Supporting Information

Porphyrinoid Chemistry in Hemoprotein Matrix: Detection and Reactivities of Iron(IV)-Oxo Species of Iron Porphycene Incorporated into Horseradish Peroxidase

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Instruments. UV-visible experiments were conducted on a Shimadzu UV-3150 double beam spectrophotometer equipped with a thermostat cell holder within 0.1 °C deviation. pH values were monitored by a HORIBA F-52 pH meter The EPR spectra were measured by a Varian E-12 spectrometer equipped with an Oxford ESR-900 liquid helium cryostat. Kinetics measurements were conducted with a rapid scan stopped-flow system constructed by Unisoku Co., Ltd. (Osaka, Japan). A Xe Arc lamp was employed as a source of the probe light to follow the spectral changes. Gas chromatography analysis was conducted on a Shimadzu GC-2014 Chromatograph equipped with a DB-1 30 m × 0.251 mm × 0.25 μm J&W GC Column Agilent Technologies.

Materials. 2,7-Diethyl-3,6,12,17-tetramethyl-13,16-bis(2-carboxyethyl)porphycenatoiron (III) chloride (**2**) was synthesized by the same method described in the previous paper.[S1] Native horseradish peroxidase (HRP) was purchased from Sigma (P 8415, hydrogen-peroxide oxidoreductase, Type XII), salt-free powder and used without further purification. The native enzyme exhibited an A_{402}/A_{280} ratio (R_Z value) of 3.3. Catalase from bovine liver was purchased from Sigma. All regents were obtained from commercial sources and were used as received. Distilled water was demineralized by Milli-Q Academic A10. Hydrogen peroxide was purchased from Wako Pure Chemical Industries. The concentration was determined from molar extinction coefficient at 240 nm of H_2O_2 (ε_{240} = 39.4 $M^{-1}cm^{-1}$). The reconstituted enzyme with **2** was prepared by the method described below**.**

Preparation of HRP Reconstituted with an Iron Porphycene, rHRP(2). ApoHRP was prepared according to the method of Teale[S2] with some modifications. HRP was dissolved in 700 μL of 100 mM sodium phosphate buffer (pH = 7.0). The pH of the solution was brought to pH = 1.9 by slowly adding 0.1 M HCl. The acidic HRP solution was extracted with 2-butanone (2 mL ×4) to remove the heme, and was then immediately neutralized with 1 M NaOH. The apoHRP in the neutral solution was dialyzed against 500 mL of 50 mM sodium phosphate buffer (pH 7.0). The concentration was

determined from the absorbance intensity at 278 nm (ε_{278} = 20000 $M^{-1}cm^{-1}$).[S3] A stock solution of **2** was prepared in dimethylsulfoxide, and the concentration was determined from the absorbance intensity at 368 nm (ε_{368} = 89,000 $M^{-1}cm^{-1}$ in CH_2Cl_2[S4]). The iron porphycene solution was added dropwise into the solution of apoHRP with gently shaking on an ice-bath. After slow shaking for 30 min at 4 ºC, the mixture was immediately concentrated, diluted and concentrated again by ultrafiltration to remove low-weight materials. After centrifuging (12,000 rpm, 10 min, 4 ºC), the reconstituted HRP having the iron porphycene, rHRP(**2**), was purified by passing through a HiTrap DEAE FF (1 mL, GE Healthcare) column with chilled 50 mM sodium phosphate buffer (pH 7.0). Moreover, the rHRP(**2**) was purified by passing through a Sephadex G-25 Fine (0.8 x 27 cm, GE Healthcare) column with 50 mM sodium phosphate buffer (pH 7.0). The value of A_{soret}/A_{278} for rHRP(**2**) was 2.2. The molar extinction coefficient at 382 nm (ε_{382}) was determined to be 77,000 $M^{-1}s^{-1}$ by pyridine-hemochromogen mehod.[S5] The prepared protein was sufficiently stable for a couple of days at 4 ºC

Electron Paramagnetic Resonance (EPR) Spectroscopy. The measurements of EPR spectra were carried out at the X-band (9.35 GHz) microwave frequency with 100 kHz field modulation (0.5 mT) at 5 K. After centrifuging (12,000 rpm, 10 min, 4 ℃), a solution of the native HRP or rHRP(**2**) (270 μM) in 50 mM MOPS buffer (pH 7.0) was placed in a 5 mm tube.

Spectroscopic Characterization of Oxo-ferryl Species of rHRP(2). Formation and decay of oxo-ferryl species (iron(IV)-oxo π-cation radical and its one-electron reduced form) of rHRP(**2**) were monitored by UV-vis spectroscopy. Particularly, the measurements in near infrared region at the initial stage for the reaction of rHRP(**2**) with H_2O_2 were carried out by using a stopped-flow apparatus in 50 mM sodium phosphate buffer (pH = 7.0) at 10 ºC, where the concentrations of rHRP(**2**) and H_2O_2 employed were 5.9 μM and 11.3 μM, respectively. The probe light of the lower range (<520 nm) was cut off with an optical filter (Sigma Koki Co., Ltd., Japan, SCF-50S-52Y). The measurements in near infrared region at the initial stage for the reaction of iron(IV)-oxo π-cation radical with $K_4[Fe(CN)_6]$ were carried out by using a double mixing stopped-flow apparatus in 50 mM sodium phosphate buffer (pH = 7.0) at 10 ℃. Aging time after the mixing of H_2O_2 to the protein is 1 sec.

