The Structure−Function Relationship and Reduction Potentials of High Oxidation States of Myoglobin and Peroxidase†

Bing He,‡ Robert Sinclair,‡ Bruce R. Copeland,‡ Ryu Makino,§ L. S. Powers,*,‡ and Isao Yamazaki‡

National Center for the Design of Molecular Function, Utah State University, Logan, Utah 84322-4630, and Faculty of Science, Himeji Institute of Technology, Hyogo 678-12, Japan

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ABSTRACT: In these studies, we substitute electron-withdrawing (diacetyl) or -donating (diethyl) groups at the 2- and 4-positions of the heme in sperm whale Mb and HRP, and examine the structural and biochemical consequences. X-ray absorption spectroscopy shows that increased electron density at the heme results in an increased iron−pyrrole nitrogen average distance in both HRP and Mb, while decreased electron density results in shorter average distances. In HRP, the proximal ligand is constrained by a H-bonding network, and axial effects are manifested entirely at the distal site. Conversely, in Mb, where the proximal ligand is less constrained, axial effects are seen at the proximal side. In HRP, electron density at the heme iron depends linearly on pK_3, a measure of the basicity of the porphyrin pyrrole nitrogens [Yamada, H., Makino, R., & Yamazaki, I. (1975) *Arch. Biochem. Biophys. 169,* 344−353]. Using diethyl substitution (pK_3 = 5.8) and diacetyl substitution (pK_3 = 3.3) in HRP and Mb, we measured the one-electron reduction potentials (E_o') of HRP compounds I and II and ferryl Mb. Compound I showed a decreased E_o' with increasing electron density at the heme (pK_3), similar to E_o' of ferric HRP. E_o' of HRP compound II and ferryl Mb showed an opposite dependence. This behavior of E_o', while initially surprising, can be explained by the apparent net positive charge on the iron porphyrin in each oxidation state of the hemoproteins.

The effect of heme substitution on the function of hemoproteins has been studied in horseradish peroxidase (HRP)[1] (Gjessing & Summer, 1943; Paul, 1959; Tamura et al., 1972), cytochrome *c* peroxidase (CcP) (Yonetani & Asakura, 1968; Asakura & Yonetani, 1969), myoglobin (Mb) (Rossi-Fanelli & Antonini, 1957; Brunori et al., 1969), and hemoglobin (O'Hagan, 1960; Antonini & Gibson, 1960; Antonini et al., 1964; Sugita & Yonetani, 1971). The electron density at the heme iron is closely related to the basicity of the porphyrin pyrrole nitrogens and affects the active site heme structure. The dissociation constant for the third protonation (K_3) of the four pyrrole nitrogen atoms of the porphyrin (P), has been measured:

$$PH_2 + H^+ \xrightarrow{K_3} PH_3^+ \qquad (1)$$

This pK_3 value can be varied over a wide range by substituting various electron-withdrawing (e.g., diacetyl) or -donating (e.g., diethyl) groups at the 2,4-positions of the porphyrin. The effect of pK_3 on the spectral and ligand binding properties of metal−porphyrin complexes has been studied (Falk et al., 1966; Alben et al., 1968; McLees & Caughey, 1968). Phillips (1963) described a linear relationship between pK_3 and the reduction potentials of porphyrin−metal complexes. Brunori et al. (1971) reported a preliminary study on the reduction potentials of 2,4-substituted myoglobins. Sono et al. (1976) showed a linear relationship between pK_3 and the affinity of several 2,4-substituted myoglobins for O_2 and CO. Systematic studies with 2,4-substituted hemes have shown close correlation between pK_3 and various properties of HRP and Mb, such as protonation of the alkaline forms (Makino & Yamazaki, 1972, 1973), ligand binding (Makino & Yamazaki, 1973, 1974), and the stability of compounds I and III (Makino et al., 1976). Additionally, the one-electron reduction potential (E_o') of ferric HRP isozymes A and C depended linearly on pK_3 (Yamada et al., 1975):

$$\Delta E_o'(\text{mV}) = -59\Delta\text{p}K_3 \qquad (2)^2$$

Although E_o' of the ferric states has been measured for most hemoproteins, the E_o' values of compound I and compound II (or ferryl) have been reported only for HRP isoenzymes A and C (Hayashi & Yamazaki, 1979; Farhangrazi et al., 1995), for *Arthromyces ramosus* peroxidase (Farhangrazi et al., 1994), and for ferryl Mb (George & Irvine, 1954, 1955). The question remains whether E_o' of these higher oxidation states also varies according to eq 2.

To address these questions, we studied the structural and biochemical properties of HRP and Mb containing either naturally occurring hemes or hemes substituted with electron-withdrawing groups (diacetyl) or electron-donating groups (diethyl).

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* To whom correspondence should be addressed. Fax: (801) 797-3328.

‡ Utah State University.

§ Himeji Institute of Technology.

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[1] Abbreviations: CcP, cytochrome *c* peroxidase; diacetyl, 2,4-diacetyldeutero; E_o', one-electron reduction potential; HRP, horseradish peroxidase; iridate, hexachloroiridate; Mb, myoglobin; metMb, met-myoglobin; meso, 2,4-diethyl; XAS, X-ray absorption spectroscopy.

