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Structure–activity relation of horseradish peroxidases as studied with mutations at heme distal and proximal sites

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Abstract The distal His-Asn-Glu hydrogen bond network in the heme distal site is highly conserved among various fungal and plant peroxidases. To gain an insight into the functional roles of this hydrogen network in peroxidase activity, we have mutated the Asn70 to Val (N70V) or Asp (N70D), the Glu64 to Pro (E64P), or Gly (E64G) or Ser (E64S) in horseradish peroxidase. All the mutants disrupted the distal His-Asn hydrogen bond with maintaining the heme electronic structures as revealed by ^{1}H NMR spectra and exhibited substantial depression of the peroxidase activity. The depression of the activity was ascribed to the decreased basicity and dislocation of the distal His induced by these mutations. The replacement of the distal His by Glu (H42E) to mimic the heme distal site of chloroperoxidase impaired the peroxidase activity. We have also studied F221W HRP mutant in which the proximal Phe is replaced with Trp to mimic the heme proximal structure of cytochrome *c* peroxidase and shown that the HRP-type compound I was formed, followed by transformation to the CcP-type Trp radical.

Introduction

Horseradish peroxidase (HRP), a prototypical hemoprotein peroxidase, catalytically oxidizes small substrates to free radical products, notably the conversion of phenol to phenoxy radical. The ferric state (resting state) of the enzyme reacts with hydrogen peroxide to give compound I, a two-electron oxidized species in which the heme is oxidized to a ferryl porphyrin π cation radical. The compound I is reduced back to the resting state by substrates in the two successive reactions. At first, a substrate molecule reduces the compound I to the ferryl porphyrin species termed as compound II and, subsequently, the ferryl species is reduced by another substrate to regenerate the resting state.

Rapid reactions of the resting peroxidases with peroxides to form compound I discriminate the peroxidases from other classes of hemoproteins such as globins. Among amino acid residues in the heme cavity of peroxidase, distal histidine (His) is essential as a general acid-base catalyst (1, 2). Inspection of crystal structures in the distal site of peroxidases indicates the formation of a hydrogen bond network from the distal His to a distal calcium ion through asparagine (Asn), gulutamic acid (Glu) and a water molecule (4-7) (Fig. 1). Contrary to peroxidases, the hydrogen bond network could not be found in the distal site of globins, even though they also have the distal His in their heme pockets. While this observation suggests the hydrogen bond network improves the function of the distal His as a general acid-base catalyst in formation of compound I, roles of the hydrogen bond network has not been elucidated yet. In contrast to general peroxidases, cytochrome *c*

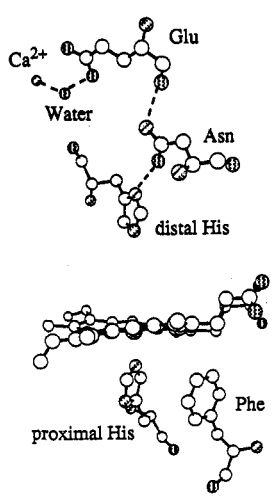

Fig. 1 Heme envirinmental structure of peanut peroxidase (7).

peroxidase (CcP) comound I contains a Trp191 radical instead of π-cation radical (8). The corresponding residue to Trp191 in CcP is Phe221 in HRP (7) (Fig. 1), suggesting these proximal residues determine the radical center in peoxidase compoiund I.

In order to examine roles of these amino acid residues in the heme environment, we have prepared several site-directed mutant HRPs and examined catalytic and structural properties utilizing various spectroscopies.

Results and Discussion

Asn70 mutants: There are highly conserved hydrogen bonds between the distal His and 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 (Fig. 1). To investigate catalytic roles of the hydrogen bond, Asn70 in HRP was replaced with Val (N70V) or Asp (N70D) (9).

