Two Types of Conformers with Distinct Fe–C–O Configuration in the Ferrous CO Complex of Horseradish Peroxidase

RESONANCE RAMAN AND INFRARED SPECTROSCOPIC STUDIES WITH NATIVE AND DEUTEROHEME-SUBSTITUTED ENZYMES*

(Received for publication, October 15, 1986)

Tadayuki Uno†, Yoshifumi Nishimura, and Masamichi Tsuboi‡
From the Faculty of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

Ryu Makino, Tetsutaro Izuka, and Yuzuru Ishimura¶
Department of Biochemistry, School of Medicine, Keio University, Shinjuku-ku, Tokyo 160, Japan

The presence of at least two types of conformers in the ferrous CO complex of horseradish peroxidase has been demonstrated with the use of native and deuteroheme-substituted enzymes. Type I conformers, predominant in acidic pH, exhibited both an Fe-CO stretching and an Fe-C-O bending Raman line together with an infrared C-O stretch band below 1920 cm⁻¹. On the other hand, type II conformers, dominant in alkaline species in alkaline pH, showed only an Fe-CO stretching Raman line with the C-O stretch above 1930 cm⁻¹. They were interconvertible either by the changes in pH or by the binding of benzhydroxamate, a substrate for the enzyme. The pKₐ value for the pH-dependent interconversion of CO complex of deuteroheme-substituted enzyme was 8.3. These findings were interpreted to mean that the bound CO molecule in type I conformers was more tilted over the heme-plane than that in type II conformers. A steric hindrance by the bound substrate or the protonated form of a distal amino acid residue, presumably of histidine, is considered to be the cause for the isomerization.

By summarizing present and previous data on the vibrational frequencies of heme-carbonyl complexes, we found that there are inverse-linear relationships between the square of Fe-CO and that of C-O stretching frequencies, while squares of Fe-CO stretching and Fe-C-O bending frequencies were linearly correlated with each other. Also found is that the dissociation rate constant of CO molecule from heme-carbonyl complexes is a linear function of the Fe-CO stretching frequency. The significance of these results is discussed.

CO complexes of hemoproteins such as oxygenases (1-4), oxidases (5-7), and oxygen carriers (8-13) as well as of model heme compounds (14-18) have been studied by resonance Raman and/or infrared spectroscopy for their Fe-CO stretching, Fe-C-O bending, and the bound C-O stretching modes. These vibrational frequencies have been shown to be sensitive to the proximal ligand field (14) as well as to the distal environment of the CO-heme complex (3, 8). Thus the CO molecule bound to the heme-iron can be a useful probe to study the fine structure of the active site in hemoproteins (17).

In the present study, we successfully measured the resonance Raman and infrared spectra of ferrous CO complex of horseradish peroxidase, where the use of a photoresistant species of the CO complex, i.e. CO-adduct of deuteroheme-substituted enzyme, permitted us to investigate vibrational spectra under various experimental conditions. The results revealed that the CO complex was in equilibrium of multiple conformers which were affected either by protonation of a distal amino acid residue or by the binding of a substrate to nearby heme. The pKₐ value of 8.3 for the interconversion between conformer I and II agreed well with that of an amino acid residue responsible for the formation of a hydrogen bond with the oxoligand in compound II (18-20). Thus, a critical role(s) of the distal amino acid in regulating the reactivity of a ligand-heme complex in horseradish peroxidase was visualized. Analyses of the data also revealed several fundamental relationships among the Fe-CO stretching, Fe-C-O bending and the bound C-O stretching frequencies and a kinetic constant.

MATERIALS AND METHODS

Enzyme Preparations—Horseradish peroxidase was obtained from Sigma (type IV) and purified by the method of Shannon et al. (21) with slight modifications; the enzyme, dissolved in 55 mM sodium acetate buffer, pH 4.4, was applied to a column of CM-Sepharose CL-6B (1.5 × 10 cm, Pharmacia), which had been equilibrated with the same buffer, and was eluted with a linear gradient of 0-30 mM NaCl. Isozyme C fraction; thus isolated from the other isozymes, was dialyzed against 10 mM sodium phosphate buffer, pH 7.0, and was concentrated with Centricon-10 (Amicon). Apoenzyme of isozyme C was prepared according to the method of Teale (22). Then deuteroheme-substituted enzyme was prepared by reconstituting the apoenzyme with deuteroheme by the method described previously (23).

