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PUTATIVE HYDROGEN BOND NETWORK IN THE HEME DISTAL SITE OF HORSERADISH PEROXIDASE

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Summary: The $N_{\delta 1}$ atom of the distal His of several peroxidases is known to make a hydrogen bond with the side chain oxygen of Asn. Thus, a mutant horseradish peroxidase, in which Asn70 is replaced by Val, has been expressed in *Escherichia coli* to disrupt the putative hydrogen bond. Substitution of Asn70 to Val reduces the rate constant for the compound I formation from 1.6×10^7 (native) to 6×10^5 $M^{-1}s^{-1}$. The rate constant for reduction of compound I of N70V by guaiacol has been also reduced from 7.8×10^6 (native) to 1.2×10^5 $M^{-1}s^{-1}$. While compound I of N70V is stable and reduced to the resting state of the mutant without apparent formation of compound II at neutral pH, compound II of N70V is obtained as a stable intermediate at alkaline pH. Similar alteration of the reactivity has been observed in the reaction with guaiacol. © 1995 Academic Press, Inc.

Peroxidases catalyze various types of oxidations by utilizing hydrogen peroxide. For instance, in the catalytic cycle of HRP, the resting ferric enzyme is first oxidized by hydrogen peroxide to yield an intermediate called compound I, which is then reduced by substrates to the resting state via the intermediate called compound II [1]. On the basis of the crystallographic structures of several peroxidases [2], the major role of the distal His in peroxidases is considered to be a general acid-base catalyst [3]. The important role of the distal His has been further demonstrated by site-directed mutagenesis studies of CcP [4]. For all the peroxidases whose crystal structures are available [2], the $N_{\delta 1}$ nitrogen atom of the distal His of the peroxidases forms a hydrogen bond to the side chain oxygen atom of Asn. Very recently, Satterlee and co-workers have shown that the disruption of the hydrogen bond by substitution of Asn82 to Asp in CcP caused disappearance of three hyperfine shifted resonances of distal His in the ^{1}H-NMR spectrum of the cyanide complex [5]. The disruption of the hydrogen bond is expected to change

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Abbreviations: CcP, cytochrome *c* peroxidase; HRP, horseradish peroxidase; ARP, peroxidase from *Arthromyces ramosus*; PCR, polymerase chain reaction.

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          42                                  70
HRP  RLHFHDC--FVNGCDASILLDNTTSFRTEKDAFGNANSARGF
     | |  |                |  |  |     | |   | |
CcP  RLAWHISGTWDKHDNTGGSYGGTYRFKKEFNDPSNAGLQNGF
          52                                  82
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Figure 1. Alignment of horseradish peroxidase and yeast cytochrome *c* peroxidase amino acid sequences in the distal His containing region [6].

the basicity of the distal His. The alteration of the basicity will affect the catalytic activity of the distal His to form compound I.

Though high resolution X-ray crystal structure of HRP is not available, inspection of the amino acid sequence alignment of HRP as compared to that of *Cc*P [6] suggests possible formation of the hydrogen bond between the distal His (His42) and Asn70 in HRP (Figure 1). Slow proton exchange of the distal His $N_{\delta 1}H$ of HRP with bulk solvent [7] supports the involvement of the $N_{\delta 1}$ nitrogen atom as a hydrogen donor. Thus, we have prepared mutant HRP (N70V) in which Asn70 has been replaced by Val to disrupt the hydrogen bond. In addition, Asn72 was also substituted by Val (N72V) and we have investigated peroxidase activity of these mutant HRPs.

MATERIALS AND METHODS

Site-Directed Mutagenesis, Protein Preparation, and Purification: HRP gene (BBG10) was purchased from British Bio-technology Ltd. The *Nde*I-*Eco*RI fragment from the BBG10 was inserted into T7 expression vector to form T7HRP expression vector. Desired mutations were introduced into the T7HRP expression vector by use of a PCR-based technique. No additional mutations were ensured by sequencing the whole HRP gene. Recombinant HRPs were prepared as described by Smith *et al.* [8], with some modifications.

Spectroscopy: Electronic absorption spectra of HRPs in resting state were recorded at 20°C in quartz cuvettes on Shimadzu UV-2200 spectrophotometer. Spectra of intermediates were recorded on Shimadzu UV-2200 spectrophotometer equipped with temperature controller (MT-602-068, Netsu Denshi). Hyperfine-shifted ^{1}H-NMR spectra were recorded at 500 MHz on a GE Omega 500 spectrometer.