[2] The minus sign was omitted in the paper of Yamada et al. (1975).

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The structural consequences of changing the electron density in the heme have not been investigated. Are the structural changes similar in HRP and Mb?

MATERIALS AND METHODS

HRP and sperm whale Mb were obtained from Toyobo (Osaka, Japan) and Sigma (St. Louis, MO), respectively. HRP and metMb substituted with meso- and diacetylhemes were prepared according to Makino and Yamazaki (1972, 1974). Potassium hexachloroiridate (K_2IrCl_6) was obtained from Aldrich (Milwaukee, WI) and was added to hemoprotein solutions as a 10 mM stock solution containing 0.01 N HCl. All other reagents used were of analytical grade. Absorption spectra were obtained using a Shimadzu UV-2101PC spectrophotometer at a scan rate of 15 nm/s. The ϵ_{mM} values used to measure HRP concentrations were 96 at 394 nm for meso-HRP, 107 at 403 nm for native HRP, and 77 at 413 nm for diacetyl-HRP (Makino & Yamazaki, 1972). Similarly, the values for Mb were 165 at 395 nm for meso-metMb, 160 at 409 nm for native metMb, and 86 at 413 nm for diacetyl-metMb (Makino & Yamazaki, 1974). The redox equilibria were obtained at 15 ± 1 °C and in 0.1 M potassium phosphate buffer (pH 7), except where otherwise mentioned. This low temperature (15 °C) was used to avoid denaturation of heme-substituted hemoproteins during their oxidation by $IrCl_6^{2-}$.

X-ray Absorption Spectroscopy. Data were collected at the Stanford Synchrotron Radiation Laboratory on beamline 4−3 using Si(111) monochromator crystals providing 2−3 eV resolution at the Fe edge (7110 eV) together with a 13 element Ge detection system. Experiments were performed at 155 K as described previously (Powers et al., 1981; Powers, 1982; Chance et al., 1982) to prevent damage caused by X-ray exposure (Chance et al., 1980). Protein and model compound data were collected under identical conditions. Analysis methods have been described previously (Lee et al., 1981; Powers, 1982; Chance et al., 1984; Powers & Kincaid, 1989). Briefly, data for each sample were compared among preparations to ensure reproducibility and averaged. Background contributions due to isolated atoms were removed, followed by multiplication by k^3 (k is the wave vector). These isolated EXAFS modulations were then normalized to one absorbing atom and are shown in Figure 1. Figure 2 shows the Fourier transforms of the data in Figure 1. The contribution of each coordination shell was isolated by Fourier-filtering, and the back-transformed data were used in the fitting procedures. Protein and model compound data were analyzed in the same manner.

A nonlinear least-squares fitting procedure was used to fit the protein data with the data of the model compounds (Lee et al., 1981; Powers & Kincaid, 1989). Each atom type in this procedure is described by the average distance (r) from the absorbing atom, the number of atoms (N) in the coordination shell, the change in the Debye−Waller factor ($\Delta\sigma^2$) which describes the thermal and lattice disorder with respect to the model compound, and the change in threshold energy (ΔE_0) with respect to the model compound. The N parameters were fixed at their known values. These procedures have been described previously (Powers & Kincaid, 1989) and correctly address the number of unknowns that can be accommodated in a meaningful fit, physically reasonable values for the fitting parameters, the difference

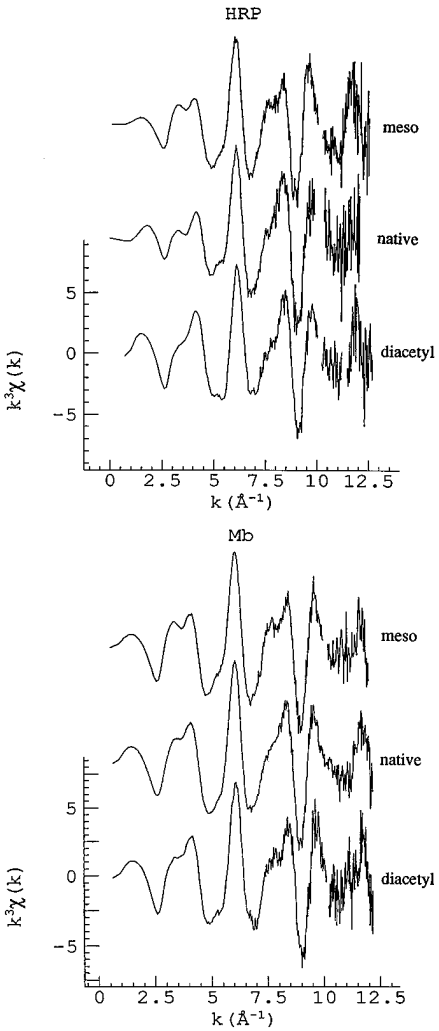

FIGURE 1: Background-subtracted EXAFS data. Heme-substituted HRP (top panel); heme-substituted Mb (bottom panel).

in the sum of residuals squared ($\sum R^2$) which describes the goodness of the fit that must be observed to distinguish two physically meaningful fits, three atom-type consistency test, and error estimation procedures.

