# Catalytic Roles of the Distal Site Asparagine−Histidine Couple in Peroxidases[†]

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ABSTRACT: There are highly conserved hydrogen bonds between the distal His and the adjacent Asn in many peroxidases. Although the crystal structure of horseradish peroxidase C (HRP) is not available, comparison of the amino acid sequence with cytochrome *c* peroxidase indicates that Asn70 is making the hydrogen bond with the distal His in the active site of HRP. To investigate the catalytic roles of the hydrogen bond, Asn70 in HRP was replaced with Val (N70V) or Asp (N70D). Though UV−vis, CD, and $^1$H-NMR spectra of native (plant enzyme), wild-type (recombinant enzyme), and mutant HRPs suggest that the active site and secondary structure are very similar even after the mutation, the mutants exhibit low $V_{max}$ values for the hydroquinone oxidation (native, 281; wild-type, 283; N70V, 18; and N70D, 33 $\mu$M·min$^{-1}$). The rates of compound **I** formation were decreased to less than 10% of that of the native enzyme. The reduction rates of compounds **I** and **II** by guaiacol also were reduced to less than 10% of that of the native enzyme. Substituent effects of various phenol derivatives on the reduction of native, wild-type, and mutant compound **I** were examined. Large negative Hammett $\rho$ values ($\rho_{N70V:fast} = -4.0$, $\rho_{N70V:slow} = -3.6$, $\rho_{N70D} = -3.8$, $\rho_{native} = -6.9$, and $\rho_{wild-type} = -6.8$) are an indication of electron transfer being the rate-determining step in the phenol oxidation. However, these results also indicate the participation of the deprotonation step in the compound **I** reduction process. The proton abstraction from phenol must be harder for the mutants due to the decrease of basicity of the distal His upon mutation. Contrary to phenol oxidation, ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] oxidation activity was substantially increased by the mutations (native, 73; wild-type, 71; N70V, 217; and N70D, 234 $\mu$M·s$^{-1}$). The redox potentials of N70V and N70D compounds **II** are 957 and 970 mV (vs NHE), which are 95 and 108 mV higher than that of native compound **II** (862 mV), respectively. Therefore, the high ABTS oxidation activities of mutants are attributed to these high redox potentials of compound **II**.

Peroxidases are found in many plants, some animal tissues, and microorganisms where they carry out a variety of biosynthetic and degradative transformations of substrates by using peroxides, especially hydrogen peroxide as an oxidant (Everse et al., 1991; Hewson & Hager, 1979). Peroxidase reactions are two-electron redox processes consisting of three distinct steps:

$$\text{POD} + \text{ROOH} \rightarrow \text{compound } \mathbf{I} + \text{ROH} + \text{H}_2\text{O} \quad (1)$$

$$\text{compound } \mathbf{I} + \text{AH}_2 \rightarrow \text{compound } \mathbf{II} + \text{AH}^\bullet \quad (2)$$

$$\text{compound } \mathbf{II} + \text{AH}_2 \rightarrow \text{POD} + \text{AH}^\bullet \quad (3)$$

$$(2\text{AH}^\bullet \rightarrow \text{A}_2\text{H}_2 \text{ or } \text{AH}_2 + \text{A})$$

In these equations, POD, ROOH, compound **I**, and compound **II** represent the ferric resting state of peroxidase ($Fe^{III}$ Por),[1] peroxide, a two-electron oxidized form of the enzyme ($Fe^{IV}$═O $Por^{•+}$), and a one-electron oxidized form of the enzyme ($Fe^{IV}$═O Por), respectively. The electron donor substrate $AH_2$ is a wide variety of organic and inorganic compounds.

Rapid reactions of the resting peroxidases with peroxides to form compound **I** discriminate the peroxidases from other classes of hemoproteins. This characteristic reactivity must be related to the distinct protein structural features, since other hemoproteins which share the same protoporphyrin IX prosthetic group exhibit very different reactivities with peroxides. For instance, while peroxidases react readily with hydrogen peroxide ($\sim 10^7$ M$^{-1}$·s$^{-1}$) (Loo & Erman, 1975), metmyoglobin reacts with hydrogen peroxide at a rate of $\sim 10^2$ M$^{-1}$·s$^{-1}$ (Yonetani & Schleyer, 1967). Poulos and Kraut suggested that the invariant distal His, which is located near the heme (Figure 1), serves as a general acid−base catalyst in the reaction with peroxide as shown in Scheme 1 (Poulos & Kraut, 1980). Erman et al. (1992, 1993) and Newmyer et al. (1995) have demonstrated a critical role for the distal His in the rapid reaction with peroxide. Substitution of the distal His to Leu (C*c*P) (Erman, et al., 1992, 1993) and to Ala or Val (HRP) (Newmyer & Ortiz de Montellano, 1995) greatly reduced the compound **I** formation rates by

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[1] Abbreviations: Por, porphyrin; $Por^{•+}$, porphyrin $\pi$ cation radical; HRP, horseradish peroxidase C; ARP, *Arthromyces ramosus* peroxidase; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NHE, normal hydrogen electrode.