Determination of Formation Rate of the Iron(IV)-oxo π-Cation Radical (k_1). After mixing of HRP (2.3 μM for the nHRP, 1.3 μM for rHRP(**2**)) with excess H_2O_2 in 50 mM sodium phosphate buffer (pH = 7.0) at 10 ºC by using a stopped-flow apparatus, the spectral changes in the range of 300-700 nm were monitored. The absorbance change at 403 nm (for the native HRP) or 380 nm (for rMb(**2**)) was analyzed by one-phase exponential kinetics to yield the pseudo-first-order rate constant (Figure S2). Plotting the rate constants against [H_2O_2] yielded k_1 from the slope (Figure S3).

Rate of the Iron(IV)-oxo Formation (k_2) and Regeneration of Ferric State (k_3). The rate constants

k_2 and k_3 were obtained by analyzing the spectral changes (300–700 nm) after the rapid mixing of iron(IV)-oxo π-cation radical with excess guaiacol in 50 mM sodium phosphate buffer (pH = 7.0) at 10 ºC. Iron(IV)-oxo π-cation radical was generated by the addition of 1.1 equiv. of H_2O_2 into the incubated ferric protein solution in the sample reservoir of the stopped-flow apparatus (2.0 μM for the native HRP and 2.8 μM for rHRP(**2**)). The absorbance change observed was composed of two exponential components (Figure S4), and the first rapid stage and the second slow stage were attributed to the iron(IV)-oxo formation and the regeneration of a ferric state, respectively. The absorbance change at 415 nm (for the native HRP) or 580 nm (for rMb(**2**)) was analyzed by two-phase exponential kinetics to yield the two pseudo-first-order rate constants. Plotting each rate constant against [guaiacol] yielded k_2 and k_3 from the slopes (Figure S3).

Peroxidase Activity toward Guaiacol Oxidation. The protein (1 μM, 10 μL) and guaiacol solution were added into 970 μL of 50 mM sodium phosphate buffer (pH = 7.0) at 25 ºC. The concentrations of guaiacol employed were in a range from 20 to 300 μM. H_2O_2 aq (400 mM, 10 μL) was added into the mixed solution and stirred. The final volume of the mixed solution was 1 mL. The oxidation rate was determined by following the increase in the absorbance at 470 nm. The oxidation rate constants were calculated by using a molar absorption coefficient of the oxidation product of 26,600 $M^{-1}cm^{-1}$.[S6]

Peroxygenase Activity toward Thioanisole Sulfoxidation. The protein (5 μM, 1 mL) was incubated in 50 mM sodium phosphate buffer (pH = 7.0) for 5 min at 25 ºC. H_2O_2 aq (900 mM, 5 μL) was added into the protein solution after the addition of thioanisole (89 mM, 52 μL) in methanol to the protein solution. After the incubation for 5–20 min at 20 ºC, catalase (1 mg/mL, 5 μL) was added into the mixed solution to stop the reaction. Then, benzyl alcohol (10.3 mM, 10 μL) as an internal standard and diethyl ether were added to the solution. To extract thioanisole, methylphenyl sulfoxide and benzyl alcohol, the mixed solution was shaken hard by vortex. The separated ether phase was concentrated by a N_2 flow, and analyzed by gas chromatography equipped with DB-1. The volatile materials were detected by GC/FID or GC/MS.

References

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(S2) Teale, F. W. J. *Biochim. Biophys. Acta* **1959**. *35*, 543.

(S3) (a) Tamura, M.; Asakura, T.; Yonetani, T. *Biochim. Biophys. Acta* **1972**, *268*, 292–304. (b) ε_{403} = 102,000 $M^{-1}cm^{-1}$ was used as the absorbance intensity at 403 nm of native HRP. Schonbaum, G. R.; Lo, S. *J. Biol. Chem*. **1972**, *247*, 3353–3360.

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(S6) DePillis, G. D.; Sishta, B. P.; Mauk, A. G.; Ortiz de Montellano, P. R. *J. Biol. Chem*. **1991**, *266*, 19334–19341.

