Oxidation–Reduction Properties of Compounds I and II of *Arthromyces ramosus* Peroxidase†

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Received November 24, 1993; Revised Manuscript Received February 14, 1994®

ABSTRACT: At neutral pH, compound I of *Arthromyces ramosus* peroxidase (ARP) was stable and was reduced to ferric ARP without apparent formation of compound II upon titration with ascorbate or hydroquinone. In the titration experiments, compound II was seen as an intermediate only at alkaline pH. However, measuring a difference spectrum in the Soret region by a stopped-flow method, we found that compound II was formed during the catalytic oxidation of ascorbate even at neutral pH. Using an EPR spectrometer with a microflow system, we measured the steady-state concentration of benzosemiquinone formed in the ARP-catalyzed oxidation of hydroquinone. The results clearly showed that ARP catalyzes the oxidation of hydroquinone by a one-electron-transfer mechanism, as does horseradish peroxidase. These observations led to the conclusion that compound I is reduced to compound II through a one-electron reduction by ascorbate or hydroquinone. Therefore, we concluded that ARP compound II is unusually unstable and is rapidly reduced to ferric enzyme without accumulation in the titration experiment. The unusual instability of ARP compound II is explained in terms of the high reduction potential of compound II. The reduction potentials (E_0') of compounds I and II were measured at several pH values from redox equilibria with potassium hexachloroiridate on the basis of $E_0' = 0.90$ V for the $IrCl_6^{2-}$–$IrCl_6^{3-}$ couple. These values were determined to be 0.915 and 0.982 V at pH 7, respectively, and decreased with increasing pH. This pH dependence was markedly changed by the buffer concentration. The change of E_0' associated with buffer concentration had little effect on the pH activity profiles of ARP-catalyzed oxidations of hydroquinone and guaiacol.

Horseradish peroxidase (HRP)[1] has long been used in clinical examination for colorimetric determination of biological materials by means of oxidative coupling of phenol with 4-aminoantipyrine (Allain et al., 1974). Akimoto et al. (1990) reported that *Arthromyces ramosus* peroxidase (ARP) gives 100 times higher chemiluminescence in the oxidation of luminol than does HRP, and suggested advantages of ARP in clinical tests.

ARP is isolated from the hypomycete *Arthromyces ramosus* (fungi imperfecti), which produces a large amount of peroxidase extracellularly. Baunsgaard et al. (1993) have proposed that ARP belongs to the same class as other extracellular fungal lignin and manganese peroxidases. ARP is a single-polypeptide protein of M_r = 38 000 with an isoelectric point at pH 3.5. It contains one iron protoporphyrin IX complex as a prosthetic group and about 5% carbohydrates (Kjalke et al., 1992; Shinmen et al., 1986). Preliminary X-ray structure analysis of ARP has been reported by Kunishima et al. (1993).

George (1953a) and Fergusson (1956) reported that some inorganic compounds, such as potassium hexachloroiridate (iridate), cause direct oxidation of HRP to compound I. Later, Hayashi and Yamazaki (1979), confirming a two-step oxidation of HRP to compound I via compound II, measured the reduction potentials (E_0') of compounds I and II from equilibria coupled with the K_2IrCl_6–K_3IrCl_6 system in a narrow pH region.

In this paper, we show that ARP is easily oxidized to compound I by K_2IrCl_6 at alkaline pH. Although the reaction of ARP with iridate is similar to that of HRP, there are differences in the redox properties between these two peroxidases.

MATERIALS AND METHODS

ARP was prepared at Suntory Laboratories (Osaka, Japan) according to the method of Shinmen et al. (1986). Potassium hexachloroiridate (K_2IrCl_6) was obtained from Aldrich and was added to ARP solutions from its 1 mM stock solution containing 0.01 N HCl. All other reagents used were of analytical grade. The buffer system was sodium succinate for pH 5–5.5, potassium phosphate for pH 6–8, and sodium carbonate for pH 8.5–10. The reactions were carried out at room temperature (24 ± 1 °C). Absorption spectra were scanned from 700 to 250 nm with a Shimadzu UV-2101PC spectrophotometer at a speed of 15 nm/s. The stopped-flow apparatus was constructed at the National Center for the Design of Molecular Function. The dead time was 2 ms, and the sample volume used was 25 μL per one-shot experiment.

Measurements of E_0' from Redox Equilibria. E_0' values of compounds I and II were measured from redox equilibria of ARP with iridate according to Hayashi and Yamazaki (1979). The molar concentrations of ferric ARP and compounds I and II at equilibrium were measured on the

† This work was supported by NIH Research Resource Grant 5P41 RR06030-03 and NIH Grant ES04922.

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® Abstract published in *Advance ACS Abstracts*, April 1, 1994.