Measurements of E_o' from Redox Equilibria. E_o' of ferryl Mb was measured from equilibria with iridate according to a modified method of George and Irvine (1954, 1955). Since the equilibrium between Mb and iridate was not stable, the time dependence of the apparent equilibrium constant was examined. The concentration of $IrCl_6^{2-}$ was measured from the increase in absorbance at an isosbestic point of metMb and ferryl Mb, appearing at 508 nm for meso-Mb, 519.5 nm for native Mb, and 528.5 nm for diacetyl-Mb. $IrCl_6^{2-}$ has absorbance in the visible region which is lost upon reduction. The E_o' values of HRP compounds I and II were also obtained from equilibria with iridate according to Hayashi and Yamazaki (1979). The equilibrium concentrations of ferric, compound I, and compound II forms of both meso-HRP and native HRP were calculated on the basis of $\Delta\epsilon_{mM}$ values listed in Table 1. The $\Delta\epsilon_{mM}$ values for native HRP were obtained from Hayashi and Yamazaki (1979), and those for meso-HRP were calculated from Figure 3 of the paper of Makino and Yamazaki (1972). For example, the equations used to calculate millimolar concentrations of three components (ferric, compound I, and compound II) of meso-HRP are as follows; [ferric] = $A/96 + \Delta A/38.5$; [compound II]

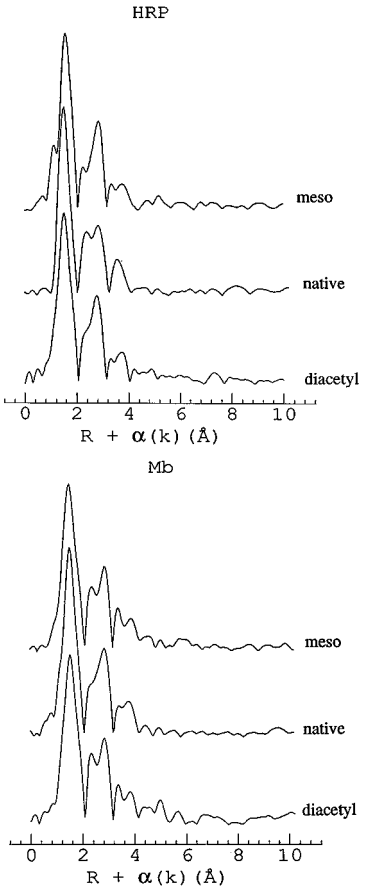

FIGURE 2: Fourier transform of the EXAFS modulation shown in Figure 1. Heme-substituted HRP (top panel); heme-substituted Mb (bottom panel).

Table 1: Millimolar Difference Absorption Coefficient ($\Delta\epsilon_{mM}$[a]) Used for Calculation of the Equilibrium Concentrations of Ferric HRP, Compound I, and Compound II[b]

heme	ferric	compound II	compound I
meso-HRP	395 nm (−38.5)[c]	419 nm (31.5)	404.5 nm (−24.4)
native HRP	398 nm (−42.3)[c]	430 nm (40.8)	410 nm (−38.9)

[a] The difference from the absorbance of ferric HRP. [b] Each wavelength is isosbestic for two components except the one to be measured. [c] The ferric concentration calculated with this value is the decrease from the initial concentration (see Materials and Methods).

$= \Delta A/31.5$; and [compound I] $= -\Delta A/24.4$, where A is the initial absorbance of ferric meso-HRP at 394 nm and ΔA is the difference from the initial absorbance of ferric meso-HRP at the isosbestic points listed in Table 1. The concentration of residual $IrCl_6^{2-}$ was measured from the absorbance at wavelengths where the absorbance of the enzyme was small and not significantly changed during its conversion between ferric, compound I, and compound II. The wavelengths used were 446 nm for meso-HRP (Makino & Yamazaki, 1972) and 448 for native HRP (Yokota & Yamazaki, 1977). The absorbance of $IrCl_6^{2-}$ at these wavelengths was obtained from an absorption spectrum of 100 μM $IrCl_6^{2-}$.

In the reaction of diacetyl-HRP with iridate at neutral pH, the enzyme apparently existed as an equilibrium mixture of ferric and compound II. Therefore, the calculation procedure was essentially the same as that used for Mb. The concentration of remaining $IrCl_6^{2-}$ was measured at an isosbestic point for ferric and compound II of diacetyl-HRP, appearing at 466 nm.

Table 2: X-ray Absorption Spectroscopy Results for HRP

heme	Fe−Np[b]	Fe−N_ϵ[c]	Fe−X[d]
meso-	2.06	1.94	
native[a]	2.04	1.91	2.38
diacetyl-	2.02	1.89	2.08

[a] Chance et al. (1984). [b] Error ± 0.015 Å. [c] Error ± 0.015 Å. [d] Error ± 0.05 Å.

Table 3: X-ray Absorption Spectroscopy Results for Mb

heme	Fe−N_p[b]	Fe−N_ϵ[c]	Fe−X[d]
meso-	2.06	2.21	1.91
native[a]	2.04	2.14	1.92
diacetyl-	2.02	2.10	1.90

[a] Powers (1989). [b] Error ± 0.015 Å. [c] Error ± 0.05 Å. [d] Error ± 0.015 Å.