While UV-vis and CD spectra of native (plant enzyme), wild-type (recombinant enzyme), and mutant HRPs in the resting states suggest that the secondary structure is similar even after the mutation, the mutants exhibit low *V*max values for the hydroquinone oxidation (native: 281, wild-type: 283, N70V: 18, N70D: 33 $\mu M \cdot min^{-1}$). The rates of compound I formation were decreased to less than 10% of the native enzyme (TABLE 1). The reduction rates of compounds I and II by guaiacol also were reduced to less than 10% of the native enzyme. Substituent effects of various phenol derivatives on the reduction of compounds I of native, wild-type, and the mutants were examined. Large negative Hammett ρ-values (ρ_{N70V}: fast = -4.0, ρ_{N70V}: slow = -3.6, ρ_{N70D} = -3.8, ρ_{native} = -6.9, $\rho_{wild\text{-}type}$ = -6.8) are the indication of electron transfer being the rate determining step in the phenol oxidation. However, these results also indicate participation of the deprotonation step in compound I reduction process. Resonance Raman experiments found that basicity of the distal His in the ferrous state was decreased from 7.2 (native and wild-type) to 5.9 (N70V) and 5.5 (N70D) by the mutations (Fig. 2) (10). Thus, the proton abstraction from phenol was harder for the mutants due to the decreased basicity of the distal His upon mutation. Contrary to phenol oxidation, ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) oxidation activity was substantially increased by the mutations (native: 73, wild-type: 71, N70V: 217, N70D: 234 $\mu M \cdot sec^{-1}$). 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. Thus, the high ABTS oxidation activities of mutants are attributed to these high redox potentials of compound II.

TABLE 1. Reaction Rates ($M^{-1} \cdot s^{-1}$) for Compound I Formation of HRPs

HRP	k_1
native	1.4×10^7
wild-type	1.4×10^7
N70V	1.2×10^6, 3.0×10^5
N70D	1.5×10^6
E64S	4.3×10^5
E64P	4.4×10^5
E64G	4.3×10^5
H42E	4.9×10^3
H42Q	9.6×10^1

The paramagnetic ^1H NMR spectrum clearly showed the large upfield shift of the resonance of distal His $N_\varepsilon H$ (signal a) from 31.8 ppm (wild-type) to 25.5 ppm (N70D) and substantial broading

of the signal of distal His $N_\delta H$ (signal b) at 16.7 ppm in the N70D mutant (Fig. 3) (11). These results indicated reorientation of the distal His and disruption of the hydrogen bond between the distal

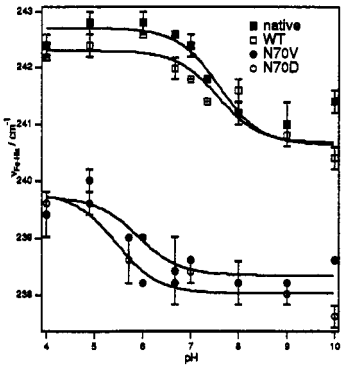

Fig. 2 pH dependence of Fe^{2+}-His frequencies of reduced HRPs.

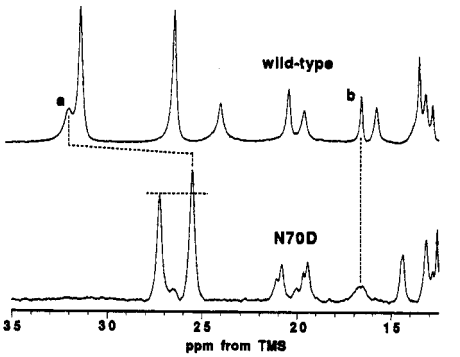

Fig. 3 ^1H NMR spectra of cyanide-ligated HRPs.

His and Asp70 in the mutant. The disappearance of the splitting in the ^1H NMR signal of heme peripheral 8-methyl group observed in 50 % H_2O / 50 % ^2H_2O solution of N70D-CN also suggests that the hydrogen bond between the distal His and heme-bound cyanide is disrupted by the mutation, which was supported by the low C-N vibration frequency and large dissociation constant of the heme-bound cyanide in the mutant. These observations led us to the conclusion that the improper positioning of the distal His induced the cleavages of the hydrogen bonds around the distal His, resulting in the substantial decrease of the catalytic activity without large structural alterations of the enzyme. The His-Asn hydrogen bond in the distal site of peroxidases, therefore, is essential for the catalytic activity by not only increasing basicity of the distal His but also by controlling the precise location of the distal His .

In the N70D mutant, it would not be surprising if Asp70 can form a hydrogen bond with the distal His, since Asp has a carboxyl group. However, the present study revealed that distal His42 is not hydrogen-bonded to Asp70. Asn70 in HRP forms a hydrogen bond not only with the $N_\delta H$ of the distal His but also with the peptide carbonyl oxygen of Glu64 (Fig. 1) which is also highly conserved in peroxidases, suggesting that Asn70 is fixed at the optimal position by the Glu64-Asn70 hydrogen bond. In the N70D mutant, however, the hydrogen bond between Asp70 and Glu64 would be disrupted and the elimination of the Glu64-Asp70 interactions would cause the positional change of Asp70, resulting in the breakage of the His-Asp hydrogen bond and thereby reorientation of the distal His. Thus, our results suggest that Glu64 as well as Asn70 affects reactivity of the heme through the hydrogen bond network in the distal site, Glu64-Asn70-His42.

