Low Catalytic Turnover of Horseradish Peroxidase in Thiocyanate Oxidation

EVIDENCE FOR CONCURRENT INACTIVATION BY CYANIDE GENERATED THROUGH ONE-ELECTRON OXIDATION OF THIOCYANATE*

(Received for publication, August 17, 1996, and in revised form, January 19, 1997)

Subrata Adak‡, Abhijit Mazumdar, and Ranajit K. Banerjee§

From the Department of Physiology, Indian Institute of Chemical Biology, Calcutta 700 032, India

The catalytic turnover of horseradish peroxidase (HRP) to oxidize SCN− is a hundredfold lower than that of lactoperoxidase (LPO) at optimum pH. While studying the mechanism, HRP was found to be reversibly inactivated following pseudo-first order kinetics with a second order rate constant of 400 m−1 min−1 when incubated with SCN− and H2O2. The slow rate of SCN− oxidation is increased severalfold in the presence of free radical traps, 5–5-dimethyl-1-pyrroline N-oxide or tert-phenyl-tert-butylnitrone, suggesting the plausible role of free radical or radical-derived product in the inactivation. Spectral studies indicate that SCN− at a lower concentrations slowly reduces compound II to native state by one-electron transfer as evidenced by a time-dependent spectral shift from 418 to 402 nm through an isosbestic point at 408 nm. In the presence of higher concentrations of SCN−, a new stable Soret peak appears at 421 nm with a visible peak at 540 nm, which are the characteristics of the inactivated enzyme. The one-electron oxidation product of SCN− was identified by electron spin resonance spectroscopy as 5–5-dimethyl-1-pyrroline N-oxide adduct of the sulfur-centered thiocyanate radical (απ = 15.0 G and αβ = 16.5 G). The inactivation of the enzyme in the presence of SCN− and H2O2 is prevented by electron donors such as iodide or guaiacol. Binding studies indicate that both iodide and guaiacol compete with SCN− for binding at or near the SCN−-binding site and thus prevent inactivation. The spectral characteristics of the inactivated enzyme are exactly similar to those of the native HRP-CN− complex. Quantitative measurements indicate that HRP produces a 10-fold higher amount of CN− than LPO when incubated with SCN− and H2O2. As HRP has higher affinity for CN− than LPO, it is concurrently inactivated by CN− formed during SCN− oxidation, which is not observed in case of LPO. This study further reveals that HRP catalyzes SCN− oxidation by two one-electron transfers with the intermediate formation of thiocyanate radicals. The radicals dimerize to form thiocyanogen, (SCN)2, which is hydrolyzed to form CN−. As LPO forms OSCN− as the major stable oxidation product through a two-electron transfer mechanism, it is not significantly inactivated by CN− formed in a small quantity.

Horseadish peroxidase (HRP)1 (EC 1.11.1.7; donor H2O2 oxidoreductase) catalyzes the oxidation of a wide variety of organic and inorganic electron donors by H2O2 through intermediate formation of compound I and compound II (1–4). The oxidation of aromatic donors proceeds through these intermediates by two one-electron oxidations with the formation of the substrate free radicals (3). The enzyme also catalyzes one-electron oxidation of various plant electron donors such as indoleacetic acid and ascorbate (5, 6). Among inorganic substrates, HRP catalyzes the oxidation of iodide, thiocyanate, nitrite, and bisulfite (1, 7–9), of which the mechanism of oxidation of iodide has been extensively studied (7, 8, 10, 11). Iodide is oxidized through a two-electron transfer directly to compound I with the intermediate formation of enzyme-hypoiodous complex (7, 8, 10, 11). Electron donors appear to bind at the exposed heme edge close to the heme methyl C1H3 and C18H3 to promote electron transfer to the C20 heme edge for oxidation by heme ferryl group (12–20). Recently, the plausible role of some conserved residues in aromatic donor binding in the heme distal pocket has also been reported (18–22).

Thiocyanate, a pseudohalide, is oxidized by various mammalian peroxidases (10, 23–27). Lactoperoxidase-H2O2-SCN− is a potent bacteriostatic-bacterialidical system also (28–31). The antibacterial activity might be due to various oxidation products of SCN− such as CN− (32), (SCN)2 (31), cyanosulfuric acid, or cyanosulfuric acid (33) or OSCN− (23). Recently, OSCN− has been identified by NMR studies as the major stable oxidation product at equimolar concentrations of H2O2 and SCN−, but CN− may be formed if the ratio of [H2O2]/[SCN−] exceeds 1 (28). The pathway for SCN− oxidation has been proposed (23) at equimolar concentrations of H2O2 and SCN− as follows.