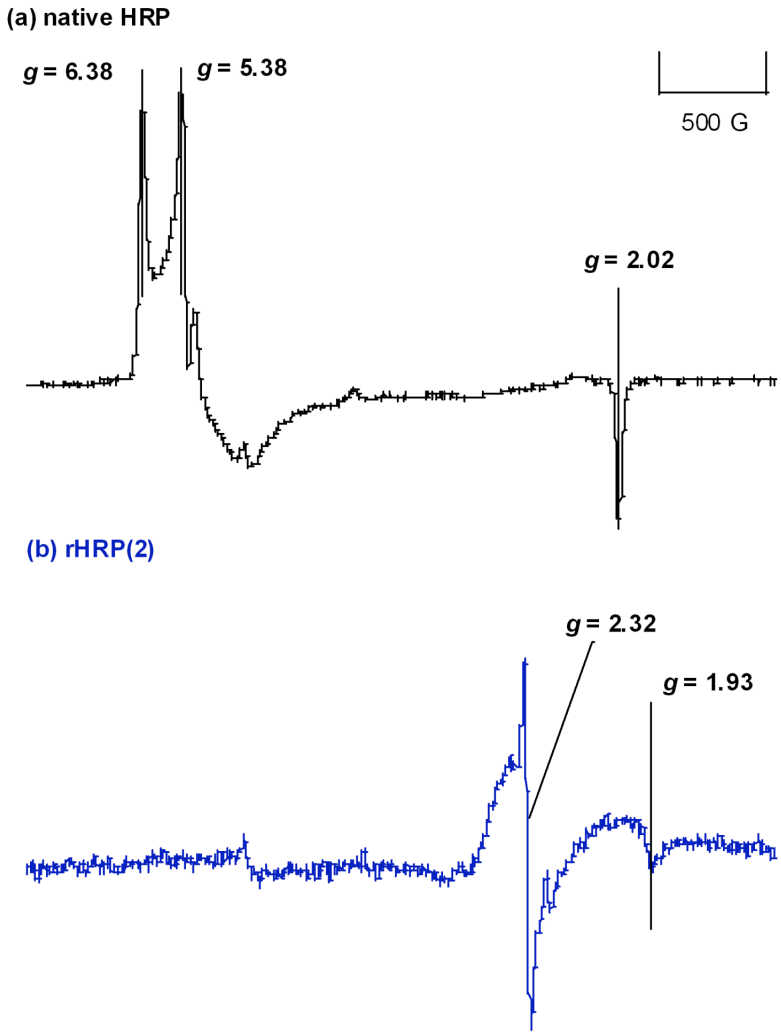


Figure S1. EPR spectra of native HRP (spectrum (a)) and rMb(**2**) (spectrum (b)) in 50 mM MOPS buffer (pH = 7.0) at 5 K.

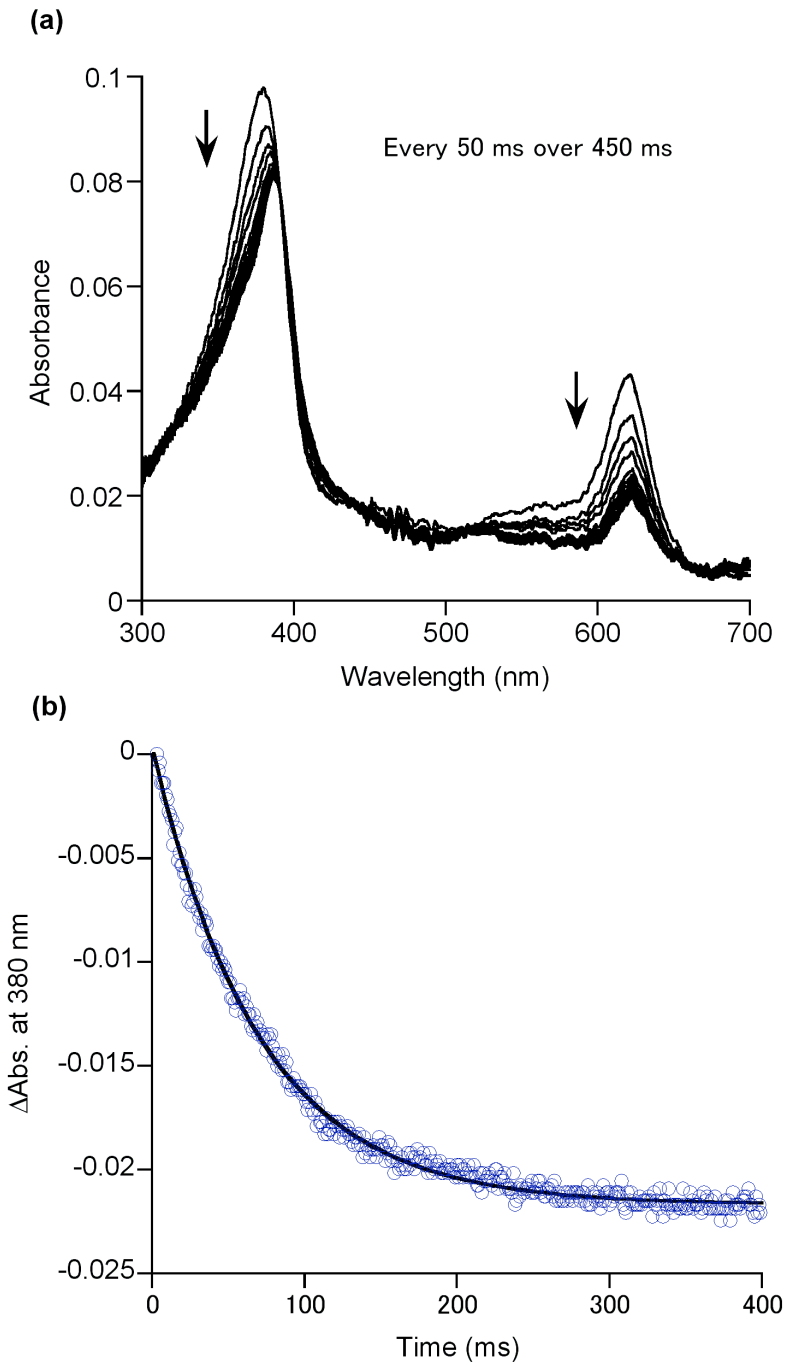


Figure S2. (a) Spectral changes in the reaction of the ferric state rHRP(**2**) with H_2O_2. (b) Absorbance change at 380 nm and single exponential fit of the reaction of rHRP(**2**) with H_2O_2 for kinetics measurement. Experimental conditions : [rHRP(**2**)] = 1.3 μM, [H_2O_2] = 12 μM, in 50 mM sodium phosphate buffer (pH 7.0) at 10 ºC.

