Articles

Formation of a Porphyrin π-Cation Radical in the Fluoride Complex of Horseradish Peroxidase†

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ABSTRACT: Horseradish peroxidase (HRP) was oxidized by $IrCl_6^{2-}$ to a mixture of compounds I and II, the rate of oxidation and the ratio of the mixture being greatly affected by pH (Hayashi & Yamazaki, 1979). Oxidation of HRP by $IrCl_6^{2-}$ in the presence of fluoride was significantly accelerated. This resulted in the formation of a new compound which is a ferric fluoride complex containing a porphyrin π-cation radical. The spectrum of the new compound showed a decreased absorption band in the Soret region and a broad band at 570 nm; which was converted to that of the original ferric fluoride complex by addition of ascorbate or hydroquinone. Addition of cyanide slowed down the oxidation of HRP by $IrCl_6^{2-}$, and the oxidation product was the same as that obtained in the absence of cyanide. Compound I was formed when H_2O_2 was added to HRP in the presence of fluoride or cyanide. The one-electron reduction potential (E_o') of the oxidized HRP−fluoride complex was measured at several pH values, the E_o' value at pH 7 being 861 ± 4 mV. The ratio of $\Delta E_o'$ to ΔpH was 49 mV/pH unit.

The formation of a porphyrin π-cation radical was first demonstrated in organic solvents by using chemical oxidants (Fuhrhop & Mauzerall, 1969) and by electrochemical oxidation (Fajer et al., 1970). Comparing these spectra with those of compounds I of horseradish peroxidase (HRP)[1] and catalase, Dorphin et al. (1971) proposed that these compounds I contain a porphyrin π-cation radical. The reduction potentials of porphyrin π-cation radical compounds and their pH dependence have been measured in HRP (Hayashi & Yamazaki, 1979), Zn-substituted HRP (Kaneko et al., 1980), Mg-substituted HRP (Kuwahara et el., 1982), and *Arthromyces ramosus* peroxidase (ARP) (Farhangrazi et al., 1994). Fuhrhop et al. (1973) have shown a linear relationship between the reduction potential of the porphyrin π-cation radical and the electrostatic action of the metal center in metallooctaethyl porphyrins. It has also been suggested from theoretical calculations (Fujita et al., 1983) and a nuclear Overhauser study (Thanabal et al., 1988) that the porphyrin π-cation radical spin is delocalized on the axial ligand. Forman et al. (1971) observed a significant change in the EPR and optical absorption spectra of porphyrin π-cation radicals when the ligand was changed from Br^- to ClO_4^- or BF^{4-} and suggested that the shift in the *g*-value is consistent with increasing spin-orbit coupling with the heavier halide ion. Porphyrin π-cation radicals are also formed in ruthenium(II) porphyrin complexes with CO and bromide (Barley et al., 1984; Morishima et al., 1986). The delocalization of the porphyrin π-cation radical spin onto a soft base does not seem to destabilize the π-cation radical. The pH dependence of the reduction potentials of HRP and Mg-substituted HRP is closely related to the proton dissociation at the distal base. In aqueous solutions of an Fe-tetraphenylporphyrin, however, the midpoint potential for the porphyrin radical formation is pH-independent (Kaaret et al., 1991).

We report here an investigation of the effect of ligands at the sixth distal position on the formation of a porphyrin π-cation radical in HRP and the pH dependence of the one-electron reduction potential (E_o') of the oxidized HRP-fluoride complex (HRP^+−F) containing a porphyrin π-cation radical.

MATERIALS AND METHODS

HRP was obtained from Toyobo (Osaka, Japan) and was used without further purification. The absorbance ratio at 403 nm to 280 nm was 3.0. *Arthromyces ramosus* peroxidase (ARP) was donated from Suntory Laboratories (Osaka, Japan). Potassium hexachloroiridate (K_2IrCl_6) was obtained from Aldrich and was added to HRP solutions from a 10 mM stock solution which also contained 0.01 N HCl. Sodium fluoride, potassium cyanide, and all other reagents were of analytical grade. The final fluoride concentration of 0.5 M was obtained by addition of 0.125 mL of a 4 M NaF stock solution. The final volume used for spectrophotometric measurements was 1 mL. Absorption spectra were scanned with a Shimadzu UV-2101 PC spectrophotometer at a speed of 15 nm/s.

