Characterization of Polyethylene Glycolated Horseradish Peroxidase in Organic Solvents: Generation and Stabilization of Transient Catalytic Intermediates at Low Temperature

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Abstract: Polyethylene glycolated horseradish peroxidase (PEG-HRP) can catalyze one- and two-electron oxidation reactions in organic solvents as well as in aqueous buffer. Even though the oxidation of guaiacol in benzene and chlorobenzene is 5 orders of magnitude slower than in phosphate buffer, compounds I and II are involved in the catalytic cycle in organic media. Factor analysis and global fittings of rapid scan data set reveal that the formation of compound I of PEG-HRP in organic media consists of two steps (the first fast and the second slow) and suggest the involvement of a H_2O_2−HRP complex in the catalytic cycle. The labile precursor of compound I is stabilized when PEG-HRP reacts with hydrogen peroxide in chlorobenzene at −20 °C. The absorption spectrum of the precursor does not exhibit the features of hyperporphyrin spectrum but has a normal Soret as previously observed in R38L HRP. More importantly, compound I of PEG-HRP can be maintained for more than an hour at −20 °C in chlorobenzene.

Introduction

Horseradish peroxidase (HRP), a member of the plant peroxidase containing heme as a prosthetic group, catalyzes the oxidation of a variety of substrates utilizing hydrogen peroxide.[1] It has been established that the enzymatic reactions in aqueous buffer normally proceed through the mechanism depicted in Figure 1,[1a,2] where compounds I and II represent the ferryl intermediates, and AH_2, the peroxidase substrate such as guaiacol. Compound I is reduced back to the ferric resting state either by the two sequential one-electron-transfer processes from peroxidase substrates or by the two-electron oxidation processes associated with the ferryl oxygen transfer to substrates such as thioethers.[3]

A couple of intermediates have been proposed in the formation of compound I (Figure 2).[1b] On the basis of the X-ray crystal structure of cytochrome *c* peroxidase (C*c*P) and homologies of amino acid sequences of C*c*P and HRP, the distal histidine in the heme pocket presumably functions as a general acid−base catalyst in formation of the compound I.[4] Thus, a postulated mechanism (Figure 2) includes (1) the formation of peroxide−heme complex, (2) the deprotonation of peroxide by the distal histidine to form compound 0 (a peroxoanion−heme species), and (3) the heterolytic O−O bond cleavage with a help of the protonated imidazole and a positively charged arginine residue in the active site.

Kinetic evidence for the precursor of HRP compound I was first provided by the low-temperature stopped-flow experiment

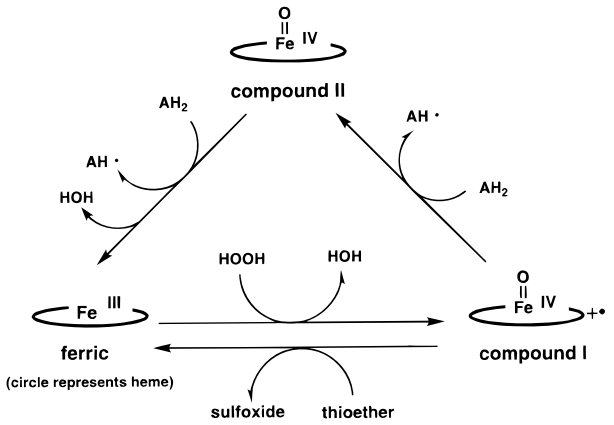

Figure 1. Catalytic cycle of Horseradish peroxidase (HRP). AH_2 represents peroxidase substrate such as guaiacol.

