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Resonance Raman evidence for oxygen exchange between the Fe^{IV}=O heme and bulk water during enzymic catalysis of horseradish peroxidase and its relation with the heme-linked ionization

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ABSTRACT **Raman spectroscopic studies of compound II of horseradish peroxidase show that the oxygen atom in the Fe^{IV}=O group of the heme is rapidly exchanged in H_2O at pH 7.0 but not in an alkaline solution (pH 11.0). This conclusion is based on studies of shift in the Fe^{IV}=O stretching mode of compound II in $H_2{}^{18}O$; further studies show that the Fe^{IV}=O heme is hydrogen-bonded to an amino acid residue of the protein in neutral solutions but not in the alkaline solution. Deprotonation of this residue takes place with the midpoint pH at 8.8 and accordingly corresponds to the so-called heme-linked ionization. It is concluded that this hydrogen-bonded proton plays an important part in the oxygen exchange mechanism. From this it seems clear that this hydrogen-bonded proton has an essential role in the acid/base catalysis of this enzyme and that alkaline deactivation of this enzyme can be attributed to the lack of a hydrogen-bonded proton at high pH.**

A peroxidase, a heme-containing enzyme, catalyzes oxidation of various organic molecules with H_2O_2 as the specific oxidant, but its physiological function is considered to protect biomembrane from attack by H_2O_2. Reaction mechanisms of a peroxidase have been explored mainly with horseradish peroxidase (HRP) (see ref. 1 for a review), whereas reactions of animal (2) and yeast peroxidases (3) are known to be similar to those of HRP. In the catalytic cycle of HRP, the native ferric enzyme is first oxidized by H_2O_2 into an intermediate called compound I, which is then reduced twice by substrates to the native state through the other intermediate, compound II. Therefore, compound II has an oxidation state for the Fe atom higher than the ferric state by one oxidative equivalent.

A heme structure of compound II has long been a matter of debate, but recently Mossbauer (4), NMR (5), electron nuclear double resonance (6), extended x-ray absorption fine structure (7), and resonance Raman (RR) (8, 9) studies have agreed that compound II contains the ferryl heme with an oxene ligand (Fe^{IV}=O) instead of a hydroxy ligand (Fe^{IV}—OH). The latter structure, however, was favorable to explain the pH and mass ($^1H/^2H$) dependencies of the rate constant (10–12), which required involvement of a deprotonating group with $pK_a = 8.6$ in acid/base catalysis by compound II (13–15). Although the importance of ionization of a particular amino acid residue has been repeatedly stressed as the heme-linked ionization for ferric and ferrous HRP on the basis of pH dependence of the redox potential (14, 15), the visible absorption (14), and the RR spectra (16, 17), its practical role in catalytic reactions still remains to be explained satisfactorily for compound II with the Fe^{IV}=O structure.

Previously we assigned the Raman line for the Fe^{IV}=O stretching mode of compound II on the basis of the ^{18}O isotopic frequency shift (8); the assignment was consistent with the results by Terner *et al.* (9). In this paper we report a very unexpected but important finding—namely, that the oxygen atom of the Fe^{IV}=O heme is rapidly exchanged with that of bulk water under the acid/base catalysis by the specific proton involved in the heme-linked ionization (18).§

EXPERIMENTAL PROCEDURES

HRP isozyme C (Toyobo, Osaka, Japan, grade I-C), with the absorbance ratio of $A_{408}/A_{280} = 3.2$, was dissolved in a phosphate or glycine buffer for the measurements at neutral or alkaline pH, respectively. The concentration in mmol/liter of the enzyme was determined spectrophotometrically at pH 6 by measuring absorbance at 403 nm, using $\epsilon_{mM} = 107.7$ (19). Compound II was prepared by mixing equal moles of H_2O_2 and the ferric enzyme (HRP) in the presence of the same number of moles of ferrocyanide. Its formation in the nearly pure form was confirmed with the visible absorption spectrum in a separate experiment.