Reactions were carried out at room temperature (20 ± 1 °C) in 50 mM sodium succinate for pH 5, potassium phosphate for pH 6.0−8.7, and sodium carbonate for pH 9.

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[1] Abbreviations: ARP, *Arthromyces ramosus* peroxidase; E_o', one-electron reduction potential; HRP, horseradish peroxidase; HRP−F, HRP−fluoride complex; HRP^+−F, oxidized HRP−F.

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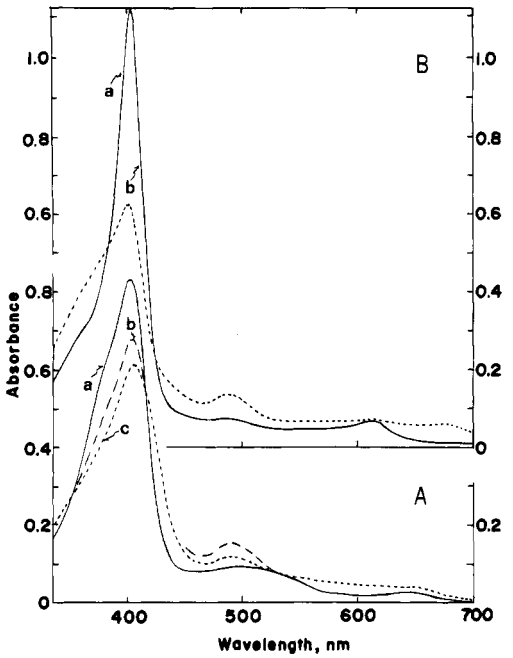

FIGURE 1: Absorbance changes observed when $IrCl_6^{2-}$ was added to HRP at pH 7 in the presence and absence of fluoride. (A) 7.8 μM HRP (a). Spectra were scanned 1 min (b) and 4 min (c) after addition of 25 μM $IrCl_6^{2-}$ to (a). (B) 7.8 μM HRP in the presence of 0.5 M fluoride (a), and the spectrum (b) was scanned immediately after addition of 25 μM $IrCl_6^{2-}$ to (a).

The E_o' value for HRP^+−F was measured from redox equilibrium with the iridate couple. A value of 900 mV was used for E_o' of $IrCl_6^{2-}$. The amount of HRP^+−F was measured from the absorbance increase at 680 nm. The residual $IrCl_6^{2-}$ concentration was measured at 488 nm, where $IrCl_6^{2-}$ exhibited maximum absorbance and the absorbance of the HRP−fluoride complex (HRP−F) did not change significantly upon oxidation. The ϵ_{mM} value used for calculation of the $IrCl_6^{2-}$ concentration was 3.88 at 488 nm.

RESULTS

HRP is oxidized by $IrCl_6^{2-}$ to compound II and further to compound I (Hayashi & Yamazaki, 1979). At pH 7, oxidation was slow, and redox equilibrium was attained in a few minutes after $IrCl_6^{2-}$ was added to HRP (Figure 1A). The equilibrated mixture consisted of three redox states of HRP (ferric, compound II, and compound I) and two states of iridate ($IrCl_6^{2-}$ and $IrCl_6^{3-}$). When fluoride was added to this system, the reaction of HRP with $IrCl_6^{2-}$ changed drastically (Figure 1B). The spectral change became much faster, and the resultant spectrum was different from that obtained in the absence of fluoride. In both cases, the intensity of the Soret band decreased, but in the presence of fluoride no red shift of the Soret band was observed and a significant broad absorption band appeared at 670 nm. Upon addition of 100 μM ascorbate or hydroquinone to the final products, spectra of the ferric enzyme and the fluoride complex were completely restored in the absence (Figure 1A) and presence (Figure 1B) of fluoride, respectively. Comparing the results of Figure 1A and Figure 1B, we concluded that $IrCl_6^{2-}$ reacts directly with the HRP−fluoride complex to yield a new oxidation product of HRP.