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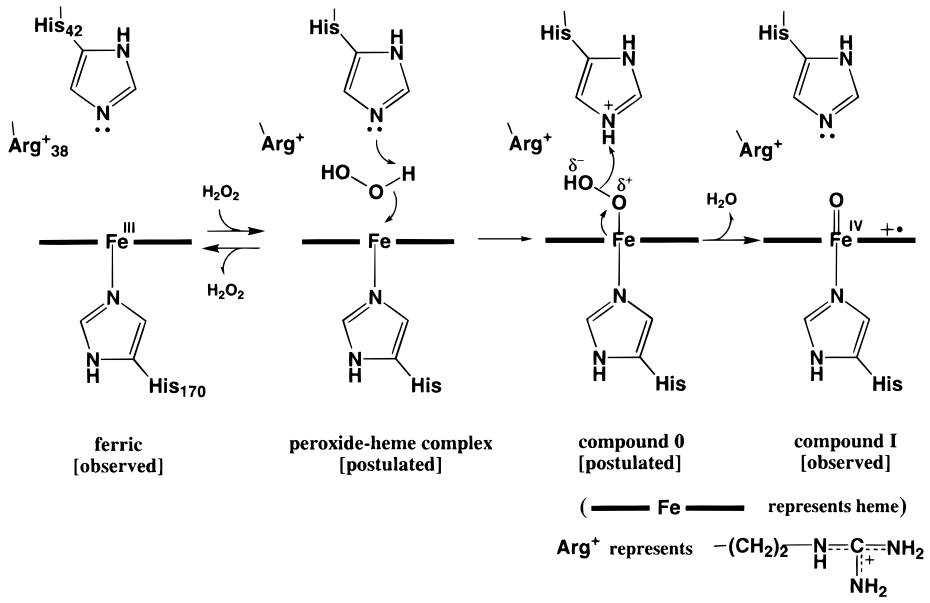

Figure 2. Proposed steps in the formation of compound I of peroxidases. The mechanism includes two postulated intermediates, a peroxide−heme cmplex and compound 0. Amino acid residues in the active site (His-42, His-170, and Arg-38 in Horseradish peroxidase) are shown.

in 50% v/v methanol/phosphate buffer.[5] The new intermediate, so-called compound 0, was proposed to be a hyperporphyrin formed by deprotonation of a H_2O_2−HRP complex and had a spectrum with two intense absorptions at 330 and 400 nm. The replacement of Arg-38 of HRP with a nonpolar leucine residue inhibited the O−O bond cleavage, and the intermediate was successfully observed at 10 °C in aqueous buffer (Figure 2).[6] The spectrum of the species, however, resembled that of the ferric state, and no intermediates having a spectrum similar to that of compound 0 were detected. Recent calculations using the INDO/ROHF/CI quantum chemical method suggested that a hyperporphyrin spectrum, or a split Soret, was originated from the peroxoanion−iron(III) complex.[7] The authors further proposed the spectrum of a neutral H_2O_2−HRP complex is similar to that of the resting enzyme. Together with these results, the new intermediate observed for R38L HRP would be a neutral peroxy−iron complex and an anionic form might not be stable in the less polar environment. Thus, we have been motivated to investigate biochemical processes of native HRP in virtually nonpolar reaction media.

During the last two decades polyethylene glycol (PEG) was widely used as a covalent modifier of a variety of proteins and provided a wide range of solubilities in both organic and aqueous media.[8] HRP conjugated with PEG (PEG-HRP) was originally prepared by Y. Inada in 1984, and a variety of strategies have been used by a number of groups.[9] The studies have been focused on measurements of catalytic activities of PEG-HRP in organic media;[9] however, in 1995, P. A. Mabrouk provided the evidence that the active site of PEG-HRP remains structurally intact in benzene on the basis of ESR, absorption, circular dichroism, and resonance raman measurements of the ferric state.[10] Furthermore, Mabrouk successfully stabilized PEG-HRP compound II for more than 2 h in benzene at room temperature and proposed a novel idea that "the use of nonaqueous media may provide an unparalleled opportunity to probe intermediates in complex biochemical mechanisms."[10] Since compound I and some postulated intermediates such as compound 0 have not been identified in organic media as far as we know, we report herein the generation of PEG-HRP compound I in benzene and chlorobenzene, utilizing a stopped-flow apparatus equipped with a rapid scanning spectrometer. More importantly, a possible peroxy−iron intermediate of PEG-HRP, whose spectrum is similar to that observed for R38L HRP, has been stabilized in chlorobenzene at −20 °C.

Methods and Materials

Materials. Native HRP (type VI) was purchased from Sigma. Activated PEG_2 (average molecular weight = 10 000) was obtained from Seikagaku Corporation. Hydrogen peroxide, *tert*-butyl hydroperoxide, thioanisole, styrene, and guaiacol were commercially available. Benzene and chlorobenzene were purified and dried as described before use.[11] The Karl Fischer titration method was used to determine the amount of water in organic solvent. Unless indicated, "dry organic solvent", of which water content was 4.4 ± 0.3 ppm in benzene or 9.0 ± 2 ppm in chlorobenzene, was used for the experiments in this work.