[1] Abbreviations: ARP, *Arthromyces ramosus* peroxidase; E_0', reduction potential; EPR, electron paramagnetic resonance; HRP, horseradish peroxidase; iridate, potassium hexachloroiridate.

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Table 1: Millimolar Difference Absorption Coefficients ($\Delta\epsilon_{mM}$) Used for Calculation of Concentrations of Ferric ARP and Compounds I and II at Equilibrium[a]

wavelength	component to be measured	$\Delta\epsilon_{mM}$[b]
397[c]	−[ferric][d]	−71.6
412.5[c]	[compound I]	−60.0
434.5	[compound II]	51.6

[a] Each wavelength is isosbestic for two components, excluding the one to be measured. [b] The difference from the absorbance of ferric ARP. [c] The isosbestic points for compounds I and II (397 nm) and for ferric ARP and compound II (412.5 nm) were determined by using the spectrum of compound II obtained upon addition of equimolar H_2O_2 to ARP at pH 10 (Figure 2A). [d] The ferric concentration is obtained as the decrease from the initial concentration.

basis of the $\Delta\epsilon_{mM}$ values listed in Table 1. The absorbance in the equilibrated systems was corrected by subtracting the absorbance of the remaining K_2IrCl_6. The concentration of remaining K_2IrCl_6 was measured at 462 nm where the absorbance change was negligible during the conversion of ARP between ferric ARP, compound I, and compound II. The equations used to calculate the millimolar concentration of each component of ARP at equilibrium were as follows: [ferric] = $A/108 - \Delta A/71.6$; [compound II] = $\Delta A/51.6$; and [compound I] = $\Delta A/60$, where A was the initial absorbance of ferric ARP at 403.5 nm and ΔA was the difference from the initial absorbance of ferric ARP at the isosbestic points listed in Table 1. [$IrCl_6^{3-}$] was calculated as the difference between the initial and the remaining [$IrCl_6^{2-}$].

Catalytic Activity. Oxidations of hydroquinone and guaiacol were measured by following the decrease in absorbance at 250 nm and the increase at 470 nm, respectively. For hydroquinone, we used a value of 8.28 for $\Delta\epsilon_{mM}$, and for guaiacol, we used a value of 6.65 for the ϵ_{mM} of oxidized guaiacol formed per millimolar H_2O_2 (Farhangrazi et al., 1992).

Electron Paramagnetic Resonance (EPR) Experiments. EPR experiments were done using two solutions (each 0.5 mL) in a microflow system connected to a Medical Advances X-band EPR loop gap resonator sample cavity (Hubbell et al., 1987). The first solution contained 0.2 μM HRP or 1.1 μM ARP, and 2 mM hydroquinone in 20 mM potassium phosphate buffer (pH 7.0 or 6.0). The second solution contained 1 mM H_2O_2 in 20 mM of the same buffer. The two solutions were mixed at a constant flow speed for 15 s, during which the EPR spectrum of benzosemiquinone was scanned. The magnetic field of 25 G was scanned over 15 s with a modulation amplitude of 0.2 G and a time constant of 30 ms.

Determination of Extinction Coefficient. An ARP solution (1 mL) was mixed with 3 mL of a pyridine solution consisting of 100 mL of pyridine, 30 mL of 1 N NaOH, and 300 mL of water. To this mixture were added a few crystals of solid $Na_2S_2O_4$, and the absorbance of the alkaline pyridine hemochromogen was measured at 557.5 nm. The ϵ_{mM} of ARP at 403.5 nm was determined to be 108 ± 3 on the basis of ϵ_{mM} = 32.0 at 557.5 nm for the alkaline pyridine hemochromogen of protoheme (de Duve, 1948).

RESULTS

At neutral pH, the addition of an equimolar amount of H_2O_2 to ARP yielded stable compound I. The spectrum of this compound was similar to that of HRP compound I (Figure 1). However, when titrated with ascorbate or hydroquinone, compound I of ARP was apparently reduced directly to the ferric enzyme. When an equimolar amount of H_2O_2 was added to ARP at pH 10, compound I was formed (Figure 2A), but

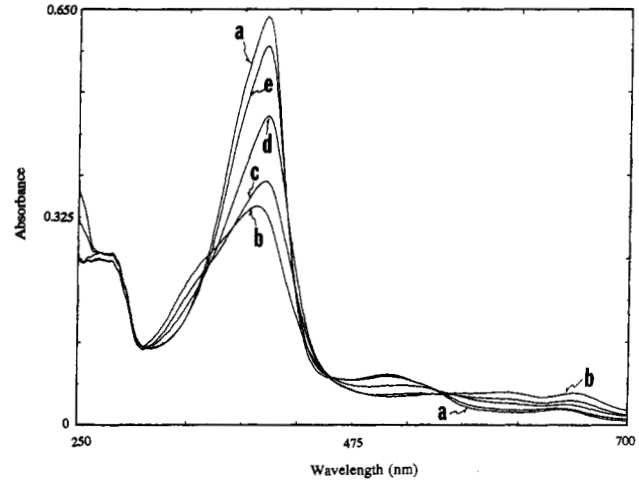

FIGURE 1: Spectral change in the reduction of ARP compound I at pH 7.0 (20 mM phosphate). Spectra: a, 5.9 μM ARP; b, immediately after addition of 6 μM H_2O_2 to (a); c, 15 min after (b); d, after 2 μM hydroquinone was added to (c); e, after another 2 μM was added to (d).