Rate Constants of the Compound I Formation and Reduction: Formation and reduction of compound I were monitored on a stopped-flow spectrophotometer (RA401, Otsuka Electronics) at 25°C in 50 mM sodium phosphate buffer, pH 7.0. Rate constants were obtained by fitting the recorded data to exponential functions by using a least square minimization procedure.

Assay of Peroxidase Activity: The activities of guaiacol oxidation by native, [1]wild-type,[1] and mutant HRPs were determined on a stopped flow spectrophotometer at 25°C. H_2O_2 (2 mM) in 50 mM buffer in one syringe was mixed with an equal volume of the same buffer containing 10.2 mM guaiacol and 20 nM enzyme in the other syringe. The reaction was monitored at 470 nm, and the rate of guaiacol oxidation was determined from the data obtained in the initial 1 sec.

RESULTS AND DISCUSSION

Electronic Absorption and ^{1}H-NMR Spectra of Resting Forms of HRPs: Electronic absorption spectra of carefully purified native, wild-type, and N70V HRPs in the

[1] Native HRP: peroxidase (mainly isozyme C) isolated from *horseradish*;
wild-type HRP: recombinant horseradish peroxidase C expressed in *Escherichia coli*.

resting forms are shown in Figure 2. N70V HRP shows similar spectral features (λ_{max} = 637, 499, 404 nm) to those of the native (λ_{max} = 642, 492, 402 nm) and wild-type (λ_{max} = 644, 497, 402 nm) HRPs. The RZ values (A_{Soret}/A_{280}) of native and N70V HRPs are 3.2 and 4.0, respectively, which are sufficient for assay of peroxidase activities. Alteration of Asn70 to Val caused significant increase of the extinction coefficient of the Soret band (ε_{Soret} = 102, 103, 120 mM^{-1} cm^{-1} for native, wild-type, and N70V HRPs, respectively).

In the hyperfine-shifted ^{1}H-NMR spectra of the resting forms of native and N70V HRPs, appearance of the four heme peripheral methyl peaks of these HRPs at the same chemical shifts (82.9, 75.7, 72.0, 55.2 and 82.6, 75.1, 72.6, 55.4 ppm for native and N70V HRPs, respectively) suggests that the electronic structure of the heme in N70V HRP is not altered by the mutation (spectra not shown). Further, the spectra of cyanomet forms of native and wild-type HRPs are essentially identical [9], indicating the heme environmental structures of the HRPs used in this study are highly similar.

Formation and Electronic Absorption Spectra of Compound I and II of HRPs: At neutral pH, the addition of a small excess of H_2O_2 to a resting form of N70V HRP yielded stable compound I at 2°C with half-life of 4 hours. The electronic absorption spectrum of compound I of N70V HRP is similar to those of native and wild-type HRP compound I. When titrated with ferrocyanide, compound I of N70V HRP was reduced to the resting form without showing the one electron reduced intermediate, compound II (data not shown). It is commonly observed that HRP compound I is reduced stepwise to compound II and the resting enzyme even without addition of reductant [10]. Thus, we have measured time-dependent spectral changes of compounds I of wild-type and N70V HRPs without addition of any reductants. The compound I of wild-type HRP was gradually reduced to ferric enzyme via compound II (Figure 3a). Formation of compound II of N70V HRP was not so clear as in the case of wild-type HRP.

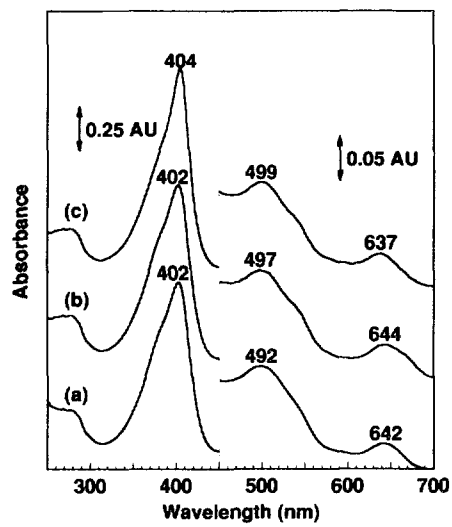


Figure 2. Electronic absorption spectra of (a) native, (b) wild-type, and (c) N70V HRPs in 50 mM sodium phosphate buffer at pH 7.0 and 20°C. Concentration of samples is 10 μM.

