Detection of a Tryptophan Radical as an Intermediate Species in the Reaction of Horseradish Peroxidase Mutant (Phe-221 → Trp) and Hydrogen Peroxide

(Received for publication, February 10, 1998, and in revised form, March 27, 1998)

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The crucial reaction intermediate in the reaction of peroxidase with hydrogen peroxide (H₂O₂), compound I, contains a porphyrin π-cation radical in horseradish peroxidase (HRP), which catalyzes oxidation of small organic and inorganic compounds, whereas cytochrome c peroxidase (CcP) has a radical center on the tryptophan residue (Trp-191) and oxidizes the redox partner, cytochrome c. To investigate the roles of the amino acid residue near the heme active center in discriminating the function of the peroxidases in these two enzymes, we prepared a CcP-like HRP mutant, F221W (Phe-221 → Trp). Although the rapid spectral scanning and stopped-flow experiments confirmed that the F221W mutant reacts with H₂O₂ to form the porphyrin π-cation radical at the same rate as for the wild-type enzyme, the characteristic spectral features of the porphyrin π-cation radical disappeared rapidly, and were converted to the compound II-type spectrum. The EPR spectrum of the resultant species produced by reduction of the porphyrin π-cation radical, however, was quite different from that of compound II in HRP, showing typical signals from a Trp radical as found for CcP. The sequential 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 the function of peroxidases.

Peroxidases catalyze one-electron oxidation of various substrates by using H₂O₂ as an oxidant. In the first step of the catalytic cycle, horseradish peroxidase (HRP), 1 one of the typical peroxidases, yields an intermediate called compound I, in which the ferric enzyme (Fe³⁺) undergoes 2 equivalent oxidation to yield an oxoferryl porphyrin π-cation radical (2). Compound I in HRP affects one-electron oxidation of the substrates, small organic or inorganic molecules, producing the second intermediate, compound II. The remaining oxidized site, ferryl iron (Fe⁴⁺), again oxidizes substrates to be reduced back to the resting state. On the other hand, the compound I of another typical peroxidase, cytochrome c peroxidase (CcP), can catalyze the redox reaction with cytochrome c (Cyt c), a sole substrate of CcP (3, 4). Although the resting state of CcP reacts with H₂O₂ to form the oxidized intermediate, compound I, as does HRP, CcP compound I has a UV-visible spectrum similar to that of HRP compound II (5, 6). Several spectroscopic studies combined with site-directed mutagenesis (7–9) have revealed that CcP compound I has a stable indolyl cation radical at Trp-191 as one of the oxidized site. The formation of the Trp radical would facilitate one electron transfer from Cyt c to the porphyrin, inasmuch as Cyt c cannot be directly accessible to the CcP heme active center (10, 11). Thus, the position of the radical center in compound I is closely related to function of peroxidase and amino acid residues that control the radical center can be considered to be one of key factors to discriminate the functions of peroxidases.

On the basis of sequence alignment between HRP and CcP, an amino acid residue corresponding to Trp-191 in CcP is a Phe residue (Phe-221) in HRP (12). To investigate the control mechanism for the position of the radical center in the reaction intermediates, some mutagenic studies focused on Trp-191 in CcP have been carried out and shown that a “HRP-type” CcP mutant, in which the proximal Trp was replaced with Phe (Trp-191 → Phe), yields an oxyferryl porphyrin π-cation radical intermediate similar to that of HRP compound I, suggesting that the proximal Trp controls the position of the radical center (8, 9, 13). However, the radical transfer from the porphyrin ring to the proximal Trp has not yet been confirmed, which is one of the most crucial processes in the formation of CeP compound I.

In this paper, we have prepared a complementary HRP mutant enzyme to the CeP mutant, a “CcP-type” HRP mutant having a tryptophan radical at the position of Phe-221 (F221W) (Fig. 1), in order to elucidate the regulation mechanism of radical transfer in peroxidase reaction intermediates. NMR, EPR, and ICP emission spectroscopies were used in this study to characterize the heme environmental structure of the mutant. These spectroscopies, demonstrating formation of the Trp radical during the oxidation of the mutant by hydrogen peroxide after the porphyrin π-cation radical formation, provide strong evidence that the radical center on the porphyrin ring can be transferred to the Trp residue near the heme in peroxidase, indicating that the Trp residue is the key amino acid residue to differentiate the function of peroxidases.