The CO complex of the enzymes was prepared by adding a minimum amount of fresh sodium dithionite (Nakarai Chemicals Co., Japan) under CO atmosphere. In some experiments, ~5-fold excess of sodium benzhydroxamate (Tokyo Kasei Co., Japan) was added to a CO complex. ΔCO² (99 atom % for $^{13}$C), ΔCO² (99 atom % for $^{18}$O), and ΔCO² (99 atom % for $^{13}$C, $^{18}$O) were used to calculate the concentration of native and deuteroheme-substituted horseradish peroxidase, respectively.

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Resonance Raman Spectroscopy—Resonance Raman spectra were measured by using a JASCO R-800 UV spectrophotometer. Detection was made with the use of a Hamamatsu Photonics R-585 photomultiplier, or a Tracer Northern IDARSS detector system which consisted of a TN-1223-4SF photodiode array-head equipped with an intensifier (Hamamatsu Photonics, model V1830S) and a TN-1710-4K analyzer with a TN-1710-21 optical spectrometer module (20). The spectra in Figs. 1 and 3 were obtained by the former detection system, and spectra in Figs. 2, 4, 5, and 6 were by the latter system. The data acquisition times are given in each figure legend.

The 406.7- and 413.1-nm lines of a Kr laser (Spectra Physics, SP 164-01 equipped with an ultra high-field magnet), and a 457.9-nm line of an Ar laser (NEC, model GLS 3300) were employed for excitation with the laser power of approximately 10–20 mW at the sample. Raman cell (volume: 0.4 ml, 1 cm in diameter) was spun throughout the measurements under the flush of ice-cooled N2 gas. The laser beam was defocused onto the cell to lessen the photodissociation of CO from the CO complex (3, 7). For the experiments with CO isotopes at 406.7-nm excitation, the laser power was reduced to less than 5 mW to minimize the production of photodissociated species.

IR Spectroscopy—Infrared spectra were recorded on a JASCO IR spectrophotometer, model IR-810, in the absorbance mode. The data points were accumulated at every 0.1 cm⁻¹. To improve the signal to noise ratio, nine successive scans were averaged. The temperature of the infrared cell was maintained at 15 °C by thermomodule elements attached to the cell, monitoring the temperature with a Pt thermister mounted into a drilled hole of the cell adjacent to the windows. The cell had CaF2 windows and a light-path length of 0.08 mm. Enzymes which were dialyzed against 10 mM Tris-HCl buffer at pH 7.4 were concentrated to ~4 mM and were diluted to approximately 2 mM with a desired buffer solution for use. The sample thus prepared was introduced into the cell with a gas-tight microsyringe (Hamilton). Other details are described under appropriate figure legends.

RESULTS

Resonance Raman Spectra of Native Enzyme—In Fig. 1 are shown the resonance Raman spectra of ferric, ferrous, and ferrous CO forms of isozyme C of native horseradish peroxidase in the region between 1700 and 100 cm⁻¹. The spectra were obtained upon either 406.7- or 413.1-nm excitation at pH 7.0. In the top spectrum, the ferric form of the enzyme exhibited a number of strong lines in the region between 1650 and 1350 cm⁻¹, around 700 cm⁻¹, and below 400 cm⁻¹. The spectral pattern coincided well with that reported previously (25, 26). The spectral feature below 400 cm⁻¹ was somewhat peculiar as an iron-protoporphyrin derivative in that many strong lines were present in the region; it may be caused in part by symmetry-lowering to D₄h due to ruffling of the porphyrin core (27) and by the enhancement of some Raman modes which are inactive under the D₄h point group. Upon reduction to the ferrous state, the peculiar feature below 400 cm⁻¹ almost disappeared, and resultant spectrum showed a number of lines specific to the ferrous form (bottom). Among the lines newly appeared, one at 244 cm⁻¹ has been assigned to the stretching mode of Fe-histidine residue (28).