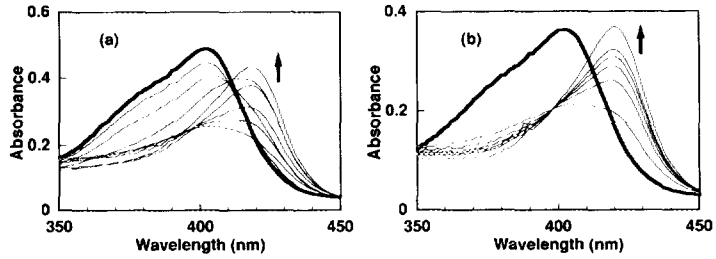


Figure 3. Time dependent spectral changes of wild-type HRP after addition of a small excess of hydrogen peroxide. The thick line indicates the spectrum before the addition of peroxide. (a) 4.8 μM wild-type HRP in 50 mM sodium phosphate buffer,pH 7.0, at 20°C. (b) 3.6 μM wild-type HRP in 50 mM sodium borate buffer,pH 10.0,at 20°C. Repetitive scans showing the reduction of compound I of native HRP were recorded at (a) 0, 2, 35, 55, 60, 65, 72,85 min and (b) 0, 1, 2, 3, 4, 5, 14 min after addition of peroxide.

However, the presence of shoulder around 420 nm even 1 hour after the formation of N70V compound I suggests the accumulation of a significant amount of compound II during the reduction (Figure 4a). On the contrary, the addition of a small excess amount of H_2O_2 to the wild-type (Figure 3b) and N70V (Figure 4b) HRPs *at pH 10* gave compounds I, followed by the complete conversion to compounds II. The stability of compounds I and II of N70V HRP is thus pH dependent; compound I is stable at neutral pH, whereas compound II is stable at alkaline pH. Very interestingly, similar pH dependency was reported by Farhangrazi *et al.* for ARP [11].

With the fact that a distal basic group, probably the distal His, forms hydrogen bond to the oxo-ligand of compound II in HRP [12], the present results imply that the reactivity of HRP compound II would be further influenced through a hydrogen bond network.

Rate Constants for the Reaction between HRPs and Hydrogen Peroxide: The distal His in peroxidase has been shown to participate in the reaction with hydrogen peroxide [4]. Scheme 1 depicts a proposed role of the distal His for the compound I formation [3]. In the first step, hydrogen peroxide approaches the heme pocket (step 1) and the

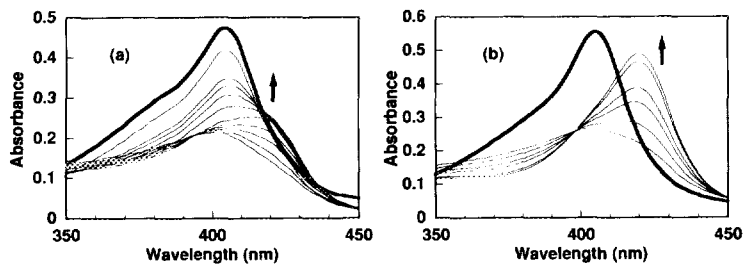
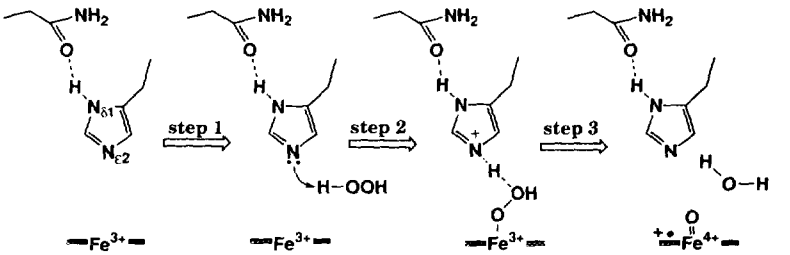


Figure 4. Time dependent spectral changes of N70V HRP after addition of a small excess of hydrogen peroxide. The thick line indicates the spectrum before the addition of peroxide. (a) 3.8 μM N70V HRP in 50 mM sodium phosphate buffer,pH 7.0,at 4°C. (b) 4.6 μM N70V HRP in 50 mM sodium borate buffer,pH 10.at 4°C. Repetitive scans showing the reduction of N70V compound I were recorded at (a) 0, 4, 20, 60, 180, 195, 210, 245, 260, 275 min and (b) 0, 4, 26, 46, 66, 170 min after addition of peroxide.