E_o' was calculated by using a value of 0.900 V for E_o' ($IrCl_6^{2-}/IrCl_6^{3-}$) as an approximate average of the values reported at 20−25 °C and ionic strength of about 0.1 (George & Irvine, 1955; Margerum et al., 1975; Nord et al., 1978).

RESULTS

Structure of the Local Heme Environment. The results of the fitting procedures for the X-ray absorption spectroscopy data are shown in Tables 2 and 3. Substitution of the electron-withdrawing diacetyl groups in the 2- and 4-positions on the heme results in a decrease in the average heme pyrrole nitrogen distance (Fe−N_p) in both HRP and Mb. However, substitution of the electron-donating meso groups in both HRP and Mb results in an increase in this average distance. While the differences in these average distances (0.02Å) between meso-substituted and native heme and between native and diacetyl-substituted heme are nearly the same as our conservative error estimate, the differences observed between meso- and diacetyl-substituted heme are outside this estimate. It is clear that these show a lengthening of the average heme pyrrole nitrogen distance in both HRP and Mb with increasing electron density on the heme.

No distal ligand (Fe−X) is observed for HRP substituted with meso-heme, while the diacetyl-heme substitution results in the clear presence of an O(N) ligand in the distal position and the native protein has a long distance associated with a water oxygen (Chance et al., 1984). However, the proximal histidine distance (Fe−N_ϵ) remains unchanged within error. In Mb, the distal water oxygen distance is unchanged within error, but the proximal histidine distance appears to increase with electron density on the heme.

Optical Absorption Spectra and E_o' Measurements. Figure 3 shows visible absorption spectra of meso-metMb (A), native metMb (B), and diacetyl-metMb (C). We observed a clear red shift in the charge transfer bands around 500 and 630 nm with increasing electron-withdrawing capacity of the 2,4-substituents. A similar red shift of the Soret bands of heme-substituted metMb was reported by Makino and Yamazaki (1974). As reported by George and Irvine (1954), the oxidation of native metMb by $IrCl_6^{2-}$ was fast. The oxidation was also fast for meso-metMb, but was slow for diacetyl-metMb. $IrCl_6^{2-}$ was not stable under the conditions used in these experiments and slowly disappeared, probably through reactions with amino acid residues. In buffer solutions without Mb and at neutral pH, $IrCl_6^{2-}$ was stable. George and Irvine (1954) described extra consumption of

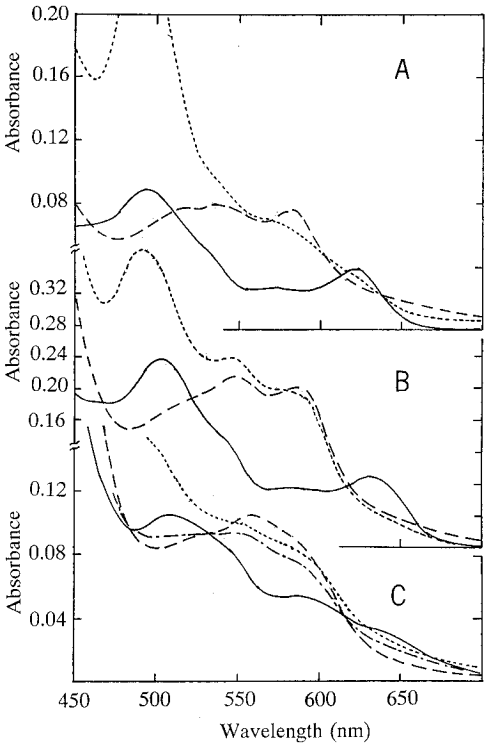

FIGURE 3: Absorption spectra used for calculation of E_o' for the ferryl/ferric couple of Mb at pH 7. (A) 10.4 μM meso-metMb. (B) 25.3 μM metMb. (C) 11.9 μM diacetyl-metMb. MetMb (—); ferryl Mb (− −), spectra were taken 1 min after addition of 50 μM H_2O_2 (5% dilution effect was not corrected); and the spectra used for E_o' calculation (•••) were taken 1 min (A and B) and 6 min (C) after addition of 100 μM K_2IrCl_6. In panel C, the spectrum (—•—) of a mixture of ferric and ferryl Mbs was obtained upon addition of 100 μM ascorbate to the spectrum shown as a dotted line.

Table 4: Time-Dependent Changes in the Concentrations of Ferryl Mb [Mb(IV) in Percent] and Residual $IrCl_6^{2-}$ [a]

	min[b]	Mb(IV) (%)[c]	$[IrCl_6^{2-}]$ (μM)	K[d]	E_o' (V)
meso	1	39	50.5	0.63	0.912
	2	30	37.7	0.72	0.908
	3	27	31.0	0.81	0.905
native	1	68	64.4	1.19	0.896
	2	66	55.2	1.54	0.889
	3	62	48.3	1.71	0.886
diacetyl	1	32	56.1	0.36	
	3	45	34.7	1.54	0.889
	5	54	24.5	3.57	0.867
	6	57	17.5	5.46	0.857

[a] Experimental conditions are described in the legend of Figure 1. The real E_o' values were obtained from data at reaction times (after addition of 100 μM $IrCl_6^{2-}$) when the ferryl Mb concentration reached a maximum. [b] Time in minutes after addition. [c] Percent concentration of ferryl myoglobin. [d] $K = [IrCl_6^{3-}][\text{ferryl Mb}]/[IrCl_6^{2-}][\text{metMb}]$.