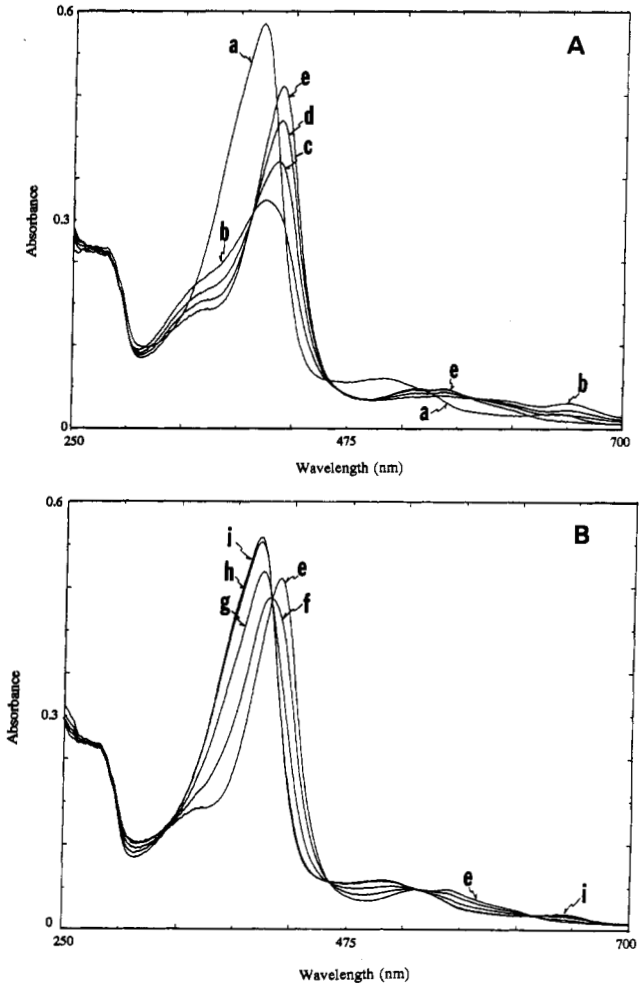

FIGURE 2: Spectral change of ARP upon addition of H_2O_2 at pH 10.0 (20 mM carbonate). Panel A: spetrum a, 5.4 μM ARP; spectrum b, immediately after addition of 6 μM H_2O_2 to (a). Time-dependent change of b; 52 (c), 110 (d), and 220 s (e) after addition of H_2O_2. Panel B: Hydroquinone (1 μM) was added successively to spectra e (f), f (g), g (h), and h (i). The spectra were taken immediately after the additions of hydroquinone.

was converted spontaneously to compound II with a half-time of a few minutes (Figure 2A). At pH 10, compound II was stable and reduced back to ferric ARP stoichiometrically upon

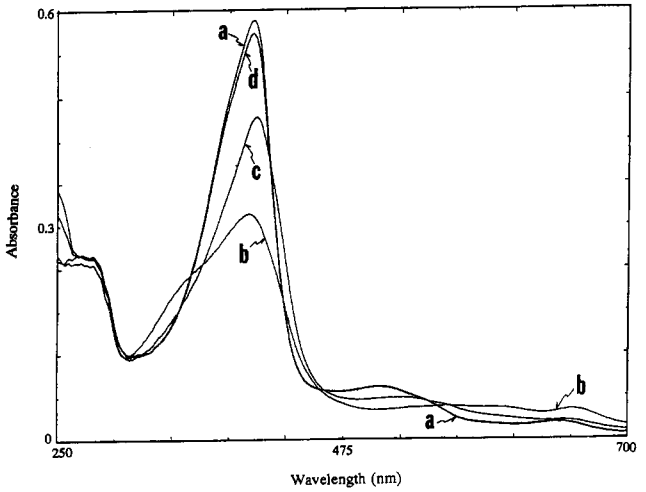

FIGURE 3: The reduction of ARP compound I at pH 10.0 (2 mM carbonate). Spectra, a, 5.4 μM ARP; b, 6 μM H_2O_2 added to (a); c, 2 μM hydroquinone added to (b); d, another 2 μM hydroquinone added to (c).

