb (59). Therefore, it is highly likely that H$_2$O-595 happens to occupy the 6th coordination position of the heme iron and that it is hydrogen bonded to a neighboring amino acid residue such as Trp-51 but is not directly interacting with the heme iron.

The x-ray crystallographic analysis on the fluoride complex of cytochrome c peroxidase pointed out a small adjustment in local arrangements of distal residues such as Arg-48 and His-52 upon binding of fluoride (60). The Fe(III)–F distance (2.0 Å) is clearly shorter than the Fe(III)–H$_2$O distance (2.4 Å) of native cytochrome c peroxidase, which is consistent with the spectral change from (B) to (C) in Fig. 4 caused by a change of the coordination number.

It was previously pointed out from the magnetic measurements that ferricytochrome c peroxidase is a mixture of two chemical compounds ("acidic" and "alkaline" forms), each of which is a thermal mixture of the high-spin and low-spin species (61). Although the "acidic" to "alkaline" transition of ferricytochrome c peroxidase has not been readily monitored by spectrophotometry at ambient temperatures due to a small spectral difference, the pK value of this transition was estimated to be ~6 from the cryogenic spectrophotometry (61). On the other hand, the RR spectra shown in Fig. 5 demonstrated that the 5- and 6-coordinate high-spin hemes are in equilibrium which is biased to the former at pH 6 but to the latter at pH 4.3. Therefore, it is highly likely that the high-spin species of the "acidic" and "alkaline" forms correspond to the 6- and 5-coordinate hemes, respectively.

Protonation of the pK$_a = 5.5$ residue changes significantly the relative stability of the 5- and 6-coordinate hemes. The water molecule (H$_2$O-595) near the 6th coordination position is hydrogen bonded to Trp-51 at pH 6.0 (60) and hinders other ligands from binding to the heme iron. Therefore, only when a ligand interacts relatively strongly with the heme iron, the water molecule is replaced by the ligand. The binding of fluoride to ferricytochrome c peroxidase corresponds to this case. In fact, the Fe(III)–F distance in cytochrome c peroxidase (2.0 Å) is shorter than the estimated Fe(III)–F distance (2.3 Å) for MbF (62). The hydrogen bonding of H$_2$O-595 is presumably destabilized by the protonation of the pK$_a = 5.5$ residue. Then H$_2$O-595 may be replaced by an exogenous ligand, if any, or coordinated to the heme iron. Acidification by HCl may correspond to the former case. Since the acidification by HNO$_3$, H$_2$SO$_4$, HBr, HI, and CH$_3$COOH resulted in a similar spectrum, the coordination of a water molecule to the heme iron seems most likely.

The pK$_a = 5.5$ residue is assigned to His-52 (distal histidine) or Asp-235. The pK$_a$ value of distal histidine of Mb-F and Hb-F were determined to be 5.5 and 5.1, respectively (48). In this case, the dissociable proton of distal histidine is strongly hydrogen bonded to the bound fluoride ion, lowering the Fe(III)–F stretching frequency by 60 cm$^{-1}$ more in the acidic form than in the alkaline form. In contrast, the fluoride ion of cytochrome c peroxidase fluoride is hydrogen bonded to Arg-48 but not to distal histidine (60). The pK$_a$ value of the distal histidine of carbon monoxide Mb is reported to be 5.2 from $^1$H NMR (63) and between 5 and 6 from $^{13}$C NMR (64). Accordingly, the pK$_a$ value does not depend on the oxidation state of the heme iron for Mb. However, there is no transition between pH 4.8 and 6.2 for ferrocytochrome c peroxidase.

It is not clear whether the pK$_a = 5.5$ ionization of ferricytochrome c peroxidase corresponds to the pK$_a = 7.3$ transition of ferricytochrome c peroxidase or not. Since the CO stretching infrared band of carbonmonoxy cytochrome c peroxidase is reported to exhibit a large change at pH 7.5 (65, 66), the pK$_a = 7.3$ ionization of ferricytochrome c peroxidase is most likely to be associated with distal histidine. Then, the pH-dependent frequency shift of the Fe(II)–histidine (proximal) stretching infrared band shown in Fig. 2 is caused by the protonation of distal histidine. This implies an overall structural change of the protein moiety upon this ionization. On the other hand, the pK$_a$ value of the heme-linked histidine is considered to be higher by 3.5–3.8 pH units in the ferric state than in the ferrous state for horseradish peroxidase isozymes (14). If this were also applicable to cytochrome c peroxidase, then the ionization of distal histidine would result in the spin state transition for ferricytochrome c peroxidase, which yields the RR spectral difference between Fig. 4A and 4B. In this case, the ionization of ferricytochrome c peroxidase corresponding to pK$_a = 5.5$ might be attributed to Asp-235.

REFERENCES