Glu64 mutants: There is a highly conserved Glu residue in the heme distal site of various fungal and plant peroxidases. The carboxylate in the side chain of the Glu interacts with a distal calcium ion through a hydrogen bond with a water molecule (Fig. 1), implying that the Glu residue is related to the stabilization of the calcium ion. On the other hand, the peptide carbonyl oxygen in the main chain of the Glu residue hydrogen-bonds with an adjacent Asn residue, which in turn makes a hydrogen bond with distal His (Fig. 1). Our study on the Asn70 mutants study suggested that Glu64 controls the precise location and the basicity of the distal His through the Glu64-Asn70-distal His hydrogen bond network, improving catalytic activity of preoxidase. In order to examine roles of the Glu residue highly conserved in the distal site of peroxidases, we have prepared three HRP

mutants. An E64S (Glu64 → Ser) mutant was prepared to eliminate the carboxylate in the side chain of Glu64 without perturbation on the configuration of the main chain and the hydrogen bond with Asn70. In addition, E64P (Glu64 → Pro) and E64G (Glu64 → Gly) mutants were constructed to perturbe the hydrogen bond with Asn70 by deviating the configuration of the main chain at the position of 64 (12).

All the Glu64 mutants exhibited quite similar catalytic and spectral properties. These mutations substantially depressed oxidation activities for hydroquinone and elementary reaction rates in the catalytic cycle (TABLE 1). The ^{1}H NMR spectra of the mutants revealed that the replacements of Glu64 reoriented the distal His and disrupted the hydrogen bond between distal His and Asn70 as found for N70D mutant, whereas structural changes for other heme environments were subtle. The ICP emission spectroscopy revealed that one calcium ion was deleted in all the mutants (TABLE 2).

TABLE 2. Calcium Contents of Wild-type, E64S, E64P, and E64G HRPs

HRP	mol Ca^{2+} / mol HRP
wild-type	2.1
E64S	1.0
E64P	1.1
E64G	1.1

TABLE 3. Peroxygenase Activities ($nmol^{-1} \cdot s^{-1} \cdot \mu mol^{-1}$) of Native, Wild-type, H42E and H42Q HRPs at pH 7.0 and 25.0 °C

HRP	thioanisole (%S)	styrene (%S)
native	8.0 (52)	trace
wild-type	48 (61)	0.057 (15)
H42E	2.6×10^3 (45)	40 (15)
H42Q	16 (36)	0.69 (53)

This result indicates that the elimination of the carboxylate in the side chain of Glu64 by the mutaions removed the distal calcium ion and thereby rearranged amino acid residues in the distal site, which caused similar catalytic and structural features of the mutants. Thus, we can conclude that the interaction of the Glu residue with the distal calcium ion through the water molecule improves peroxidase activity by maintaining the structural integrity in the distal cavity.

His42 mutants: The distal histidine (His) is highly conserved in peroxidases and has been considered to play a major role as a general acid-base catalyst for peroxidase reaction cycle (Fig. 1). Recently, however, the X-ray structure of chloroperoxidase from the marine fungus *Caldariomyces fumago* has revealed that a Glu residue is located at the position where most of the peroxidase has a histidine residue, suggesting that the carboxyl group in the Glu residue can also assist cleavage of O-O bond in peroxides (13). In order to investigate catalytic roles of the glutamic acid at the distal cavity, two horseradish peroxidase mutants were prepared, in which the distal His42 has been replaced by Glu (H42E) or Gln (H42Q) (14).

The formation rate of compound I in the H42E mutant was significantly greater than that for the H42Q mutant, indicating that the distal Glu can play a role as a general acid-base catalyst (TABLE 1). However, the peroxidase activity of the H42E mutant was still lower, compared to that for native enzyme. On the basis of the CD, ^{1}H NMR, resonance Raman spectra and EPR spectra, it was suggested that the basicity of the distal Glu is lower than that of the distal His and the position of the distal Glu is not fixed at the optimal position as a catalytic amino acid residue, although no prominent structural changes around heme environment were detected. Our preliminary X-ray crystal analysis of the H42E mutant also suggested that the distal Glu moved away from the heme, relative to the distal His in wild-type enzyme. Therefore, we can conclude that the less basicity and improper

positioning of the distal Glu would destabilize the heme-H_2O_2-distal Glu ternary intermediate in formation of compound I, resulting in depression of the reaction rate with hydrogen peroxide. Another characteristic feature in the mutants was the enhancement of the peroxygenase activity (TABLE 3). Since the peroxygenase activity was remarkably enhanced in the H42E mutant, the distal Glu is also crucial to facilitate the peroxygenase activity as well as the enlarged distal cavity caused by the amino acid substitution. These observations indicate that the distal amino acid residue is essential for function of peroxidases and subtle conformational changes around the distal cavity would control the catalytic reactions in peroxidase.