\[
\text{LPO} \quad 2 \text{SCN}^- + \text{H}_2\text{O}_2 + 2 \text{H}^+ \rightarrow (\text{SCN})_2 + 2 \text{H}_2\text{O} \quad (\text{Eq. 1})
\]
\[
(\text{SCN})_2 + \text{H}_2\text{O}_2 \rightarrow \text{HOSCN} + \text{SCN}^- + \text{H}^+ \quad (\text{Eq. 2})
\]
\[
\text{HOSCN} \rightarrow \text{H}^+ + \text{OSCN}^- \quad (\text{Eq. 3})
\]

Alternatively, SCN− may be directly oxidized to OSCN− as follows.

\[
\text{LPO} \quad \text{SCN}^- + \text{H}_2\text{O}_2 \rightarrow \text{OSCN}^- + \text{H}_2\text{O} \quad (\text{Eq. 4})
\]

As (SCN)2 is unstable in aqueous solution and readily hydrolyzed to HOSCN (34), OSCN− is the major oxidation product in LPO-catalyzed SCN− oxidation (23, 28, 35). The binding of SCN− to LPO is facilitated by protonation of an ionizable group

---

* 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.

† Recipient of the Senior Research Fellowship of the Council of Scientific and Industrial Research (CSIR), New Delhi, India.

§ To whom correspondence should be addressed: Dept. of Physiology, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Rd., Calcutta 700 032, India. Tel.: 91-33-473-3491/492/6793; Fax: 91-33-473-0284/94-3333.

1 The abbreviations used are: HRP, horseradish peroxidase; LPO, lactoperoxidase; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; PBN, tert-tert-butylnitrone; ESR, electron spin resonance; IAA, indole acetic acid.
of pK₆ 6.4, which is presumably distal histidine (36). It binds with the $K_w$ value of 90 ± 5 mM (36) and is oxidized by compound I through two-electron transfer to form OSCN⁻ (37). Although LPO-catalyzed SCN⁻ oxidation has been extensively studied (10, 23–37), the literature is very scanty on the mechanism of HRP-catalyzed SCN⁻ oxidation. $^{15}$N NMR studies indicate that SCN⁻ binds to HRP away from the distal histidine, near the heme methyl C18H and C18H₂ with the $K_w$ value of 158 ± 19 mM and the binding is facilitated by protonation of an acid group with pK₆ 4.0 (38, 39). The oxidation of SCN⁻ and identification of the oxidation product have been studied and compared with LPO (9). While HOSCN/OSCN⁻ is the major oxidation product in the LPO system (28), (SCN)₂ is the oxidation product in HRP as evidenced by NMR studies (9). However, the mechanism of formation of (SCN)₂ is not known yet. Moreover, the detailed kinetic and spectral evidence for SCN⁻ oxidation by HRP and its comparison with that of LPO are still lacking. In this paper, we present evidence to show that SCN⁻ is mainly oxidized by HRP through two one-electron transfer mechanisms to form thiocyanate radicals. The radicals may dimerize to form (SCN)₂ (9), which is hydrolyzed to yield CN⁻. CN⁻ binds with the heme iron concurrent with oxidation and lowers the catalytic turnover of HRP. In contrast, LPO forms very little CN⁻ due to direct two-electron oxidation of SCN⁻ to form stable OSCN⁻ (28), and therefore it oxidizes SCN⁻ with much higher turnover.

MATERIALS AND METHODS

Horseradish peroxidase (Type VI A, RZ = 3.0), lactoperoxidase (RZ = 0.88), PBN, diethylenetriamine pentaacetic acid, 5,5-dimethyl-1-pyrroline N-oxide (DMPO), indoleacetic acid, ascorbate, and guaiacol were obtained from Sigma. KSCN, KI, and KNO₂ were from Merck (India). Other chemicals were of analytical grade. The concentration of HRP and LPO was determined from $ε_{240} = 102$ cm⁻¹ mM⁻¹ (40) and $ε_{412} = 112$ cm⁻¹ mM⁻¹ (41), respectively.

Horseradish Peroxidase- and Lactoperoxidase-catalyzed Oxidation of SCN⁻—SCN⁻ oxidation by HRP (0.5 μM) or LPO (0.01 μM) was measured in an incubation mixture containing 1 mM H₂O₂ and 1 mM SCN⁻ in 50 mM sodium acetate buffer, pH 4.5 or 5.6, respectively, in a final volume of 2 ml. The reaction mixture contained 50 mM sodium acetate buffer, pH 4.5 or 5.6, respectively, in a final volume of 2 ml. The reaction was stopped by adding 100 mM catalase. To 0.2 ml of the reaction mixture, 1.6 ml of 0.1 M HCl and 0.2 ml of 0.1 M FeCl₃ were added and the absorbance of the FeSCN²⁺ complex was measured at 450 nm (42). The concentration of SCN⁻ was calculated from a standard curve.