The resonance Raman spectrum of the ferrous CO complex was shown in the middle. As a whole, it was distinct from the spectra of other forms but partly resembled that of the ferric form. As seen, both ferric and ferrous CO forms had their ν₈ lines at the same frequency, 1376 cm⁻¹. Such a coincidence of ν₈ lines between the two forms has been reported for those of other hemoproteins such as hemoglobin and myoglobin (30) and has been considered to denote the "ferric-like" structure of the ferrous CO complex. When spinning of the Raman cell was stopped the spectrum was readily converted to that of the ferrous form, indicating that continuous illumination of the laser beam caused photodissociation of CO molecule. As judged from the intensities of the ν₈ line at 1359 cm⁻¹ and the ν₂ line at 1473 cm⁻¹ which were characteristic of the ferrous form, contamination of the ferrous form was less than 20% under the experimental conditions.

Assignments of ν(Fe–CO) Stretching and δ(Fe–C–O) Bending Modes—Fig. 2 shows effects of CO isotopes (13C¹⁶O, 13C¹⁸O or 13C¹⁸O) on the resonance Raman spectrum of ferrous CO complex of horseradish peroxidase. At pH 7.0 (left), three isotope-sensitive Raman lines were detected between 600 and 500 cm⁻¹. The 541 cm⁻¹ line and the shoulder peak at 519 cm⁻¹ of ¹³C¹⁸O complex (top) down-shifted monotonously upon increasing the total mass of the CO isotopes (middle and bottom) and were therefore assigned to the ν(Fe–CO) stretching modes. As seen, the frequency shift of the shoulder peak at 519 cm⁻¹ was obscure especially with ¹³C¹⁸O, because of an overlapping of a porphyrin mode at 496 cm⁻¹ (bottom spectrum). Nevertheless, the monotonous down shift was obvious from the fact that the trough between the 519 and 496 cm⁻¹ lines became less marked. Thus, the ferrous CO form had two ν(Fe–CO) stretching modes at a neutral pH, suggesting that it consisted of at least two conformers. Besides them, an appearance of another new Raman line at 574 cm⁻¹ was noticed upon employment of ¹³C¹⁸O together with a slight decrease in the intensity of the Raman line at 590 cm⁻¹ (middle). The new Raman line could be assigned to the δ(Fe–
The spectrum of ferrous CO complex of native horseradish peroxidase. At pH 10.0, respectively. Other conditions were the same as those in Fig. 1. The asterisk indicates the plasma line of the laser.

C–O bending mode, since the line appeared only with $^{13}$C$^{18}$O but not with $^{13}$C$^{16}$O, and the amount of shift by $^{13}$C$^{16}$O, 16 cm$^{-1}$, agreed with the value reported for the isotope shift of the bending mode of CO complexes of hemes and other hemoproteins (3, 10, 14, 15). It is considered therefore that the bending mode of the original $^{13}$C$^{16}$O complex had been hidden in a strong porphyrin skeletal vibration at 590 cm$^{-1}$. These and other isotope shifts observed in this study were summarized in Table I.

When pH was raised to 10 (Fig. 2, right), the 541 cm$^{-1}$ line in the above mentioned spectrum changed to a broad Raman line around 531 cm$^{-1}$. The line down-shifted to 513 cm$^{-1}$ upon substitution by $^{13}$C$^{16}$O, indicating that it was assignable to a $\nu$(Fe–CO) stretching mode. On the other hand, positions of other lines at 679, 590, and 496 cm$^{-1}$ were not affected by the changes in pH or by the isotope substitution. For this reason, they were not Fe–CO stretching nor Fe–C–O bending modes but porphyrin skeletal vibrations. Thus, only a single $\nu$(Fe–CO) stretching mode was detectable in an alkaline pH.