EXPERIMENTAL PROCEDURES

Materials—General molecular biology supplies were obtained from Takara and Perkin-Elmer. All buffer materials and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. and Nacalai

‡ This work was supported in part by Grants-in-aid 07309006 and 08249102 for Scientific Research (to I. M.) from the Ministry of Education, Science, Culture and Sports. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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§ Supported by research fellowships from the Japan Society for the Promotion of Science for Young Scientists.

The abbreviations used are: HRP, horseradish peroxidase isozyme C; wild-type HRP, recombinant horseradish peroxidase isozyme C expressed in E. coli; CeP, cytochrome c peroxidase; Cyt c, cytochrome c; ABTS, 2,2′-azinobis(3-ethylbenothiazoline-6-sulfonic acid)diammonium salt; ICP, inductively coupled plasma; mW, milliwatt(s).

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of compound I ($k_f$) was measured by following decrease of the absorbance at 395 nm, the isosbestic point between compound I and compound II. For the F221W mutant, the electron transfer rate from Trp-221 to the porphyrin ($k_{ET}$) was measured at 412 nm, the isosbestic point between the ferric state and the oxyferriyl species. The reduction rate of compound II ($k_r$) was obtained by almost the same method as the formation rate of compound I. The $k_r$ values for the wild-type and mutant HRPs were acquired by adding guaiacol or ferrocyanide 5 min after addition of a slight excess of $H_2O_2$. The rate of formation of compound II was followed at 424 nm for wild-type HRP and 419 nm for the mutant.

**Electron Paramagnetic Resonance (EPR) Spectroscopy—**EPR spectra of HRPs were measured by a Varian E-12 spectrometer equipped with an Oxford ESR-900 liquid helium cryostat. Measurements were carried out at the X-band (9.22 GHz) microwave frequency at 5 and 77 K. The range of the microwave power is 0.01 mW to 100 mW. The sample concentration was about 400 μM, and the volume was 50 μL. The sample solution was frozen in EPR tube by submersion in liquid nitrogen over 1 min after addition of a small excess of $H_2O_2$ to ferric wild-type enzyme. To observe the EPR signal from the unstable species in the F221W mutant, the rapid mixing, freeze-quench technique was used (19). The photolysis experiments were performed with a tungsten light.

**Quantitative Analysis for Calcium Ion Bound in HRP—**The amount of calcium ion bound in the HRP samples in deionized water at pH 7.0 was determined by ICP emission spectroscopy (Jarrel Ash ICP-AES). The samples analyzed here contained 1.2 mg of calcium/liter.

**Peroxidase Activity—**Oxidation activities for guaiacol and ABTS were measured by monitoring the increase of the absorbance at 470 and 405 nm, respectively on a Perkin-Elmer UV-visible spectrophotometer (Lambda 19) at 25.0 °C. For the guaiacol oxidation, 1 mM $H_2O_2$ was mixed with 5.1 mM guaiacol in 50 mM sodium phosphate buffer at pH 7.0, after which 5 mM enzyme was added to initiate the reaction. For the ABTS oxidation, 5 mM enzyme was added to the solution containing 1 mM $H_2O_2$ and 0.9 mM ABTS in 50 mM sodium phosphate buffer per pH 7.0.

**Redox Potential of Fe$^{2+}$/Fe$^{3+}$ Couple—**The redox potentials of HRPs were monitored at 435 nm with platinum electrode on a Perkin-Elmer UV-visible spectrophotometer (Lambda 19). The mediator was a mixture of safranine T, phenosafranine, benzylviologen, and α-hydroxy-phenazine. The ferric enzymes were photoreduced at 25.0°C in 50 mM sodium phosphate buffer containing 50 mM EDTA, pH 7.0. The reductive titration of HRP was carried out with a short irradiation with white light. This measurement was repeated until HRP could not be reduced further by the irradiation.

The midpoint potential of HRP ($E_m$) was determined by the plot of the monitored electrode potential ($E_o$) against the percentage of reduced HRP estimated from the absorbance change at 435 nm by using the following Nernst equation.

$$E_o = E_m + \frac{RT}{2F} \ln\left(\frac{[\text{oxidized HRP}]}{[\text{reduced HRP}]}\right)$$

(1)

$s$ and $F$ correspond to a number of electrons involved in the redox reaction and Faraday constant, respectively. The midpoint potential value of HRP was corrected by that of a phenosafranine ($-252$ mV).