Determination of H₂O₂ Consumption during SCN⁻ Oxidation—H₂O₂ consumption was assayed by measuring the concentration of H₂O₂ (43, 44). The assay system contained in a final volume of 1.2 ml: 50 mM sodium acetate buffer, pH 4.5, 2 mM SCN⁻, 0.5 μM HRP, and 2.0 mM H₂O₂ added last to start the reaction. At different time intervals, an 0.1-ml aliquot was added to 3 ml of 80 mM HCl followed by the addition of 20 μl of 100 mM ferromonochrome sulfate and 0.2 ml of 2.5 M KSCN to measure the absorbance at 480 nm (43). The concentration of H₂O₂ was calculated from a standard curve. The amount of SCN⁻ oxidized under identical conditions was determined (42) as already described.

Inactivation of HRP in the Presence of SCN⁻ and H₂O₂—HRP (2 nm) was incubated with varying concentrations (0–2 mM) of SCN⁻ in 50 mM sodium acetate buffer, pH 4.5, in the presence or absence of 0.6 mM H₂O₂ in a final volume of 1 ml in a spectrophotometric cuvette. At various times of incubation, the peroxidase activity of the whole reaction mixture was measured by $I_{3-}$ assay at 553 nm after addition of 2.0 mM KI followed by 0.6 mM H₂O₂ to start the reaction (45). $I_{3-}$ formation was measured in a spectrophotometer at 280 nm (48) for 10 min with 1 mM SCN⁻ and 1 mM H₂O₂ in 50 mM sodium acetate buffer, pH 4.5, for complete inactivation. Residual H₂O₂ was decomposed by a small amount of catalase. The reaction mixture was then passed through a GF/C filter, and the filtrate was mixed with LPO or HRP to get the final enzyme concentration of 1 μM. The optical absorbance spectrum of the mixture was then recorded.

Identification of Inactivating Species—HRP (1.0 μM) was incubated for 10 min with 1 mM SCN⁻ and 1 mM H₂O₂ in 50 mM sodium acetate buffer, pH 4.5, for complete inactivation. Residual H₂O₂ was decomposed by a small amount of catalase. The reaction mixture was then passed through a GF/C filter, and the filtrate was mixed with LPO or HRP to get the final enzyme concentration of 1 μM. The optical absorbance spectrum of the mixture was then recorded.

Quantitation of CN⁻ Production of HRP–H₂O₂–SCN⁻ and LPO–H₂O₂–SCN⁻ Systems—The amount of CN⁻ produced in the HRP or LPO system as recovered in the filtrate was determined from the absorbance of the peroxidase-cyanide complex at 428.5 nm using lactoperoxidase (23). The concentration of CN⁻ was calculated from the plot of 1/Aₘ versus 1/Δλ. Donors competing for binding to HRP at the same site as that of SCN⁻ affect the apparent dissociation constant, $K_{obs}$ of SCN⁻ in the presence of the inhibitor (1-) and is related to the inhibitor concentration [I] by (38, 47, 48)

$$K_{obs} = K_f [I] + K_d$$

where $K_f$ is the apparent dissociation constant of the HRP-I⁻ complex in the absence of SCN⁻ and $K_d$ is the apparent dissociation constant for the binding of SCN⁻ to HRP as defined in Equation 5. All kinetic and spectral studies were carried out in a Shimadzu UV-2201 computerized spectrophotometer.

Detection of SCN⁻ Radicals by ESR Spectroscopy—Thiocyanate free radicals were detected as spin adduct with DMPO by ESR spectroscopy. The reaction mixture contained 100 mM sodium acetate buffer, pH 5.5, 100 mM SCN⁻, 100 mM DMPO, 1 mM diethylenetriamine pentaacetic acid, 30 μM H₂O₂, and 2 mM H₂O₂ added last to start the reaction. ESR spectra were recorded on a Varian E-112 spectrometer fitted with a TM-110 cavity operating at 9.45 GHz with 100 kHz modulation frequency.

RESULTS

HRP-catalyzed SCN⁻ Oxidation by H₂O₂—The catalytic turnover of HRP for SCN⁻ oxidation was compared with that of LPO at their optimum pH. From the initial rate of SCN⁻ oxidation, the catalytic turnover of HRP was found to be a hundredfold lower than that of LPO (Fig. 1). While studying the mechanism of this significantly lower turnover of HRP, we observed that preincubation of HRP with increasing concentrations of SCN⁻ in the presence of a fixed concentration of H₂O₂ resulted in concentration and time-dependent inactivation of the enzyme following pseudo-first order kinetics (Fig. 2A). Catalytic activity could be recovered by dilution, dialysis, or by passage through the Sephadex G-25 column indicating reversibility of the inactivation. $K_{obs}$ values obtained from the slope of each line (Fig. 2A) when plotted against SCN⁻ concentrations yielded a straight line (Fig. 2A, inset) from which a second order rate constant for inactivation was calculated to be $400 \text{ m}^{-1} \text{ min}^{-1}$. Inactivation of HRP is also dependent on H₂O₂ concentration and the presence of a catalytic concentration of SCN⁻. A plot of the percent inhibition against the turnover number ([H₂O₂]/[HRP]) ratio as shown in Fig. 2B indicates that the percent inhibition is directly dependent on the number of turnovers of the enzyme. The enzyme is completely inactivated after 2 × $10^4$ turnovers consuming 20 nmol of H₂O₂/pmol of HRP.
concentrations of DMPO on the turnover of SCN− 4-fold stimulated by 50 mM of DMPO. The data indicate that the number was calculated from nanomoles of H2O2 consumed per nanomole of 2 ml. After 5 min of incubation, 1 ml of the reaction mixture was used for the measurement of the residual activity and H2O2 consumption as described under “Materials and Methods.” The turnover was used for the measurement of the residual activity and H2O2 consumption as described under “Materials and Methods.”

