Absence of a Detectable Intermediate in the Compound I Formation of Horseradish Peroxidase at Ambient Temperature\(^*\)\(^{\dagger}\)

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A microsecond-resolved absorption spectrometer was developed to investigate the elementary steps in hydrogen peroxide (\(\text{H}_2\text{O}_2\)) activation reaction of horseradish peroxidase (HRP) at ambient temperature. The kinetic absorption spectra of HRP upon the mixing with various concentrations of \(\text{H}_2\text{O}_2\) (0.5–3 mM) were monitored in the time range from 50 to 300 \(\mu\)s. The time-resolved spectra in the Soret region possessed isosbestic points that were close to those between the resting state and compound I. The kinetic changes in the Soret absorbance could be well fitted by a single exponential function. Accordingly, no distinct spectrum of the putative intermediate between the resting state and compound I was identified. These results were consistent with the proposal that the \(\text{O}–\text{O}\) bond activation in heme peroxidases is promoted by the imidazolium form of the distal histidine that exists only transiently. It was estimated that the rate constant for the breakage of the \(\text{O}–\text{O}\) bond in \(\text{H}_2\text{O}_2\) by HRP is significantly faster than \(1 \times 10^4\ \text{s}^{-1}\).

Heme peroxidases are oxidative metabolizing enzymes ubiquitously distributed in plants, animal tissues, and micro-organisms and oxidize a broad range of substrates by utilizing hydrogen peroxide (\(\text{H}_2\text{O}_2\)) as an oxidant (1, 2). The substrates are oxidized by two common intermediates of heme peroxidases called compounds I and II. Compound I is formed upon the activation of \(\text{H}_2\text{O}_2\) with the resting enzymes and contains an oxidant (1, 2). The substrates are oxidized by two common intermediates of heme peroxidases called compounds I and II. Compound I is formed upon the activation of \(\text{H}_2\text{O}_2\) with the resting enzymes and contains an oxidant. Compound I oxidizes the substrate and converts to compound II containing an oxyferryl heme, which returns to the resting state by oxidizing another molecule of the substrate. Heme peroxidases develop an efficient mechanism to capture and activate \(\text{H}_2\text{O}_2\) rapidly and specifically. The bimolecular rate constant for the formation of compound I (\(1.7 \times 10^7\ \text{M}^{-1}\text{s}^{-1}\)) is close to that of the diffusion-limited reactions (\(\sim 10^7\ \text{M}^{-1}\text{s}^{-1}\)) (4). The \(\text{H}_2\text{O}_2\) activation conducted by heme peroxidases produces little reactive oxy-

gen radicals as side products. Thus, intensive experimental and theoretical investigations have been conducted to reveal the molecular mechanism for the \(\text{H}_2\text{O}_2\) activation steps of heme peroxidases (5–7). The heme-peroxidase reactions are also important as models for other heme enzymes, such as catalase-peroxidase, cytochrome P450, and cytochrome oxidase, whose catalytic reactions involve heme species similar to those found in compounds I and II.

The authors propose that the \(\text{O}–\text{O}\) bond activation in heme peroxidases was first hypothesized by Poulos and Kraut (8, 9) based on the crystal structure of cytochrome c peroxidase. They propose that the conserved His-52 in the distal heme pocket assists the formation of a putative iron-peroxide complex by deprotonating \(\text{H}_2\text{O}_2\) and the heterolytic cleavage of the \(\text{O}–\text{O}\) bond by protonating to the outer oxygen (Fig. 1A). They further propose that an electrostatic interaction between Arg-48 and the outer oxygen atom promotes the \(\text{O}–\text{O}\) bond heterolysis. The proposal has been examined by numerous mutational studies on the distal His and Arg residues of cytochrome c peroxidase (10, 11) and of horseradish peroxidase (HRP)\(^6\) (12–18). In addition, the roles of the hydrogen bonding network that regulates the protonation state of the distal His were investigated for HRP (19–22). These studies establish the detailed functions of the distal residues and basically support the Poulos-Kraut mechanism (2, 23).