**RESULTS**

**Reaction with Hydrogen Peroxide—**The resting state of wild-type HRP reacts with $H_2O_2$ to form the oxidized intermediate, compound I. This oxidized species, the porphyrin π-cation radical is characterized by a diminished absorbance in the Soret region (9) as shown in Fig. 2a. Compound I was then gradually one electron-reduced to compound II with shift of the Soret peak from 402 to 420 nm and finally back to the resting state (20). In the F221W mutant, the addition of $H_2O_2$ also decreased the absorbance at the Soret band, which is characteristic of the porphyrin π-cation radical formation (Fig. 2b). However, the absorbance of the Soret band increased again faster than in the course of the wild-type enzyme. In the resultant spectrum ($\lambda_{max} = 419$ nm) corresponds to that of HRP compound II ($\lambda_{max} = 420$ nm). The rapid restoration of the absorbance at the Soret band indicates that the porphyrin π-cation radical in the F221W mutant is highly unstable and easily reduced. Even for the parent enzyme in the absence of the substrates, compound I is converted to compound II with the donation of one electron from impurities in the solution (21).
the porphyrin π-cation radical in the wild-type without the substrate is typically about 30 min (21), which is much longer than that of the porphyrin π-cation radical in the F221W compound I (≈5 s). Because the spectral pattern in the Soret band for CcP compound I (oxyferryl-Trp radical) is quite similar to that of compound II (oxyferryl) in HRP and were quite similar in this mutant (22), we could not confirm a radical formation on the newly introduced Trp residue in the second reaction intermediate (λmax = 419 nm) for the HRP mutant by the absorption spectrum.

EPR Spectrum of the Intermediate Species—In order to follow the radical center formed by the reaction of the F221W mutant with hydrogen peroxide, we used the EPR spectroscopy at 5 and 77 K in the presence of an equimolar amount of H2O2. In the parent enzyme, a broad EPR signal characteristic of compound I, oxyferryl π-cation radical (Fig. 3a), was gradually diminished with the same rate as restoration of the absorbance of the Soret band, which corresponds to reduction of the porphyrin π-cation radical and formation of compound II, oxoferryl species, inasmuch as compound II is EPR silent. In contrast to that of the parent enzyme, the F221W mutant exhibited a rather sharp EPR signal at g∥ = 2.036 and g⊥ = 2.007 (Fig. 3, b and c), characteristic of the Trp radical in CcP compound I (g∥ = 2.037 and g⊥ = 2.005) (23–25). Although we have not yet succeeded in the quantitative analysis for the EPR measurements after mixing the mutant with hydrogen peroxide, the second reaction intermediate in which the Soret peak appeared at 419 nm seems to afford these EPR signals characteristic of the Trp radical. These signals lost their intensity with time and the radical center was quenched within 1 min after mixing with hydrogen peroxide (data not shown). On the other hand, the absorption spectra of the mutant in 1 min after the mixing had the Soret peak at 419 nm (results not shown), indicating that the heme iron is still in the oxyferryl state as found for native HRP (1). These results indicate that the mutant HRP has another radical center such as the Trp radical in CcP compound I.

Despite the close similarity of the EPR signal between F221W HRP and native CcP, some significant differences were also detected. The EPR spectrum of the Trp radical of CcP undergoes substantial spectral changes on warming to 77 K. At this temperature, the EPR signal from the Trp radical was broadened, which is attributable to the multiple radical species corresponding to the thermally excited states (25, 26). However, the EPR signal from the Trp radical of the mutant at 77 K (Fig. 3d) was almost same as that at 5 K under the low microwave power (Fig. 3c). The thermally excited states for the Trp radical in CcP were also manifested in the photoillumination EPR measurements. By repeating illumination, a new and much more intense EPR signal with complex structures emerged and the characteristic EPR signal disappeared again after the sample had been warmed to 193 K for 20 min and cooled to 77 K (25). The reversible formation of the radical center on the Trp residue via an intermediate species by illumination also suggests the possible existence of several thermally excitable energy levels near the ground state of the Trp radical in CcP (25). The EPR spectrum of F221W HRP mutant, however, was almost insensitive to heating and photoillumination (results not shown). Another distinct difference of the EPR spectra between the two proteins is the power dependence of the EPR signal. The EPR signal from the Trp radical of the F221W mutant was significantly saturated at 0.1 mW (Fig. 3b), whereas more microwave power was required to saturate the wavenumber of the Soret band, which corresponds to reduction of the porphyrin π-cation radical and formation of compound II, oxoferryl species, inasmuch as compound II is EPR silent.