$IrCl_6^{2-}$ upon oxidation of metMb, but did not observe time-dependent changes in the apparent equilibrium constant. Considering the difference in the rate of oxidation of metMb by $IrCl_6^{2-}$ and the instability of $IrCl_6^{2-}$ in Mb solutions, we compared the time dependence of the amount of ferryl Mb produced by these reactions and of the calculated values of E_o' (Table 4). The amount of ferryl-Mb reached a maximum after 1 min and gradually decreased in the reactions of native metMb and meso-metMb with $IrCl_6^{2-}$. Diacetyl-metMb oxidation by $IrCl_6^{2-}$ reached equilibrium 6 min after the reaction started. Since the reaction of ferryl diacetyl-Mb with ascorbate was very slow, we could selectively reduce $IrCl_6^{2-}$ to obtain the spectrum of a mixture of ferric and ferryl Mbs

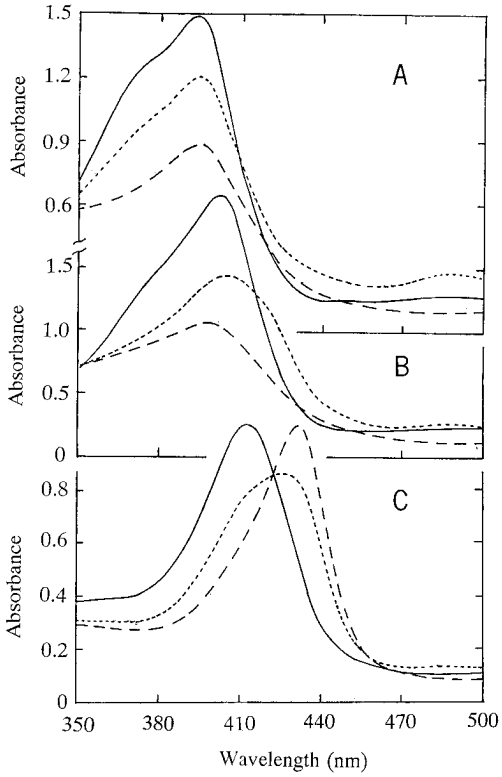

FIGURE 4: Absorption spectra used for calculation of E_o' of HRP compounds I and II. (A) 15.7 μM meso-HRP (—); compound I (− −), the spectrum was taken immediately after addition of 25 μM H_2O_2; and the spectrum at equilibrium with iridate (•••) was taken 3 min after addition of 75 μM K_2IrCl_6 at pH 7. (B) 20.3 μM HRP (—); compound I (− −), the spectrum was taken immediately after addition of 50 μM H_2O_2; and the spectrum at equilibrium with iridate (•••) was taken 3 min after addition of 100 μM K_2IrCl_6 at pH 7. (C) 13.6 μM diacetyl-HRP (—); compound II (− −), the spectrum was taken immediately after addition of 16 μM H_2O_2; and the spectrum at equilibrium with iridate (•••) was taken 3 min after addition of 100 μM $IrCl_6^{2-}$ at pH 6.

without spectral interference from $IrCl_6^{2-}$ (Figure 3C). The spectrum was consistent with that obtained by subtracting the absorbance of the residual $IrCl_6^{2-}$ in reactions without added ascorbate.

George and Irvine (1955) reported a value of 0.887 V for native Mb at 20 °C, using a value of 0.898 V for E_o' of $IrCl_6^{2-}$. From Table 4, we determined the E_o' of ferryl Mb to be 0.912 V for meso-Mb, 0.896 V for native Mb, and 0.857 V for diacetyl-Mb. The spectra we used for calculation of these E_o' values at 15 °C are shown as dotted lines in Figure 3. The dashed lines show the spectra of the ferryl Mbs obtained by addition of a slight excess of H_2O_2.

Figure 4 shows the Soret spectra of meso-HRP (A), native HRP (B), and diacetyl-HRP (C). The spectral characteristics were discussed by Makino and Yamazaki (1972). The Soret peaks are significantly red-shifted with decreasing electron density at the heme iron. Addition of H_2O_2 to these enzymes resulted in conversion of meso-HRP and native HRP to a stable compound I. However, diacetyl-HRP was converted to compound II, and under these conditions, we could not detect the formation of diacetyl-HRP compound I. The spectra, scanned immediately after addition of H_2O_2, are shown as dashed lines in Figure 4. The rate of oxidation of ferric HRP by $IrCl_6^{2-}$ was relatively slow as reported by Hayashi and Yamazaki (1979). The redox equilibrium was attained 3 min after addition of $IrCl_6^{2-}$ and was stable for

Table 5: Calculation of E_o' of HRP Compounds I and II

	concentrations (μM)							
	ferric	compound II	compound I	$IrCl_6^{2-}$	K_I[a]	E_o' of compound I	K_{II}[a]	E_o' of compound II
meso	7.3	1.1	7.6	29.0	10.96	0.841	0.239	0.935
native	3.0	3.7	12.8	59.4	2.36	0.879 (0.880)[b]	0.90	0.903 (0.900)[b]
diacetyl	6.8	6.8	<0.3	11.1	<0.054	>0.975[c]	8.0	0.849 (pH 6)
diacetyl value calcd at neutral pH								0.790[d] (pH 7)