An aqueous solution of $H_2{}^{18}O_2$ was prepared by oxidation of 2-ethyl anthraquinol (20) in a mixed solvent [xylene/acetophenone/octanol, 13.1:43.5:34.4 (weight %)] with an excess of $^{18}O_2$ (purity 99%, Prochem, Summit, NJ), followed by extraction with degassed H_2O under a nitrogen atmosphere. The 2-ethyl anthraquinol solution was obtained *in situ* from hydrogenation of 2-ethyl anthraquinone in the presence of 2% Pd on Al_2O_3. The formation of $H_2{}^{18}O_2$ was confirmed by observation of the Raman line due to the $H^{18}O$—^{18}OH stretching mode, and its concentration was determined by iodometry. $H_2{}^{18}O$ (98.4%) was purchased from Amersham.

Raman scattering was excited at 406.7 nm with a Kr^+ ion laser (Spectra Physics, Santa Clara, CA, model 165) and detected with a diode array detector (PAR 1420) attached to Spex 1404 double monochromator and with an OMA II console (PAR 1215). The frequency calibration was performed with indene (500–1650 cm^{-1}) and carbon tetrachloride (200–500 cm^{-1}) as standards (21). Since compound II was found to be photolabile at this excitation wavelength, all of the Raman spectra were measured within 4 min after the formation of compound II using a homemade spinning cell (1800 rpm, diameter = 2 cm) and minimal laser power (<10 mW).

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Abbreviations: HRP, horseradish peroxidase; RR, resonance Raman.

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RESULTS

Fig. 1 shows the RR spectra of native HRP and of compound II at pH 7 derived from samples prepared with four different combinations of $H_2{}^{18}O_2$ or $H_2{}^{16}O_2$ and $H_2{}^{18}O$ or $H_2{}^{16}O$. The Raman lines of native enzyme (spectrum a) at 679 (p) and 757 cm^{-1} (dp) (p and dp denote polarized and depolarized, respectively) are assigned to the ν_7 and ν_{16} modes of a porphyrin (22), respectively. They are insensitive to the formation of compound II. When compound II was formed with $H_2{}^{16}O_2$ in $H_2{}^{16}O$, a new line appeared at 774 cm^{-1} (spectrum b), and this line did not exhibit a frequency shift upon replacement of $H_2{}^{16}O_2$ with $H_2{}^{18}O_2$ (spectrum c), although a slight intensity increase around 743 cm^{-1} was noticed. In contrast, when $H_2{}^{16}O$ was replaced with $H_2{}^{18}O$ without using $H_2{}^{18}O_2$, the intensity of the 774-cm^{-1} line decreased significantly and instead a new line appeared at 740 cm^{-1} (spectrum d). When $H_2{}^{18}O_2$ and $H_2{}^{18}O$ were used to form compound II, the 774-cm^{-1} line disappeared completely while the 740-cm^{-1} line remained.

On the other hand, the corresponding new line of compound II in an alkaline solution was identified previously at 787 cm^{-1} (8) and this line exhibited frequency shifts of +3 and −34 cm^{-1} upon isotope replacements of ^{56}Fe with ^{54}Fe and of $H_2{}^{16}O_2$ with $H_2{}^{18}O_2$, respectively (8). Since the frequency shifts closely agreed with those expected for the Fe=O two-body harmonic oscillator (+3.3 cm^{-1} and −35 cm^{-1} for the ^{54}Fe and ^{18}O substitutions, respectively), the 787-cm^{-1} line was assigned to the Fe^{IV}=O stretching mode ($\nu_{Fe=O}$). Similarly, the new RR line in Fig. 1 characteristic of compound II at pH 7.0 (774 cm^{-1}) is believed to arise from the $\nu_{Fe=O}$ mode. The fact that this line exhibits an isotopic frequency shift to 740 cm^{-1} for compound II derived from $H_2{}^{16}O_2$ in the $H_2{}^{18}O$ solution suggests that the heme-bound oxygen exchanges with an oxygen atom from bulk water.