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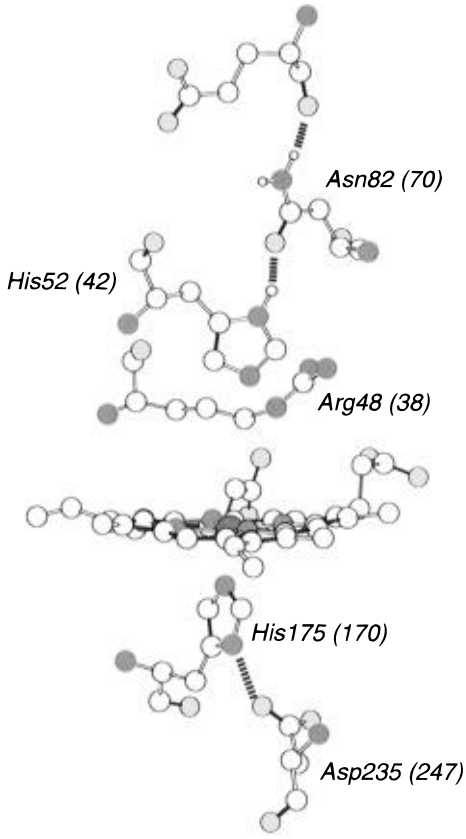
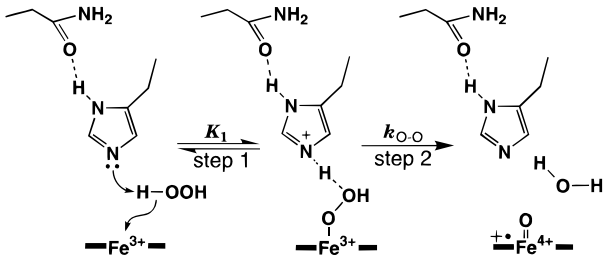



FIGURE 1: Proximal and distal site structure of C*c*P. The hydrogen bond is as |||||| . The amino acid numbering is for C*c*P, but the numbers in parentheses denote the numbering for HRP.

Scheme 1: Plausible Mechanism for Compound I Formation[a]




[a] Poulos and Kraut (1980).

5−6 orders of magnitude. Therefore, the distal His is one of the most important residues in the catalytic cycle of the peroxidase reaction.

Inspection of a crystal structure of C*c*P indicates the formation of a hydrogen bond between the distal His and Asn, an amino acid a bit far from the immediate vicinity of the heme (Figure 1). Amino acid sequence alignments (Baunsgaard et al., 1993) and X-ray crystal structures (Finzel et al., 1984; Poulos et al., 1993; Edwards et al., 1993; Kunishima et al., 1994; Fukuyama et al., 1995; Sundaramoorthy et al., 1994; Petersen et al., 1994; Patterson & Poulos, 1995) show that the hydrogen bond is highly conserved among many plant and fungal peroxidases. Contrary to peroxidases, the Asn−His couple could not be found in the distal site of myoglobin and hemoglobin, even though they also have the distal His in their heme pockets. Recently, Satterlee et al. (1994) and Delauder et al. (1994) reported the replacement of distal Asn82 of C*c*P with Asp by site-directed mutagenesis. While they have examined $^1$H-NMR spectroscopic and thermodynamic properties of the mutant C*c*P, effects of the disruption of the His−Asn couple on its peroxidase activities have not been reported.

In order to examine the roles of the highly conserved hydrogen bond in the catalytic activities of peroxidases, we have prepared two site-directed mutant HRPs, including N70V (Asn70 → Val) and N70D (Asn70 → Asp). The basicities of the distal His and peroxidase activities were examined through elementary reaction rates. We also have examined redox potentials of compounds **I** and **II** in order to gain further insight into the instability of N70V compound **II** (Nagano et al., 1995).

## EXPERIMENTAL PROCEDURES

*Materials.* Plant horseradish peroxidase C (native HRP) was obtained from Sigma (type VI) and used without further purification. The native enzyme exhibited an $A_{402}/A_{280}$ ratio (RZ value) of 3.2. Monosubstituted phenols, ABTS, $H_2O_2$, $K_2IrCl_6$, and $K_3IrCl_6$ were purchased from Wako. General molecular biology supplies were obtained from Takara and Toyobo.

*Site-Directed Mutagenesis.* Site-directed mutagenesis of HRP has been reported elsewhere (Nagano et al., 1995).

*Expression and Purification of Wild-Type*[2] *and Mutant HRPs.* BL21 was used as a host strain for expression of apoHRP. An overnight culture of BL21 (20 mL of 2xTY) was inoculated into 3 L of 2xTY. Cultures were grown for 16 h at 37 °C and were harvested. Extraction of apoHRP was performed according to a procedure previously described (Adachi et al., 1993). The protein pellet was solubilized in 8 M urea/20 mM Tris-HCl (pH 8.2)/1 mM EDTA / 20 mM DTT. The solubilized apoHRP was brought to 40 mM DTT and left at 30 °C for 2 h. The reduced apoHRP was applied to a 3.5 × 100 cm column of Sephacryl S-200 HR equilibrated with 20 mM Tris-HCl (pH 8.0)/1 mM EDTA/6 M urea/2 mM DTT. Reactivation of reduced apoHRP was performed according to the method described by Gazaryan et al. (1994). The reactivated holoHRP solution (2 L) was loaded onto a 5 × 10 cm CM-52 column equilibrated with 20 mM sodium acetate (pH 4.3). At this stage, a large excess of heme was removed from holoHRP. The eluate was concentrated to 50 mL with an Amicon YM10 membrane and dialyzed against 2.0 L of 20 mM sodium acetate containing urea at pH 4.3. The concentration of the urea in the dialysis buffer was decreased stepwise (1.5, 1.0, 0.5, and 0 M, 3 h for each concentration). The protein was loaded onto a 2.5 × 10 cm CM-52 column that had been equilibrated with 20 mM sodium acetate (pH 4.3), and eluted with a linear NaCl gradient from 0 to 1.0 M. Fractions (RZ > 3.0) were pooled and brought to 50 mM sodium phosphate (pH 7.0). SDS−PAGE was utilized to check the purity of the final sample, which consisted of a single band.