[a] $K_I = [IrCl_6^{3-}][\text{compound I}]/[IrCl_6^{2-}][\text{compound II}]$ for E_o' of compound I. $K_{II} = [IrCl_6^{3-}][\text{compound II}]/[IrCl_6^{2-}][\text{ferric}]$ for E_o' of compound II. [b] The E_o' values in parentheses were reported by Hayashi and Yamazaki (1979). The values have been corrected using a value of 0.900 V for E_o' of $IrCl_6^{2-}$. [c] The lowest limit, calculated by assuming that the detectable amount of compound I is less than 2%. [d] E_o' of diacetyl-HRP compound II at pH 7 at 15 °C was calculated from the value measured at pH 6 according to the equation: $E_o' = E_o + 0.057 \log \{(K_{red}[H^+] + [H^+]^2)/(K_{ox} + [H^+])\}$, where $pK_{red} = 9.1$ (Makino & Yamazaki, 1972) and $pK_{ox} = 8.5$ (Makino et al., 1986).

several minutes in each HRP reaction. The spectra obtained after 3 min are shown as dotted lines in Figure 4. Addition of $IrCl_6^{2-}$ to ferric HRP and meso-HRP gave a mixture of ferric, compound I, and compound II. The concentrations of the three components were measured as described under Materials and Methods after the absorbance of residual $IrCl_6^{2-}$ was subtracted. Table 5 shows the measured concentration of $IrCl_6^{2-}$ at equilibrium in these reactions and the calculated concentrations of ferric, compound I, and compound II of meso-HRP and native HRP. The sum of the calculated concentrations of the three HRP components agreed well with the amount of added ferric HRP, having errors of only a few percent. The equilibrium constant and the E_o' values are also listed in Table 5. In the present study, the E_o' values of native HRP compounds I and II at pH 7 were 0.879 and 0.903 V, respectively. The corresponding values reported by Hayashi and Yamazaki (1979) were 0.880 and 0.900 V.

E_o' of diacetyl-HRP compound II was measured in the same way as that of ferryl Mb. No compound I formation was observed as judged from the decrease of absorbance at pH 6 and 7 at 423 nm (an isosbestic point for ferric enzyme and compound II of diacetyl-HRP). In this case, assuming that the amount of compound I is less than 2%, we determined the lower limit for E_o' of diacetyl-HRP compound I to be 0.975 V. The redox equilibrium between diacetyl-HRP and iridate at pH 7 was not suitable for calculating the E_o' of compound II because of the large difference in the E_o' values between the two redox couples. We measured E_o' from the redox equilibrium at pH 6 where the E_o' values are closer. We then calculated E_o' at pH 7 from E_o' values at pH 6 and 6.5 according to the equation described in Table 5 (see footnote *d*).

DISCUSSION

E_o' of Ferric Hemoproteins and the Electron Density and Structure of the Heme. XAS shows that substitution of electron-withdrawing diacetyl groups at the 2,4-positions of the heme results in a decrease in the average $Fe-N_p$ distance in both HRP and Mb while substitution of electron-donating meso groups results in an increase in this average distance. Similar results are observed for these proteins having electron-withdrawing and -donating ligands in the distal position (Powers, 1989; Chance et al., 1984). These results are not surprising, based on the considerable literature of heme model compounds [for example, see Hoard (1975)].

In HRP, heme substitution results in changes at the distal side of the heme. However, the proximal histidine distance ($Fe-N_\epsilon$) is unaltered by either electron-withdrawing or electron-donating groups. This distance is also unchanged when electron-donating and -withdrawing ligands are bound (Chance et al., 1984) and is consistent with the fact that the proximal histidine in HRP is probably constrained by an H-bonding network as seen in CcP (Finzel et al., 1984; Poulos & Finzel, 1984). Conversely, in Mb, the distal oxygen distance is unchanged by heme substitution, while the proximal histidine distance appears to increase with increased electron density in the heme. Again, while this increase is close to our error estimate, a similar lengthening of the proximal histidine distance with increasing electron density on the heme is observed in native Mb bound with electron-donating ligands such as fluoride and formate (Powers, 1989). In addition, the average heme pyrrole nitrogen distance is also lengthened on binding of these electron-donating ligands. Similarly, the binding of electron-withdrawing ligands such as cyanide and azide produces changes in these bond distances that are similar to those found for diacetyl heme substitution (Powers, 1989). These results are consistent with the fact that the Mb proximal histidine can adjust its length depending on the electron density at the heme and the electron-donating or -withdrawing properties of the distal ligand (Powers, 1989).

The fact that peroxidases have lower redox potentials for the ferric/ferrous couple than the globins has been explained in terms of strong negativity of the proximal base. This idea has been confirmed by site-specific mutagenesis on CcP by Goodin and McRee (1993), who reported that His-175 (proximal) is deprotonated in the wild-type enzyme through the hydrogen bond to Asp-235. E_o' of ferric CcP increases when the hydrogen bond is removed. E_o' of metMb is significantly decreased upon replacement of histidine-93 (proximal base) with glutamate or aspartate (Varadarajan et al., 1989) or with cysteine or tyrosine (Adachi et al., 1991).