Fig. 2 shows the RR spectra of compound II in ^2H_2O. The $\nu_{Fe=O}$ mode in neutral solution was observed at 776 cm^{-1} (spectrum B). When $H_2{}^{18}O_2$ was used in ^2H_2O, the intensity of the 776-cm^{-1} line was reduced and a new line appeared at 743 cm^{-1}, as shown by spectrum C. Therefore, the 776-cm^{-1} line is assigned to the $\nu_{Fe=O}$ mode in ^2H_2O. Note that the 776- and 743-cm^{-1} lines are narrower than are the corresponding lines in the ^1H_2O solution despite the fact that the absorption spectra of the two solutions are otherwise exactly the same. The $\nu_{Fe=O}$ mode appeared reproducibly at slightly higher frequency in ^2H_2O than in ^1H_2O at pH 7.0, although there was no difference in alkaline solutions.

The $\nu_{Fe=O}$ line in ^1H_2O seems too broad to determine quantitatively the amount of deuteration shift of the $\nu_{Fe=O}$ mode. To overcome this difficulty, we calculated the differences with regard to the spectrum of the ^{18}O derivative: $^1H_2{}^{16}O$ vs. $^1H_2{}^{18}O$ (spectrum D) and $^2H_2{}^{16}O$ vs. $^1H_2{}^{18}O$ (spectrum E). The positive peak in the derivative-type difference spectra appeared at higher frequency with ^2H_2O than with ^1H_2O by 2 cm^{-1}. This observation is not consistent with an Fe—OH structure, which should give rise to a shift to lower frequency upon deuterium substitution. Conse-

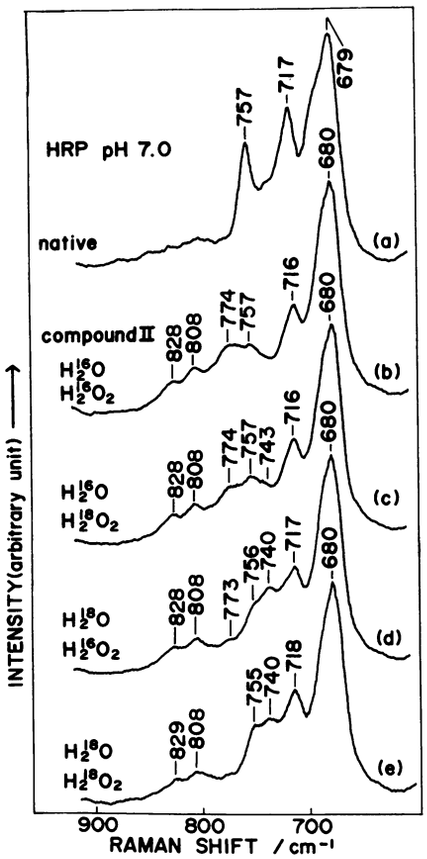

FIG. 1. RR spectra in the 600- to 900-cm^{-1} region of native HRP and compound II derived from four isotope combinations of $H_2{}^{18}O_2$ or $H_2{}^{16}O_2$ and $H_2{}^{18}O$ or $H_2{}^{16}O$. Excitation, 406.7 nm; laser power, <10 mW; accumulation time, 3 min; enzyme concentration, 100 μM. Spectrum a, native HRP. Compound II derived from $H_2{}^{16}O_2$ in $H_2{}^{16}O$ (spectrum b), from $H_2{}^{18}O_2$ in $H_2{}^{16}O$ (spectrum c), from $H_2{}^{16}O_2$ in $H_2{}^{18}O$ (spectrum d), and from $H_2{}^{18}O_2$ in $H_2{}^{18}O$ (spectrum e).