We measured the EPR spectrum of the F221W mutant at 15 K, which is also similar to that at 5 and 77 K. The EPR pattern was almost insensitive to temperature between 5 and 77 K, which is quite different from that of CcP (25).
signal from CeP. This is interpreted by a longer spin lattice relaxation time \( T_1 \) (27) and weak interactions between the radical and oxyferryl heme (28) in the HRP mutant.

**1H NMR Spectroscopy**—Although prominent changes in the reactivity toward hydrogen peroxide and formation of the other radical center were induced by the mutation at Phe-221, the structural alterations monitored by the hyperfine-shifted NMR of the heme peripheral groups and amino acid residues near the heme iron were less drastic (Fig. 4). For the ferric high spin wild-type HRP, the resting state of peroxidases, the four peaks from the heme peripheral methyl groups were observed at 84, 77, 73, and 56 ppm (5-, 1-, 8-, and 3-methyl groups, respectively) (29, 30). In the spectrum for the F221W mutant, the corresponding peaks were slightly shifted (80, 77, 70, and 55 ppm). A single resonance assignable to the \( \text{N}^1 \text{H} \) of the proximal His (His-170) in the mutant was also observed with a slight shift at 99 ppm, compared with that (101 ppm) of wild-type HRP. These results suggest that the heme environmental structure of the F221W mutant is not altered largely by the mutation.

**Quantitative Analysis for Calcium Ion Bound in HRP**—It should be also noted here that calcium ion bound near the heme in HRP is one of the distinct structural difference between the two peroxidases, HRP and CeP. HRP has one calcium ion at both of the proximal and distal site, whereas none is contained in CeP. The bound calcium ion in HRP stabilizes the protein structure and enhanced the catalytic reactivity of HRP (31–34), whereas, in CeP, the absence of the calcium ion is essential for the formation of the stable radical center at the Trp residue (35, 36). Mutations near the heme distal sites of HRP resulted in the loss of the bound calcium ion (distal calcium) and the severe functional defects (37). In order to confirm the binding of calcium ions in the mutant, we used ICP measurement (38). The results from these experiments clearly showed that the mutant can bind 2 mol of calcium ion/mol of mutant protein, as found for wild-type HRP (Table I).

**Peroxidase Activity**—In order to clarify the effect of the mutation on catalytic oxidation activities for guaiacol and ABTS, their activities under the steady-state condition were investigated. Relative oxidation activities for guaiacol and ABTS were determined by observing the formation of the oxidation product of guaiacol and ABTS at 470 and 405 nm, respectively (14). Although the activities of the wild-type enzyme were virtually the same as those reported previously (14), significant depression of the activities (the remaining activities were about 30% of the wild-type enzyme) were observed for both of the substrates in the mutant as summarized in Table II.

To gain further insights into the effect of the mutation on the catalytic activity of HRP, we estimated the elementary reaction rates in the catalytic cycle. The rate of formation of compound I was determined by monitoring the decay of the absorption at 395 nm, an isosbestic point between compound I and compound II for the wild-type enzyme. The value found with the mutant was \( k_1 = 1.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \) at pH 7.0 and 25 °C, which is virtually the same as that of the parent enzyme \( k_1 = 1.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \) (Table III). The radical transfer rate from the porphyrin ring \( k_{\text{ET}} \) which corresponds to the electron transfer rate to the porphyrin ring, was monitored at 417 nm (Fig. 5), the isosbestic point between the ferric state and the oxyferryl species. The time course can be fitted by a single exponential as shown in Fig. 5, and the rate was estimated to be \( 65 \text{ s}^{-1} \). The reduction of compound II to the ferric state \( k_3 \) process was examined in the reaction of compound II with guaiacol or ferrocyanide which is an electron donor to HRP. The \( k_3 \) values for guaiacol and ferrocyanide in the wild-type enzyme were \( 2.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \) and \( 7.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1} \), respectively, corresponding to those in the native enzyme (39). As listed in Table III, the \( k_3 \) values for the reaction of the F221W mutant with both of the two substrates were depressed to about 30% of that for the wild-type enzyme.