The effect of electron density on E_o' of ferric HRP A and C has been studied quantitatively using 2,4-substituted hemes. The basicity of the pyrrole nitrogens was concluded to directly control the electron density at the heme iron, because there is a linear correlation between pK_3 and pK_a (proton dissociation constant for neutral forms) in ferric HRP and metMb (Makino & Yamazaki, 1972, 1973). For HRP and metMb, respectively:

$$\Delta pK_a \text{ of HRP} = 1.02(\Delta pK_3) \quad (3)$$

$$\Delta pK_a \text{ of metMb} = 0.69(\Delta pK_3) \quad (4)$$

The ligand at the sixth position of the heme iron in the

alkaline hemoproteins is a hydroxyl ion. Direct thermodynamic correlation between pK_3 and E_o' of ferric HRP was demonstrated in eq 2 from a proportionality constant of about −59 for the ratio of $\Delta E_o'$ (mV) to ΔpK_3 (Yamada et al., 1975).

Of four 2,4-substituted artificial hemes used in the previous experiments, meso- and diacetyl-hemes were chosen in the present experiments for the following reasons. The reconstituted HRP and metMb with meso- and diacetyl-hemes are stable, and their properties have been reliably demonstrated to depend on the pK_3 values (Yamazaki et al., 1978). The pK_3 is 5.8 for mesoporphyrin and 3.3 for diacetylporphyrin (Falk et al., 1966; Caughey et al., 1966). The difference of 2.5 in the pK_3 values of the two artificial porphyrins is sufficiently large to see the effect of pK_3, and the pK_3 value of 4.85 for the natural protoporphyrin falls between these values.

E_o' of Higher Oxidation States and pK_3. Numerous papers have already examined the relationship between heme protein reduction potentials and function. The discussion, however, has so far been limited to E_o' values of ferric hemoproteins. To further understand the functions of peroxidases and other hemoprotein enzymes, the redox properties of compounds I and II must be considered. The dependence of E_o' on the electron density at the heme iron for these higher oxidation states differs from that found for the ferric/ferrous couple. Plotting these E_o' values at 15 °C against pK_3, we can roughly formulate the following relationships:

$$\Delta E_o' \text{ of HRP compound I} = -57(\Delta pK_3) \quad (5)$$

$$\Delta E_o' \text{ of HRP compound II} = 57(\Delta pK_3) \quad (6)$$

$$\Delta E_o' \text{ of ferryl Mb} = 17(\Delta pK_3) \quad (7)$$

Although the relationships between $\Delta E_o'$ and ΔpK_3 in the above equations are rather qualitative, we can make the following conclusions: (a) E_o' of compound I depends on pK_3 in the same manner as does E_o' of ferric HRP (compare eqs 2 and 5); (b) E_o' of compound II shows the opposite dependence on pK_3 to that of ferric HRP or compound I; (c) E_o' of ferryl Mb behaves similarly to that of HRP compound II, but the proportionality constant is smaller; (d) at neutral pH, compounds I and II of native HRP have similar E_o' values, but those of meso- and diacetyl-HRP have very different values from native HRP and from each other; and (e) the two-electron reduction potentials for the HRP compound I/ferric couple, namely, the mean values of E_o' of HRP compounds I and II, are approximately the same regardless of the corresponding pK_3 value.

The values of E_o' of compound I and ferric HRP are similarly affected by the electron density at the heme, while that of compound II shows the opposite relationship. This result suggests that an increase in electron density at the heme does not always decrease the E_o' value of a certain oxidation state. While our E_o' results stand on their own, we have given some thought to why the variation in E_o' with heme electron density should differ for the compound II/ferric couple relative to the compound I/compound II and ferric/ferrous couples. A simple way to explain this phenomenon is in terms of the apparent net charge in the vicinity of the heme iron. The apparent net charge on the iron porphyrin is known to be ∼0 ($Fe^{2+}N_4^{2-}$) in the ferrous state and ∼+1 ($Fe^{3+}N_4^{2-}$) in the ferric state. N_4^{2-} denotes that two of the four pyrrole nitrogens of porphyrin are negatively charged in the heme. Similarly, the apparent net charge on the iron porphyrin of compound II (or ferryl) and compound I is considered to be ∼0 ($Fe^{4+}O^{2-}N_4^{2-}$) and ∼+1 ($Fe^{4+}O^{2-}N_4^{2-}$-porphyrin$^+$), respectively. Although the excess charge is delocalized, the net positive charge on the iron porphyrin changes alternately in the three-step reduction of compound I to the ferrous form (Figure 5). The reductions of HRP compound I and ferric HRP are accompanied by a decrease in the apparent net positive charge on the iron porphyrin. In contrast, the reductions of HRP compound II and ferryl Mb result in an increase in the apparent net positive charge. This process could cause an opposite dependence of E_o' on pK_3 in compound II and ferryl Mb compared to compound I and ferric HRP.