**Resonance Raman Spectra of Deuteroheme-substituted Enzyme**—Fig. 3 shows resonance Raman spectra of ferric, ferrous, and ferrous CO forms of the deuteroheme-substituted enzyme at pH 7.0. Each spectral pattern was considerably different from that of the corresponding form of the native enzyme in Fig. 1. In the ferric form (top), $v_2$ and $v_4$ lines were found at 1486 and 1374 cm$^{-1}$ which were lower in frequency than those of native enzyme by 16 and 2 cm$^{-1}$, respectively. On the other hand a $v_1$ line, which was at 679 cm$^{-1}$ in the native enzyme, was found at 692 cm$^{-1}$ with concomitant appearance of a strong line at 634 cm$^{-1}$. The 634 cm$^{-1}$ line has been assigned to $\delta$(Cb-H) mode by Desbois et al. (31). The appearance of a strong line at 344 cm$^{-1}$ was also noticed. In the ferrous form (bottom), $v_2$ mode down-shifted to 1467 cm$^{-1}$ from that of native enzyme at 1473 cm$^{-1}$. The $\nu$(Fe–His) mode also down-shifted slightly (Table I). The effect of heme-substitution was thus evident in the Raman spectra.

Upon ligation of CO to the ferrous deuteroheme-substituted enzyme (middle), the appearance of strong Raman lines at 535 and 521 cm$^{-1}$, which were unique to the CO complex, was noticed. When effects of CO isotopes on these Raman lines were examined (Fig. 4), both 535 and 521 cm$^{-1}$ lines down-shifted monotonously upon increasing the total mass of CO, indicating that they were assignable to the $\nu$(Fe–CO) stretching modes. On the other hand, the 586 cm$^{-1}$ line showed a "zig-zag" isotope-shift pattern characteristic of a $\delta$(Fe–C–O) bending mode (10). It should be noted that these stretching and bending modes were remarkably enhanced in the CO complex of the heme-substituted enzyme. These findings together with the aforementioned results on the native enzyme establish that two $\nu$(Fe–CO) stretching modes and one $\delta$(Fe–C–O) bending mode are present in the CO complex of horse-

**TABLE I**

| Vibrational modes | $\nu$(Fe–CO) | $\delta$(Fe–C–O) | $\nu$(Fe–His)$^*$ |
|------------------|-------------|-----------------|------------------|
|                  | $^{13}$C$^{16}$O | $^{12}$C$^{16}$O | $^{13}$C$^{18}$O | $^{12}$C$^{18}$O | $^{13}$C$^{16}$O | $^{12}$C$^{18}$O | $^{13}$C$^{16}$O | $^{12}$C$^{18}$O |
| Native enzyme without BHA$^b$ | | | | | | | | |
| pH 7.0 | 541 | 537 | 530 | | 590 | 574 | 590 | | 244$^d$ |
| pH 10.0 | 519 | 514 | 508 | | 513 | | | | |
| with BHA | | | | | | | | |
| pH 7.0 | 547 | | | | 590 | | | | |
| Deuteroheme without BHA | | | | | | | | |
| pH 7.0 | 535 | 513 | 525 | | 586 | 570 | 583 | | 241$^f$ |
| pH 10.0 | 521 | 517 | 512 | | | | | | |
| with BHA | | | | | | | | |
| pH 7.0 | 544 | | | | 530 | 582 | | | 565 |

$^a$ $\nu$(Fe–His) denotes the stretching frequency between heme-iron and proximal histidine in the ferrous state.

$^b$ BHA denotes benzhydroxamic acid.

$^c$ Not determined.

$^d$ From Ref. 28.

$^e$ At pH 4.4.

$^f$ At pH 9.0.
The enzyme concentration was 40 mM sodium phosphate buffer, pH 7.0. Excitation was by 406.7-nm light with the power output of 10 mW.

The pH-titration curve described above conformed to a single proton-coupled equilibrium with a pK_a value of 8.3.