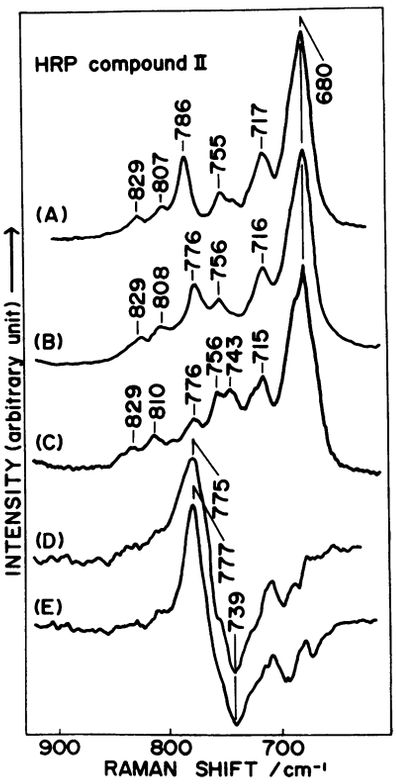

FIG. 2. RR spectra in the 600- to 900-cm^{-1} region of compound II in ^2H_2O solutions and their difference spectra with regard to the spectrum of the ^{18}O derivative. Instrumental conditions are the same as those in the legend of Fig. 1. Spectrum A, compound II in ^2H_2O at p^{2}H 11.2 derived with $^1H_2{}^{16}O_2$; spectrum B, compound II in ^2H_2O at p^{2}H 7.0 derived with $^1H_2{}^{16}O_2$; spectrum C, compound II in ^2H_2O at p^{2}H 7.0 derived with $^1H_2{}^{18}O_2$; spectrum D, difference spectrum of spectrum b (Fig. 1) − spectrum e (Fig. 1); spectrum E, difference spectrum of spectrum B (this figure) − spectrum e (Fig. 1).

quently, we conclude that there is no exchangeable proton bound to the heme-bound oxygen.

Fig. 2, spectrum A, displays the RR spectrum of compound II in an alkaline solution in ^2H_2O. The Fe^{IV}=O stretching mode is observed at 786 cm^{-1}, which is not shifted from the frequency observed in the ordinary H_2O solution but is appreciably shifted from the values observed in neutral solutions. Since the intensity of the Raman line at 680 cm^{-1} is not altered by the pH change, the intensity of the 786-cm^{-1} line relative to that of the 680-cm^{-1} line was plotted against pH (Fig. 3). This plot can be well fitted by a theoretical curve (solid line) calculated for ionization of a residue with pK_a = 8.8; this value is in close agreement with the reported pK_a value (= 8.6) for the heme-linked ionization of compound II (13–15). The lower frequency of the $\nu_{Fe=O}$ mode in neutral solutions compared with alkaline solutions may suggest the presence of a hydrogen bond between this protonated residue, probably distal histidine (1, 15), and the heme-bound oxygen or alternatively may suggest that the protonation has induced a tertiary structure change of the protein, distorting the Fe—histidine bond in the proximal side and thus introducing some *trans* effect on the Fe^{IV}=O bond. To determine which alternative is correct, the RR spectra in the low-frequency region were investigated.

Fig. 4 shows the RR spectra of compound II in H_2O at pH 7 (spectrum A) and at pH 11.2 (spectrum B). The upper and lower frequency regions were obtained in the same exposure, although they are presented separately to adjust the background level. The Raman lines at 774 (spectrum A) and 787 (spectrum B) cm^{-1} at pH 7.0 and 11.2, respectively, confirm the formation of compound II. The Raman spectra in the lower frequency region (350–200 cm^{-1}) are nearly identical in the two solutions at pH 7 and 11.2. Although the Raman line around 370 cm^{-1} seems to be slightly shifted in frequency between the two spectra, this Raman line is broad in alkaline solution, and therefore the shift is not definite. In the case of deoxy hemoglobin (Hb), distortion of the proximal histidine was sensitively reflected by changes in the Fe—histidine stretching mode around 220 cm^{-1} (23–25) so that this line is used as an indicator of the quaternary structure (see ref. 26 for a review). For various plant peroxidases in the reduced state, only the Raman line for the Fe—histidine stretching mode around 240 cm^{-1} exhibited pH-dependent frequency shifts (17, 27). The RR spectra of intestinal peroxidase (28) and lactoperoxidase (29), which are considered to have a protoheme, displayed a peculiar pattern in the low-frequency region below 450 cm^{-1}, whereas the spectrum in the high-frequency region (1200–1650 cm^{-1}) is similar to that of Hb. The peculiar spectrum is presumably due to unusual protein–heme interactions. Therefore, the identical low-frequency RR spectra of compound II obtained at different pH values suggest that the protein–heme interactions, including the Fe—histidine bond, are altered little by a pH change. In consequence, the observation of a lower Fe^{IV}=O stretching frequency in neutral solutions than in alkaline solutions is ascribed to hydrogen bonding between Fe^{IV}=O and the residue with pK_a = 8.8.