Effect of Spin Trap on the Catalytic Activity of HRP on SCN− Oxidation—The kinetics of the HRP-catalyzed SCN− oxidation was further studied in the absence or presence of the spin trap as shown in Fig. 3. No significant SCN− oxidation and H2O2 consumption were evident in the absence of HRP. However, the initial rate of SCN− oxidation or H2O2 consumption was significantly increased in the presence of the free radical traps such as PBN or DMPO. The inset shows a plot of varying concentrations of DMPO on the turnover of SCN− which is 4-fold stimulated by 50 mM of DMPO. The data indicate that free radicals derived from the oxidation of SCN− are involved in limiting the catalytic turnover of the enzyme.

Figure 1. Oxidation of SCN− by HRP and LPO in the presence of H2O2. HRP (0.5 μM (○) or 0.01 μM LPO (△) was incubated with 1 mM SCN− and 1 mM H2O2 in 50 mM sodium acetate buffer, pH 4.5 or 5.6, respectively. After a fixed time, the reaction was stopped by catalase and SCN− concentration was determined as described under “Materials and Methods.”

Spectral Evidence for SCN− Oxidation and Enzyme Inactivation—The spectral evidence for the oxidation of SCN− by the HRP-H2O2 system is shown in Fig. 4A. Addition of a 5-fold excess of H2O2 to native HRP (trace a) produces a mixture of compound I and compound II as shown in trace b. Low concentrations of SCN− (4 μM) immediately reduced compound I to compound II (trace c), which was then slowly (20 min) reduced to the native enzyme (trace h) as evidenced by a time-dependent spectral shift from 417 nm to 402 nm through an isosbestic point at 408 nm. It is interesting to note that the broad spectrum of the mixture of compound I and II (trace b) increases in height for the initial few minutes due to complete reduction of compound I to compound II (trace c), which is then slowly reduced to the native state. However, in the presence of higher concentrations of SCN− (50 μM), a new Soret peak appears at 421 nm after the addition of H2O2 (Fig. 4B, trace b) with the visible peak at 540 nm (Fig. 4B, inset). This enzyme never returns to the native state in the presence of iodide (trace c) or guaiacol (trace d), indicating its inability to oxidize these electron donors. However, if SCN− (50 μM) is added to a mixture of compound I and compound II produced by a 5-fold molar equivalent of H2O2 (Fig. 4C), it causes an immediate spectral shift from 412 nm (mixture of compound I and compound II) to 417 nm (not shown) as a result of reduction of compound I to compound II with the increase of its visible peaks (trace b to c) at 527 and 556 nm (4B). After 3 min, the visible peaks are diminished (trace d) and a new peak appears at 540 nm (trace e) characteristic of the inactivated enzyme. Fig. 4D shows the effect of varying concentrations of H2O2 on the formation of the inactivated enzyme at 421 nm. Addition of single equivalent of H2O2 to native HRP (trace a) does not cause the formation of the inactivated enzyme (trace b). However, a gradual increase in H2O2 concentration causes a gradual decrease in the Soret peak at 402 nm with the increase in 421 nm peak for the inactive enzyme indicating its dependence on H2O2 concentration at a fixed concentration of SCN−. As most of the studies were carried out with an excess of H2O2, the results may be interpreted as the observation under steady-state conditions at the particular time.
induced inactivation of HRP in the presence of H₂O₂, spectral studies were carried out in the presence of the spin trap, DMPO. When SCN⁻ was added to the reaction mixture containing HRP and H₂O₂ in the presence of DMPO, it immediately reduced compound II to the native state with peaks at 402, 500, and 650 nm. The enzyme is also protected by aromatic electron donors such as guaiacol, or 10 mM iodide, 50 mM ascorbate, or 50 mM indoleacetic acid.

The inactivation of HRP absorbs at 421 nm (Fig. 5C) when passed through a Sephadex G-25 column is converted back to the native active enzyme absorbing at 402 nm, indicating the reversible nature of the inactivation.