Guaiacol oxidation was measured on a Shimazu UV-2400 PC. HPLC analysis was carried out on a Shimazu LC-10AD equipped with a SPD-10A UV−visible detector. Rapid scan spectra were measured on a Hi-Tech SF-43 stopped-flow apparatus equipped with a MG 6000 diode array spectrometer. Hiranuma AQ-3 was used for the Karl Fischer titration.

Preparation of PEG-HRP. HRP type VI (10 mg) and activated PEG_2 (500 mg) were stirred together in 1.5 mL of 50 mM sodium borate buffer, pH 10 at 37 °C for 2 h. The reaction was quenched by the addition of 60 mL of 50 mM sodium phosphate buffer, pH 7.0. The

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reaction mixture was ultrafiltrated with Amicon YM 30 membrane to approximately 5 mL. To remove unreacted PEG and sodium borate, 100 mL of distilled water was added to the residual solution, and the ultrafiltration was repeated three times. The completion of the modification of three lysine residues in HRP was confirmed by the SDS−PAGE analysis. While trace amounts of singly (MW 53 kD) and doubly (MW 63 kD) modified HRP were observed, triply pegylated HRP (MW 73 kD) was the major product. PEG-HRP in distilled water was lyophilized and stored at −20 °C. Freeze-dried PEG-HRP was dissolved in buffer or dry organic solvents before use.

Preparation of Hydrogen Peroxide Solution in Benzene and Chlorobenzene. A hydrogen peroxide saturated benzene solution was prepared by mixing 1 mL of 30% hydrogen peroxide and 4 mL of benzene. The aliquot (normally 10 μL) of hydrogen peroxide in benzene was added to a 50 mM potassium acetate buffer solution containing potassium iodide (10 mM) and HRP (0.05 μM), and the change in absorbance at 353 nm in the buffer was monitored to determine the concentration of hydrogen peroxide by using a molar absorption coefficient of $\epsilon = 2.62 \times 10^4$ M^{-1} cm^{-1}.

Guaiacol Oxidation. The reaction mixture contained 10 μM PEG-HRP, 1 mM H_2O_2, and 10 μL of guaiacol in benzene or chlorobenzene. Since guaiacol oxidation was too fast to measure in 50 mM phosphate buffer (pH 7), the concentration of PEG-HRP was reduced to 10 nM. The rate of oxidation was determined by measuring the increase in absorbance at 470 nm with a molar absorbance coefficient of $\epsilon = 2.6 \times 10^4$ M^{-1} cm^{-1}.

Thioanisole Sulfoxidation. To a 0.5-mL solution of PEG-HRP (3 μM) and thioanisole (1 mM, added in 10 μL of methanol) in 50 mM sodium phosphate buffer (pH 7.0) was added H_2O_2 (1 mM) at 25 °C. After 5 min of incubation acetophenone was added as an internal standard, and the solution was extracted with CH_2Cl_2. The extract was concentrated nearly to dryness under a stream of nitrogen. The residue, taken up in the HPLC solvent, was analyzed by isocratic HPLC on a Daicel chiral column. The column was eluted with 85% hexane/15% 2-propanol at a flow rate of 0.5 mL/min. The HPLC effluent was monitored at 254 nm. The retention times for the *S* and *R* isomers for methyl phenyl sulfoxide were 16.7 and 20.0 min, respectively.

For sulfoxidation in organic solvents, the reaction mixture containing PEG-HRP (3 μM), thioanisole (1 mM, added in 10 μL of methanol), and H_2O_2 (1 mM) in 0.5 mL of benzene or chlorobenzene was incubated at 25 °C for 5 min. The mixture was frozen to stop the reaction, and the solvent was removed under vacuum. The internal standard was added to the residue, and the sulfoxide products were analyzed by HPLC.

The amount of racemic sulfoxide from control incubations without the enzyme was subtracted from the experimental values to obtain the true enantiomeric excess.