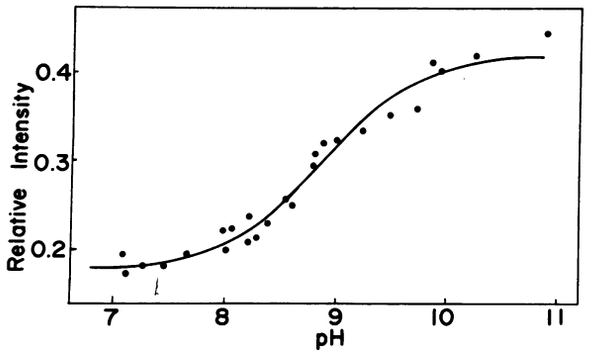

FIG. 3. pH-dependent intensity change of the Fe^{IV}=O stretching RR line of compound II in ^1H_2O. The maximum intensities at 787 cm^{-1} relative to those at 680 cm^{-1} are plotted against pH. The solid line is a theoretical curve expected for dissociation of one proton with pK_a = 8.8.

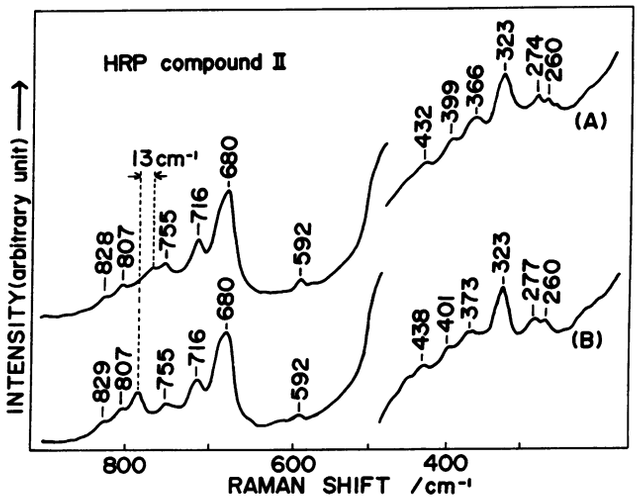

FIG. 4. RR spectra in the 200- to 900-cm^{-1} region of compound II in ^1H_2O in the lower frequency region. The upper and lower frequency regions were obtained in an identical exposure but are represented separately to adjust background levels. Spectrum A, pH 7.0; spectrum B, pH 11.2. Excitation, 406.7 nm; <10 mW; accumulation time, 2 min.

DISCUSSION

The hydrogen-bonded Fe^{IV}=O heme is distinguished from the hydroxy form. The Fe^{III}—OH stretching frequencies of myoglobin (Mb) and Hb, reported at 490 and 495 cm^{-1} (30), respectively, are distinctly lower than the Fe^{IV}=O stretching frequency, implying that the Fe^{III}—OH bond is much weaker than the Fe^{IV}=O bond; from the simple diatomic approximation, the stretching force constants are k_{Fe-OH} = 1.8 and $k_{Fe=O}$ = 4.5 millidynes/Å (1 dyne = 10 μN). The Fe^{II}—O_2 stretching force constant derived from the Fe^{II}—O_2 stretching frequency of oxyHb (568 cm^{-1}) (23) is 3.8 millidynes/Å and is appreciably smaller than the Fe^{IV}=O stretching force constant. Therefore, the Fe^{IV}=O bond is considered to have some double-bond character. However, the Fe^{IV}=O bond of HRP compound II seems weaker than the Fe^{IV}=O bond of a model iron-porphyrin, which has its $\nu_{Fe=O}$ mode at 852 cm^{-1} (31), corresponding to $k_{Fe=O}$ = 5.3 millidynes/Å. This change in bond strength may be partly due to the chemical difference in the fifth coordination position of the Fe atom in these different compounds; it is imidazolic nitrogen of histidine for HRP and vacant for the porphyrin complex.