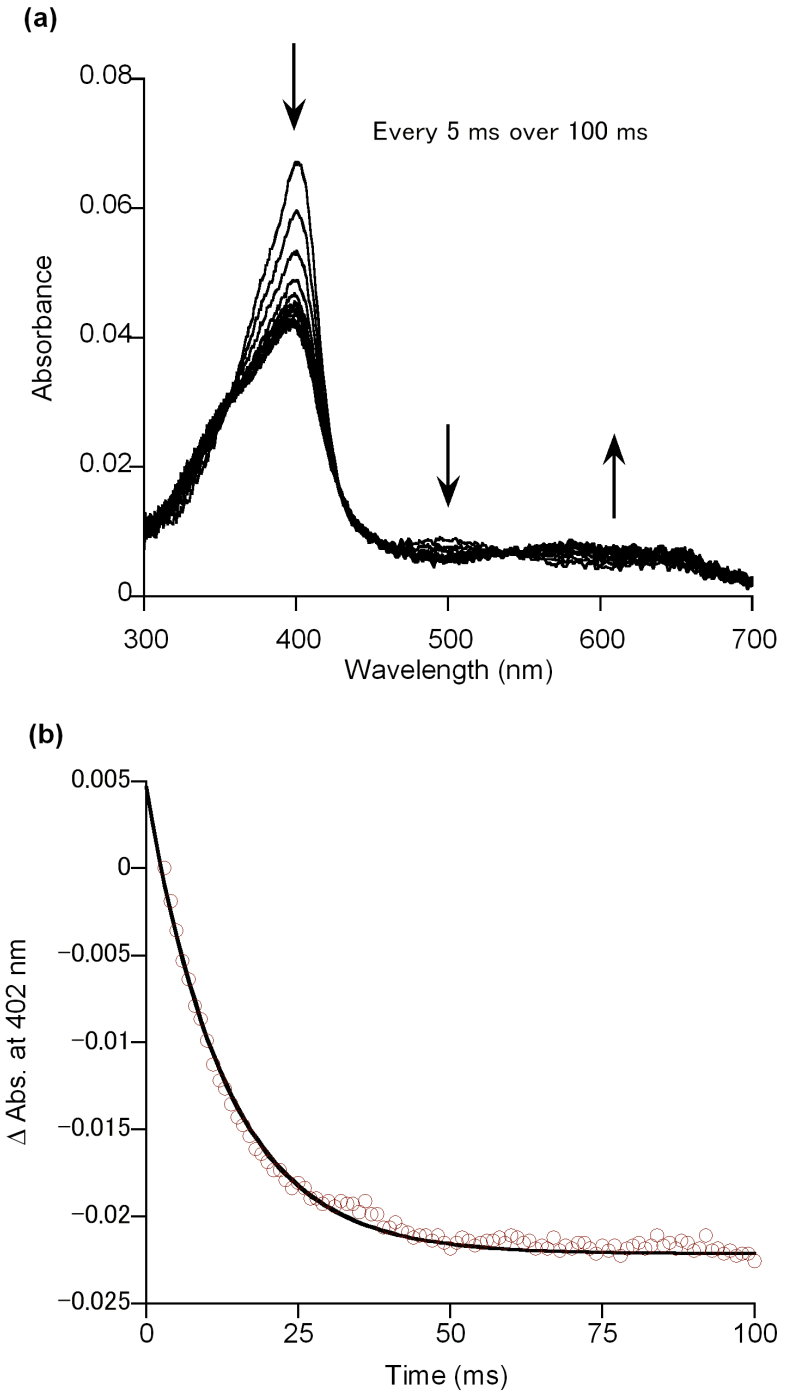


Figure S3. (a) Spectral changes in the reaction of ferric native HRP with H_2O_2. (b) Absorbance change at 402 nm and the single exponential fit of the reaction of native HRP with H_2O_2 for kinetics measurement. Experimental conditions: [native HRP] = 0.76 μM, [H_2O_2] = 8.1 μM, in 50 mM sodium phosphate buffer (pH 7.0) at 10 ºC.