The new product was tentatively quantified from the intensity of the absorbance at 680 nm, because neither the ferric fluoride complex nor $IrCl_6^{2-}$ absorbs in this region. Figure 2 shows that the formation of the new compound was affected by pH in two ways. At high pH, the amount of new compound formation increased, and at the same time nonspecific loss of $IrCl_6^{2-}$ took place. The amount of nonspecific loss of $IrCl_6^{2-}$ was roughly equivalent to the molar concentration of the enzyme at pH 8. The nonspecific loss of $IrCl_6^{2-}$ is due to the oxidation of an amino acid (probably Tyr) residue, and the rate increases with increasing pH. At pH 8, the addition of equimolar $IrCl_6^{2-}$ did not change the spectrum of the HRP−fluoride complex. Since the new compound was not stable under these conditions, each point in Figure 2 was obtained from a separate experiment.

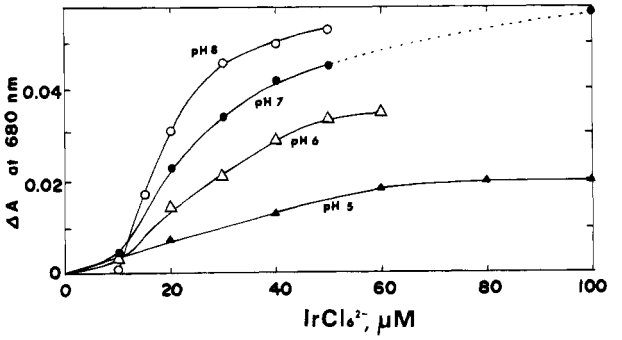

FIGURE 2: Effect of the $IrCl_6^{2-}$ concentration on the increase of absorbance at 680 nm. The absorbance reached a maximum immediately after addition of $IrCl_6^{2-}$ to 9.2 μM HRP and then slowly decreased. The maximum value was plotted against the $IrCl_6^{2-}$ concentration. Each point was obtained from separate experiments.

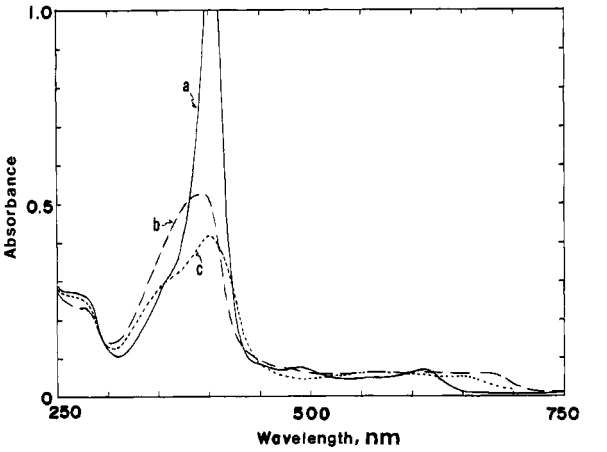

FIGURE 3: Spectra of HRP compound I and HRP^+−F at pH 7. (a) 8.0 μM HRP in the presence of 0.5 M fluoride. (b) Obtained by subtracting the spectrum of the residual $IrCl_6^{2-}$ (74 μM) from that scanned immediately after addition of 92 μM $IrCl_6^{2-}$ to (a). See the text. (c) The spectrum of compound I was obtained immediately after addition of 15 μM H_2O_2 to (a).