*Hydroquinone Peroxidase Activity.* HRP (2 $\mu$L of a 5 $\mu$M solution) was added to a cuvette containing 50 $\mu$M hydrogen peroxide and hydroquinone in 50 mM sodium phosphate buffer (pH 7.0) at 25 °C in a final volume of 2.0 mL. The concentration of the hydroquinone was in a range from 10 to 200 $\mu$M. The oxidation rate was determined by following the increase in absorbance at 250 nm on a Shimadzu UV-2200 spectrophotometer equipped with a temperature con-

[2] Wild-type HRP is recombinant horseradish peroxidase C expressed in *Escherichia coli.*

troller (MT-602-068, Netsu Denshi). Oxidation rates were expressed as $\mu M \cdot min^{-1}$ by using a molar absorption coefficient of the hydroquinone oxidation product of 8.28 $mM^{-1} \cdot cm^{-1}$ (Farhangrazi at el., 1994).

*ABTS Peroxidase Activity.* HRP (2 $\mu$L of a 5 $\mu$M solution) was added to a cuvette containing 1.0 mM hydrogen peroxide and ABTS in 50 mM sodium phosphate buffer (pH 6.0) at 25 °C in a final volume of 2.0 mL. The concentration of the ABTS was in a range from 100 to 700 $\mu$M. The oxidation rate was determined by following the increase in absorbance at 405 nm. Oxidation rates were expressed as micromolar per second by using a molar absorption coefficient of the oxidation product of 36.8 $mM^{-1} \cdot cm^{-1}$ (Smith at al., 1990).

*Spectroscopy.* Electronic absorption spectra of HRPs in the resting state were recorded at 20 °C in quartz cuvettes. Spectra of intermediates were recorded at 2−8 °C.

Circular dichroism spectra were measured using a J-720 spectropolarimeter (JASCO) at room temperature. The light path of a sample cell was 0.2 mm. The concentration of the samples was 20 $\mu$M. α-Helical content was estimated by using the mean residue ellipticity at 222 nm.

$^1$H-NMR spectra of HRPs in their resting states (0.5−1.0 mM protein in 50 mM sodium phosphate buffer at pH 7.0) were recorded at 500 MHz on a GE Omega 500 spectrometer (Ishimori & Morishima, 1986, 1988).

*Rate Constants for the Formation and Reduction* of *Compounds **I** and **II**.* Formation and reduction of compound **I** were monitored on a stopped-flow spectrophotometer (RA401, Otsuka Electronics) at 25 °C in a 50 mM sodium phosphate buffer solution at pH 7.0. The enzyme concentration was 2.0 $\mu$M. A greater than 10-fold excess of hydrogen peroxide or phenol relative to the enzyme concentration was used to ensure pseudo-first-order kinetics. Compound **I** formation rates were monitored at absorption maxima of the Soret band (402 nm for native and wild-type and 404 nm for mutants). Reaction rates of compound **I** reduction by phenols were monitored at 412 nm. Solutions of compound **I** were prepared just before the kinetic measurement by adding a slight excess of hydrogen peroxide to a resting state enzyme solution. Rate constants were obtained by fitting the recorded data to single- or double-exponential function by using an IgorPro program (WaveMetrics). Because of the instability of mutant compounds **II** at neutral pH, the rate constants for mutant compound **II** reduction ($k_3$) could not be measured by the stopped-flow technique. Thus, the $k_3$ values of the mutants were calculated by using $k_1$, $k_2$, and the overall reaction rate ($V$). The overall reaction rate ($V$) under the steady-state condition was expressed as follows (Cormier & Prichard, 1968; Hasinoff & Dunford, 1970).

$$V = (2k_1k_2k_3[\text{HRP}][\text{substrate}][H_2O_2])/[(k_1k_2 + k_1k_3) \times [H_2O_2] + k_2k_3[\text{substrate}]]$$

*Redox Potentials of Compounds **I** and **II**.* Redox potentials of compounds **I** and **II** were measured from redox equilibria of HRP with iridate according to a method by Hayashi and Yamazaki (1979). Two milliliters of ferric HRP (10 $\mu$M) in 50 mM sodium phosphate buffer at pH 7.0 was incubated at 2 °C for 20 min. A solution of $K_2IrCl_6$ in the same buffer containing 0.01 N HCl was added (40 $\mu$M final concentration) and the mixture incubated at 2 °C for 15 min. The molar concentration of ferric HRP, $K_2IrCl_6$, and compounds **I** and **II** was calculated by deconvolution of the spectrum at equilibrium into four spectra (ferric HRP, compound **I**, compound **II**, and $K_2IrCl_6$). The deconvolution was performed using an IgorPro program. The concentration of $K_3IrCl_6$ was calculated as the difference between the initial and the remaining concentration of $K_2IrCl_6$. The redox potentials of compounds **I** and **II** are calculated using the equilibrium data and the Nernst equation. A value of 0.90 V for $E_0'(K_2IrCl_6/K_3IrCl_6)$ was used for calculation (Farhangrazi et al., 1994). All redox potentials reported herein are referenced vs NHE.

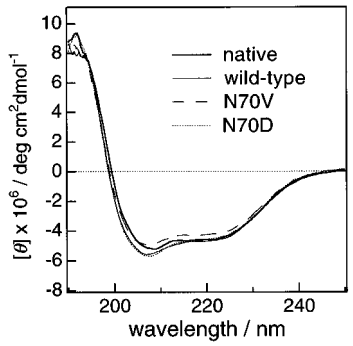



FIGURE 2: Circular dichroism spectra of resting state native (thick line), wild-type (thin line), N70V (broken line), and N70D (dotted line) HRPs in 50 mM sodium phosphate at pH 7.0 and 20 °C. The concentration of the sample is 20 $\mu$M.

## RESULTS

*Electronic Absorption Spectroscopy.* The wavelength and extinction coefficients at the absorption maxima for various oxidation and ligation states of HRPs are listed in Table 1. Inspection of Table 1 shows that the visible spectral properties of mutant HRPs in the ferric resting and ferrous states are essentially the same as those of native and wild-type HRPs. The only anomalies in the ferric resting state appear to be the increase of the extinction coefficient of Soret bands.