**Redox Potential of \( \text{Fe}^{2+}/\text{Fe}^{3+} \) Couple**—Redox potential of \( \text{Fe}^{2+}/\text{Fe}^{3+} \) couple is also one of the factors reflecting the formation of compounds I and II in the peroxidases (40). The monitored electrode potentials against the ratio of the reduced form to the total amount of enzyme were fitted well by the theoretical Nernst equation (Equation 1) (data not shown). The midpoint potentials of the wild-type enzyme (–261 mV) was almost identical with that of HRP reported by Yamada et al. (41). The redox potential for the F221W mutant (–178 mV) was significantly higher than that of the parent enzyme and rather close to that of native CeP (–182 mV).

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**Table I**

| HRP           | Ca\(^{2+}\) | \(\text{mol/mol}\) |
|---------------|-------------|---------------------|
| Wild type     | 2.0 ± 0.1   |                     |
| F221W mutant  | 1.9 ± 0.2   |                     |

**Table II**

| HRP           | Guaiacol \(\times 10^{-6}\) | ABTS \(\times 10^{-7}\) |
|---------------|-----------------------------|------------------------|
| Wild type     | 4.1                         | 2.9                    |
| F221W mutant  | 1.3                         | 0.68                   |

**Table III**

| HRP          | \(k_1 \times 10^{-7}\) | \(k_3 \times 10^{-5}\) (guaiacol) | \(k_3 \times 10^{-4}\) (ferrocyanide) |
|--------------|------------------------|-----------------------------------|--------------------------------------|
| Wild type    | 1.2                    | 2.5                               | 7.9                                  |
| F221W mutant | 1.0                    | 0.71                              | 4.2                                  |
F221W mutant and hydrogen peroxide are mixed. The concentration of enzyme and hydrogen peroxide were 2 and 20 μM, respectively. The upper panel of the figure shows the residuals from the best one-experimental fit to the time course.

**DISCUSSION**

**Characterization of the Second Intermediate in the Reaction of the F221W HRP Mutant with Hydrogen Peroxide**—The time course of the absorbance during the reaction between HRP F221W and hydrogen peroxide was characterized by the initial decrease in absorbance changes, which was followed by the increase in absorbance at the Soret band. These spectral changes were also encountered for native HRP, which corresponds to the successive formation of the reaction intermediates, compounds I and II. In the mutant, however, the life time for compound I was extremely short and very rapid restoration was observed for the absorbance of the Soret band. Another distinct difference between wild-type and mutant HRP was found in their EPR spectra. In the wild-type enzyme, a broad EPR signal characteristic of porphyrin π-cation radical appeared by addition of hydrogen peroxide (Fig. 3a) and gradually diminished by formation of compound II. The F221W mutant, however, exhibited a completely different EPR signal from that of the wild-type enzyme with the restoration of the absorbance of the Soret band. Although the quantitative analysis of the time course for the EPR spectrum of the F221W HRP mutant in the presence of hydrogen peroxide has not been successful, the EPR signal appeared concomitant with the restoration of the absorbance at the Soret band, suggesting that the second reaction intermediate still has a radical center, as found for CcP (7–9). The shape and g-values for the EPR signal of the mutant were also quite similar to those of the Trp radical in CcP compound I.4 A tyrosine residue, which is also susceptible to the oxidation (19), would be another candidate for the oxidized residue, but the EPR signal is quite different from that observed for a CcP mutant, W191F (19). It is, therefore, concluded that the second reaction intermediate still has a radical center, as found for CcP compound I.4

The x-ray structure for wild-type HRP has revealed that HRP originally has one Trp residue at position 117 between the D and D' helix (12). However, the distance between Trp-117 and heme periphery is about 12 Å, and rapid mixing EPR experiment showed that this Trp residue never formed a stable intermediate such as the Trp radical for CcP compound I in catalytic cycle. Thus it is highly unlikely that the large structural changes are induced by the mutation we introduced in this study and the distance between Trp-117 and heme periphery is drastically shortened, inasmuch as the perturbations on the NMR spectrum by the mutation were moderated. Moreover, according to the x-ray structure for wild-type CcP, Trp-51 is also close to the heme active site (10). Although the Trp-51 residue lies almost the same distance as the Trp-191 and parallel to the heme periphery, a previous study has unambiguously identified that the radical site can only be Trp-191, not Trp-51 (42). This finding in CcP also supports the absence of the radical at Trp-117 in HRP. It is, therefore, the Trp residue introduced by the mutation and located near the heme periphery (5 Å) that is oxidized to form the Trp radical as is Trp-191 in CcP.