Upon addition of a sufficient excess of $IrCl_6^{2-}$ to HRP in the presence of fluoride at pH 7, the resultant spectrum indicated a mixture of the spectra of the new compound and the residual $IrCl_6^{2-}$. The residual $IrCl_6^{2-}$ concentration was approximately estimated from the absorbance increase at 488 nm. Approximately 2 equiv of $IrCl_6^{2-}$ per equivalent of enzyme was consumed, 1 equiv being used to oxidize the enzyme to the new compound and the other being lost for nonspecific oxidation of the enzyme. Figure 3 shows an approximate calculated spectrum of the new compound. When H_2O_2 was added to HRP in the presence of fluoride,

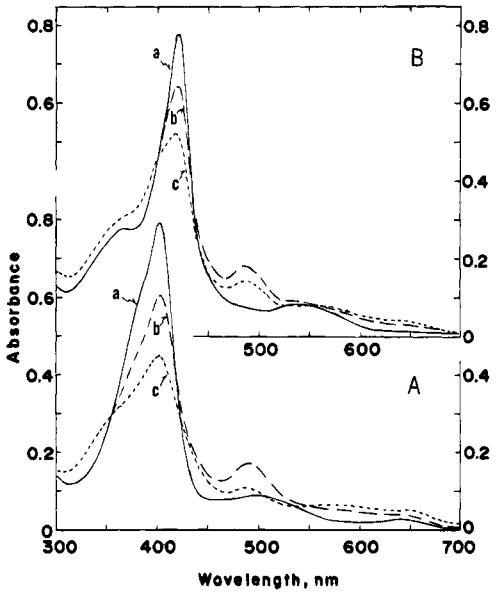

FIGURE 4: Effect of cyanide on the oxidation of HRP by $IrCl_6^{2-}$. HRP (7.5 μM), $IrCl_6^{2-}$ (50 μM), pH 8.0. (A) HRP (a). The spectra were scanned immediately (b) and 2 min (c) after addition of $IrCl_6^{2-}$ to (a). (B) (a) HRP in the presence of 20 μM cyanide. The spectra were scanned immediately (b) and 4 min (c) after addition of $IrCl_6^{2-}$ to (a).

the resultant spectrum was the same as that of compound I obtained in the absence of fluoride. This spectrum is also shown in Figure 3. Since the formation of a porphyrin π-cation radical is known to result in a decrease in the Soret band intensity and the formation of a broad absorption band at 670 nm, we concluded that the new compound is an HRP–fluoride complex containing a porphyrin π-cation radical. We also investigated this reaction when fluoride is replaced by cyanide. When $IrCl_6^{2-}$ was added to HRP in the presence of cyanide at pH 7, the spectral change was slow and small as compared to that shown in Figure 1A. The result suggested that cyanide inhibited the reaction of HRP with $IrCl_6^{2-}$. To clarify the effect of cyanide, we carried out the reaction at pH 8. HRP was oxidized by $IrCl_6^{2-}$, mostly to compound I, faster and more effectively at pH 8 than at pH 7 (Figure 4A). When cyanide was added to this system, the reaction clearly slowed down (Figure 4B). The isosbestic point at 395 nm apparently reflects conversion of the cyanide complex to compound I. The result shown in Figure 4B can be explained by assuming that $IrCl_6^{2-}$ reacts only with free HRP to produce compound I, but does not react with the cyanide complex. The oxidation of cyanide by $IrCl_6^{2-}$ was negligible under these conditions. When H_2O_2 was added to HRP in the presence of cyanide, the resultant spectrum was the same as that of compound I shown in Figure 3.

The E_o' value for HRP^+–F was measured from equilibrium with the iridate redox couple according to the equation:

$$E_o'(HRP^+–F) - E_o'(IrCl_6^{2-}) = 59 \log\{[IrCl_6^{3-}][HRP^+–F]/[IrCl_6^{2-}][HRP–F]\} \quad (1)$$

Problems in measuring the above redox equilibrium constant are (1) $IrCl_6^{2-}$ oxidizes not only the porphyrin but also protein amino acid residues (Figure 2) and (2) HRP–F is

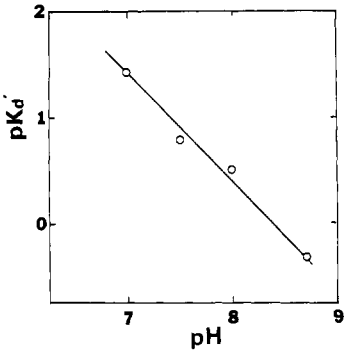

FIGURE 5: pH dependence of the dissociation constant (K_d) of HRP–F. K_d' was approximately measured from the amount of HRP–F in the presence of 0.5 M fluoride.