Upon raising the pH from 6.5 to 10.2, the ν(Fe-CO) Raman line at 535 cm⁻¹ disappeared, while the 521 cm⁻¹ line was intensified. The ν(Fe-CO) Raman line at 586 cm⁻¹ disappeared concurrently, but the other Raman lines including the ν_s, line were not altered significantly (data not shown). It should be noted that these pH titration experiments became possible by the use of a CO complex of the deuteroheme-substituted enzyme, where the ν(Fe-CO) modes were considerably enhanced. The CO complex was also found to be more photoresistant against laser illumination than that of native horseradish peroxidase, as expected from the fact that the binding rate constant (k_on) of CO to the ferrous deuteroheme-substituted enzyme was 10 times greater than that with the ferrous native enzyme (32).

Fig. 5B shows pH-dependent changes in C-O stretch bands in the infrared spectra; as pH was raised from 6.5 to 9.5, the 1906 cm⁻¹ band diminished in its intensity, while the 1932 cm⁻¹ band gained its intensity. When pH-dependent changes in the infrared spectra were analyzed by computer-generated difference spectrum, which was recorded against the spectrum at pH 6.5, the decrease in the intensity of the 1906 cm⁻¹ band were proportional to the increase in the band intensity at 1932 cm⁻¹. Similar pH-dependent changes in infrared spectra for the CO complex of native enzyme are described under Fig. 7.

In Fig. 5C, the changes in intensity of ν(C-O) band at 1906 cm⁻¹ as well as the Raman intensity of ν(Fe-CO) at 535 cm⁻¹ are plotted as a function of pH. As seen, both plots coincided well with each other indicating that they were originated from the same species in the CO complex of horseradish peroxidase. Likewise, the species with ν(C-O) band at 1932 cm⁻¹ and ν(Fe-CO) Raman line at 521 cm⁻¹ was attributable to another single entity. Hereafter, on this basis, we call the former species present at an acidic pH the type I conformer, while the latter species, predominant in an alkaline pH, is the type II conformer. This classification can also be applied to the conformers in the CO complex of native horseradish peroxidase, although their ν(Fe-CO) modes were found at slightly different frequencies.

The pH-titration curve described above conforms to a single proton-coupled equilibrium with a pK_a value of 8.3.
This value agreed well with the $pK_a$ value of 8.25 reported for an acid-base transition in ferrous-CO complex of native horseradish peroxidase (33). These findings indicate that the transition between type I and type II conformers is caused by protonation of an amino acid residue at the distal side. As judged from the $pK_a$ value, the amino acid residue involved is likely to be a histidyl residue. The structures of both conformers deduced from these findings are discussed later.

**Effects of Benzhydroxamate Binding**—In Fig. 6A are shown resonance Raman spectra between 670 and 400 cm\(^{-1}\) of the CO complex of deuteroheme-substituted enzyme in the presence of benzhydroxamate, a known substrate for horseradish peroxidase (34). At pH 7.0, two isotope-sensitive Raman lines were found at 544 and 582 cm\(^{-1}\), which were assignable to $v(\text{Fe-CO})$ stretching and $\delta (\text{Fe-C-O})$ bending modes, respectively, as judged from their isotope-induced shifts by \(^{13}\text{C}\)\(^{18}\text{O}\). Fig. 6B shows resonance Raman spectra in the region between 700 and 450 cm\(^{-1}\) for the CO complex of native enzyme in the presence of benzhydroxamate. Among several lines, the Raman line at 547 cm\(^{-1}\) could be assignable to $v(\text{Fe-CO})$, in comparison with the result of the heme-substituted enzyme.

Fig. 7 compares infrared C–O stretch bands of the CO complex in native horseradish peroxidase in the presence and absence of benzhydroxamate. In the absence of benzhydroxamate (Fig. 7, left), the CO complex showed two stretch bands at a neutral pH, while essentially a single band was observed at pH 9.8. The results are in good agreement with those of Barlow et al. (35) and Smith et al. (36). In the presence of benzhydroxamate at a neutral pH, the CO complex showed a sharp single band at 1911 cm\(^{-1}\) (Fig. 7, right). The deuteroheme-substituted enzyme in the presence of benzhydroxamate at a neutral pH showed its C–O stretching frequency at 1910 cm\(^{-1}\) (Table II). The Fe–CO and C–O stretching frequencies at an alkaline pH were not measurable for the benzhydroxamate-bound form of the enzymes, due to a low affinity of both native and heme-substituted enzymes toward benzhydroxamate.