Although the present spectral data clearly showed that both of F221W mutant HRP and CcP have a radical center on the Trp residue near the heme, the environment of the radical center is significantly different between the two peroxidases. A characteristic feature for the Trp radical in the F221W HRP mutant is the rapid decay of the EPR signal, which completely disappears within 60 s. On the other hand, the Trp radical in CcP is quite stable and survives for more than 5 min in the absence of the substrate, cytochrome c. One of the structural factors responsible for the unstable Trp radical in the HRP mutant would be the existence of a cation near the Trp residue. A mutagenetic study on the CcP has demonstrated that the introduction of the cation binding site into the proximal site significantly enhanced electrostatic potential near the Trp residue, resulting in the destabilization of the radical on the Trp residue (33). In the F221W mutant, the structural effects of the mutation was localized near the mutation site and two calcium ions are still bound as revealed by means of ICP measurement, implying the electrostatic potential around the radical on the Trp is much higher in the HRP mutant than that in CcP and the enhanced electrostatic potential would destabilize the radical on the Trp residue as found for the CcP mutant.5

Another factor destabilizing the Trp radical would be the orientation of Trp-221. On the basis of the x-ray crystal structure of CcP (10), Trp-191 is in van der Waals contacts with Met-230 and Met-231 and is hydrogen-bonded with Asp-235 to fix the indole ring at the optimal position for the radical formation. In fact, several CcP mutants (M230L, M230I, M230Y, M231L, and D235N), in which these interactions are perturbed by the mutations (16, 18), exhibited unstable Trp radicals. In the present work, the HRP mutant would not form hydrogen bonds or van der Waals contacts to fix the Trp residue at the optimal orientation as found for CcP, because there are no amino acid residues to interact with the Phe-221 in HRP (12). Therefore, Trp-221 in the HRP mutant is not fixed at the optimal orientation as is Trp-191 in CcP, which eventually destabilizes Trp-221 radical.

The temperature and power dependence values of the EPR signal from the Trp radical in the HRP mutant are also indic-

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4 The peroxo radical localized on tryptophan in myoglobin also affords the similar EPR signal (57, 58). In this point, we performed the same experiment under the condition which completely excluded the molecular oxygen by the reported method (58). The EPR signal observed in anaerobic system is almost identical to the characteristic signal of the Trp radical spectrum of the F221W HRP mutant ($g_\parallel = 2.036$ and $g_\perp = 2.007$) in the aerobic system. Thus, the EPR spectrum of the F221W HRP mutant is not due to a tryptophan peroxyl radical as formed in myoglobin.

5 To confirm that the Trp newly introduced by the mutation was oxidized by addition of hydrogen peroxide, we also performed the amino acid analysis, because native HRP has one naturally occurring Trp. Unfortunately, however, the amino acid analysis for Trp residue of the mutant HRP has not been successful, due to the low sensitivity of Trp residue.

6 To examine the effect of the calcium ion binding on the stability of the Trp radical, we have tried to remove the calcium ion from the F221W mutant (26, 27). However, the mutant protein was so unstable to removal of the calcium ion that it was precipitated by denaturation.
ative of the different environment for the Trp radical from that in CeP. The long spin-lattice relaxation time observed for the HRP mutant was also encountered for the Tyr radical formed in the W191F CeP mutant (12). Because the Tyr residue trapping the radical in the mutant CeP is located at more than 10 Å from the heme iron, the weak electronic interaction results in the long spin-lattice relaxation. Although the distance from the heme iron to Trp-221 in the mutant would be comparable to that from the heme to Trp-191 in CeP, the configurational changes of the indole ring such as alteration of the orientation may affect the interaction with the heme. The different environment around the Trp radical in the HRP mutant from that in CeP is manifested in the EPR spectral changes upon photo-illumination and heat up. As previously reported, the photoillumination-dependent EPR spectrum of the Trp radical would correspond to several thermally excitable energy levels near the ground state of the Trp radical in CeP and broadening at 77 K also supports the thermally excited multiple radical center (25, 26). In a sharp contrast to the Trp radical in CeP, photoillumination and heating did not significantly affect the EPR spectrum of the HRP mutant, supporting the notion that the environmental structure and interactions with adjacent amino acid residues near the Trp radical are different in the HRP mutant and CeP.