Oxidation of the mutants in the resting state with 1 equiv of hydrogen peroxide produced compound I that differed little from native and wild-type compounds **I**. We have already reported that N70V compound **II** is unstable at pH 7 (Nagano et al., 1995). A similar instability of N70D compound **II** was observed at pH 7, while N70V (Nagano et al., 1995) and N70D compounds **II** were very stable at pH 9 for more than 1 h at 4 °C.

*Circular Dichroism and Proton Nuclear Magnetic Resonance ($^1$H-NMR) Spectroscopy.* In native HRP, there are eight carbohydrate chains attached through surface Asn residues and there are four intramolecular disulfide bridges. The HRPs expressed in *Escherichia coli* are not glycosylated (Smith at al., 1990). The apoHRPs were reconstituted with heme in the presence of 2 M urea and were reoxidized with the oxidized form of glutathione to make disulfide bridges. It is important to confirm structural intactness of recombinant HRPs. Figure 2 shows circular dichroism spectra in the far-UV region of native and recombinant HRPs in the resting state. Essentially identical spectral shapes of the recombinant HRPs show that the secondary structures of them are comparable to those of native HRP (Table 2). This confirms that the removal of the carbohydrate and the mutations have less effect on the folding of HRP and proper disulfide bonds are formed in recombinant enzymes.

Table 1: Wavelength of Absorption Maxima in Visible Absorption Spectra of Some Derivatives for Native, Wild-Type, and Mutant HRPs at pH 7

| | enzyme | Soret | | visible | | | |
|---|---|---|---|---|---|---|---|
| $Fe^{III}$ | native | 402 | (102) | 492 | (11.3) | 642 | (2.9) |
| | wild-type | 402 | (103) | 497 | (11.4) | 644 | (3.1) |
| | N70V | 404 | (120) | 499 | (10.3) | 637 | (3.1) |
| | N70D | 404 | (125) | 499 | (11.4) | 637 | (3.4) |
| $Fe^{III}CN$ | native | 421 | (110) | | 540 | (11.4) | |
| | wild-type | 421 | (112) | | 540 | (11.9) | |
| | N70V | 421 | (122) | | 539 | (12.4) | |
| | N70D | 422 | (116) | | 538 | (12.0) | |
| $Fe^{II}$ | native | 436 | (89.8) | | 557 | (11.9) | |
| | wild-type | 436 | (89.1) | | 557 | (12.4) | |
| | N70V | 437 | (91.0) | | 557 | (11.9) | |
| | N70D | 436 | (99.0) | | 557 | (12.9) | |
| $Fe^{II}CO$ | native | 422 | (159) | 542 | (12.9) | 572 | (12.1) |
| | wild-type | 422 | (159) | 542 | (13.7) | 572 | (13.1) |
| | N70V | 422 | (169) | 541 | (13.4) | 571 | (11.9) |
| | N70D | 429 | (178) | 541 | (13.9) | 570 | (12.3) |
| compound **I** | native | 404 | (52.8) | 578 | (7.2) | 654 | (6.2) |
| | wild-type | 403 | (53.0) | 577 | (7.5) | 657 | (6.1) |
| | N70V | 398 | (50.3) | 581 | (7.2) | 649 | (6.7) |
| | N70D | 401 | (59.4) | 561 | (8.2) | 650 | (6.1) |
| compound **II** | native | 420 | (105) | 527 | (9.6) | 554 | (9.4) |
| | wild-type | 420 | (103) | 527 | (9.4) | 555 | (9.2) |
| | N70V | | | [a] | | | |
| | N70D | | | [a] | | | |

[a] Very unstable at neutral pH.

Table 2: Mean Residue Ellipticity at 220 nm and Estimated α-Helical Contents of Native, Wild-Type, and Mutant HRPs in Their Resting State

| enzyme | $-[\theta]_{222} \times 10^{-4}$ [a] | α-helix (%) |
|---|---|---|
| native | 1.48 | 41 |
| wild-type | 1.50 | 42 |
| N70V | 1.36 | 37 |
| N70D | 1.45 | 40 |

[a] Mean residue ellipticity in deg·cm$^2$·dmol$^{-1}$.

In order to gain further insight into the structural effects of the mutations, we have measured $^1$H-NMR spectra in the resting state. Figure 3 shows the hyperfine-shifted $^1$H-NMR spectra of resting state HRPs at pH 7.0 and 23 °C. The intense peaks in these spectra are due to heme methyl groups. For native HRP, four heme peripheral methyl peaks are observed at 54.6, 71.1, 74.9, and 81.9 ppm (5-, 1-, 8-, and 3-Me, respectively) (La Mar et al., 1980). The spectrum of wild-type HRP is almost identical to that of native HRP. Although resonances of 1- and 8-methyl groups of the mutants are slightly shifted and are overlapped, two methyl signals (5- and 3-methyl groups) are observed at nearly the same chemical shifts for native, wild-type, and mutant HRPs.

*Peroxidase Activities.* We have utilized hydroquinone and ABTS as substrates for the peroxidase assay. Figure 4 shows the initial oxidation rate vs substrate concentration profiles, and evaluated kinetic parameters are summarized in Table 3. While wild-type HRP exhibited hydroquinone oxidation activity comparable to that of native enzyme, the replacement of Asn70 with Val or Asp markedly depressed the activities. The most obvious difference in the parameters for hydroquinone oxidation is a lower apparent $V_{max}$ value for the mutant HRPs. Upon oxidation of hydroquinone, the $V_{max}$ values for N70V and N70D mutants are only 6 and 10% of that for the native enzyme, respectively. In contrast to the oxidation of phenols, ABTS oxidation activities of the