Single and Double Mixing Rapid Scan Experiments. Rapid scan spectra were measured on a Hi-Tech SF-43 stopped-flow apparatus equipped with a MG 6000 diode array spectrometer. To determine the rate of compound I formation, single mixing experiments (mixing of PEG-HRP and hydrogen peroxide) were performed. In the single mixing mode, compound I in the cell was subjected to the photoreduction by Xenon lamp.[12]

By double mixing experiments, the rate of compound I reduction with guaiacol was measured. In the double mixing mode, compound I was generated in the first mixing of PEG-HRP and hydrogen peroxide, and guaiacol was added to compound I by the second mixing. The delay time, defined as the interval between the first and the second mixing, was set depending on the rate of compound I formation. In the double mixing mode, compound I formed in a loop not in the cell; therefore, the intermediate is not exposed to light until the second push is excused. To minimize the photoreduction of compound I by Xenon lamp,[12] the shutter of the light source was closed while spectra were not being collected. Factor analysis and global fitting were performed using SPECFIT from Spectrum Software Associates (NC).

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Table 1. The Oxidation of Guaiacol and Thioanisole by PEG-HRP

		thioanisole oxidation		
	guaiacol oxidation: rate (turnover/min)	rate (turnover/min)	% ee	dominant isomer
in phosphate buffer, pH 7	6.1×10^3	1.4	28	*R*
in benzene	1.4×10^{-2}	6.2×10^{-2}	26	*R*
in chlorobenzene	3.1×10^{-2}	4.6×10^{-2}	24	*R*

Results and Discussion

Catalytic Activity of PEG-HRP. To confirm the catalytic activity of polyethylene glycolated HRP, we used guaiacol and thioanisole as substrates. The oxidations by PEG-HRP are slower in organic solvents than in phosphate buffer (Table 1). Guaiacol oxidation in benzene and chlorobenzene is 5 orders of magnitude slower than in phosphate buffer. Although the rate of sulfoxidation is 100-fold slower in the organic solvents than in buffer, the oxidation proceeds with modest enantioselectivity, and the values of enantiomeric excess (%) are not significantly different regardless of the reaction media. The results imply that the active site of PEG-HRP remains essentially the same both in organic and aqueous media. Interestingly, native HRP oxidizes thioanisole with 70% ee (dominant isomer *S*;[3] therefore, it appears that the PEG modification altered the binding orientation of thioanisole.

Oxidation of a wide variety of substrates by PEG-HRP has been reported; however, the sulfoxidation of thioanisole presented here is the first example of the two-electron oxidation associated with the ferryl oxygen transfer to substrates in organic media. Since compound II retains only one oxidation equivalent and cannot perform the two-electron oxidation processes, our results indicate that compound I of PEG-HRP is involved in the oxidations in organic solvents. To confirm the formation of PEG-HRP compound I in organic solvents, we have examined details of the reactions as follows.

Generation of Catalytic Intermediates in Aqueous Buffer and Benzene. The absorption spectra of ferric PEG-HRP in benzene and sodium phosphate buffer are essentially identical to the spectra previously reported.[9a,10] The addition of a stoichiometric amount of hydrogen peroxide to PEG-HRP in sodium phosphate buffer causes the decrease in the absorbance of the Soret band to give a compound I-like spectrum (λ_{max} = 403 nm) (Figure 3, Table 2). The addition of 2 equiv of guaiacol with respect to the compound I-like species affords compound II, followed by the ferric state of PEG-HRP. Thus, we have concluded that the species is compound I of PEG-HRP. In the absence of guaiacol, compound I is subsequently converted into compound II under the Xenon lamp of rapid scanning spectroscopy; however, the rate of reduction by photolysis (k'_{obs2}) is ∼200-fold slower than that by guaiacol (k_{obs2}) (Table 3). The further photoreduction of PEG-HRP compound II is not observed in the buffer. The Soret bands of PEG-HRP for ferric, compound I, and compound II in sodium phosphate buffer are observed at 403, 403, and 420 nm, respectively.

Similar spectral changes are also observed in benzene (Figure 4).[13] PEG-HRP reacts with hydrogen peroxide to generate a compound I-like species, which is subsequently photoreduced

(13) Although extinction coefficients of ferric HRP and its compound I in buffer were previously reported as 102 and 53.8 mM^{-1} cm^{-1} (in ref 1a), respectively, the intensity of Soret of PEG-HRP compound I in benzene (Figure 4) is greater than we expected. Since the extinction coefficient of compound I for PEG-HRP in buffer is approximately half of that of ferric PEG-HRP (Figure 3), the nature of compound I in organic media could be different.