Radical Transfer from the Porphyrin Ring to the Trp Residue—As discussed in the previous section, the F221W HRP mutant has a radical center in Trp-221 as the second reaction intermediate in the reaction with hydrogen peroxide. Because the rapid decrease of the absorbance at the Soret band immediately after addition of hydrogen peroxide unambiguously indicates the formation of the oxyferryl-porphyrin \( \pi \)-cation radical at the first step of the reaction of the mutant with hydrogen peroxide (43, 44), the radical center would be transferred from the porphyrin ring to the Trp residues to yield oxyferryl-Trp radical species as the second intermediate.

It is quite interesting to compare the present results with those from the complimentary mutation in CeP, a HRP-like CeP (9, 45). The HRP-like CeP has a phenylalanine residue at the position of Trp-191 in which the radical center is located. Because a phenylalanine residue is fairly resistant to the ring oxidation compared with a tryptophan residue (12), the oxidation of Phe-191 was not detected. Instead of the oxidation of the Phe residue, a transient porphyrin \( \pi \)-cation was observed and the radical center was successively transferred to a tyrosine residue located 11 Å from the heme iron, which affords a typical EPR signal of a tyrosine radical. The results from the complimentary two mutants clearly showed that the oxidation sensitive residue, Trp residue, near the heme periphery plays crucial roles in the formation and transfer of the radical center generated in the reaction of peroxidase with hydrogen peroxide.

It should be noted here that the radical transfer from the porphyrin ring to the Trp residue would be also facilitated by the destabilization of the \( \pi \)-cation radical on the porphyrin ring. As the previous studies have proposed, the high valent porphyrin species such as oxyferryl porphyrin \( \pi \)-cation radical are stabilized by the electronic donation from the anionic axial ligands (46, 47). Because the increase of the electronic donation has been considered to be correlated with the decrease of the redox potential between ferric and ferrous states (48), the redox potential for Fe\(^{3+}\)/Fe\(^{3+}\) couple serves as an indicator for the stability of the high valent porphyrin species. In fact, heme enzymes having porphyrin \( \pi \)-cation radical as the reaction intermediate such as HRP (250 mV) (41), catalase (−500 mV) (49), and P-450cam (−170 mV) (48), have their redox potentials much lower than that of human myoglobin (+50 mV) (47) and sperm whale myoglobin (+55 mV) (50). For the F221W mutant, the redox potential of the Fe\(^{3+}\)/Fe\(^{3+}\) couple (−178 mV) was much higher than that for the wild-type enzyme (−260 mV), rather close to that of CeP (−182 mV). The higher redox potential corresponds to the decreased electron donation from the axial ligand, which leads to the destabilization of the high valent porphyrin species, oxyferryl \( \pi \)-cation radical. In the F221W mutant having the oxidation sensitive Trp residue near the heme periphery, the destabilized porphyrin \( \pi \)-cation radical would readily be transferred to the Trp residue, resulting in formation of the radical center on the Trp residue.

The radical transfer from the porphyrin ring to the Trp residue would shed light on the reaction mechanism of CeP. Despite extensive efforts to detect the radical transfer from the porphyrin ring to Trp-191, neither radical transfer nor porphyrin \( \pi \)-cation radical was confirmed in the wild-type enzyme. Although the formation of the porphyrin \( \pi \)-cation radical in CeP was supported by use of the HRP-like mutant, W191F CeP (9, 45), the radical transfer has not yet been shown in peroxidases. In this paper, we have demonstrated the radical transfer in HRP, of which structural features are quite similar to that of CeP, strongly suggesting the radical transfer from the porphyrin ring to the Trp residue in CeP. As discussed in the previous section, however, the Trp radical in the HRP mutant is quite unstable, compared with that in CeP. In other words, the radical transfer from the porphyrin ring to Trp-191 in CeP would be much faster than that in the HRP mutant we obtained here, which has prevented us from directly observing the porphyrin \( \pi \)-cation radical and the radical transfer to the Trp residue in CeP.