Despite the progress understanding on the functions of the distal residues of heme peroxidases, the putative precursor for compound I (the iron-peroxide complex) has never been identified at ambient temperature for wild type enzymes. It has been known that the lifetime of the precursor at room temperature is shorter than the dead time of conventional stopped-flow devices (several ms). The cryogenic stopped-flow experiments on HRP, using methanol or Me\(_2\)SO as cryosolvents, suggest the presence of the precursor state (24–26). The subsequent theoretical study assigned the spectrum of the state to the ferric peroxy anion complex (Fe\(^{3+}\)-\(\text{OOH}\)) (27). The cryorationalysis experiment on the ferrous oxygen-bound form of HRP identifies the Fe\(^{3+}\)-\(\text{OOH}\) species in the low spin form, which however, cannot convert to compound I and directly returns to the resting state upon the temperature increase (28, 29).

A similar cryorationalysis experiment based on x-ray crystallographic analysis, however, detected the formation of compound I (30). The stopped-flow experiments at room temperature for the formation process of compound I in the Arg-38→Leu mutant (R38L) of HRP suggest the existence of an intermediate in the ferric high spin state (17, 18), which is assigned to the ferric hydrogen peroxide complex (Fe\(^{3+}\)-\text{HOOH}) (27). The low temperature results for the polyethylene-glycolated HRP suggests the high spin intermediate (31). Thus, although several cryogenic experiments have detected the putative precursory state, the information is conflicting, and no evi-

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\(^6\) The abbreviations used are: HRP, horseradish peroxidase; BCP, bromocresol purple.
A clue to understand the conflicting information is proposed by Denisov et al. (29) and Egawa et al. (32). To explain the observation that the Fe$^{3+}$/H$_{11001}$-OOH species created by the cryoradiolysis of the ferrous oxygen-bound HRP cannot convert to compound I, Denisov et al. point out that the species lacks a proton required for the O–O bond heterolysis (29). Egawa et al. investigated the Fe$^{3+}$/H$_{11001}$-OOH species of metmyoglobin (32, 33), which was first identified in the reaction of H$_2$O$_2$ with a mutant of metmyoglobin (34). They detected the Fe$^{3+}$/H$_{11001}$-OOH species only in alkaline conditions and explain that the absence of the N$^{5}$/H$_{9254}$ proton from the distal His decelerates the conversion of the Fe$^{3+}$/H$_{11001}$-OOH species (Fig. 1B, (c)) to compound I (Fig. 1B, (e)) due to the lack of a proton for the O–O bond heterolysis. Egawa et al. (32) implemented the Poulos-Kraut mechanism by adding the deprotonation equilibrium of the N$^{5}$/H$_{9254}$ proton (Fig. 1B, (b) and (c)), which is biased to the protonated form in heme peroxidases due to the conserved hydrogen bonding.

To confirm the Poulos-Kraut mechanism and its implementation by Denisov et al. and Egawa et al., it is important to demonstrate the absence of the Fe$^{3+}$/H$_{11001}$-OOH species in the formation process of compound I upon the mixing of HRP and H$_2$O$_2$. In our previous study, we utilized a rapid freeze-quench device that can initiate and terminate biological reactions within 200 μs and monitored the compound I formation process of HRP (35). The EPR spectrum for the sample trapped at 200 μs after mixing H$_2$O$_2$ and HRP shows the complete formation of compound I (35). In the current study, we further improved the time resolution of the observation system, and developed a new kinetic absorption spectrometer having a 50-μs time resolution. The spectrometer consisted of a continuous fast-flow mixer using a stainless mixing plate sandwiched by quartz glasses (36) and an imaging spectrograph attached with a charge-coupled device. Utilizing this apparatus to the reaction of HRP with H$_2$O$_2$, we confirmed that the Fe$^{3+}$/H$_{11001}$-OOH species could not be identified, even with the improved time resolution.