The above findings on the effects of substrate to the Fe–CO and C–O stretching frequencies indicated that the substrate binds to a proximity of heme-iron in the distal side and alters the Fe–C–O configuration through the distal steric effect. Similar changes in the configuration of Fe–C–O due to the steric hindrance by a bound substrate have been described for the CO complex of cytochrome P-450cam (camphor 5-monoxygenase) (3) and tryptophan 2,3-dioxygenases (2). Finally, it should be noted that the $v(\text{C–O})$ bands downshifted by 2–3 cm\(^{-1}\) irrespective of pH when the medium was changed from H\(_2\)O to D\(_2\)O, as demonstrated in the same figure. Significance of this finding is discussed later.

**Relationships among the Vibrational Frequencies and $k_{\text{off}}$ Value**—The results so far obtained by the resonance Raman and infrared spectroscopic methods are summarized and compared with those of other hemoproteins hitherto described in Table II. Upon examination of these parameters, we found several correlations among them (Figs. 8 and 9). First, plots of squares of $v(\text{C–O})$ values against squares of $v(\text{Fe–C–O})$ values gave two sets of inversely linear relationships which seemingly parallel with each other: one for the hemoproteins with a nitrogenuous base as the proximal ligand, and the other for those with cysteinyl thiolate anion as the ligand (Fig. 8A). Thus, they are called hereafter as “N” and “S” families of hemoproteins or heme compounds for convenience. As can be seen, all the data points fitted well to either one of the lines except for cytochrome aa\(_3\), indicating that the force constants for Fe–CO and C–O bondings are linear functions of each other within respective families. Then such correlations are utilized to infer the structures of these conformers under “Discussion.”

On the other hand, plots of squares of $\delta(\text{Fe–C–O})$ frequency against squares of $v(\text{Fe–C–O})$ frequency give rise to another linear correlation irrespective of the kinds of their proximal ligands (Fig. 8B). Furthermore, the logarithms of the dissociation rate constant of the CO-heme complex ($k_{\text{off}}$) was found to be a linear function of the $v(\text{Fe–C–O})$ stretch value irrespective of the kinds of proximal ligand (Fig. 9). Thus, the $k_{\text{off}}$ value can be considered as a direct function of the strength of the Fe–CO bond.

**Discussion**

The x-ray structural analysis on the CO complex of mesotetraphenylporphyrin with pyridine as the 5th ligand has
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TABLE II

| No. | Heme-CO                 | pH | \(\nu(Fe-CO)\) | \(\Delta(Fe-C-O)\) | \(\nu(C-O)\) | Reference |
|-----|-------------------------|----|----------------|---------------------|-------------|-----------|
| 1a  | Native HRP              | 7.0| 541            | 590                 | 1906        | This paper|
| 1b  | Native HRP              | 7.0| 519            | -                   | 1934        |           |
| 2   | Native HRP (+BHA)       | 10.0| 531            | -                   | 1933        |           |
| 3   | Native HRP (+BHA)       | 7.0| 547            | 590                 | 1911        |           |
| 4a  | Deutero-HRP             | 7.0| 535            | 586                 | 1906        |           |
| 4b  | Deutero-HRP             | 7.0| 521            | -                   | 1932        |           |
| 5   | Deutero-HRP (+BHA)      | 10.0| 521            | -                   | 1934        |           |
| 6   | Deutero-HRP (+BHA)      | 7.0| 544            | 582                 | 1910        |           |
| 7   | Mb                      | 7.0| 512            | 577                 | 1944        | 17        |
| 8   | Mb                      | 7.0| 507            | 577                 | 1951        | 17        |
| 9   | Mb                      | 7.0| 505            | 580                 | 1957        | 11, 37    |
| 10  | CTT III                 | 7.0| 500            | 574                 | 1960        | 13        |
| 11  | TPP (py)                | 484| 1976           | 13                  |            |
| 12  | TPP (N-Melm)            | 489| 1969           | 14, 16              |            |
| 13  | Heme 5                 | 495| 1964           | 15                  |            |
| 14  | SP-15                   | 509| 1945           | 15                  |            |
| 15  | SP-14                   | 512| 1939           | 15                  |            |
| 16  | SP-13                   | 514| 1932           | 15                  |            |
| 17  | Cytochrome aox          | 520| 1963.5         | 5, 6                |            |
| 18  | P-450cam (+camphor)     | 481| 1940           | 1, 3                |            |
| 19  | P-450cam (-camphor)     | 484| 1963           | 1, 3                |            |
| 20  | P-450cam (+norcamphor)  | 473| 1946           |                     |            |
| 21  | P-450cnc                | 477| 1963           | 4                   |            |