The frequency shift of the $\nu_{Fe=O}$ mode of compound II (13 cm^{-1}) from neutral to alkaline solutions has been ascribed to the presence or absence of the hydrogen bond to the Fe^{IV}=O. Simultaneously, the same explanation was proposed independently by Sitter *et al.* (32). However, the size of the frequency shift observed for $\nu_{Fe=O}$ is much smaller than the corresponding frequency shift of the Fe—F stretching mode of HbF (61 cm^{-1}) and MbF (62 cm^{-1}) (33) from acid to neutral solutions, where the heme-bound F^- is hydrogen bonded to the distal histidine at low pH but not at pH 7.4. Accordingly, the hydrogen bonding to the Fe^{IV}=O heme in the present case might be very weak. This means that the vibrational motion of the oxygen atom in the Fe^{IV}=O

stretching mode is not necessarily accompanied by a simultaneous displacement of the hydrogen atom, consistent with the fact that the $\nu_{Fe=O}$ mode shows no shift toward lower frequency upon deuteration of the protein. A model of the active site of compound II deduced from this study is schematically illustrated in Fig. 5.

The heme-linked ionization (A ⇄ B) of the native enzyme occurs with pK_a = 5.5 (34). The deprotonated native form (Fig. 5, step A) reacts with H_2O_2, giving rise to protonated compound II (step D). The strength of the Fe^{IV}=O bond as well as the heme structure are nearly the same for step D at pH 7 and for step E at pH 11.2, except for the presence of the weak hydrogen bond in neutral solutions. Nevertheless, the heme-bound oxygen atom in D at pH 7 is rapidly exchanged with bulk water but that in E at pH 11 is not exchanged at all. Therefore, it seems that the hydrogen-bonded proton in D plays an essential role in the exchange phenomenon. In the absence of this proton, at alkaline pH, the enzyme is inactive. Presumably, the heme-bound oxygen and the hydrogen-bonded proton combine and exchange with bulk water through structure C. This exchange reaction continues until an electron is donated from a substrate. Such an oxygen exchange has never before been suggested for HRP, although it was noticed for cytochrome P-450 (35) and its model compounds (36, 37). It is stressed here that the exchange phenomenon is not an intrinsic property of the Fe^{IV}=O heme but is enabled by participation of a hydrogen-bonded proton in the acid/base catalysis.

The $\nu_{Fe=O}$ line was more intense in ^2H_2O than in ^1H_2O. This trend is more pronounced for neutral solutions than for alkaline solutions and is therefore considered to be relevant to the exchange reaction. If the heterogeneous surroundings of the Fe^{IV}=O bond during the exchange reaction cause broadening of the RR line and thus an apparent weak peak intensity for the 778-cm^{-1} line, this explanation should also be applied to the neutral ^2H_2O solution. One may suspect that the native form might have already formed during the measurements of the ^1H_2O solution because of a higher rate constant for reduction of compound II in ^1H_2O than in ^2H_2O. However, the RR spectra of the ^1H_2O and ^2H_2O solutions of compound II in the higher frequency region (1300–1650 cm^{-1}) (not shown) indicate that contamination by the native form is negligible under the present experimental conditions.

As one of plausible interpretation, we assume that there is a water molecule near the Fe^{IV}=O bond, as pointed out for crystalline cytochrome *c* peroxidase from x-ray crystallographic analysis (38). A libration mode is reported at 710 cm^{-1} for liquid water; it shifts to higher frequency as temperature is lowered (39). If the frequency of the corresponding vibration of the water molecule adjacent to the Fe^{IV}=O bond in HRP is located near 750 cm^{-1}, then it is possible that vibrational energy transfer takes place from the $\nu_{Fe=O}$ mode to this vibration of the water, thus broadening the $\nu_{Fe=O}$ line. Since this libration is shifted to lower frequency with ^2H_2O (530 cm^{-1}) (39), the energy transfer would no longer be important in ^2H_2O and thus no broadening would occur. A similar consideration has been proposed previously for broadening observed for the C=NH stretching vibration (1640 cm^{-1}) of the protonated Schiff base of bacteriorhodopsin (40), although in this case its counterpart is a bending vibration of water.