unstable at higher pH. Makino and Yamazaki (1973) reported the pH dependence of K_d' for HRP–F in the pH region below 7. K_d' is formulated as

$$K_d' = [HRP][HF + F^-]/[HRP–F] = K_d(K_a/H^+ + 1) \quad (2)$$

where $K_d = [HRP][HF]/[HRP–F]$, $[HF + F^-]$ is the total free fluoride concentration, and K_a is the proton dissociation constant for HF, the pK_a value being 3.14 (Nicholls & Schonbaum, 1963). The pK_d is then calculated to be 4.89 from the data of Makino and Yamazaki (1973). In the presence of 0.5 M fluoride, all HRP is regarded as HRP–F at pH below 6. At pH values above 7, a significant amount of HRP exists as the aquo ferric form (free) even in the presence of 0.5 M fluoride. The amount of HRP–F was measured from the absorbance increase in the Soret band in the presence of 0.5 M fluoride, and the K_d' values calculated from eq 2 are plotted against pH in Figure 5. Although the estimated pK_d' values are approximate, it might be concluded that the slope of pK_d' vs pH is about 1.0 at pH values up to 8.7. When $IrCl_6^{2-}$ was added to HRP in the presence of 0.5 M fluoride at high pH, total [HRP] = $[HRP^+–F]$ + [HRP–F] + [free HRP]. We measured $[HRP^+–F]$ from the absorbance increase at 680 nm and the [HRP–F]/[HRP] ratio from the data shown in Figure 5. The residual $IrCl_6^{2-}$ concentration was measured as described under Materials and Methods. According to eq 1, we measured the E_o'-$(HRP^+–F)$ values at different pH values, each from several measurements in the middle region of the Nernst plots. The experimental error was minimum at pH 6 and increased with increasing difference between the E_o' values of the measured and the standard iridate couples. The values for $E_o'(HRP^+–F)$ in millivolts were 950.3 ± 4.3 at pH 5, 902.7 ± 2.4 at pH 6, 861.0 ± 3.5 at pH 7, and 807.6 ± 7.8 at pH 8. These are plotted in Figure 6. The ratio of $\Delta E_o'(HRP^+–F)$ to ΔpH was 48.7 mV/pH unit.

ARP reacted with $IrCl_6^{2-}$ similarly as HRP did both in the presence and in the absence of fluoride. The spectra of the oxidized fluoride complexes of ARP and HRP were similar except that the 680 nm band for HRP was replaced by the 656 nm band in the case of ARP (Figure 7). The reduction potential of the oxidized ARP–fluoride complex at pH 7 was measured to be about 871 mV on the basis of the assumption that ARP exists as the fluoride complex in the presence of 0.5 M fluoride. If only half the ARP present is converted to the fluoride complex, the reduction potential will be 18 mV less than 871 mV.

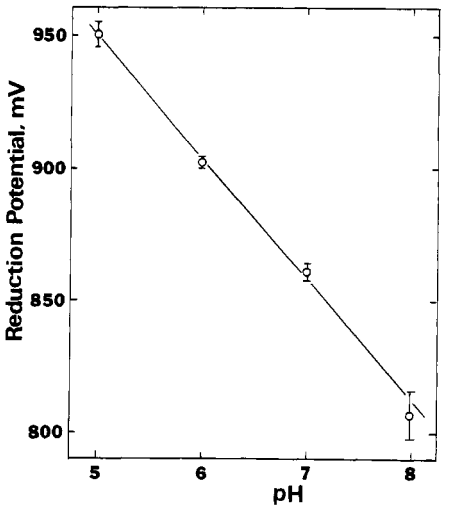

FIGURE 6: pH dependence of E_o' for HRP^+−F.