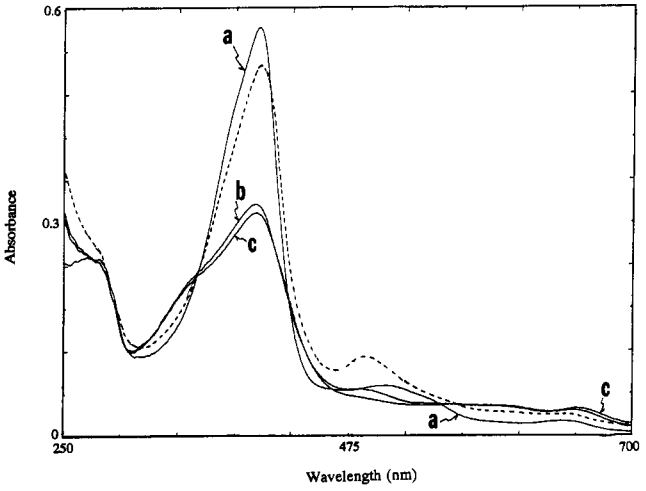

FIGURE 4: Spectral change of ARP upon addition of 20 μM K_2IrCl_6 at pH 9.0 (20 mM sodium carbonate): 5.3 μM ARP (a), immediately (b) and 5 min (c) after addition of 20 μM K_2IrCl_6 to (a). The dotted-line spectrum was taken 1 min after addition of 20 μM K_2IrCl_6 to 5.3 μM ARP at 2 mM carbonate and did not change over several minutes. The pH shift caused by addition of 20 μl of the K_2IrCl_6 stock solution (1 mM) was negligible.

addition of ascorbate or hydroquinone (Figure 2B). The stability of compounds I and II was therefore pH dependent: compound I was stable at neutral pH, and compound II was stable at alkaline pH. This pH profile of the stability of compounds I and II was observed at a buffer concentration of 20 mM. When the buffer concentration was reduced to 2 mM, compound I was stable at pH 10 as well as at pH 7 (Figure 3).

In other peroxidases, compound II is more stable than compound I. Why is ARP exceptional? We thought that the E_0' values of compounds I and II might be crucial factors in controlling their stabilities. The E_0' values of peroxidase compounds I and II have been reported only for HRP isozymes A and C (Hayashi & Yamazaki, 1979), in spite of the importance of these values for comparative analysis of the catalytic activity of various peroxidases. The difficulty in measuring these E_0' values could be ascribed to their high potentials of about 1 V. Like HRP, ARP was also oxidized by K_2IrCl_6 to compounds I and II. At pH 9 and at a carbonate concentration of 20 mM, about a 4 times molar excess of the iridate converted ARP to a mixture of 88% compound I and

Table 2: Calculation of Reduction Potentials of ARP Compounds I and II[a]

		compound II		compound I	
buffer concn	pH	log K^b	E_0', V	log K^c	E_0', V
20 mM	6	2.44	1.044	0.91	0.954
	7	1.39	0.982	0.26	0.915
	8	0.38	0.922	−0.32	0.881
	9			−1.27	0.825
	9[d]		0.894		0.841
2 mM	6	2.69	1.059	0.54	0.932
	7	2.06	1.022	0.29	0.917
	8	1.80	1.006	0.39	0.923
	9	1.27	0.975	0.31	0.918
	9.5	0.54	0.932	−0.25	0.885

[a] E_0' was calculated using a value of 0.90 V for $E_0'(IrCl_6^{2-}$–$IrCl_6^{3-})$. If the same value is used to calculate the E_0' values of HRP compounds I and II, they become 30 mV less than those values reported previously (Hayashi & Yamazaki, 1979), being 0.880 and 0.900 V at pH 7, respectively. [b] K = $[IrCl_6^{3-}][\text{compound II}]/[IrCl_6^{2-}][\text{ferric}]$. [c] K = $[IrCl_6^{3-}][\text{compound I}]/[IrCl_6^{2-}][\text{compound II}]$. [d] Calculated from the rate constants.

12% compound II (Figure 4). But at pH 9 and at a carbonate concentration of 2 mM, the addition of the same amount of the iridate caused only a partial change of ARP, forming 13% compound II and 25% compound I (dotted line in Figure 4). According to the procedures described in Materials and Methods, at 20 mM carbonate we estimated the E_0' of compound I as 0.825 V, but the E_0' of compound II could not be measured because the concentration of ferric ARP was nearly zero. At 2 mM carbonate the E_0' values of compounds I and II were estimated as 0.918 and 0.975 V, respectively. Similarly, we measured these reduction potentials under various conditions. The results are listed in Table 2, and the reduction potentials are plotted against pH in Figure 5, panel A, for 20 mM buffer and Figure 5, panel B, for 2 mM buffer. At pH 9 (20 mM carbonate), the equilibrium constant was obtained by measuring rate constants (M^{-1} s^{-1}) for forward and reverse reactions involved in the following redox equilibrium of ARP with iridate (Hayashi & Yamazaki, 1979):

$$\text{ferric} + IrCl_6^{2-} \underset{4.1 \times 10^3}{\overset{5.1 \times 10^3}{\rightleftharpoons}} \text{compound II} + IrCl_6^{3-} \quad (1)$$

$$\text{compound II} + IrCl_6^{2-} \underset{7.5 \times 10^4}{\overset{7.0 \times 10^5}{\rightleftharpoons}} \text{compound I} + IrCl_6^{3-} \quad (2)$$