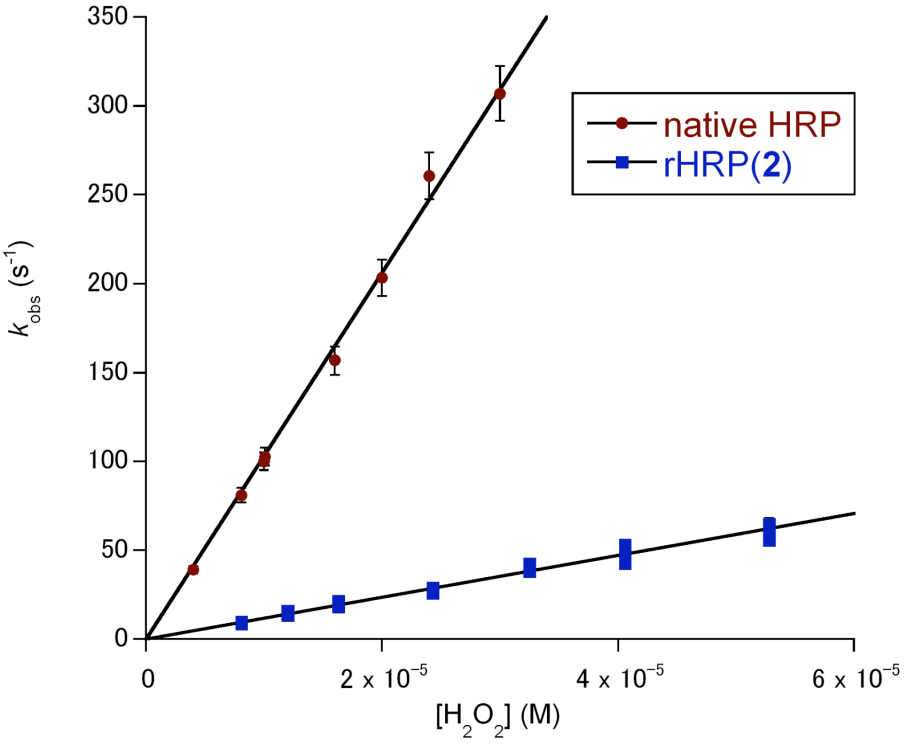


Figure S4. Pseudo-first-order plots in the reaction of HRPs with H_2O_2 in 50 mM sodium phosphate buffer (pH = 7.0) at 10 ºC.

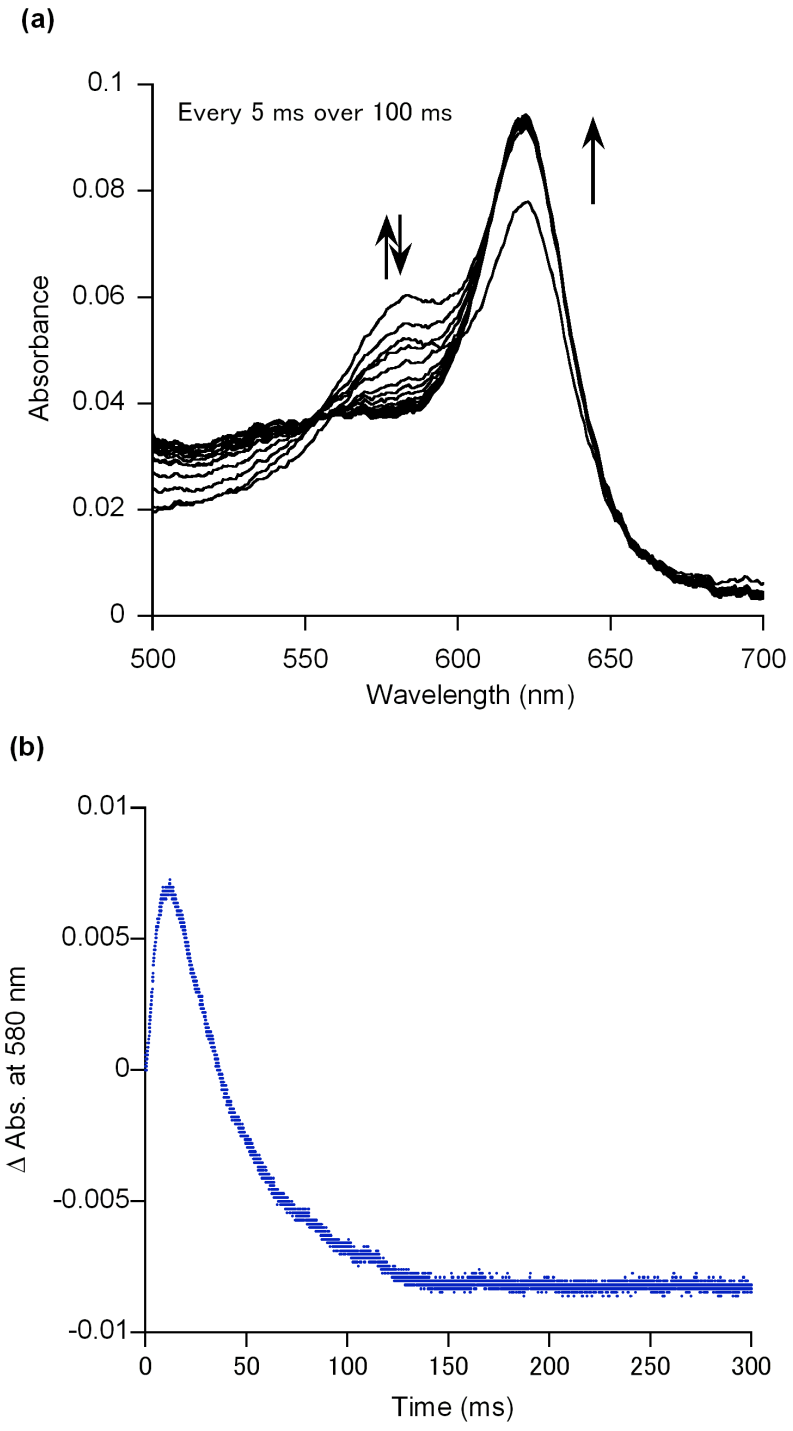


Figure S5. (a) Spectral changes in the reaction of Compound I of rMb(**2**) with guaiacol. (b) Absorbance change at 580 nm. Experimental conditions : [rHRP(**2**)] = 2.8 μM, [H_2O_2] = 2.8 μM, [guaiacol] = 30 μM in 50 mM sodium phosphate buffer (pH 7.0) at 10 ºC.