Another possibility for explaining the broadening is that the life time of species (D) in Fig. 5 is longer in ^2H_2O than in ^1H_2O. When we reexamine the RR spectra of compound II derived from $^1H_2{}^{18}O_2$ in $^1H_2{}^{16}O$ (Fig. 1, spectrum c) and in $^2H_2{}^{16}O$ (Fig. 2, spectrum C), the intensity increase of the Fe^{IV}=^{18}O stretching mode in ^2H_2O is noteworthy. Since the intensities of the $\nu_{Fe=O}$ modes in ^1H_2O and ^2H_2O are alike in alkaline

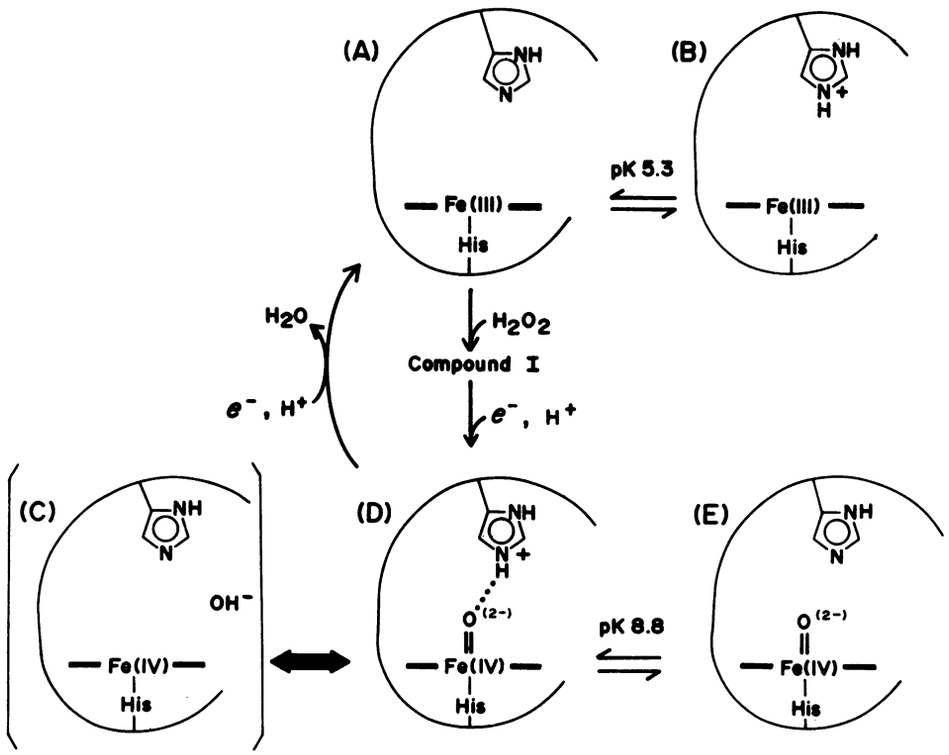

FIG. 5. Schematic diagram for molecular mechanism of HRP catalysis. A ⇄ B represents the equilibrium for the heme-linked ionization in the native ferric form, and D ⇄ E represents that for compound II. C ↔ D represents a hypothetical mechanism for the oxygen exchange between the Fe^{IV}=O heme and bulk water. In this figure, distal histidine is considered to be responsible for the heme-linked ionization. In the acidic form of compound II (D), the proton of distal histidine is hydrogen bonded to the heme-bound oxygen (O^{2-}) and they combine to yield a hydroxy anion upon the oxygen exchange reaction (C).

solutions (8), the intensity difference between the ^1H_2O and ^2H_2O solutions might be related to the exchange reaction. Furthermore, the $\nu_{Fe=O}$ frequency is slightly lower in ^1H_2O than in ^2H_2O in neutral solutions, whereas there is no difference in alkaline solutions, suggesting that the hydrogen bond may be slightly stronger in ^1H_2O than in ^2H_2O. The stronger hydrogen bond may result in more effective catalysis and thus a higher rate constant for the exchange reaction. Since the exchange reaction always takes place in the actual catalytic reaction, its rate should be directly related to the rate of enzymic reaction. In fact, the rate constant of the enzymic reaction is smaller in ^2H_2O than in ^1H_2O (10). Thus, the structural implication of the $^1H/^2H$ and pH dependencies of the rate constant can be reinterpreted consistently with the Fe^{IV}=O structure.

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