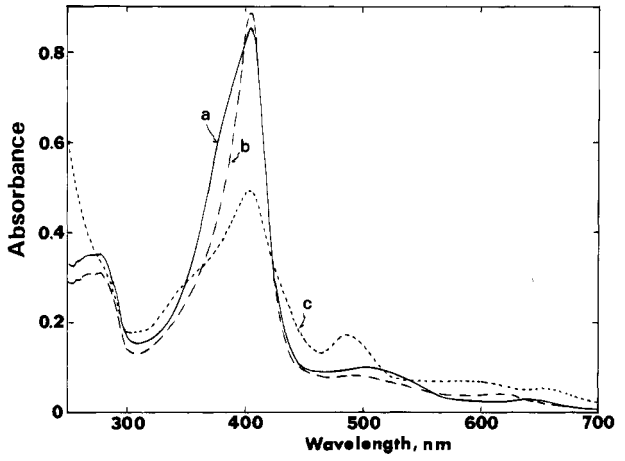

FIGURE 7: Oxidation of the ARP−fluoride complex by $IrCl_6^{2-}$. (a) 0.75 μM ARP. (b) 0.67 μM ARP in the presence of 0.5 M fluoride. (c) The spectrum scanned immediately after addition of 50 μM $IrCl_6^{2-}$ to (b).

DISCUSSION

The reaction of HRP with $IrCl_6^{2-}$ was affected quite differently by fluoride and cyanide. Fluoride accelerates the reaction to yield a new product. The product is concluded to be an oxidized HRP because it can be completely reduced back to the HRP−fluoride complex by hydroquinone or ascorbate. Acceleration of the oxidation reaction by fluoride suggests that $IrCl_6^{2-}$ reacts directly with the fluoride complex. The optical absorption spectrum of HRP^+−F is similar to that of compound I, but distinct differences are observed in the Soret band and in the absorption intensity around 680 nm. The porphyrin π-cation radicals of Zn- and Mg-substituted HRPs exhibit distinct absorption bands around 686 nm (Kaneko et al., 1980; Kuwahara et al., 1982). The possibility that Fe(III) of the fluoride complex is oxidized to Fe(IV) by $IrCl_6^{2-}$ in addition to the porphyrin is excluded. Upon oxidation of the iron, we would expect the fluoride to be replaced with a hydroxyl ion which will result in the formation of stable oxoferryl with a porphyrin π-cation radical (compound I). This result is inconsistent with the spectral properties of the new compound. Therefore, we suggest that the new product is a ferric HRP−fluoride complex which contains a porphyrin π-cation radical.

On the other hand, addition of cyanide slows the reaction of HRP with $IrCl_6^{2-}$. When the reaction is carried out at pH 8 (Figure 3), the product is mostly compound I, both in the presence and in the absence of cyanide. Therefore, it is concluded that $IrCl_6^{2-}$ reacts only with free HRP to form compound I via compound II. Details of the reaction of free HRP with $IrCl_6^{2-}$ have been described by Hayashi and Yamazaki (1979).