The reaction of compound II with $IrCl_6^{2-}$ was measured by using a stopped-flow method. The other rate constants were measured with a normal spectrophotometer. The E_0' values obtained are plotted in Figure 5A. This kinetic method could not be applied at neutral pH because of the instability of ARP compound II.

The instability of compound II might be explained in terms of its high E_0' value. It was 67 mV higher than that of compound I at pH 7. It has been reported that HRP compounds I and II have similar E_0' values at neutral pH (Hayashi & Yamazaki, 1979). Therefore, it seemed that the apparent direct reduction of ARP compound I to the ferric enzyme shown in Figures 1 and 3 was ascribable to the instability of compound II.

From the E_0' data, however, we could not exclude the possibility that compound I undergoes two-electron reduction with ascorbate or hydroquinone under experimental conditions where compound I was reduced to the ferric enzyme without accumulation of compound II. In order to make this point clear, we carried out the following two experiments. Using

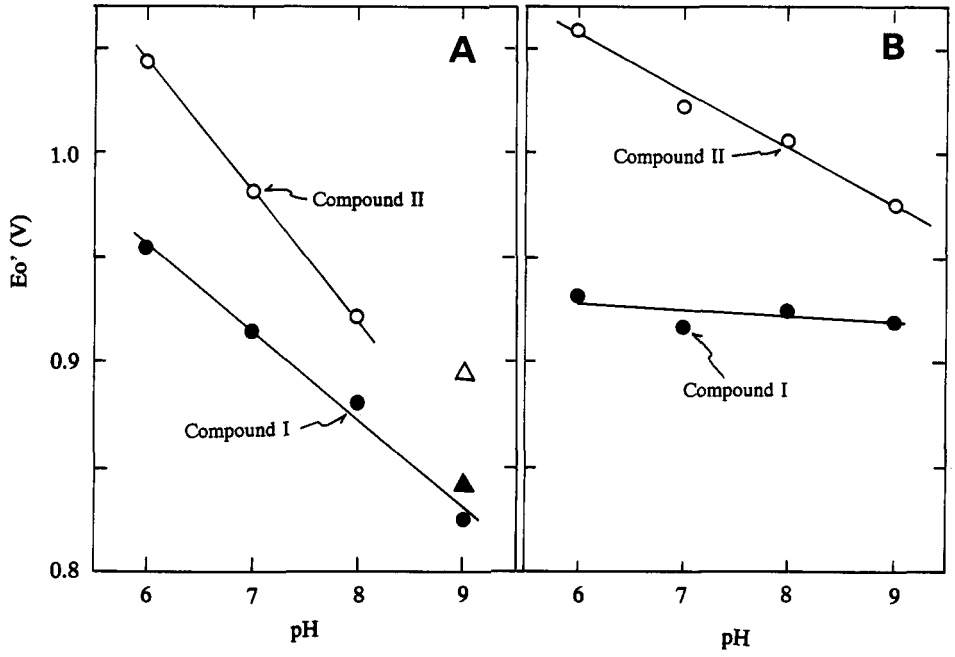

FIGURE 5: pH dependence of E_0' of ARP compounds I and II at buffer concentrations of 20 (A) and 2 mM (B). The E_0' values were obtained by measuring the concentrations of components (●, ○) and the rate constants involved in redox equilibria (▲, △).

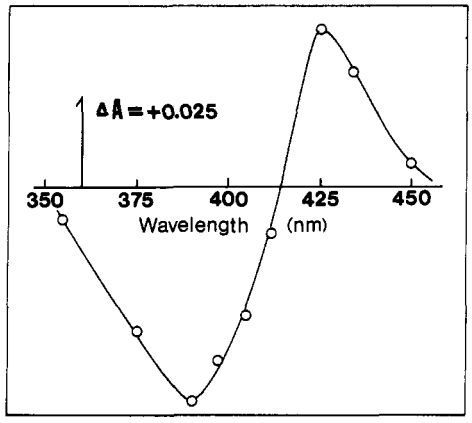

FIGURE 6: Difference spectrum of ARP observed during the steady state of its catalytic oxidation of ascorbate at pH 7.0 (20 mM phosphate). The absorbance change was followed at the indicated wavelengths after two solutions of ARP + ascorbate and H_2O_2 were mixed. Final concentrations were 1.8 μM ARP, 0.1 mM H_2O_2, 0.5 mM ascorbate, and 20 mM phosphate.