Protection of HRP against SCN⁻ Inactivation by Various Electron Donors—Since iodide is optimally oxidized at a pH of about 4, where SCN⁻ is oxidized, the effect of iodide on the formation of the inactive enzyme was studied spectrally. Table I shows that in the presence of I-, SCN⁻ cannot inactivate the enzyme as evidenced by the absence of 421- and 540-nm peaks for the inactive enzyme. Instead, the enzyme remains in the native state with peaks at 402, 500, and 650 nm. The enzyme is also protected by aromatic electron donors such as guaiacol and other natural substrates such as ascorbate (6). Indoleacetic acid is, however, ineffective and causes its conversion to compound III with a visible peak at 670 nm (49). Protection studies could not be done kinetically, as the colored oxidation products of iodide and guaiacol during preincubation interfere with the final enzyme assay.

Binding of SCN⁻ by Optical Difference Spectroscopy in the Presence or Absence of Iodide or Guaiacol—The binding of SCN⁻ gives a characteristic difference spectrum of HRP-SCN⁻ complex versus HRP, having a maximum at 416 nm and a minimum at 395 nm (Fig. 6A). The equilibrium dissociation constant, K_d, for the HRP-SCN⁻ complex as calculated from the plot of 1/ΔΔ absorbance = 1/K_d SCN⁻ was 125 mM. SCN⁻ binding was also studied in the presence of varying concentrations of iodide. The plot (Fig. 6B) indicates that iodide competitively inhibits SCN⁻ binding. The inset shows the plot of K_d obs of SCN⁻ as a function of iodide concentration. Using Equation 6, the K_d of iodide at this site was calculated to be 110 mM.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Spectral changes in HRP-catalyzed SCN⁻ oxidation by H₂O₂. A: Soret spectrum of 0.8 mM HRP in 50 mM sodium acetate buffer, pH 4.5 (trace a); spectrum of a mixture of compound I and II (trace b) obtained immediately after addition of a 5-fold excess of H₂O₂ to native HRP; trace c, the complete compound II formation 30 s after the addition of 4 mM SCN⁻. Traces c–h were recorded 1–20 min after addition of SCN⁻. B: trace a, the spectrum of native HRP + SCN⁻ (50 μM); trace b, a + 10 mM H₂O₂; trace c, b + 100 μM I⁻; trace d, c + 10 mM guaiacol. C: trace a, the visible spectrum of a 2 μM mixture of compound I and compound II in presence of 10 mM H₂O₂; traces b–e were recorded at a 1-min interval after the addition of 50 mM SCN⁻. Note the initial increase in absorption at 527 and 556 nm (trace b to c) due to complete formation of compound II which is followed by the appearance of the visible peak at 540 nm (trace e) due to formation of inactive HRP. D: trace a, the spectrum for native HRP + SCN⁻ (50 μM); trace b, a + one equivalent H₂O₂ (1 mM); traces c–k, after further additions of 1 mM H₂O₂ for each trace until the final concentration of 10 mM is reached (trace k). Traces l and m were obtained at final H₂O₂ concentration of 15 and 20 μM, respectively.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Protection of HRP by DMPO against inactivation by SCN⁻/H₂O₂ system (A), spectral evidence for the oxidation of SCN⁻ by LPO compound II (B), and spectral evidence for the reversibility of the inactivated HRP (C). A: trace a, the Soret spectrum of 0.8 mM HRP in 50 mM acetate buffer, pH 4.5; trace b, a + H₂O₂ (10 mM); trace c, b + 50 mM DMPO; trace d, c + 25 mM SCN⁻. B: LPO (1.4 mM) in 50 mM acetate buffer, pH 5.6 (trace a); a + 10-fold excess of H₂O₂ (trace b); b + 50 mM SCN⁻ (trace c). C: Soret spectrum of 0.9 mM inactivated HRP (trace a). Trace b was recorded after passing the inactivated enzyme through the Sephadex G-25 column.

**Table I**

Spectral evidence for the protection of HRP by electron donors against inactivation by SCN⁻/H₂O₂ system

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Soret peak (nm)</th>
<th>Visible peak (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP</td>
<td>402</td>
<td>500, 650</td>
</tr>
<tr>
<td>HRP + SCN⁻</td>
<td>402</td>
<td>500, 650</td>
</tr>
<tr>
<td>HRP + H₂O₂</td>
<td>418</td>
<td>527, 556</td>
</tr>
<tr>
<td>HRP + SCN⁻ + H₂O₂</td>
<td>421</td>
<td>540</td>
</tr>
<tr>
<td>HRP + I⁻ + SCN⁻ + H₂O₂</td>
<td>402</td>
<td>500, 650</td>
</tr>
<tr>
<td>HRP + guaiacol + SCN⁻ + H₂O₂</td>
<td>402</td>
<td>500, 650</td>
</tr>
<tr>
<td>HRP + ascorbate + SCN⁻ + H₂O₂</td>
<td>402</td>
<td>500, 650</td>
</tr>
<tr>
<td>HRP + IAA + SCN⁻</td>
<td>410</td>
<td>555, 670</td>
</tr>
</tbody>
</table>
The stable oxidation product of SCN\(^{-}\)—SCN\(^{-}\) or CN\(^{-}\)—is formed through a one-electron transfer from the enzyme to the substrate, which is not observed in LPO. The inactivating species has a formation of sulfur-centered thiocyanate radical through one-electron oxidation of SCN\(^{-}\), which is not observed in LPO.