MATERIALS AND METHODS

Preparations—All chemicals were purchased from Wako, Nacalai Tesque, and ICN as the highest quality available. Horseradish peroxidase was purchased from Wako, and isoforms B and C were purified using the reported method at 4 °C (37). In brief, the enzyme was dialyzed against 5 mM acetate buffer at pH 4.4, transferred to a CM-cellulose column, and eluted with a gradient of the acetate concentration until reaching a concentration of 100 mM. The eluted sample was dialyzed against 5 mM Tris buffer at pH 8.4 and applied to a DEAE-cellulose column and eluted with the same buffer. The purified enzyme contain-
ing isozymes B and C possessed the RZ value of 3.3 and was used for the kinetic measurements.

**Experimental Setup**—The T-shaped flow mixer originally reported by Akiyama et al. (36) was employed (Fig. 2A). Two solutions to be mixed were introduced in syringes and were supplied continuously to the mixing cell, which was constructed with a stainless mixing plate and two quartz plates. The two solutions were accelerated in the narrow channel (35 × 100 μm), creating turbulent flow at the center of the T-shaped channel, and were mixed. The mixed solution flowed inside the 100-μm square channel, where reactions were monitored using optical absorption spectroscopy.

The efficiency of the mixer was initially evaluated by observing the acid-base discoloration reaction. A solution containing 1 mg/ml bromocresol purple (BCP) and 10 mM sodium hydroxide at pH 12 and a solution of 50 mM sodium succinate at pH 4.0 were injected into the mixer at the flow rate of 0.084 ml/s for each solution (Fig. 2A, inset). The discoloration of BCP implied the mixing. The line speed of the mixed solution was 16.8 mm/ms along the flow channel. The distance along the channel required for the complete discoloration was <500 μm, which corresponded to 30 μs. Thus, kinetic absorption spectra in the time domain after 50 μs can be reliably observed using the system.

The optical setup of the developed system was shown schematically in Fig. 2B. The defocused white light from a xenon lamp (Hamamatsu, 150 W) was passed through the flow channel, and the image of the channel was focused at the entrance slit of an imaging spectrograph (Chromex, model number 250iS). A heat absorbing filter was placed in front of the slit. The image of the channel was dispersed by the spectrograph and was detected by a cooled charge-coupled device (Princeton Instruments, TE/CCD 1340/400) as the two-dimensional image. The axes of the image parallel and perpendicular to the entrance slit correspond to the time after the mixing and wavelength, respectively. The kinetic absorption spectra (A) were calculated by comparing the two-dimensional images for the sample (I) and for the buffer reference (I₀) after subtracting the dark image (D) based on the following equation.

\[
A = \log \left( \frac{I}{I₀ - D} \right) \quad (\text{Eq. 1})
\]

Approximately 25 s were required for a measurement of the time-resolved spectra in the Soret region, with optical densities of ~0.04, which was achieved at the HRP concentration of 50 μM. The wavelength calibration of the spectra was performed with the bright lines of a mercury lamp.

**Reaction of HRP with Peroxides**—A buffered solution containing the ferric form of HRP and another buffered solution containing various concentrations of H₂O₂ were passed through cellulose syringe filters (0.22 μm pore) and were introduced into 5-ml gas-tight syringes. They were injected into the mixer at the rate of 0.084 ml/s for each solution. The line speed of the mixed solution in the observation channel was 16.8 mm/ms. The buffer employed for the kinetic measurements was 50 mM sodium phosphate at pH 7. All kinetic measurements were performed at room temperature (~20 °C).