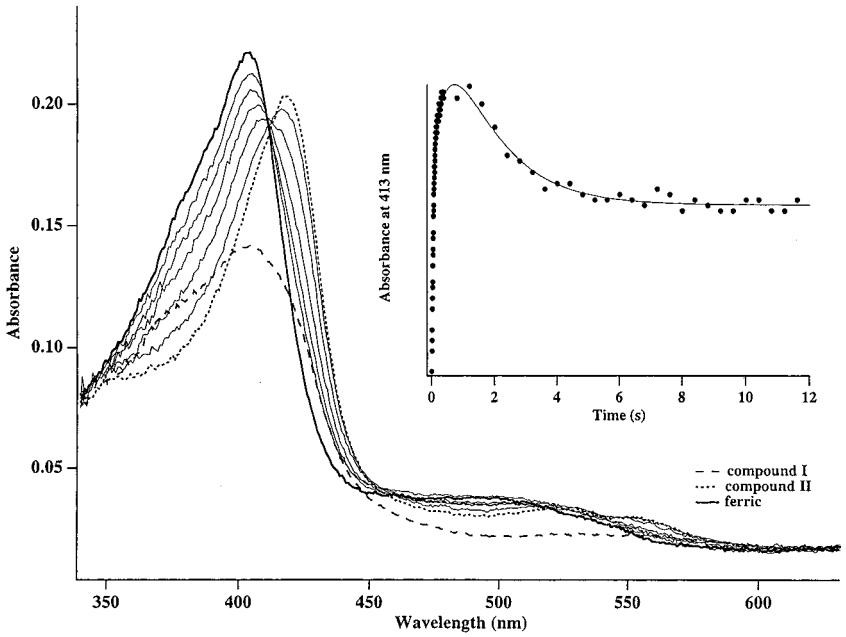

Figure 3. The reduction of compound I of PEG-HRP with 2 equiv of guaiacol in 50 mM sodium phosphate buffer, pH 7.0. The experiment was performed in the double mixing mode. The dashed line (- - -), 0-s scan initiated at the start of second mixing to add guaiacol, represents compound I. The dotted line (•••) (0.8-s scan) and the thick solid line (11-s scan) are the spectrum of compound II and ferric, respectively. Thin solid lines indicate the spectral changes from compound II to ferric at 2-s intervals. Inset: Time course at 413 nm.

Table 2. Electronic Absorption Maxima for PEG-HRP in Buffer and Benzene

	Soret (nm)	visible (nm)
PEG-HRP in buffer		
ferric	403	502, 640
compound I	403	528, 557, 640
compound II	420	522, 553
PEG-HRP in benzene		
ferric	405	502, 640
compound I	405	530, 573, 638
compound II	413	540, 574

Table 3. The Observed Rate Constants for the Compound I Formation (k_{obs1}) and Reduction of Compound I (k'_{obs2}, k_{obs2}) and II (k_{obs3}) (at 7.5 °C)[a]

	compound I formation: k_{obs1} (s^{-1})	compound I reduction		compound II reduction by guaiacol k_{obs3} (s^{-1})
		by photo-reduction: k'_{obs2} (s^{-1})	by guaiacol: k_{obs2} (s^{-1})	
PEG-HRP in buffer	120 ± 4	0.078 ± 0.02	16 ± 0.66	0.68 ± 0.07
PEG-HRP in benzene	7.2 ± 0.4	0.12 ± 0.01	0.19 ± 0.014	ND[b]

[a] PEG-HRP (3 μM) and a stoichiometric quantity of hydrogen peroxide were used to form compound I. Compound I was reduced in the presence of 2 equiv of guaiacol or by photolysis. [b] Too slow to determine the rate constant.

to compound II.[10] Although 2 equiv of guaiacol with respect to the enzyme reduces the compound I-like species at the rate of 1.5-fold greater than that of reductive photolysis, compound II is not converted to the ferric resting state (Table 3). In the presence of a 2500-fold excess of guaiacol, the spectrum of a compound I-like species is converted to a compound II chromophore, and compound II is reduced back to the ferric PEG-HRP. The observed spectral changes clearly indicate the first intermediate to be compound I, and two ferryl species (compounds I and II) are catalytic intermediates in organic media. Factor analysis and global fitting on the data set collected in benzene with a two-step model (compound I → compound II → ferric) provides the rate constants of 0.18 and 0.012 s^{-1}, respectively. The conversion of compound II to the ferric enzyme is slower than the reduction of compound I by guaiacol.