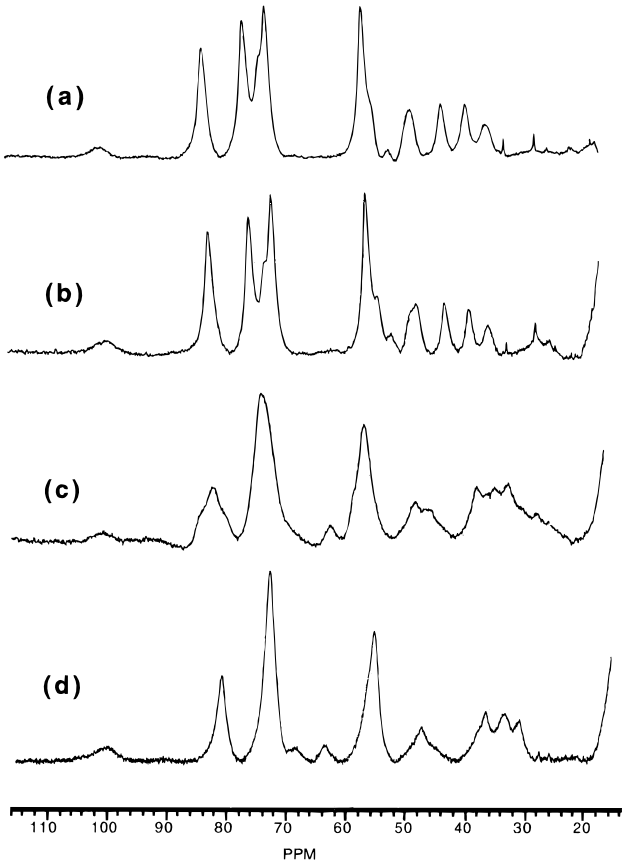



FIGURE 3: $^1$H-NMR spectra of resting state (a) native, (b) wild-type, (c) N70V, and (d) N70D HRPs in 50 mM sodium phosphate at pH 7.0 and 23 °C. The concentration of the sample is 1 mM.

mutants were significantly increased. The $V_{max}$ values for the mutants are 3-fold increased compared to those for native and wild-type HRPs. For both hydroquinone and ABTS oxidation, $K_m$ values of the mutants are almost identical to

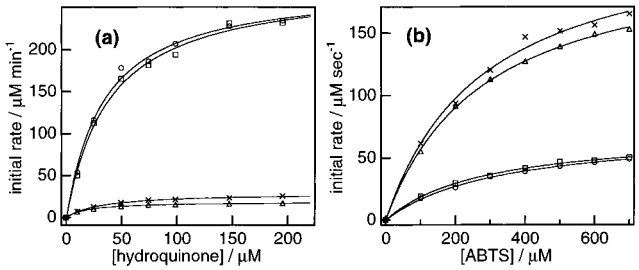



FIGURE 4: Initial reaction rate−substrate concentration profiles. (a) Hydroquinone and (b) ABTS: (○) native, (□) wild-type, (△) N70V, and (×) N70D HRPs.

Table 3: Kinetic Parameters for the Oxidation of Hydroquinone and ABTS by Native, Wild-Type, and Mutant HRPs

| | hydroquinone | | ABTS | |
|---|---|---|---|---|
| enzyme | $K_m$ ($\mu$M) | $V_{max}$ ($\mu$M·min$^{-1}$) | $K_m$ ($\mu$M) | $V_{max}$ ($\mu$M·s$^{-1}$) |
| native | 33 | 281 | 339 | 73 |
| wild-type | 37 | 283 | 286 | 71 |
| N70V | 19 | 18 | 279 | 217 |
| N70D | 33 | 29 | 278 | 234 |

Table 4: Elementary Reaction Rate Constants [a] of Native, Wild-Type, and Mutant HRPs

| enzyme | $k_1 \times 10^{-7}$ | $k_2 \times 10^{-6}$ [b] | $k_3 \times 10^{-5}$ [c] |
|---|---|---|---|
| native | 1.5 | 6.6 | 5.2 |
| wild-type | 1.4 | 6.2 | 5.7 |
| N70V | 0.12,[d] 0.03[d] | 0.12 | 0.20 |
| N70D | 0.15 | 0.45 | 0.58 |

[a] Rate constants are in M$^{-1}$·s$^{-1}$. [b] Guaiacol was used as a reducing substrate. [c] Calculated from the steady-state reaction rate ($V$) and $k_1$ *and* $k_2$ as noted in Experimental Procedures. [d] Fraction of fast and slow phases = 52:48.

those of native and wild-type HRPs.

*Kinetic Measurements. Rate Constants of Compound* ***I*** *Formation ($k_1$), Reduction ($k_2$), and Compound* ***II*** *reduction ($k_3$).* In order to clarify the effect of the mutation on the peroxidase activities, kinetic measurements were made at pH 7 for native, wild-type, and mutant HRPs. The rate constants $k_1$, $k_2$, and $k_3$ are listed in Table 4. As already reported, $k_1$ and $k_2$ of N70V were reduced to less than 10% of that of the native enzyme (Nagano et al., 1995). A very similar decrease in $k_1$ and $k_2$ of N70D was also observed. Due to the instability of mutant compounds **II** at pH 7, we calculated the rate constants of the compound **II** reduction ($k_3$) on the basis of expression of the overall reaction rate ($V$) under the steady state condition. Calculated $k_3$ values for N70V and N70D HRP were reduced to 7 and 11% of the value of the native enzyme, respectively.

All three elementary reaction rate constants of the mutants were reduced to nearly the same extent compared to those of native HRP. Keeping in mind that reduction of compound **II** is the rate-determining step in general peroxidase cycles, a decrease in $k_3$ mainly contributes to the apparent lower phenol oxidation activities under the steady state condition.