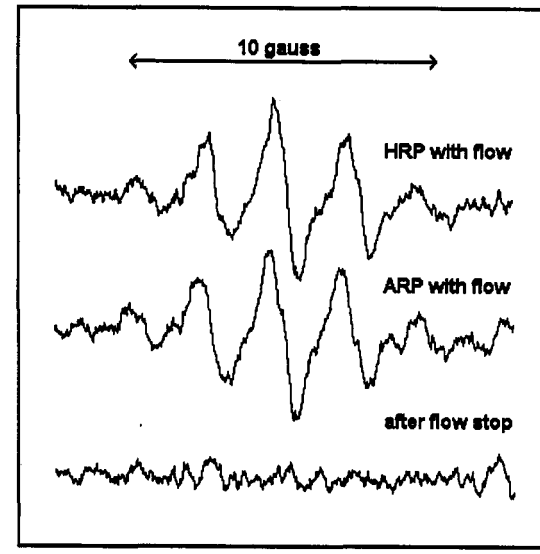

FIGURE 7: EPR spectra of benzosemiquinone observed 120 ms after the reaction started (see Materials and Methods). Final concentrations were 0.1 μM HRP or 0.55 μM ARP, 0.5 mM H_2O_2, 1 mM hydroquinone, and 20 mM phosphate, pH 6.0. Under these conditions, hydroquinone was oxidized at the same rate in both HRP and ARP reactions.

a stopped-flow optical method, we found that, at steady state of an ARP–H_2O_2–ascorbate reaction, the enzyme existed as a mixture of ferric ARP (0.97 μM), compound II (0.61 μM), and compound I (0.22 μM) at pH 7 (Figure 6). If the two-electron reduction of compound I occurred, compound II should not be observed (Ohtaki et al., 1982). This result showed that one-electron reduction of compound I by ascorbate did occur through a peroxidase cycle (Yamazaki et al., 1960).

$$\text{ARP} + \text{H}_2\text{O}_2 \xrightarrow{k_1} \text{compound I} \quad (3)$$

$$\text{compound I} + \text{AH}_2 \xrightarrow{k_2} \text{compound II} + \text{AH}^\bullet \quad (4)$$

$$\text{compound II} + \text{AH}_2 \xrightarrow{k_3} \text{ARP} + \text{AH}^\bullet \quad (5)$$

By applying Chance's equation (Chance, 1957) to a kinetic trace at 434.5 nm, we estimated k_3 to be 1.1×10^6 M^{-1} s^{-1}. Since $k_1[\text{ARP}][\text{H}_2\text{O}_2] = k_2[\text{compound I}][\text{AH}_2] = k_3$-[compound II][$AH_2$] at steady state, k_1 and k_2 were estimated as 3.6×10^6 and 3.0×10^6 M^{-1} s^{-1}, respectively. A similar value for k_1 has been reported by Kjalke et al. (1992).

In order to determine whether or not hydroquinone was oxidized to benzosemiquinone through a one-electron oxidation mechanism, we carried out EPR measurements with the use of a microflow system. In the experiments described in Figure 7, we compared the steady-state concentrations of benzosemiquinone formed in HRP and ARP reactions at enzyme concentrations giving the same rate for the hydroquinone oxidation at pH 6.0. Since it has been established that HRP catalyzes the oxidation of hydroquinone by way of a 100% one-electron-transfer mechanism (Yamazaki, 1977), the result that ARP gave the same concentration of benzosemiquinone at steady state led us to conclude that ARP also uses the same mechanism. Therefore, we concluded that the mechanism of

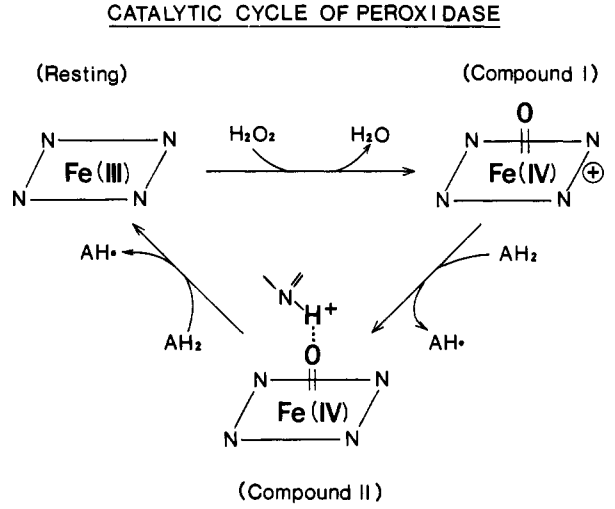

FIGURE 8: Catalytic cycle of peroxidase reactions. ARP catalyzes the oxidation of hydroquinone and ascorbate according to this mechanism, as does HRP. The existence of hydrogen bonding between FeO and a distal base has been suggested in HRP compound II (Sitter et al., 1985; Hashimoto et al., 1986).