*Not measurable.

*Norcamphor-bound form of cytochrome P-450cam, unpublished result.

![Fig. 8. Correlations between squares of \(\nu(Fe-CO)\) and \(\nu(C-O)\) values and between squares of \(\nu(Fe-CO)\) and \(\Delta(Fe-C-O)\) values. All the data are from those in Table II and the numerals in the figure correspond to those in Table II. The \(\Delta(Fe-C-O)\) values plotted were from Ref. 38 for native horseradish peroxidase, Ref. 39 for Mb, Ref. 40 for Lb, and Ref. 32 for cytochrome P-450cam.](image)

![Fig. 9. A correlation between the \(\nu(Fe-CO)\) and the logarithm of the \(k_{eq}\) values of CO from the CO complex. The numerals in the figure correspond to those in Table II. The \(k_{eq}\) values shown that the CO molecule without steric hindrance binds perpendicularly to the heme plane (41). A similar Fe-C-O configuration has been postulated from the CO complex of other synthetic heme models such as heme 5 (15) and meso-tetra(a,a,a,a-,pivalamidophenyl) (14, 16). As shown in Table II, their \(\nu(Fe-CO)\) values were the lowest among those in the N family, while their \(\nu(C-O)\) values were the highest. Accordingly, the data points for them are located in the uppermost portion of the plot in Fig. 8A (11-13). When a steric hindrance had been offered on the ligated CO molecule of heme 5, however, the corresponding data points moved to the middle of the plot for the N family (14-16); they moved along the line toward a right-lower direction as steric hindrance was increased by the shortening of the chain length of the bridge covering the heme-iron. On the other hand, theoretical calculations on the structures of these CO complexes (15) have indicated that such a steric hindrance to the ligated CO caused a tilting of the Fe-CO linkage, rather than bending. Thus, the plots in Fig. 8A can be used to evaluate the degree of tilting
of Fe-CO linkage in the heme-CO complexes.

When the data on the CO complex of hemoproteins were included in the plot, two linear relationships were obtained, N and S families. This finding may indicate that the force constant between heme-iron and proximal ligand is different in two families. Among the N family, only the point for cytochrome a2 was dissatisfied with the relationship. The reason for this is unknown but is probably related to the effect of another heavy metal, copper, which sits nearby heme, as discussed previously (7). No other hemoprotein nor heme compound listed here has such an additional metallic center in the vicinity of heme-iron.

In the above plot, the data points for horseradish peroxidase were found in the lower portion of the N family indicating that the CO complex of horseradish peroxidase has a more tilted configuration than those of other hemoproteins and model-heme compounds. Furthermore, they could be classified into two subgroups corresponding to type I (1a, 3a, 4a, and 6) and II (1b, 2a, 4b, and 5) conformers, respectively, and the configuration of Fe-CO linkage was considered to be more tilted over the heme-plane in type I than that in type II. As to the cause for such a tilted configuration, we consider the steric hindrance offered by the protonated form of a distal amino acid residue or by the substrate bound to a site nearby heme as illustrated below.