*Substituent Effect on Compound* ***I*** *Reduction by Phenol.* Rate constants for the reduction of compound **I** by various *meta*- or *para*-substituted phenols were measured. The $k_2$ values were plotted against Hammett $\sigma$ (Figure 5), and $\rho$ values are summarized in Table 5. As reported by Job and Dunford (1976), the reduction rate of compound **I** by phenols is greatly dependent on the substituent effects. Inspection

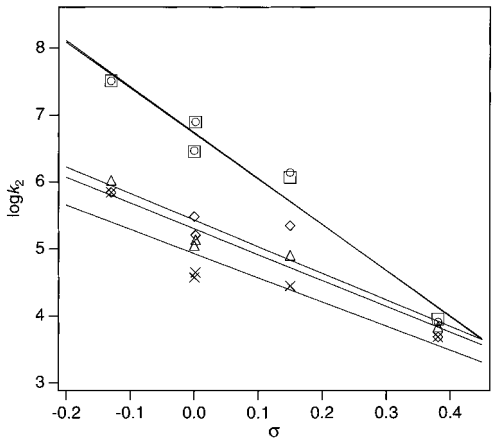



FIGURE 5: Hammett plots for the rate constants of compound **I** reduction by various phenol derivatives. For clarity, log $k_2$ instead of log($k_2^X/k_2^H$) was plotted on the ordinate. Biphasic reactions were observed for the reaction with N70V compound **I** and *p*-OMe-, *m*-OEt-, and, *m*-CHO-substituted phenols: (○) native, (□) wild-type, (◇) N70V fast phase, (×) N70V slow phase, and (△) N70D $HRP_5$.

of Figure 5 and Table 5 shows that the rate constants for the reactions of methoxyphenol with N70V and N70D compounds **I** were only 2 and 3% of that for native compound **I**, respectively, whereas the mutant compounds I can oxidize hydroxybenzaldehyde as slowly as native compound **I**.

*Redox Potentials of Compounds* ***I*** *and* ***II*****.** Like compound **II** of N70V HRP (Nagano et al., 1995), N70D compound **II** was unstable at neutral pH. Farhangrazi et al. (1994) also reported an unstable compound **II** of ARP[3] and attributed the instability to the high redox potential of ARP compound **II**. To gain insight into the instability of the mutant compounds **II**, we have measured redox potentials of compounds **I** and **II** (Table 6). The redox potentials of native and wild-type HRPs are in close agreement with those measured electrochemically (Farhangrazi et al., 1995). As expected, redox potentials of N70V and N70D compounds **II** are 95 and 108 mV higher than that of native compound **II**, respectively. The higher redox potentials imply that mutant compounds **II** are readily reduced to the resting state by substrates. The instability of mutant compounds **II** and enhanced ABTS oxidation activities of the mutants are explained by the high reduction potentials of mutant compounds **II**.

## DISCUSSION

*Reaction of Resting State HRP with Hydrogen Peroxide ($k_1$ Process).* The distal His in peroxidases has been shown to participate in the reaction with hydrogen peroxide (Poulos & Kraut, 1980; Erman et al., 1992, 1993; Newmyer & Ortiz de Montellano, 1995). Scheme 1 depicts a proposed role of the distal His compound **I** formation (Poulos & Kraut, 1980). In the first step, the distal His serves as a base to accept a proton from a hydrogen peroxide to form a peroxide intermediate as a reversible process ($K_1$, equilibrium constant for step 1). Dunford et al. (1978) measured a significant

[3] Another candidate for a hydrogen bond partner in compound **II** is the Arg residue in the distal site. However, the p$K_a$ value of the acid−base transition is 8.5 for HRP C and 6.9 for HRP $A_1$ (Hashimoto et al., 1986; Makino et al., 1986; Sitter et al., 1985), respectively. These p$K_a$ values are too low to be assigned to an Arg residue, since the distal pocket has a polar atmosphere.

Table 5: Rate Constants[a] for the Reduction of Compounds I by Various Substituted[b] Phenols

| | substituent | | | | | |
|---|---|---|---|---|---|---|
| enzyme | *p*-OMe | *m*-OH | H | *m*-OEt | *m*-CHO | $\rho$[c] |
| native | $3.25 \times 10^7$ | $7.90 \times 10^6$ | $2.93 \times 10^6$ | $1.38 \times 10^6$ | $8.01 \times 10^3$ | −6.9 |
| wild-type | $3.24 \times 10^7$ | $7.86 \times 10^6$ | $2.85 \times 10^6$ | $1.16 \times 10^6$ | $8.96 \times 10^3$ | −6.8 |
| N70V (fast) | $6.94 \times 10^5$ | $1.60 \times 10^5$ | $3.01 \times 10^5$ | $2.20 \times 10^5$ | $4.85 \times 10^3$ | −4.0 |
| N70V (slow) | | $4.46 \times 10^4$ | $3.77 \times 10^4$ | $2.81 \times 10^4$ | | −3.6 |
| N70D | $1.05 \times 10^6$ | $1.36 \times 10^5$ | $1.12 \times 10^5$ | $8.03 \times 10^4$ | $6.86 \times 10^3$ | −3.8 |

[a] Rates are in $M^{-1}\cdot s^{-1}$. [b] Substituent constants ($\sigma$): −0.13 (*p*-OMe), −0.002 (*m*-OH), 0.15 (*m*-OEt), and 0.381 (*m*-CHO). [c] Reaction constant.

Table 6: Redox Potentials[a] of Compounds **I** and **II** of Native, Wild-Type, and Mutant HRPs

| | native[b] | native | WT | N70V | N70D |
|---|---|---|---|---|---|
| $E_0'$ (compound **I**/compound **II**) | 0.891 | 0.886 | 0.887 | 0.829 | 0.860 |
| $E_0'$ (compound **II**/ferric) | 0.869 | 0.862 | 0.879 | 0.957 | 0.970 |

[a] Redox potentials are in volts. [b] At pH 7.0 and 25 °C; Farhangrazi et al., 1995.

kinetic isotope effect ($k_H/k_D = 1.6$), which supports proton abstraction from peroxide in the $k_1$ process. The following heterolytic O-O bond cleavage of the peroxide intermediate affords compound I ($k_{O-O}$: rate constant for step 2). In the transition state of the O−O bond cleavage, the hydrogen bond between the leaving hydroxide and the distal His favors heterolysis of the O−O bond. Thus, the rate constant for compound **I** formation ($k_1$) is expressed by using $K_1$ and $k_{O-O}$.