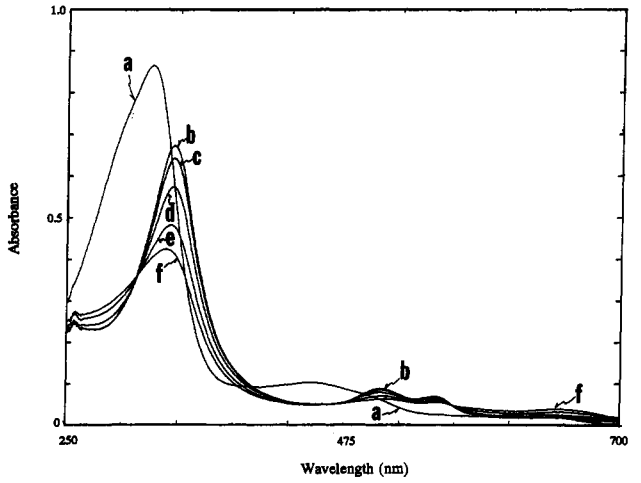

FIGURE 9: Formation of ARP compound III. Compound III (b) was formed immediately after addition of 1 mM H_2O_2 to 0.8 μM ARP solution containing 50 μM ascorbate and 20 mM phosphate, pH 7.0 (a). Spectra c, d, e, and f were scanned after spectrum b at time interval of 50 s.

ARP catalysis is essentially the same as reported for HRP and other peroxidases (Figure 8). A similar result was obtained at pH 7.0.

Compound III of peroxidase is formed from the reaction of compound II with excess H_2O_2 (Nakajima & Yamazaki, 1987). When compound I is stable, the addition of excess H_2O_2 alone can hardly convert peroxidase to compound III. An electron donor is needed to first convert compound I to compound II. At neutral pH, as expected, ARP compound III was formed when excess H_2O_2 was added with a small amount of ascorbate (Figure 9). The compound III spectrum was unstable and converted to that of compound I. This result was explained by assuming that the recycling of compound III through a compound III–ferric–compound I–compound II–compound III route was inhibited because of the stability of compound I.

The catalytic activity of ARP has been reported with iodide and guaiacol (Kjalke et al., 1992). In the reactions with hydroquinone and guaiacol, there was no essential difference in the pH activity curves at buffer concentrations of 2 and 20 mM (panels A and B of Figure 10). The change in E_0' of compounds I and II might not be reflected in the overall

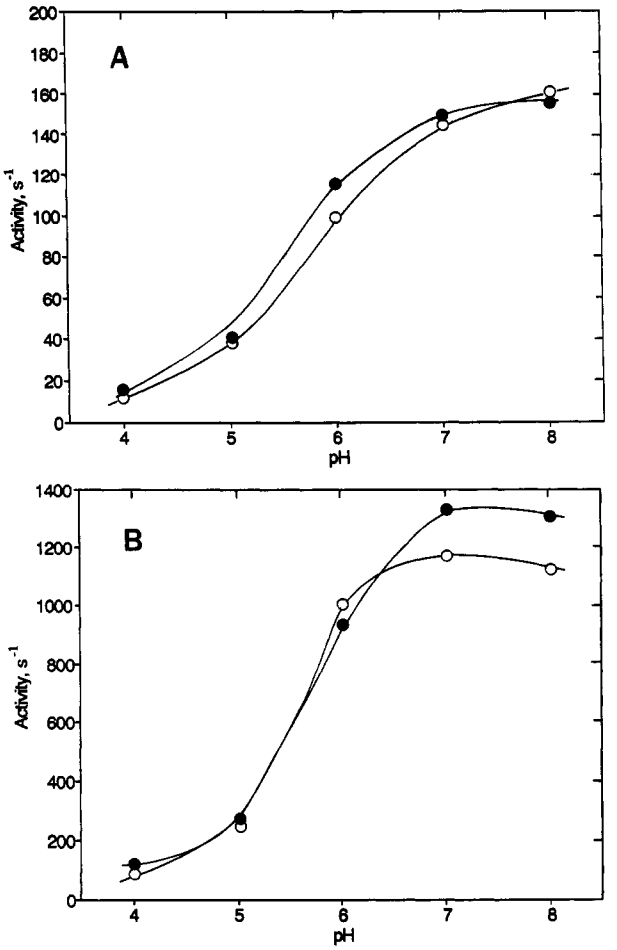

FIGURE 10: pH dependence of the rates of ARP-catalyzed oxidations of hydroquinone (A) and guaiacol (B). Panel A: 0.05–0.2 μM ARP, 200 μM H_2O_2, and 100 μM hydroquinone in the presence of 2 (●) and 20 mM buffer (○). Panel B: 0.1–0.15 μM ARP, 200 μM H_2O_2, and 1 mM guaiacol in the presence of 2 (●) and 20 mM buffer (○).

catalytic reaction. For the detailed analysis, it is necessary to measure the pH dependence of k_1, k_2, and k_3 at the two buffer concentrations.