The rate of compound I formation in buffer (k_{obs1} = 120 s^{-1} = 7.2 × 10^3 min^{-1}) (Table 3) is comparable to the turnover number of guaiacol oxidation in aqueous medium (6.1 × 10^3 min^{-1}) (Table 1). In benzene, however, the rate of guaiacol oxidation (1.4 × 10^{-2} min^{-1}) (Table 1) is 4 orders of magnitude slower than that of compound I generation (k_{obs1} = 7.2 s^{-1} = 4.3 × 10^2 min^{-1}) (Table 3). Furthermore, a large excess of guaiacol is necessary to reduce compound I to the ferric state in benzene. Thus, the results suggest that the access for the substrates to the active site appears to be the slow step in benzene.

The decrease in absorbance at 405 nm, corresponding to the compound I formation in benzene, roughly fits to the single exponential curve to give k_{obs1} = 7.2 ± 0.4 (s^{-1}) (Figure 4a); however, the systematic deviation in the residuals suggests that a single step and first-order formation of compound I would be oversimplified. Factor analysis and global fittings of the rapid scan data set reveal that the formation of compound I of PEG-HRP in benzene could consist of two steps with the rate constants of 11 and 1.3 s^{-1}. The first step might correspond to the formation of an iron−peroxy complex, and the subsequent slow step would be the O−O bond cleavage (Figure 2). Although the second step is slower than the first step, the UV−vis spectrum corresponding to compound 0, a hyperporphyrin species previously proposed as a new intermediate formed from a HRP−H_2O_2 complex,[5] is not accumulated in benzene at 7.5 °C. Thus, we have attempted to react PEG-HRP with peroxide at low temperature in chlorobenzene, of which freezing point is lower than that of benzene (melting point of benzene is 5.5 °C and of chlorobenzene is −45 °C).

Generation and Stabilization of Catalytic Intermediates in Chlorobenzene. The solubility of polyethylene glycol modified HRP in chlorobenzene versus in benzene increases approximately 2-fold. Mixing of PEG-HRP and stoichiometric amounts of hydrogen peroxide in chlorobenzene provides the

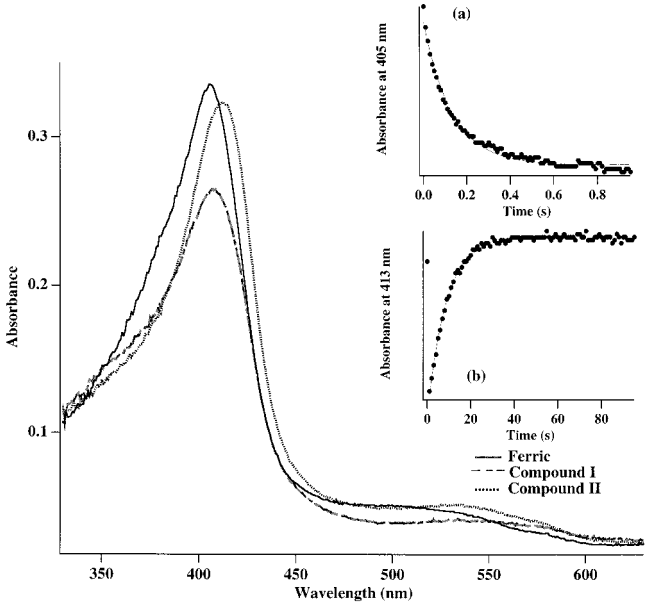

Figure 4. Absorption spectra of ferric PEG-HRP (3.3 μM) reacted with 1 equiv of hydrogen peroxide in benzene: (a) absorbance changes at 405 nm and (b) absorbance changes at 413 nm.

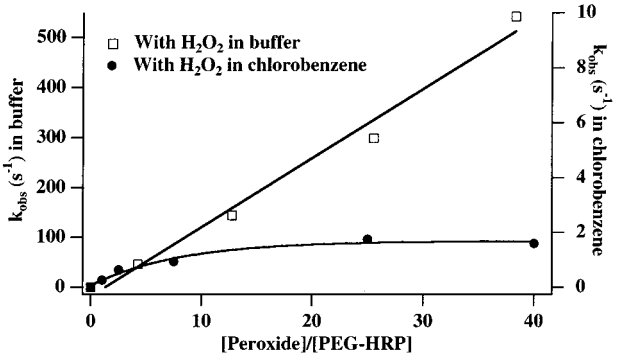

Figure 5. Variation in k_{obs} for the reaction of ferric PEG-HRP with H_2O_2 to form compound I in phosphate buffer (□) and chlorobenzene (●) as a function of [peroxide]/[PEG-HRP] at 5 °C. The concentration of PEG-HRP is 1.2 μM in the buffer and 2 μM in chlorobenzene.