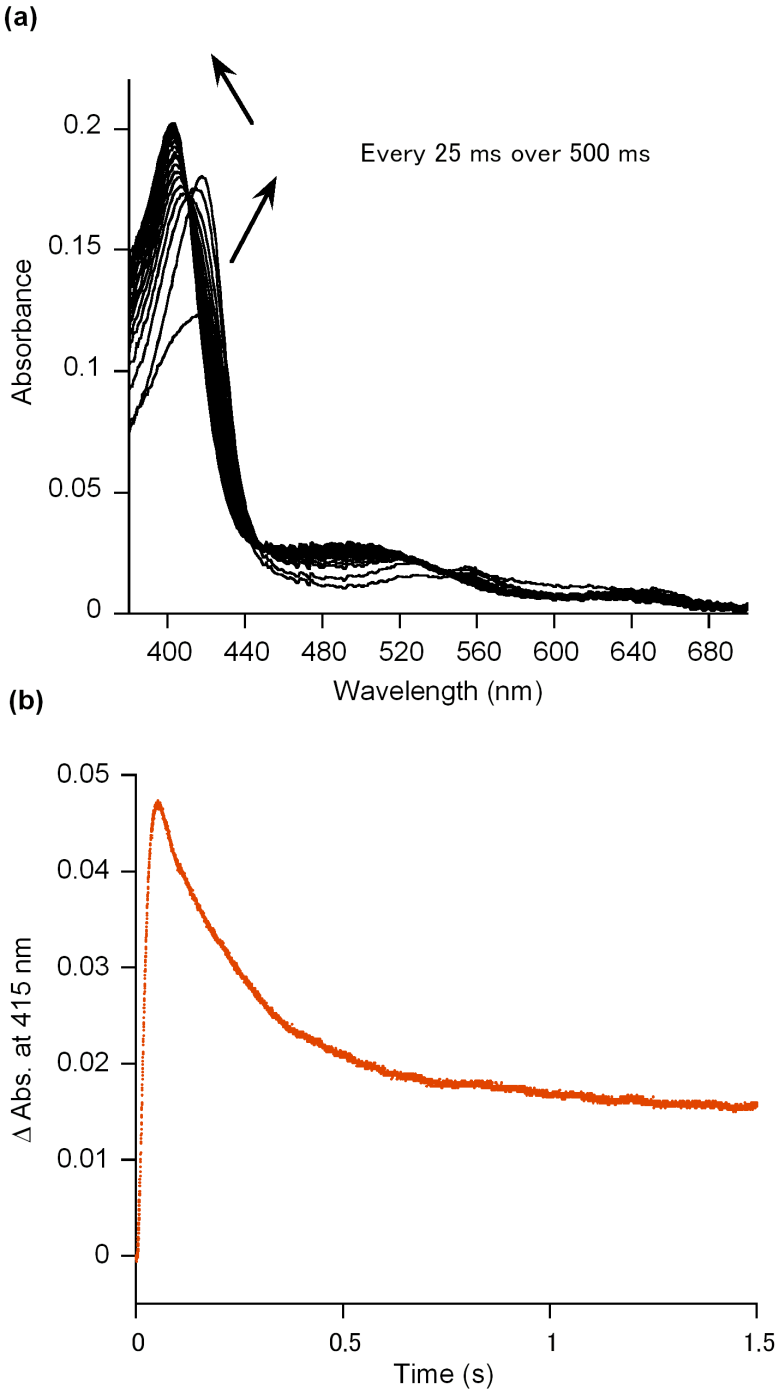


Figure S6. (a) Spectral changes in the reaction of Compound I of the native HRP with guaiacol. (b) Absorbance change at 415 nm. Experimental conditions: [native HRP] = 1.9 μM, [H_2O_2] = 1.9 μM, [guaiacol] = 20 μM in 50 mM sodium phosphate buffer (pH = 7.0) at 10 ºC.