DISCUSSION

E_0' of Compounds I and II. The E_0' value for the $IrCl_6^{2-}$–$IrCl_6^{3-}$ couple has been reported by several groups (Latimar, 1952; George & Irvine, 1955; Fergusson, 1956; Margerum et al., 1975), and there is discrepancy between these results. In the estimation of E_0' of HRP compounds I and II (Hayashi & Yamazaki, 1979), a value of 0.93 V (Fergusson, 1956) has been used. Considering the discussion of Margerum et al. (1975), we have adopted a value of 0.90 V in this paper. Therefore, care should be taken when we compare the E_0' values of compounds I and II between ARP and HRP (see footnote *a* in Table 2).

Using ARP covalently immobilized on a graphite electrode, Kulys and Schmid (1990) have measured the steady-state potential produced in the presence of H_2O_2, which was reported to be 908 mV at pH 7. This value is similar to the E_0' determined by us for ARP. It is still difficult to measure the E_0' of compounds I and II for peroxidases by using direct (unmediated) electrochemical measurements.

Rate and E_0'. The unusual instability of ARP compound II can be explained in terms of its high E_0' value of 0.982 V at pH 7. This E_0' value appears to be the highest one-electron reduction potential ever reported for biological oxidants. In

many cases, the one-electron reduction potential controls the oxidizing activity or the rate of reactions with electron donors. The E_0' of HRP compound I was once believed to be considerably higher than that of compound II because the k_2/k_3 ratio is usually 20–100 (George, 1953b). But this belief is not true. The E_0' values of HRP compounds I and II are about the same (Hayashi & Yamazaki, 1979). The difference in the oxidizing activity between compounds I and II is explained in terms of the mobility of a transferable electron. The mobility of a porphyrin π-electron is assumed to be higher than that of an Fe valence electron. In other words, a peroxidase gets an electron in and out of the porphyrin much easier than it gets an electron in and out of the heme iron. At pH 9 (20 mM carbonate), where ARP behaves like other peroxidases with respect to the stability of compounds I and II, the kinetic parameters shown in eqs 1 and 2 are similar to those of HRP (Hayashi & Yamazaki, 1979). At neutral pH, however, the high E_0' value of ARP compound II enhances its activity as oxidant and the k_2/k_3 ratio decreases from 320 for HRP (Yamazaki et al., 1973) to about 3 for ARP in the oxidation of ascorbate at pH 7. In the titration of compound I with an electron donor at pH 7 (Figure 1), compound II, which is formed from the one-electron reduction of compound I, would be immediately reduced by the neighboring electron donor to ferric ARP because of transient heterogeneity during mixing with a rod. This reduction of compound II takes place at a low k_2/k_3 ratio.

Dependence of E_0' on pH and Buffer Concentration. The pH dependence of the E_0' values of ARP compounds I and II is quite different at 2 and 20 mM buffer concentrations. At 20 mM concentration, the pH dependence is similar to that of HRP, but no evidence suggests the existence of a dissociable proton involved in the hydrogen bonding between the oxene atom and a distal base in compound II (Figure 8). Such hydrogen bonding is known in HRP compound II (Sitter et al., 1985; Hashimoto et al., 1986). HRP compound II is active only in its protonated form (Dunford & Stillman, 1976). It is important to question whether the involvement of this proton in peroxidase catalysis is a universal mechanism. ARP is the second peroxidase of which the reduction potentials of compounds I and II have been measured. Unfortunately, however, from our present results we cannot compare peroxidase catalysis between HRP and ARP since unexpected complications in the effect of buffer concentration occur in ARP catalysis. It should be noted that the E_0' of compound I becomes nearly pH independent when the buffer concentration is reduced from 20 to 2 mM. In the case of HRP, the E_0' of compound I becomes pH independent at pH values above the pK_a for protonation in compound II (Hayashi & Yamazaki, 1979). The effect of buffer concentration on protonation in compound II must be characterized by future studies.

ACKNOWLEDGMENT

We thank Dr. Stephanie Hallam for her help with determining the extinction coefficient of ARP. We also thank Michael Isaacson and Richard Ramires for their technical assistance in constructing and maintaining the micro-stopped-flow system. We are especially grateful to Dr. G. Czerlinski for the original design of the stopped-flow and EPR slow flow system and for his recommendations and helpful discussions. We would also like to thank Dr. Greg Schonbaum and Dr. Robert Sinclair for discussions leading to this study.

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