Reaction Mechanism of the F221W HRP Mutant with Hydrogen Peroxide—In the reaction of the F221W HRP mutant with hydrogen peroxide, an additional intermediate having a radical center on the Trp residue near the heme periphery was observed for the reaction cycle. The reaction of the mutant with hydrogen peroxide, therefore, can be explained by the mechanism shown by Scheme I.

\[
\text{Ferric(Fe}^{3+}) \rightarrow I_A \rightarrow I_h \rightarrow I_C \rightarrow \text{Ferric(Fe}^{3+})
\]

**Scheme I**

The species denoted \( I_A \) is an oxyferryl porphyrin \( \pi \)-cation radical as found for native HRP. The species \( I_h \) has a similar absorbance spectrum to that of compound II in native HRP, but the EPR signal was characteristic of the Trp radical. The EPR signal was diminished within a couple of minutes, whereas the absorption spectrum is still the HRP compound II-type (species \( I_C \)). These results indicate that the species \( I_A \) is Trp radical and the species \( I_C \) is HRP compound II. In the presence of the substrate, the species \( I_c \) was reduced back to the ferric resting state.

The kinetics for the steady-state and elementary reaction revealed the rate constants for the formation of these intermediates. The value of \( k_3 \), the formation rate of the porphyrin \( \pi \)-cation radical for the mutant, is not affected by the mutation, implying that both the deprotonation of hydrogen peroxide and the cleavage of the O-O bond are not affected by the mutation. As our previous studies have shown, the deprotonation and the O-O bond cleavage depend on the basicity and orientation of

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7 However, the EPR signals from the Trp radical in the F221W mutant showed the microwave power dependence (Fig. 3, b and c). The signal broadening at \( g \approx 2.036 \) in the F221W mutant might correspond to the saturation broadening and/or multiple components of the signals. Therefore, we cannot rule out the possibility that the Trp radical in the HRP mutant has a multiple components under the high power condition, but it is safely said that the radical character of the HRP mutant is quite different from that of CeP.
H2O2 showed the formation of a transient porphyrin 191, a HRP-type C
not determined due to the extremely rapid oxidation of Trp-
the absorption spectral change was 65 s
mutant protein and complexity in the quantitative analysis for
rocyanide. Although the depression for the
the reduction rate was decreased to about 30% of that for the

and reactivity of the oxyferryl center in the C
peroxide (44), the most prominent difference is the insertion of
by increase of the absorption at the Soret band. In the mutant
HRP, the formation rate of the Trp radical (kET)
determined by the absorption spectral change was 65 s
To estimate the value of k2, we have tried to follow the time
course of the EPR signal from the Trp radical, inasmuch as the
absorption spectrum was completely insensitive to the disappear-
appearance of the radical. Unfortunately, however, the time-
course experiments by using EPR spectroscopy have not yet
been successful due to the limitation of the amount of the
mutant protein and complexity in the quantitative analysis for
the EPR signal.

In reduction of compound II to the resting state, k3 process,
the reduction rate was decreased to about 30% of that for the
wild-type enzyme for both of the substrates, guaiacol and fer-
rocyanide. Although the depression for the k3 process was
significant in the mutant, the effects of the amino acid substi-
tion at Phe-221 on the k3 process would be rather small.
Some mutations around the heme active center severely inhibit
reduction of compound II and 10–30-fold decrease in
reduction of compound I and the absorption spectrum for compound II of the
intermediates prevent us from confirming that the mutant
cation radical would
Conclusions—In summary, we have detected the Trp-221
radical as the second intermediate species in the reaction of the
F221W mutant with hydrogen peroxide and demonstrated the
radical transfer from the porphyrin ring to the Trp residue near
the heme active center. This result clearly indicates that the
proximal Trp near the heme active site is a key amino acid
residue to control the location of the radical center in pero-
diase compound I and differentiate functions in peroxi-
dases.

The Trp radical is unstable due to lack of the inter-
actions to fix the indole ring of the Trp residue at the optimal
orientation and presence of a cation (calcium ion) locating near the
Trp residue.

Acknowledgments—We are thankful to Dr. Kiyohiro Imai (Osaka
University) for assistance with the stopped-flow experiments. We are
also thankful to Dr. Shigeo Umetani (Kyoto University) for assistance
with the ICP experiments.

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