Identification of the Inactivating Species during SCN\(^{-}\) Oxidation—The stable oxidation product of SCN\(^{-}\) as recovered from the reaction mixture after filtration when mixed with the native HRP, native LPO, or ferrous LPO shifts the Soret peak of the enzyme to 421, 430, and 434 nm, respectively, with the corresponding visible peak at 540, 555, and 570 nm (Table II).

These are identical to the peaks obtained by the addition of CN\(^{-}\) to the corresponding enzyme preparations. The spectrum obtained after addition of filterate or CN\(^{-}\) to HRP is also exactly similar to the spectrum (Fig. 4B) obtained during inactivation of HRP in the presence of SCN\(^{-}\) and \(\text{H}_2\text{O}_2\). Quantitation of CN\(^{-}\) production during SCN\(^{-}\) oxidation by the HRP or LPO System—Table III shows that CN\(^{-}\) production in the HRP-\(\text{H}_2\text{O}_2\)-SCN\(^{-}\) system is 98 \(\pm\) 10 \(\mu\)M, which is significantly inhibited by PBN, indicating its generation from thiocyanate radical. In contrast, LPO produces only 10 \(\pm\) 5 \(\mu\)M CN\(^{-}\), which is 10-fold lower than the HRP system.

**DISCUSSION**

The results of this study indicate the following. (a) HRP-catalyzed SCN\(^{-}\) oxidation occurs at a significantly lower rate than LPO due to concurrent inactivation. (b) HRP catalyzes SCN\(^{-}\) oxidation through a one-electron transfer mechanism forming sulfur-centered thiocyanate radicals which finally give rise to an inactivating species. (c) The inactivating species has been identified as CN\(^{-}\). (d) CN\(^{-}\) production is 10-fold higher in the HRP-\(\text{H}_2\text{O}_2\)-SCN\(^{-}\) system than LPO. (e) SCN\(^{-}\) oxidation by HRP is under the major constraint of product inhibition by CN\(^{-}\), which is not observed in LPO.

The catalytic turnover for SCN\(^{-}\) oxidation by HRP is a hundredfold lower than that of LPO. Although the electrochemical potential of LPO compound I is much higher than HRP compound I (10) and LPO has a higher affinity for binding of SCN\(^{-}\) compared with HRP (9), the third factor which controls the oxidation of SCN\(^{-}\) is the mode of oxidation on which the nature
(inactivating or not) of the product formation is dependent. LPO catalyzes SCN\(^{-}\) oxidation by a two-electron transfer mechanism (37) leading to the formation of the stable oxidation product, OSCN\(^{-}\) (23, 28, 35, 37), leaving very little possibility for the generation of CN\(^{-}\) unless the concentration ratio of H\(_2\)O\(_2\) and SCN\(^{-}\) exceeds 1 (28). Our spectral studies indicate that HRP catalyzes SCN\(^{-}\) oxidation through a one-electron transfer mechanism with the formation of sulfur-centered thiocyanate radical which is detected by ESR spectroscopy. Thiocyanate radicals may dimerize to form thiocyanogen, (SCN)\(_2\), which, being highly unstable in aqueous solution in the pH range of 5–8, is hydrolyzed to give rise to HOSCN (32, 34). As HOSCN has a pK\(_a\) value of 5.3 (35), at pH above 5.3, OSCN\(^{-}\) is the major stable oxidation product. Recently, Modi et al. (28) have shown that LPO catalyzes SCN\(^{-}\) oxidation at pH 6.1 to produce HOSCN and OSCN\(^{-}\). However, as HRP-catalyzed SCN\(^{-}\) oxidation occurs optimally at pH 4.0, (SCN)\(_2\) is the predominant oxidation species (9). (SCN)\(_2\) is hydrolyzed to yield CN\(^{-}\) (32, 34) without the formation of HOSCN (9). The entire sequence of HRP-catalyzed SCN\(^{-}\) oxidation may thus be represented as follows.