**RESULTS**

**Construction of the Kinetic Absorption Spectrometer for the Microsecond Time Domain**—We constructed a new kinetic spectrometer, which could monitor biological reactions in the time domain from 50 to 300 μs based on the T-shaped flow mixer originally reported by Akiyama et al. (36) and the imaging spectrograph with the two-dimensional detection system (Fig. 2A and B). We could reduce the mixing time to <50 μs by constructing the small flow channel with a width of 100 μm. A preliminary report of the improved mixer has been described previously (38). The mixing cell requires the continuous supply of sample solutions and a rather large amount of samples; however, the sample consumption was reduced considerably by utilizing imaging spectrograph that could monitor the entire observation channel simultaneously. A similar kinetic observation system developed independently has been reported recently (39).

The mixing dead time of the system could be roughly estimated to be <50 μs as demonstrated by the discoloration reaction of the pH-sensitive dye (Fig. 2A, inset). For the quantitative estimation of the mixing rates, we monitored the reduction reaction of [Ir⁴⁺(CN)₆]⁴⁻ by [Fe²⁺(CN)₆]₃⁻ (40). We confirmed that kinetic traces monitored at the absorbance peak for [Ir⁴⁺(CN)₆]⁴⁻ showed a single exponential decay in the time domain after 50 μs and that the apparent rate constants obtained by fitting the kinetic traces reproduced the reported values in the range of <1 × 10⁴ s⁻¹ (see “Supporting Information” in the on-line supplemental text). We concluded that the device could correctly monitor the process after 50 μs of mixing and at rate constants <1 × 10⁴ s⁻¹.

**Compound I Formation in the Microsecond Time Domain**—To investigate the sub-ms time range in the formation of compound I in HRP, the reaction of ferric HRP and 1 mM H₂O₂ at pH 7 was observed with the developed apparatus. The obtained spectra are shown in Fig. 3A. The absorption maximum in the Soret region of the ferric resting enzyme is 403 nm, whose intensity is reduced without changing the wavelength upon the addition of H₂O₂. An isosbestic point was observed at 430 nm, which is identical to that between ferric resting enzyme and compound I (41). There is another isosbestic point at 357 nm between the resting state and compound I (41). The corresponding isosbestic point in Fig. 3 is obscure due to the low signal to noise ratio and does not allow a further discussion. These spectral changes are characteristic of the formation of compound I. We next utilized the higher concentration of...
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H₂O₂, which would result in a larger accumulation of the intermediate; however, the time-resolved absorption spectra after 50 µs are almost identical to that of compound I, even with 3 mM H₂O₂ (Fig. 3B). Thus, we could not identify a distinct spectrum for the putative intermediate.

In an attempt to lengthen the lifetime of the intermediate, we utilized tert-butylhydroperoxide as an oxidant, because the formation rate of compound I using the oxidant is depressed >1000-fold compared with that of H₂O₂. The microsecond time-resolved absorption spectra for the reaction of HRP and 250 mM tert-butylhydroperoxide only showed the slow formation of compound I (not shown). We therefore concluded that the initial association of the alkyl peroxide to HRP is reduced without drastically affecting the lifetime of the intermediate.

Although we could not identify a distinct spectrum for the primary intermediate, it could be possible to infer the elementary steps of the compound I formation using the Michaelis-Menten analysis. We therefore investigated the H₂O₂ concentration dependence of the formation process of compound I. The time course of the absorbance at 403 nm for all concentrations of H₂O₂ utilized could be fitted well with a single exponential function (Fig. 4A). The apparent rate constants (k₁obs) were determined from the fitting and plotted against the H₂O₂ concentration as shown in Fig. 4B. The plot shows the saturation behavior similar to that expected for the Michaelis-Menten plot; however, it is more likely that the saturation was caused by the mixing process of the current mixer. In fact, the plot in Fig. 4B is very similar to that observed for the reduction reaction of [Ir⁴⁺Cl₄]²⁻ (see “Supporting Information” in the on-line supplemental text). We concluded that the lifetime of the primary intermediate should be significantly shorter than the time resolution of the device (1 × 10⁴ s⁻¹).