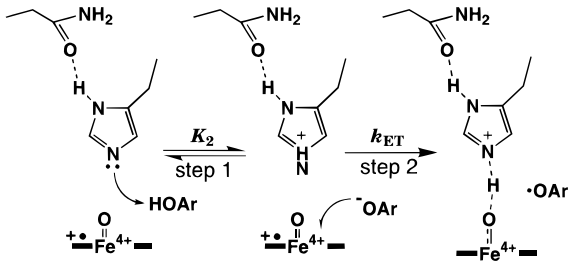
$$k_1 = K_1 k_{O-O}$$

The disruption of the hydrogen bond between Asn70 and the distal His could reduce the basicity of the distal His, resulting in a smaller equilibrium constant ($K_1$) to retard the apparent compound **I** formation (Table 4). The lower basicity of the distal His was confirmed by resonance Raman study (Mukai et al., *manuscript in preparation*). On the basis of the pH titration of the Fe−His(proximal) stretching frequency, p$K_a$ values of the mutant His were estimated to be 5.9 for N70V and 5.5 N70D. These values are much lower than those for native (7.2) and wild-type (7.2) HRPs[4] (Teraoka & Kitagawa, 1981; Kitagawa, 1988). These results indicate that the disruption of the distal site Asn−His couple makes the distal His less basic.

HRP isozyme $A_2$ is another illustration of the same relationship between the p$K_a$ value and the rate constant for the reaction with peroxide. The p$K_a$ value of HRP $A_2$ is 5.5, and its rate constant is significantly smaller than that of native HRP C (HRP C, $1.5 \times 10^7$ $M^{-1}\cdot s^{-1}$; HRP $A_2$, $0.2 \times 10^7$ $M^{-1}\cdot s^{-1}$) (Marklund et al., 1974). We, consequently, conclude that the smaller rate constants for the reaction with hydrogen peroxide are attributed to the less basic distal His arising from the disruption of the Asn−His couple.

The structural intactness of the mutant HRPs in the resting state is confirmed by the following observations. (1) The CD spectra in the resting state of the mutants are indistinguishable from that of the native enzyme. The secondary structure (α-helical contents) of the mutants is very similar to that of native enzyme (Figure 2, Table 2). (2) The Asn70 mutations exert little influence on the proton resonance peaks from two heme methyl groups (5- and 3-Me) (Figure 3). These spectroscopic properties indicate that the mutant HRPs are folded properly and the mutants have similar heme environmental structure in the resting state.

Scheme 2: Plausible Mechanism for Compound I Reduction




The resonance Raman studies indicate that six- and five-coordinated high-spin hemes coexist in the mutants (Mukai et al., manuscript in preparation). The partial occupation of the sixth coordination site of the heme, as well as the decrease of the basicity of the distal His, could account for the depression of the reaction with hydrogen peroxide, since the six-coordinated heme must undergo ligand exchange before reacting with hydrogen peroxide. However, it has been reported for the predominantly 6-coordinated C*c*P mutant (W51A) that the reaction with hydrogen peroxide was not seriously discouraged (WT, $3.03 \times 10^7$; W51A, $1.5 \times 10^7$ $M^{-1}\cdot s^{-1}$) (Goodin et al., 1991). Therefore, the existence of water molecule as a sixth ligand of the hemes in N70V and N70D HRPs cannot account for the discouragement of the reactivities with hydrogen peroxide.

*Reduction of Compounds **I** and **II** ($k_2$ and $k_3$ Processes).* In the reaction of compound **I** with phenols, the distal His abstracts a proton from the substrate (base catalysis) to form a phenoxide anion as a reversible process (Ortiz de Montellano, 1987; Dunford, 1982) (Scheme 2). The proton transfer is followed by electron transfer from the substrate to the heme, giving compound **II**. Thus, the rate constant for the reduction of compound **I** ($k_2$) would be expressed by using $K_2$ (equilibrium constant of step 1) and $k_{ET}$ (rate constant of step 2).

$$k_2 = K_2 k_{ET}$$

The involvement of an electron transfer process in the rate-determining step has been suggested by negative Hammett

[4] The reviewer pointed out that the p$K_a$ values obtained from the stretching mode of ferrous iron−proximal His are inconsistent with the pH dependence of the reaction between ferric HRP C and $H_2O_2$ (Dunford, 1991). Although we have tried to estimate the p$K_a$ values in the ferric state by use of NMR, we have not yet found any significant p$K_a$ values. This remains to be solved.

$\rho$ values for all HRPs (Table 5). However, the deprotonation process ($K_2$) should show positive Hammet $\rho$ values. Thus, the apparent $k_2$ is the result of these two inverse effects. While the observation of negative Hammet $\rho$ values for the apparent $k_2$ of HRPs indicates that the substitution effect on $k_{ET}$ is dominant, the substituent effect on the deprotonation step must be more effective in the reactions of phenols with the mutants, since it is much harder for the mutants to abstract a proton from electron rich phenols such as methoxyphenol. These considerations suggest that substituent effects on the apparent $k_2$ of phenol oxidation could show smaller negative Hammet $\rho$ values, when the mutants are employed. In fact, upon the oxidation of methoxyphenol, a less acidic phenol ($\sigma_{OMe} = -0.13$) is used as a substrate, and the decreased basicity of the distal His substantially depresses the proton abstraction (step 1) compared to that of native compound **I**. The resultant smaller $K_2$ values drastically retard the overall reduction rate of mutant compounds **I** by methoxyphenol to 2−3% of that for native enzyme. On the other hand, upon the oxidation of hydroxybenzaldehyde, an acidic phenol ($\sigma_{CHO} = 0.381$), the less basic distal His of the mutants readily abstract proton from the substrate, while the following one-electron transfer from the phenolate must be very hard for both native and mutant HRPs because of the introduction of an electron-withdrawing aldehyde. Therefore, the mutant compounds I can oxidize hydroxybenzaldehyde as *slow* as native compound **I** (Table 5, Figure 5). However, as noted above, the large negative substituent effects of phenols ($\rho = -6.9$ to $-3.8$) on the reduction of compounds **I** are the indication of the involvement of the one electron transfer process at the rate-determining step. At the same time, these results also indicate the participation of the deprotonation step in $k_2$ process.