\[
\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{compound I} \quad \text{(Eq. 7)}
\]

\[
\text{compound I} + \text{SCN}^- \rightarrow \text{compound II} + \text{SCN}^- \quad \text{(Eq. 8)}
\]

\[
\text{compound II} + \text{SCN}^- \rightarrow \text{HRP} + \text{SCN}^- \quad \text{(Eq. 9)}
\]

\[
\text{SCN}^- + \text{SCN}^- \rightarrow (\text{SCN})_2 \quad \text{(Eq. 10)}
\]

\[
3(\text{SCN})_2 + 4\text{H}_2\text{O} \rightarrow \text{CN}^- + 5\text{SCN}^- + 8\text{H}^+ + \text{SO}_4^{2-} \quad \text{(Eq. 11)}
\]

Equation 10 is the well known coupling reaction for the formation of stable oxidation product from the free radicals by dimerization (3, 53), and (SCN)\(_2\) has been shown to be formed in HRP-catalyzed SCN\(^{-}\) oxidation (9). Equation 11 is consistent with the reaction shown for the hydrolysis of (SCN)\(_2\), and may occur through the formation of some intermediates (42, 54). From the reaction sequences, thiocyanate radical, (SCN)\(_2\), SO\(_4^{2-}\), or CN\(^{-}\) may be considered for the plausible inactivating species for HRP. Our studies indicate that HRP-catalyzed SCN\(^{-}\) oxidation is increased severalfold in the presence of the free radical traps DMPO or PBN. This indicates that either thiocyanate radical or the radical-derived product is responsible for the inactivation. As the inactivation is reversible, it is unlikely that thiocyanate radical inactivates the enzyme through covalent interaction at or near the active site similar to the suicidal substrates (55, 56). Since (SCN)\(_2\) is unstable at pH 4.5 due to hydrolysis (32, 34, 54), the role of this compound in inactivation is excluded. As sulfate has no significant effect on the catalytic activity of HRP, the only stable reactive product present in the system is CN\(^{-}\), which can inactivate peroxidases by reversible interaction with the heme iron (49). Our spectral studies clearly indicate the formation of HRP-CN\(^{-}\) complex in the presence of SCN\(^{-}\) and H\(_2\)O\(_2\) as evidenced by the Soret peak at 421 nm and visible peak at 540 nm (49). However, due to remarkable similarity of the spectrum of compound II and CN\(^{-}\) complex (both low spin) at the Soret region, one cannot really distinguish between the two (9) unless their visible spectra are observed, where absorption at 540 nm is convincing evidence for the formation of the enzyme-CN\(^{-}\) complex (49). More convincing evidence for CN\(^{-}\) production comes from the observation that the enzyme-free reaction mixture when added to native HRP or LPO yields HRP-CN\(^{-}\) or LPO-CN\(^{-}\) complex having characteristic absorption maxima (49, 57). Moreover, quantitative measurement demonstrates that CN\(^{-}\) is the major reactive product in the HRP system as compared with LPO. Our kinetic and spectral studies as well as measurement of CN\(^{-}\) production indicate that in the presence of free radical trap, the enzyme remains in the highly active state because of the absence of CN\(^{-}\) production. Thus, for CN\(^{-}\) production, HRP must oxidize SCN\(^{-}\) by one-electron transfer to generate thiocyanate radical as intermediate which, when scavenged by the radical trap, relieves inhibition. LPO cannot generate sufficient CN\(^{-}\) for inactivation, as it catalyzes SCN\(^{-}\) oxidation by a single two-electron transfer (37) to form stable OSCN\(^{-}\) as the major oxidation product (9, 23, 28, 54).

It is intriguing as to why HRP and LPO catalyze SCN\(^{-}\) oxidation by two different mechanisms leading to two different oxidation products. Modi et al. (9) have suggested that this might be due to a different binding site of SCN\(^{-}\) in the heme distal pocket. HRP binds SCN\(^{-}\) at a site close to the heme peripheral C1H\(_a\) and C18H\(_b\) groups, having a pK\(_a\) of 4.0 (38), which might favor one-electron transfer, whereas the binding of SCN\(^{-}\) to LPO is facilitated by protonation of a group at pK\(_a\) 6.1, presumably contributed by the distal histidine which might favor two-electron transfer via the imidazole ring (36). However, further studies are required to substantiate it. Moreover, LPO binds CN\(^{-}\) with a K\(_a\) of 60 m\(\text{M}\) (57), which is much higher than the concentration of CN\(^{-}\) (10 m\(\text{M}\)) formed in the reaction mixture. In contrast, HRP binds CN\(^{-}\) with very high affinity of K\(_a\) of 2.3 m\(\text{M}\) (58), which is much lower than the concentration of CN\(^{-}\) (98 m\(\text{M}\)) formed in the system, making it more susceptible to inactivation by CN\(^{-}\). However, the difference in the mode of oxidation of SCN\(^{-}\) by two different peroxidases appears to be the fundamental mechanism for the differential sensitivity to CN\(^{-}\). The mechanism of SCN\(^{-}\)-induced inactivation of HRP is shown in Scheme 1. The essential feature of the scheme is the one-electron oxidation of SCN\(^{-}\) to thiocyanate radicals. This is unlike iodide, which is oxidized by a direct two-electron transfer to compound I (7, 8) but is similar to the one-electron oxidation of thiol, bisulfite, and nitrite (2, 59–61). The hyperfine splitting constants of thiocyanate radicals are comparable to the sulfur-centered thiol and bisulfite radicals formed in the HRP system (50, 51, 62–64), indicating that the