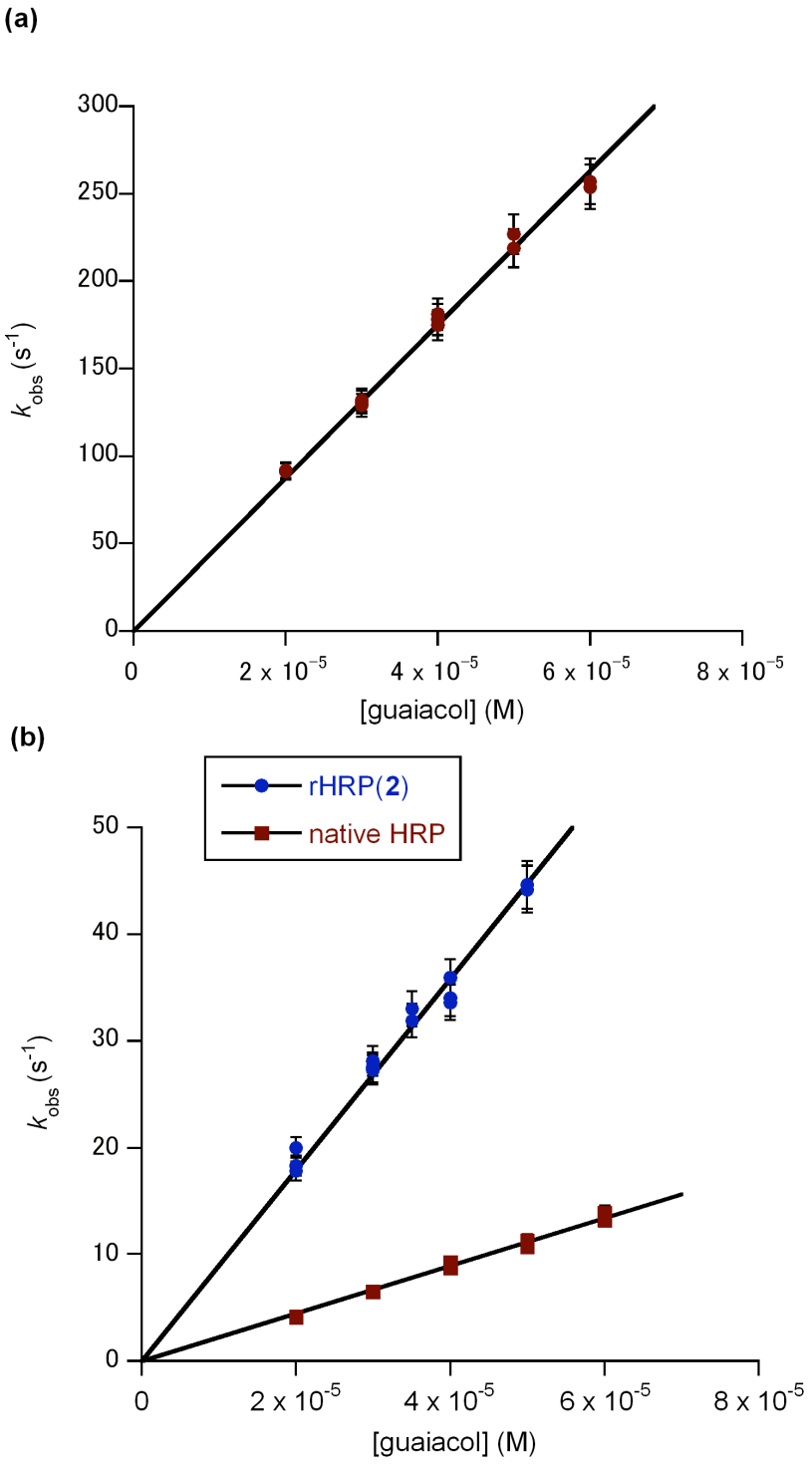


Figure S7. (a) Pseudo-first-order plot for Compound II formation (k_2 process) of the native HRP. (b) Pseudo-first-order plots for the regeneration of a ferric state (k_3 process) of HRPs in 50 mM sodium phosphate buffer (pH = 7.0) at 10 ºC.