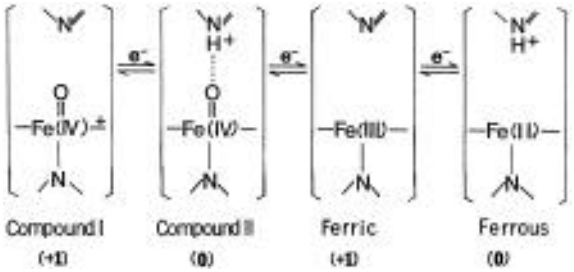

FIGURE 5: Change in the apparent net charge on the iron porphyrin in one-electron reduction processes of HRP. Two of the four pyrrole nitrogens are negatively charged. In each reduction step, the proton uptake also occurs at neutral pH. A water molecule is formed when compound II is reduced to the ferric form. The pK_a value for the distal imidazole is 8.6 for compound II and 7.3 for the ferrous state. The approximate apparent net charge on the iron porphyrin is shown at the bottom.

Protonation at the Distal Imidazole. In HRP, the distal imidazole is protonated at pK_a = 7.3 in the ferrous state (Yamada & Yamazaki, 1974) and at pK_a = 8.6 in compound II (Hayashi & Yamazaki, 1978). These pK_a values are close to neutral pH at 25 °C and control E_o' as follows:

$$E_o'(\text{V}) = E_o(\text{V}) + 0.059 \log \{(K_{red}[H^+] + [H^+]^2)/(K_{ox} + [H^+])\} \quad (8)$$

where pK_{red} and pK_{ox} denote pK_a in the reduced and the oxidized states, respectively. For E_o' of ferric HRP, pK_{red} = 7.3 and pK_{ox} = 11.0; for E_o' of compound II, pK_{red} = 11.0 and pK_{ox} = 8.6; and for E_o' of compound I, pK_{red} = 8.6. At pK_a = 11.0, alkaline ionization takes place in ferric HRP.

In ferric HRP (or metMb) and compound I, the distal imidazole exists as the deprotonated form above pH 5, probably because of electrostatic repulsion by the positive charge on the iron porphyrin. Ferric HRP and metMb are neutralized by deprotonation of water at the heme site to form their alkaline forms. Yamada et al. (1975) showed the pK_a value in ferrous HRP is only slightly affected by the pK_3 value:

$$\Delta pK_a = 0.1(\Delta pK_3) \quad (9)$$

Similarly, the pK_a value in HRP compound II is not affected by the pK_3 value, being 8.6 for native HRP (Hayashi &

Yamazaki, 1978) and 8.5 for diacetyl-HRP (Makino et al., 1986). These results show that the net positive charge present on the iron porphyrin of ferric HRP and compound I results in a larger electrostatic effect on protonation at the distal imidazole than does the difference of 2.5 in the pK_3 value.

Although the protonation at the distal base controls the E_o' value according to eq 8, it does not result in an increase of the net positive charge on the iron porphyrin in the ferrous state. However, protonation at the distal base may increase the net positive charge on the iron porphyrin of HRP compound II, due to the formation of hydrogen bonding between the distal base and the oxoferryl iron (Sitter et al., 1985; Hashimoto et al., 1986; Chang et al., 1993). This consideration, however, does not change our overall view of the effect of pK_3 on the E_o' values of HRP compounds I and II.

E_o' and Reaction Rate. Since compound II is known to be the rate-limiting intermediate in peroxidase reactions, we can easily measure the rate constant, k_3, for the reduction of compound II:

$$\text{compound II} + AH_2 \rightarrow \text{ferric} + AH \qquad (10)$$

According to Makino and Yamazaki (1972), k_3 values for the oxidation of ascorbate and guaiacol are about 2 times larger for meso-HRP than for native HRP. Considerably smaller values are obtained for diacetyl-HRP. This result can be explained from the E_o' value of compound II (Table 5). In the case of native HRP, compound I reacts with an electron donor about 100 times faster than does compound II although both compounds have nearly the same E_o' values. This has been interpreted in terms of higher mobility of a porphyrin π-electron than an iron valence electron (Hayashi & Yamazaki, 1979; Farhangrazi et al., 1995). The ratio of the rate constants for the reactions of ascorbate with HRP compounds I and II is 320 for HRP at pH 7 (Yamazaki et al., 1973) and about 3 for *Arthromyces ramosus* peroxidase. *Arthromyces ramosus* peroxidase compound II has an E_o' value which is about 70 mV higher than compound I (Farhangrazi et al., 1994).

CONCLUSION

Changes in the electron density at the heme center are reflected in the structure. Increasing the electron density results in an increase in the average heme pyrrole nitrogen distance for both HRP and Mb. In HRP, the distal oxygen distance also increases with increasing electron density at the heme center. In Mb, it is the proximal histidine whose distance increases.

In most one-electron reduction processes, the reduction results in a decrease in positive charge in the vicinity of the redox center, and the reduction potential will decrease with an increase of the electron density at the center. This is the case for reduction of metMb, ferric HRP, and HRP compound I. In the reduction of ferryl Mb and HRP compound II, however, positive charge increases at the iron porphyrin. This may explain the opposite pK_3 dependence of the E_o' values of ferryl Mb and HRP compound II we observe. The two-electron reduction potential for the compound I/ferric HRP couple will be little affected by the pK_3 value because there is no change in the net positive charge on the iron porphyrin between the two states.

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