spectrum of compound I, which is essentially identical to that observed in benzene. The k_{obs} values of compound I formation are 2.9 ± 0.2 (s^{-1}) at 7.5 °C. The photoreduction of the ferryl radical cation species gives compound II, and the spectrum is not different from that in benzene.[14] Factor analysis and global fittings of the data set again suggest that compound I formation with hydrogen peroxide in chlorobenzene consists of two steps (7.4 and 1.0 s^{-1} at 7.5 °C), but the absorption spectrum of the intermediate has not been clearly observed at 7.5 °C. Next, we have examined the effect of concentration of hydrogen peroxide on rate of compound I formation (Figure 5). While the saturation kinetics is not observed in the aqueous buffer, the k_{obs} values in chlorobenzene almost reach the maximum in the presence of 25 equiv of hydrogen peroxide with respect to PEG-HRP.[15] The results indicate that an intermediate is formed in a rapid preequilibrium prior to compound I formation;

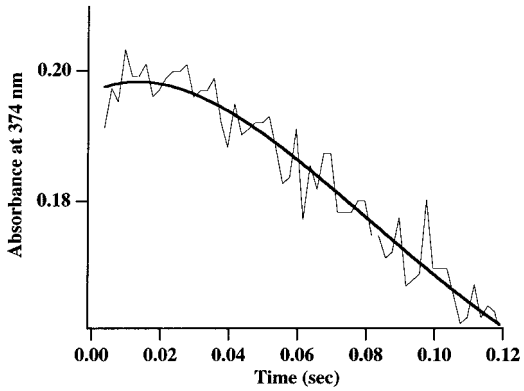

Figure 6. Time course at 374 nm of the reaction of PEG-HRP (22 μM) with H_2O_2 in chlorobenzene.

therefore, the peroxy−iron complexes would be accumulated in the presence of a large excess of hydrogen peroxide.

The mixing of PEG-HRP and hydrogen peroxide in chlorobenzene at −20 °C does not help us in identifying the hyperporphyrin intermediate, like compound 0.[5] However, the close examination of the changes in absorbance at 374 nm reveals a lag period of about 20 ms although such a lag period has not been clearly observed at 7.5 °C (Figure 6). Thus, the intermediate appears to be stabilized at lower temperature. Furthermore, the spectrum observed 10 ms after the mixing is similar to that of typical ferric resting enzyme, as observed for R38L HRP.[6] As suggested by the INDO/ROHF/CI quantum chemical method, a split Soret might originate from the peroxoanion form, like compound 0, in which HOO^- is coordinated to the heme iron, whereas a neutral H_2O_2-HRP complex would give a spectrum similar to that of the resting enzyme.[7] Thus, the new intermediate observed for R38L HRP in buffer and PEG-HRP in chlorobenzene might be assigned as a neutral peroxy−iron complex stabilized in a less-polar environment. However, the (TMP)Fe^{III}(*t*-$BuOO^-$) complex and other ferric−oxyanion heme model compounds do not exhibit the split Soret band.[16] Therefore, more detailed studies are required before a conclusive structural characterization.

Finally, compound I of PEG-HRP persists for more than an hour at −20 °C in chlorobenzene if we limit the exposure of the species under the Xenon lamp to prevent the decay (Figure 7).

Effects of Water Content on Compound I Formation in Chlorobenzene. Lyophilized PEG-HRP binds water to some extent, and the amount of water stripped off in organic solvent is correlated with the concentration of protein. When 1 μM of PEG-HRP in dry chlorobenzene (water content 9.0 ± 2 ppm) is prepared, the total amount of water in protein solution is typically 142 ± 18 ppm.[17] In order to study the influence of water on the kinetic data in organic media, the rate of compound

(14) The reaction of PEG-HRP with *tert*-butyl hydroperoxide in chlorobenzene to generate compound I is slower than with hydrogen peroxide. More than 10-fold excess of *tert*-butyl hydroperoxide with respect to the enzyme is required to observe compound I because the compound I formation (k_{obs1}) has to be at least 10 times as fast as the photoreduction (k_{obs2}) to accumulate compound I. The results are consistent with the hypothesis that bulky substrates cannot readily reach the heme of PEG-HRP in organic solvents. However, the spectrum generated with the alkyl hydroperoxide is the same as that with hydrogen peroxide.