**DISCUSSION**

We succeeded in reducing the observation dead time of the kinetic absorption spectrometer to <50 µs with the continuous flow mixing system. The spectra for the resting state and compound I of HRP obtained by the new system are identical to those obtained by the conventional system (41). The absorption spectra detected by the device were reliable in the time domain after 50 µs. Furthermore, the second order rate constant estimated from k₁obs at 0.5 mM of H₂O₂, which was free from the mixing artifact, was ∼2 × 10⁷ M⁻¹ s⁻¹. The constant agreed well with the value of the stopped-flow experiment (1.7 × 10⁷ M⁻¹ s⁻¹) (4).

Using the developed system, we systematically investigated the reaction between HRP and H₂O₂; however, we could not identify a distinct absorption spectrum for the putative intermediate before the formation of compound I, even with the high concentration of H₂O₂. Considering that the Fe³⁺-OOH species of HRP possesses a Soret maximum at 419 nm and is distinct from the resting enzyme possessing a maximum at 403 nm (29), we concluded that the Fe³⁺-OOH compound did not exist in the time domain after 50 µs. There remained a possibility that the species possessing a spectrum similar to that of the resting enzyme could exist. As reported for the R38L mutant of HRP, the Fe³⁺-HOOH species is considered to possess a Soret maximum ∼404 nm (16). The saturation behavior of k₁obs against H₂O₂ concentration could also suggest the presence of the intermediate (Fig. 4B); however, the saturation was more likely caused by the limitation of the mixing time (see “Supporting Information” in the on-line supplemental text). Thus, although there existed the possibility that the Fe³⁺-HOOH species was present at a low concentration in the time domain after 50 µs, we concluded that the lifetime of the species should have been much faster than the highest rate detectable by the current device (1 × 10⁴ s⁻¹).

The absence of the Fe⁵⁺-OOH species in the time domain after 50 µs did not agree with some of the previous results. The cryogenic stopped-flow experiments detected the intermediate in the compound I formation (24), which had been later assigned to the Fe⁵⁺-OOH species (27). However, the rate constant for the O–O bond heterolysis estimated from the extrapolation of the low temperature data to room tempera-
turer is $\sim10^3$ s$^{-1}$, which is significantly smaller than the value from the current study ($>1 \times 10^5$ s$^{-1}$). It should be noted that the small rate constant is cited in several recent reviews (2, 23). Together with the observation that the Fe$^{3+}$-OOH species created by reducing the ferrous oxygen-bound HRP at cryogenic temperature cannot convert to compound I (29), we concluded that the Fe$^{3+}$-OOH species observed previously was caused by the non-physiological solvent, temperature, and the method of initiating reactions.

Although the current data do not provide direct evidence for the Fe$^{3+}$-OOH species, the absence of the Fe$^{3+}$-OOH species with a detectable lifetime is rather consistent with the recent proposals on the activation of H$_2$O$_2$ by heme peroxidases (29, 32). It is explained that the lifetime of the Fe$^{3+}$-OOH species in heme peroxidases is short, because the distal His becomes an unstable imidazolium cation after accepting a proton from H$_2$O$_2$ at the N site because of the conserved Asn residue (Asn-70 in HRP), stabilizing the N$^\bullet$OOH species with a $\sim10^3$ s$^{-1}$ rate constant for the heterolysis as the inter-atomic distance $\ldots$ (43). Our data show that some other step or steps, such as electron transfer in the distal His acting as the base catalyst (42). Furthermore, the previous rate constant ($\sim10^3$ s$^{-1}$) was a basis for considering the O$\ldots$O bond heterolysis as the rate-limiting step in turnover reactions of HRP with fast substrates (43). The rate constant for the breakage of the O$\ldots$O bond in H$_2$O$_2$ by HRP should be significantly faster than $\sim10^6$ s$^{-1}$.

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