\[
\begin{align*}
\text{(A)} & \quad \text{B-H} \quad \text{C} \\
\text{(B)} & \quad \text{PFe}^- \\
\text{(C)} & \quad \text{PFe}^- \\
\text{(D)} & \quad \text{PFe}^- \\
\text{Sub} & \quad \text{S}_{\text{sub}} \\
\end{align*}
\]

where P, B, and Sub denote porphyrin, the distal base, and the substrate (benzhydroxamate), respectively. At an alkaline pH, the distal base is in a deprotonated form and hence offers only a minute degree of steric hindrance to Fe-CO linkage as in A. Upon lowering the pH to 7.0, however, the distal base with the pKa value of 8.3 becomes fully protonated with an increasing steric hindrance to the Fe-CO linkage as in B. However, the protonated base can take another conformation such as in C which has little effect on the Fe-CO configuration. Thus, two \(\nu(C=O)\) bands at a neutral pH can be explained by assuming such two kinds of rotational isomers for the protonated base. Finally, the binding of benzhydroxamate also causes a tilting of the Fe-CO linkage over the heme-plane, as shown in D. The substrate has been known to bind near the heme without entering the first coordination sphere of the heme-iron (34).

Barlow et al. (35) and Smith et al. (36) previously reported that two distinct infrared C-O stretch bands were present for the CO complex of native horseradish peroxidase and that a band at 1906 cm\(^{-1}\) shifted to a lower wavenumber upon deuteration of the medium. From the results, they suggested that the ligated CO molecule was hydrogen-bonded to a distal amino acid residue, presumably to a histidine. In the present study, we also observed their shifts by 2-3 cm\(^{-1}\) upon deuteration of the medium as well as two infrared C-O stretch bands. As demonstrated, the shift of the peak in a type II conformer at 1933 cm\(^{-1}\) occurred even at a high pH such as 9.5, where the formation of a hydrogen bond is highly unlikely. Thus, the changes in the infrared spectrum could better be ascribed to a conformational change in protein caused by the D\(_2\)O-H\(_2\)O exchange. Such an interpretation may accord with the recent observation by Argade et al. (42) that \((\text{Fe-His})\) stretching frequency of deoxyhemoglobin down-shifted upon deuteration of the medium, which they interpreted as due to a structural change in protein induced by the deuteration of exchangeable protons in the amino acid residues.

Finally, we also noticed two other correlations among the vibrational frequencies for Fe-CO complex and the dissociation rate constant of CO from the complex in hemoproteins and heme compounds. A plot of squares of \(b(\text{Fe-CO})\) value against squares of \(\nu(\text{Fe-CO})\) value gave a linear relationship, in which the points for horseradish peroxidase (1a, 3a, 4a, and 6) were in the upper extreme corner, while those for cytochromes P-450 (18-20) occupied the opposite corner. Although the meanings of the findings are not fully understood at present, they might represent some characteristic features of the two kinds of heme-enzymes. In this connection, it is interesting to note that a recent x-ray structural study (43) indicated the absence of a distal amino acid residue in cytochrome P-450cam, while a distal base is definitely present and plays critical roles in the catalysis in peroxidases (44). On the other hand, an inverse linear correlation found between the log \(k_{\text{cat}}\) value and \(\nu(\text{Fe-CO})\) stretching frequency can be taken to prove an \(a\) priori relationship between the Fe-CO bond strength and the rate of dissociation of bound CO; the stronger the bond the slower the dissociation rate.

In conclusion, we demonstrated in this study a critical role of the distal amino acid residue with pKa value of 8.3 in determining the structure of a CO-heme adduct in horseradish peroxidase. The same residue forms a hydrogen bond with the oxo ligand in compound II, thereby regulating the reactivity of such an active reaction intermediate (20). The binding of an electron-donating substrate such as benzhydroxamate to nearby heme affected also the structure of the CO-heme complex, suggesting a direct electron transfer between a ligand-heme complex and a substrate. Then the described correlations among the vibrational and kinetic parameters for the heme-carbonyl complexes have provided new clues for the understanding of the structure-function relationships in hemoproteins and heme compounds.

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