Like the $k_1$ and $k_2$ processes, rates of the reduction of mutant compounds **II** by guaiacol were also reduced to about 10% of that of the native enzyme. Previous resonance Raman studies on HRP compound **II** suggest that the distal His donates a hydrogen bond to ferryl oxygen at neutral pH (Hashimoto et al., 1986; Makino et al., 1986; Sitter et al., 1985).[3] It has been suggested that the reactivity of native compound **II** is controlled by the hydrogen bond strength on the basis of the following observations. (1) When the pH is raised, the hydrogen bond between the distal His and the oxo ligand is disrupted and the reactivity of native compound **II** was drastically reduced (Makino et al., 1986). (2) The diacetylheme-substituted compound **II**, which has a weaker hydrogen bond between the distal His and ferryl oxygen, exhibited a lower reactivity than native compound **II** (Makino et al., 1986). The replacement of Asn70 is expected to affect the hydrogen bond between the distal His and ferryl oxygen and the $Fe^{IV}$═O stretching frequency through the hydrogen bond network (Asn70···His···O═$Fe^{IV}$). As expected, the $Fe^{IV}$═O stretching frequency of the mutant compounds **II** was 6 $cm^{-1}$ higher than that of native compound **II** at neutral pH, which indicates that the hydrogen bond between ferryl oxygen and the distal His is substantially weakened (Mukai et al., manuscript in preparation). Consequently, we attribute the lower reactivities of mutant compounds **II** to the weaker hydrogen bond between the distal His and the ferryl oxygen.

*ABTS Oxidation Activity and Redox Potentials of Compounds I and II*. The mutant compounds **II** exhibits redox potentials ∼100 mV higher than that of native compound **II**. Farhangrazi et al. (1994) have reported that the redox potential of ARP compound **II** was as high as those of the mutant compounds **II** and the $V_{max}$ value for ABTS oxidation by ARP was 3.9 times greater than that of native HRP (Akimoto et al., 1990). Accordingly, the high reduction potentials of compounds **II** result in the high ABTS oxidation activities of the mutants.

The reason why the elimination of the hydrogen bond makes their redox potential higher is still ambiguous. Some electrostatic interaction between the heme iron and the polar group(s) or bonding interaction through the proximal ligand might occur in mutant compounds **II**. The detailed active site structures of mutant compounds **II** are under investigation.

*Hydrogen Bond Network in the Distal Site*. As demonstrated in this study, replacement of Asn70 with Val disrupts the hydrogen bond between the Asn and the distal His. The disruption of the hydrogen bond alters each elementary reaction rate and redox potentials of compound **II**. There is little difference between N70V and N70D with regard to their spectroscopic properties and reactivities, even though the Asp residue is a potent hydrogen bond acceptor. Recently, similar hydrogen bond disruption by replacement of Asn with Asp has been reported for C*c*P by Satterlee et al. (1994). The crystal structure of C*c*P indicates that Asn82 donates another hydrogen bond to the peptide carbonyl oxygen atom of Glu76 (Figure 1). The hydrogen bond between Asn and Glu is also conserved among many peroxidases (Baunsgaard et al., 1993; Finzel et al., 1984; Poulos et al., 1993; Edwards et al., 1993; Kunishima et al., 1994; Fukuyama et al., 1995; Sundaramoorthy et al., 1994; Petersen et al., 1994; Patterson & Poulos, 1995). Comparison of the amino acid sequence of HRP and C*c*P indicates that the equivalent residue is Glu64 in HRP (Welinder, 1985). Because the Asp residue ($CH_2$-COOH, $pK_a = \sim 4$) is expected to be deprotonated in the polar distal site of peroxidase having the distal His and Arg, Asp cannot act as a hydrogen bond donor to the peptide carbonyl group of Glu76 (C*c*P) or -64 (HRP). In other words, aspartates at position 82 in C*c*P and at position 70 in HRP are not able to compensate for the role of Asn. This consideration suggests that the peptide carbonyl oxygen of Glu anchors the distal His through the hydrogen bond network and is required for peroxidase catalysis.

*Peroxidase Catalysis and Active Site Structure*. Strong electron donation from the anionic proximal His to the heme iron has also been regarded as a predominant structural and functional factor which discriminates peroxidases from other hemoproteins (push effect; Yamaguchi et al., 1992, 1993). Especially it has been demonstrated the critical role of the Cys ligand of P450s in the peroxide O−O bond cleavage step (Adachi et al., 1991, 1993; Higuchi et al., 1993). However, the anionic nature of the proximal His is not critical in high rates of compound **I** formation in peroxidases, though it has a direct connection to the heme iron (Sundaramoorthy et al., 1991; Choudhury et al., 1992, 1994). On the other hand, the critical role of distal site residues has been demonstrated, especially the distal His, by site-directed mutagenesis (Erman et al., 1992, 1993; Newmyer & Ortiz de Demontellano, 1995). In this study, we have demonstrated that the replacement of the distal site Asn with Val or Asp decreased the basicity of the distal His to suppress compound I formation and reduction steps, though the Asn

residue is more than 10 Å from the heme. These results lead to the conclusion that the acid−base catalysis of the distal His is controlled by the distal site Asn through the hydrogen bond network.

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