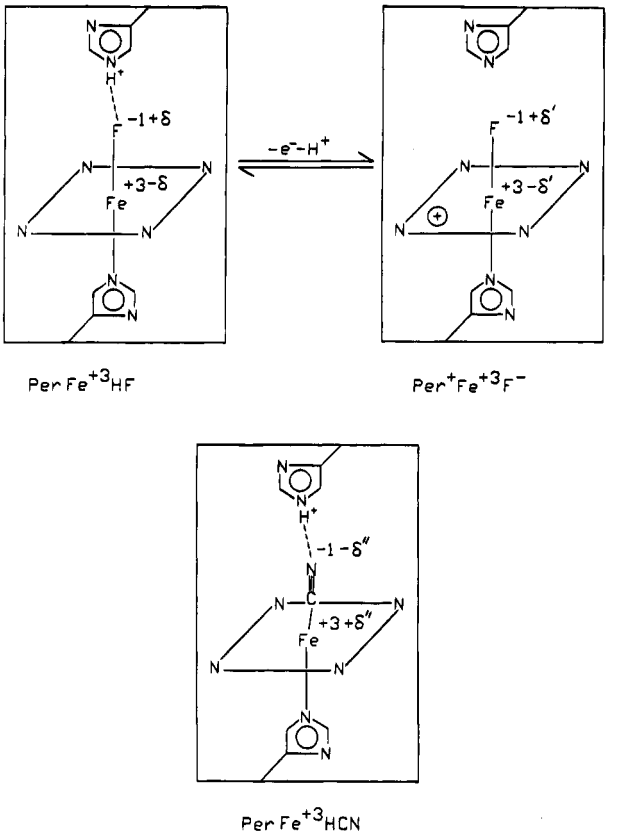

FIGURE 8: Modes of ligand−HRP interaction in HRP−F ($PerFe^{+3}$-HF), HRP^+−F ($Per^+Fe^{+3}F^-$), and HRP−cyanide complex ($PerFe^{+3}$-HCN).

Why does $IrCl_6^{2-}$ oxidize the HRP−fluoride complex but not the cyanide complex? From the pH dependence of the rate of ligand binding with catalase and peroxidase, Chance (1966) suggested that reactive species of ligands for the complex formation are the acid forms (HL) over a wide range of pH:

$$Fe^{3+} + HL \rightarrow Fe^{3+}\cdot HL \qquad (3)$$

The pH dependence of pK_d' for the HRP−ligand complexes was examined at pH values below 7 by Makino and Yamazaki (1973), and that at higher pH values is shown in Figure 5. The pK_a values for HF and HCN are 3.14 and 9.4, respectively. These ligand complexes of HRP and catalase, however, both should be formulated to be $Fe^{3+}HL$ or $Fe^{3+}(L^- + H^+)$. In the latter structure, the proton may bind to a distal base. Fluoride is a hard base and electron-donating, forming typical high-spin forms of HRP and catalase. In contrast, cyanide is a weak base and electron-withdrawing, forming low-spin hemoproteins (Brill & Williams, 1961).

Therefore, the difference between HRP complexes of fluoride and cyanide can be explained in terms of their proton dissociation ability and electronic structure shown in Figure 8. Although it is generally believed that the distal His operates as an H-bond acceptor upon ligand binding,

Edwards et al. (1984) and Edwards and Poulos (1990) reported that when fluoride binds with cytochrome *c* peroxidase, Arg-48 moved in about 2.5 Å to maximize interaction with the fluoride. In Figure 8, however, the histidine represents this general contribution. Although the distal base of HRP−F is protonated at pH up to 8.7, it is reasonable to assume that the proton is easily dissociable as compared with that present in the HRP−cyanide complex. To avoid charge repulsion, HRP−F will be deprotonated when a porphyrin π-cation radical is formed:

$$\text{HRP}-\text{F} - \text{e}^- = \text{HRP}^+-\text{F} + \text{H}^+ \qquad (4)$$

The pH dependence of E_o'(HRP$^+$−F) shown in Figure 6 is consistent with this mechanism. The electron-donating capacity of fluoride also facilitates the formation of a cation species at the iron−porphyrin center. On the contrary, a strong affinity for the proton as in the HRP−cyanide complex together with the electron-withdrawing capacity of cyanide prevents the formation of a cation species at the iron−porphyrin center.

Finally, oxidations of HRP−F and HRP compound II by K_2IrCl_6 are compared. In both reactions, the porphyrin is oxidized to a π-cation radical, and the proton at the distal base is dissociated. The pK_a value for HRP compound II is 8.6 (Hayashi & Yamazaki, 1979). The iron−ligand bond exhibits the net charge of 0 in the fluoride complex ($Fe^{3+}F^-$) and compound II ($Fe^{4+}O^{2-}$) since two of the four pyrrole nitrogens are negatively charged. This seems to be prerequisite to form a π-cation radical in the porphyrin. This is also true for Zn- and Mg-substituted HRP, which are easily oxidized to their porphyrin π-cation radical forms. The reduction potential at pH 7 is 861 mV for the HRP$^+$−F/HRP−F couple, 880 mV for the compound I/compound II couple, and 560 mV for the Mg-HRP$^+$/Mg-HRP couple. The significant low reduction potential of Mg-HRP$^+$ is ascribable to the electrostatic nature of the metal center.

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