Phe221 mutant: The crucial reaction intermediate in the reaction of peroxidase with hydrogen peroxide, compound I, contains a porphyrin π-cation radical in horseradish peroxidase, which catalyzes oxidation of small organic and inorganic compounds, whereas *Cc*P has a radical center on the tryptophan residue (Trp191) and oxidized the redox partner, cytochrome *c*. To investigate roles of the amino acid residue near the heme active center in discriminating the peroxidase function in these two enzymes, we prepared a *Cc*P-like HRP mutant (F221W) by replacing Phe221 corresponding to Trp191 in *Cc*P with Trp (15).

A ^{1}H NMR spectrum of the ferric F221W mutant found that there was no substantial structural changes in the heme environment by the mutation. However, effects of replacing Phe221 by Trp were dramatic in the time course for the reaction with hydrogen peroxide. In the F221W mutant, upon addition of hydrogen peroxide, the slight decrease of the absorbance of the Soret band characteristic of the porphyrin π cation radical formation was detected (Fig. 4). Kinetic analysis for the reaction showed that the porphyrin π cation radical was yielded at the same rate (1.3×10^7 $M^{-1}\cdot s^{-1}$) for F221W mutant as for the wild-type enzyme (1.5×10^7 $M^{-1}\cdot s^{-1}$). However, the absorbance of the Soret band rapidly increased again (rate constant: 1.7 s^{-1}) and the resultant spectrum ($\lambda_{max} = 419$ nm) corresponded to that of an oxoferryl species ($\lambda_{max} \sim 420$ nm). This result indicates that the porphyrin π cation radical in F221W mutant is unstable enough to be rapidly transformed to a *Cc*P-type compound I (oxoferryl heme and Trp radical) or compound II (oxoferryl heme) as found in

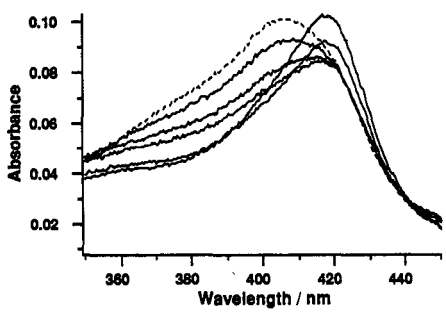

Fig.4 Time-dependent UV/vis absorption spectral changes of the F221W mutant after addition of a small excess of H_2O_2. The repetitive scans were recorded at 0 (dotted line), 21, 42, 63, 336, 1239 ms after the addition of H_2O_2.

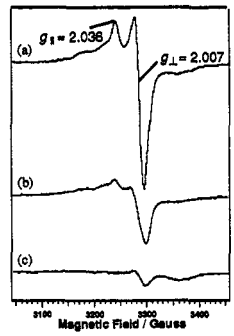

Fig 5. EPR spectra of F221W compound I (a) immediately (b) in 10 sec (c) in 1 min after the addition of H_2O_2. These spectra were taken at 5 K over a range of 400 G. Microwave power was approximately 5 mW. Each spectrum was from four accumulations of 60 s duration.

wild-type HRP, both of which exhibit the similar spectral features of the oxoferryl heme. In order to identify the intermediate species, EPR measurements were performed. The EPR spectrum of the resultant species produced by reduction of the porphyrin π-cation radical showed typical signals from a Trp radical, indicationg formation of a ferryl species with a Trp radical in the HRP mutant as found for *Cc*P compound I (Fig. 5). The formation of the Trp radical in close proximity of the ferryl heme

was supported by power saturation experiments using EPR spectroscopy. The consecutive radical formation from the porphyrin ring to the Trp residue implies that the proximal Trp is a key residue in the process of the radical transfer from the porphyrin ring, which differentiates function of peroxidase. Present observations strongly suggest that the porphyrin π-cation radical is initially produced prior to the formation of the Trp radical in CcP compound I.

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