Scheme 1. Peroxidase catalyzed heterolytic cleavage of O-O bond of hydrogen peroxide.

distal His abstracts a proton from hydrogen peroxide to give a HRP-OOH complex with concomitant formation of histidine imidazolium (step 2). The following heterolytic O-O bond cleavage of the peroxide complex affords compound I. In the transition state of the O-O bond cleavage, the leaving hydroxide could form hydrogen bond with the distal His to favor the heterolysis of the O-O bond of peroxide (step 3, *Pull effect*). Consequently, Asn70 could play an important role in the general acid-base catalysis of the distal His through the hydrogen bond between the side chain oxygen atom of Asn70 and the $N_{\delta 1}$ nitrogen atom of the distal His. Thus, we have examined effects of disruption of the hydrogen bond on the rate constant of the compound I formation (k_1) (Table 1). The value of k_1 for the native HRP is in close agreement with the values reported by Shiro *et al.* (1.6×10^7 $M^{-1}s^{-1}$) [13], Loach *et al.* (1.8×10^7 $M^{-1}s^{-1}$) [14], and Smith *et al.* (1.69×10^7 $M^{-1}s^{-1}$) [8b]. The rate constant for wild-type HRP also agrees with the value reported by Smith *et al.* (1.64×10^7 $M^{-1}s^{-1}$) [8b]. As expected, the substitution of Asn70 to Val drastically depressed k_1 to 6×10^5 $M^{-1}s^{-1}$, since the disruption of the hydrogen bond makes the distal His less basic to discourage the step 2 in Scheme 1. As well as the basicity change of the distal His, loss of appropriate geometry of the distal His in N70V HRP could be an alternative explanation, though the geometry change can not justify the pH dependent reactivity of compound II.

Peroxidase Activity: Peroxidase-catalyzed phenol oxidation involves proton abstraction from the substrate by a distal basic group, probably the distal His of peroxidase compound I [1a]. Therefore, we have examined guaiacol oxidation by native, wild-type, and mutant HRPs. Relative activities (with respect to wild-type HRP) of guaiacol oxidation by HRPs were determined by observing the oxidation product formation of guaiacol at 470 nm (steady state kinetics). Figure 5 shows the pH dependency of guaiacol oxidation activities of native, wild-type, and mutant HRPs. Native, wild-type, and N72V HRPs give almost the same activity-pH

Table 1. Rate constants for compound I formation and reduction of native, wild-type, and N70V HRPs

protein	$k_1 \times 10^7$ M^{-1} s^{-1}	$k_2 \times 10^6$ M^{-1} s^{-1}
native	1.6	7.8
wild-type	1.2	7.4
N70V	0.06	0.12

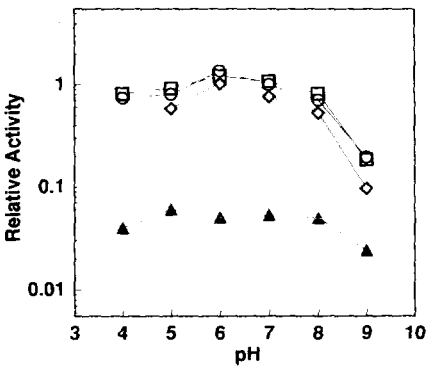


Figure 5. pH dependence of the guaiacol oxidation activities of (○) native, (□) wild-type, (◇) N72V, and (▲) N70V HRPs.

profiles. On the contrary, the activity of N70V HRP is drastically reduced at acidic and neutral pH regions. Little effect of the N72V mutation on guaiacol oxidation activity is a further support of the disruption of the hydrogen bond only in N70V HRP.

Rate Constants of Compound I Reduction by Guaiacol: In order to clarify the effect of the hydrogen bond disruption on the guaiacol oxidation catalyzed by HRP, the rate constants for compound I and compound II reduction by guaiacol (k_2 and k_3, respectively) should be measured. However, due to the instability of compound II of N70V HRP at neutral pH, we have determined k_2 at pH 7 for the native, wild-type, and N70V HRPs (Table 1). The rate constants of native and wild-type HRPs are in good agreement with those reported by Yamazaki *et al.* (9×10^6 $M^{-1}s^{-1}$) [15]. Apparently, the rate constant of N70V HRP is much smaller than those of the native and wild-type enzymes.

A recent Laue diffraction study on the structure of C*c*P shows tiny movement of the distal His (His52) away from in C*c*P compound I [16]. Thus, the hydrogen bond between Asn82 and the distal His in C*c*P compound I is conserved during the catalytic cycle of the peroxidase reactions. We expect the hydrogen bond between Asn and the distal His to be conserved even in HRP compound I. Accordingly, the replacement of Asn70 to Val have caused the suppression of the reaction rate of N70V compound I with guaiacol.

Though detailed active site structure of N70V HRP is still under investigation, we have shown for the first time that Asn70 in HRP is a very important amino acid residue for the catalytic activities of HRP. We have attributed these observations to the disruption of the hydrogen bond network from Asn70 to substrates such as hydrogen peroxide and guaiacol through the distal His.

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