(15) The second-order rate constant (k_1) in buffer was determined to be 5.9×10^6 s^{-1} M^{-1} by taking the slope of the plot, k_{obs} versus the concentration of H_2O_2. Since a nonlinear dependence of k_{obs1} on the peroxide concentration was observed in chlorobenzene, the accurate second-order rate constant could not be determined. The plot of k_{obs} versus the concentration of H_2O_2 in benzene at 7.5 °C was also found to be nonlinear, and the rate of compound I formation reached a plateau when more than 10 equiv of hydrogen peroxide were used.

(16) (a) Burstyn, J. N.; Roe, J. A.; Mikzstal, A. R.; Shaevitz, B. A.; Lang, G.; Valentine, J. S. *J. Am. Chem. Soc.* **1988**, *110*, 1382−1388. (b) Arasasgham, R. D.; Cornman, C. R.; Balch, A. L. *J. Am. Chem. Soc.* **1989**, *111*, 7800−7805. (c) Yamaguchi, K.; Watanabe, Y.; Morishima, I. *J. Am. Chem. Soc.* **1993**, *115*, 4058−4065.

(17) The amount of water in PEG-HRP benzene solution was 109 ± 3 ppm, when 1 μM of PEG-HRP in dry benzene (water content 4.4 ± 0.3 ppm) was prepared.

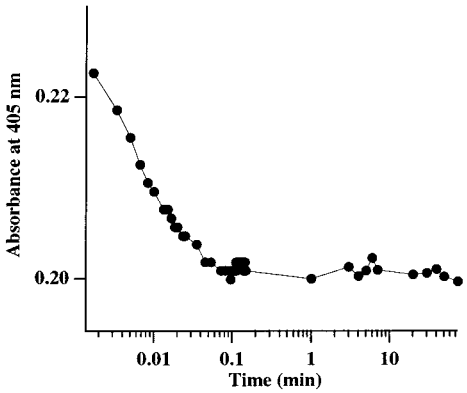

Figure 7. Time course at 405 nm of the reaction of PEG-HRP (2.2 μM) with H_2O_2 in chlorobenzene.

I formation with 10 equiv of hydrogen peroxide in chlorobenzene has been measured at 7.5 °C in the presence of various amount of water. The enhancement in the rate of compound I formation is observed when the concentration of water increases (water content = 142 ppm, $k_{obs1} = 3.9 \pm 0.9\ s^{-1}$; water content = 216 ppm, $k_{obs1} = 34 \pm 4.4\ s^{-1}$). The decay of PEG-HRP compound I to compound II is also accelerated from 0.062 to 0.55 s^{-1}. The value of k_{obs1} for compound I formation increases to $68 \pm 10\ s^{-1}$ in the presence of 305 ppm of water.[18] However, the further addition of water to chlorobenzene at 7.5 °C makes the rate determination difficult because the reaction mixture becomes turbid. Thus, the water content should be minimum for cyro-nonaqueous enzymology. In conclusion, the amount of water in organic solvent affects the formation of catalytic species of pegylated HRP, and our results are consistent with the previous reports on the important role of water in nonaqueous enzymology.[19]

In summary, the results reported in this paper indicate that (a) polyethylene glycolated HRP can catalyze not only one- but also two-electron oxidation reactions in organic solvents, (b) compounds I and II are catalytic intermediates in organic media, (c) compound I and the transient peroxy−iron intermediate are stabilized in organic media at low temperature, and (d) the water content in organic solvent affects the kinetics of compound I formation in chlorobenzene as well as the spectral nature of compound I.

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Supporting Information Available: One figure giving the absorption spectrum of ferric, compound I, and compound II of PEG-HRP in chlorobenzene (1 page, print/PDF). See any current masthead page for ordering information and web access instructions.

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(18) The water content in chlorobenzene also affects the absorption spectra of compound I. The intensity of the Soret band of PEG-HRP compound I relative to that of ferric decreased upon the addition of water (A_{Soret} (compound I)/A_{Soret} (ferric) = 0.82, 0.71, and 0.66 in the presence of 142, 216, and 305 ppm of water in chlorobenzene, respectively).

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