---

**Table III**

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>CN(^{-}) production (\mu\text{M})</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP + SCN(^{-})</td>
<td>0 (9)</td>
</tr>
<tr>
<td>HRP + SCN(^{-}) + H(_2)O(_2)</td>
<td>98 ± 10</td>
</tr>
<tr>
<td>HRP + SCN(^{-}) + PBN + H(_2)O(_2)</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>LPO + SCN(^{-})</td>
<td>0</td>
</tr>
<tr>
<td>LPO + SCN(^{-}) + H(_2)O(_2)</td>
<td>10 ± 5</td>
</tr>
</tbody>
</table>

---

**Scheme 1. Proposed mechanism for the inactivation of HRP by SCN\(^{-}\) and H\(_2\)O\(_2\).**
radical is centered on the sulfur atom of thiocyanate. Also, the stoichiometry indicates that 1 mol of CN− should be formed from the oxidation of 6 mol of SCN− of which 5 mol are regenerated with the consumption of 3 mol of H2O2. In other words, 3 mol of H2O2 should be consumed with the net oxidation of 1 mol of SCN−, which is evident from Fig. 3, and three catalytic cycles are thus required for the production of 1 mol of CN−. Thus, the formation of HRP-CN− complex will mainly depend on the H2O2 concentration at fixed enzyme and SCN− concentrations, which is evident from the kinetic and spectral studies. As 2 × 104 turnovers are required for complete inactivation of the enzyme (Fig. 2B), 40 nmol of H2O2 will be consumed by 2 pmol of HRP/ml of the reaction mixture with the formation of 13.3 nmol of CN−. Thus, 13.3 μM CN− could be formed in the system, which is compatible with the dissociation constant of the enzyme-CN− complex formation (Kd = 2.3 μM) (58) for inactivation. This somewhat higher concentration of CN− (13.3 μM) over the Kd value might be explained as due to its competition with the H2O2 for reaction with the heme iron.

Peroxidases are abundant in animal systems as well as in plants (65), which also contain SCN− (66). It is evident from this study that CN− produced from the oxidation of SCN− by HRP blocks the peroxidative activity and may thus affect plant physiology. However, the enzyme is protected against inactivation by iodoide or the aromatic electron donor guaiacol. Although iodoide is present in traces, various aromatic electron donors, including phenolic compounds, are rich in plants. It is thus highly probable that the phenolic compounds protect the enzyme against SCN−-induced inactivation. We have shown that iodoide protects the enzyme by competing with SCN− for binding at the same site. This is consistent with the earlier findings that both iodoide and SCN− bind to HRP at the same site (13, 38). However, inactivation of the enzyme is also prevented by guaiacol, which also competes with SCN− for binding at the same site or very close to it, as shown by our competitive binding studies. Although earlier studies indicate that aromatic donors may bind near the heme methyl C18H3 group (12, 18), which is away from the iodoide or SCN− binding site (12), our competitive binding data indicate that these sites are very close to each other, if not the same. Recently, we have shown that an active site arginine residue plays an obligatory role in aromatic donor binding (22) and mutant studies (21) have established that arginine-38 controls the binding of the aromatic donor in addition to its role in compound I formation. Since the positively charged arginine residue may also interact with the negatively charged substrates or cofactors (67), it is probable that the same arginine residue also controls SCN− binding, and in that case the competition of guaiacol with SCN− for binding at the same site is compatible. From the competitive binding studies it is, however, clear that the ratio of the concentration of the aromatic donors to SCN− is the determining factor for the normal functioning of the peroxidase in plant physiology. Although indoleacetic acid is the endogenous substrate of HRP (5), it cannot protect the enzyme because of the formation of compound III (5). Recently, ascorbate has been suggested to be the physiological substrate of the plant peroxidases (6), and it can completely protect HRP against SCN−-induced inactivation by CN−. It is also possible that iodoide, guaiacol, and ascorbate, being better substrates (high turnover) than SCN−, can consume H2O2 at a very high rate and thereby limiting the production of CN−. However, ascorbate might play an important role in keeping the enzyme in a fully active state in the presence of SCN− and thus helps in the decomposition of cellular H2O2, especially in the acid compartments such as vacuoles and apoplastic space (68).
Horseradish Peroxidase-catalyzed Thiocyanate Oxidation

2444–2449

68. Chance, B. (1951) Science 109, 204–208