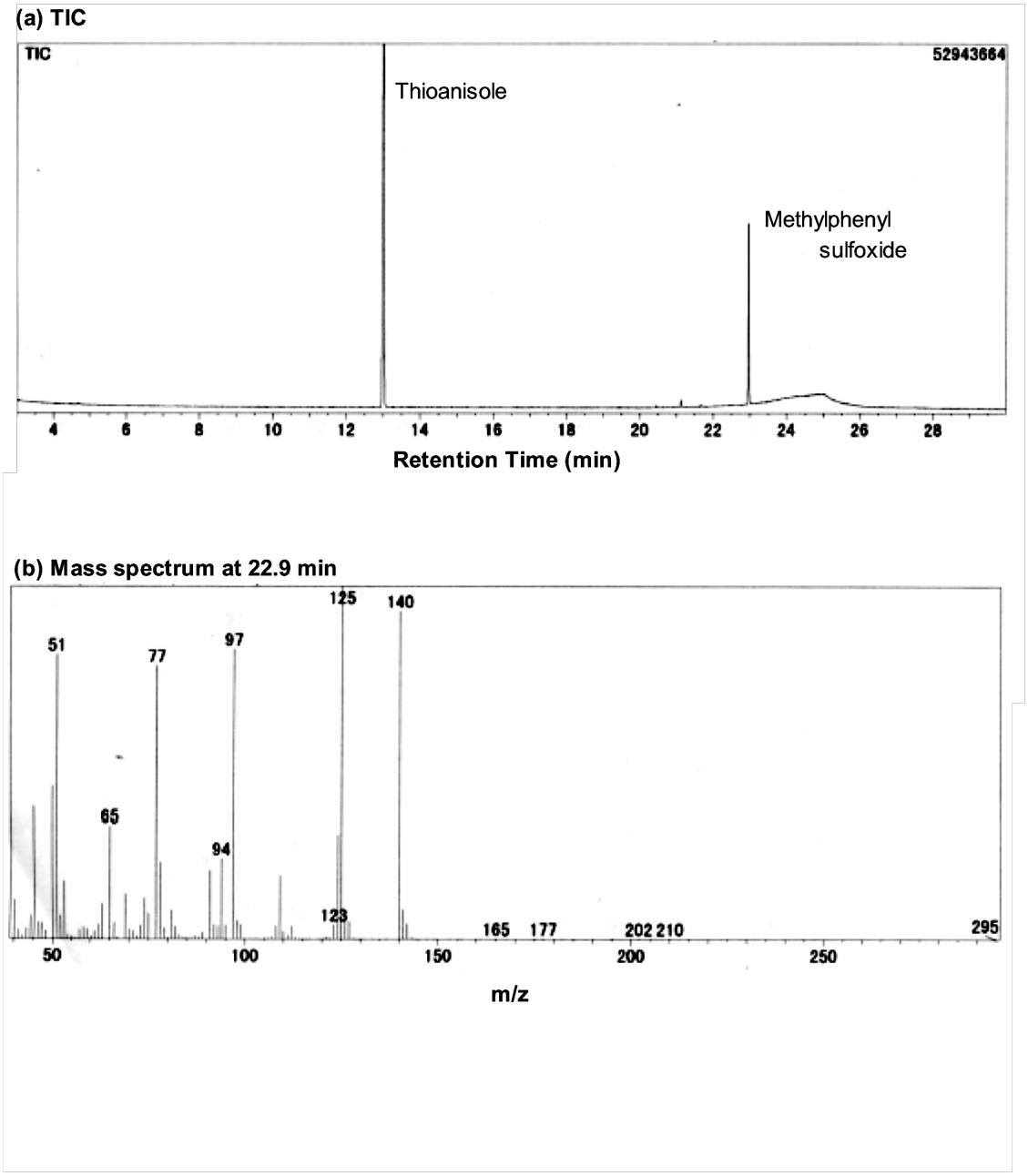
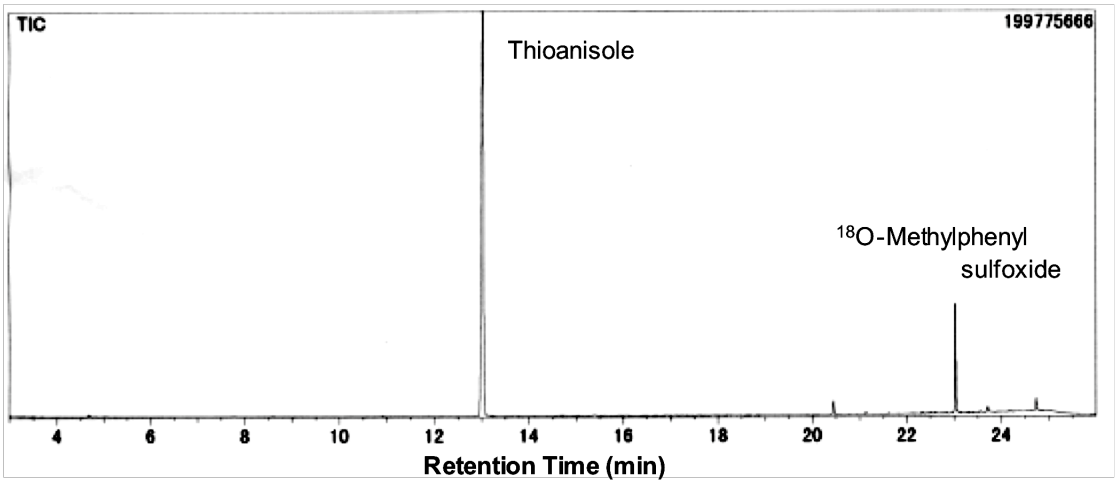


Figure S8. GC-MS data of the extracts from the reaction mixture of rHRP(**2**)-catalyzed reaction of thioanisole with $H_2{}^{16}O_2$. (a) TIC chromatogram; (b) MS spectrum detected at 22.9 min.

(a) TIC



(b) Mass spectrum at 22.9 min

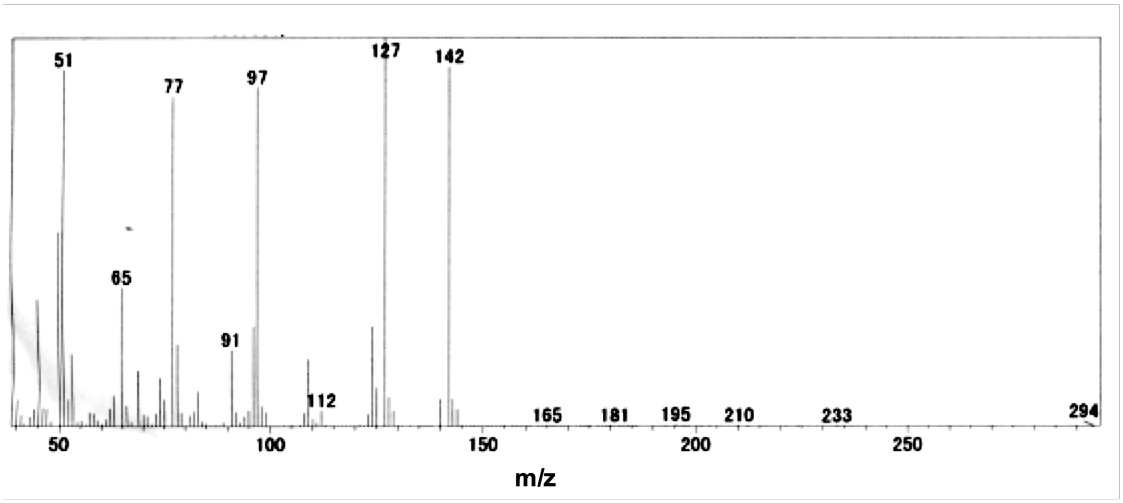


Figure S9. GC-MS data of the extracts from the reaction mixture of rHRP(**2**)-catalyzed reaction of thioanisole with $H_2{}^{18}O_2$. (a) TIC chromatogram; (b) MS spectrum detected at 22.9 min.