Low Catalytic Turnover of Horseradish Peroxidase in Thiocyanate Oxidation

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

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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 α-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 (αν = 15.0 G and αμ = 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.

Horse-radish 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-bactericidal system also (28–31). The antibacterial activity might be due to various oxidation products of SCN− such as CN− (32), (SCN)N (31), cyanosulfurous 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.

\[
\begin{align*}
\text{LPO} & \quad 2 \text{SCN}− + \text{H}_2\text{O}_2 + 2 \text{H}^+ \rightarrow (\text{SCN})_2 + 2 \text{H}_2\text{O} & \text{(Eq. 1)} \\
(\text{SCN})_2 + \text{H}_2\text{O} & \rightarrow \text{HOSC} + \text{SCN}− + \text{H}^+ & \text{(Eq. 2)} \\
\text{HOSC} & \rightarrow \text{H}^+ + \text{OSC}− & \text{(Eq. 3)}
\end{align*}
\]

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

\[
\begin{align*}
\text{LPO} & \quad \text{SCN}− + \text{H}_2\text{O}_2 \rightarrow \text{OSC}− + \text{H}_2\text{O} & \text{(Eq. 4)}
\end{align*}
\]

As (SCN)2 is unstable in aqueous solution and readily hydrolyzed to HOSC (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 abbreviations used are: HRP, horseradish peroxidase; LPO, lactoperoxidase; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; PBN, α-phenyl-tert-butylnitrone; ESR, electron spin resonance; IAA, indole acetic acid.

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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 absorption 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-cyano complex at 428.5 nm using lactoperoxidase (33). The concentration of CN⁻ was calculated from the plot of 1/ΔA versus 1/[S]. Donor competing for binding to HRP at the same site as that of SCN⁻ affect the apparent dissociation constant, Kd_absc of SCN⁻ in the presence of the inhibitor (1) and is related to the inhibitor concentration [I] by (38, 47, 48).

Kd_absc - Kd[I] + Kd (Eq. 6)

where Kd is the apparent dissociation constant of the HRP-I⁻ complex in the absence of SCN⁻ and Kd 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-2200 computerized spectrophotometer at 28 ± 2 °C.

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. Kd_absc 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 m⁻¹ min⁻¹. Inactivation of HRP is also dependent on H₂O₂ concentration and H₂O₂ to SCN⁻ concentration ratio. 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 × 10⁴ turnovers consuming 20 nmoles of H₂O₂/pmol of HRP.
concentrations of DMPO on the turnover of SCN$^{-}$ was used for the measurement of the residual activity and H$_2$O$_2$ concentration. The data indicate that 4-fold stimulated by 50 mM of DMPO. The volume of 2 ml. After 5 min of incubation, 1 ml of the reaction mixture was incubated with 1 mM SCN$^{-}$ and 1 mM H$_2$O$_2$ 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$^{-}$ and H$_2$O$_2$ concentration was determined as described under "Materials and Methods."

The kinetics of the HRP-catalyzed SCN$^{-}$ Oxidation—To study the mechanism of SCN$^{-}$ oxidation and concurrent consumption of H$_2$O$_2$ in the presence of PBN or DMPO. The concentration of H$_2$O$_2$ and the oxidation of SCN$^{-}$ was measured in the presence or absence of 1 mM PBN or 50 mM DMPO in a reaction mixture containing 0.5 $\mu$M HRP, 2 mM SCN$^{-}$, and 2 mM H$_2$O$_2$ in 50 mM acetate buffer, pH 4.5, as described under "Materials and Methods."

Fig. 1. Oxidation of SCN$^{-}$ by HRP and LPO in the presence of H$_2$O$_2$. HRP 0.5 $\mu$M (○) or 0.01 $\mu$M LPO (△) was incubated with 1 mM SCN$^{-}$ and 1 mM H$_2$O$_2$ 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."

Fig. 2. Kinetics of the inactivation of HRP on preincubation with SCN$^{-}$ and H$_2$O$_2$. A, HRP (2 nM) was preincubated with different concentrations of SCN$^{-}$ in the presence of 0.6 mM H$_2$O$_2$ in 50 mM sodium acetate buffer, pH 4.5. At different time intervals, the residual activity was measured by I$_3^-$ assay. The concentrations of SCN$^{-}$ used were 0 mM (▲), 0.25 mM (○), 0.50 mM (△), 1.00 mM (●), and 1.50 mM (△). The curves were best fit by least square analysis. The second order rate constant of inactivation was determined from the slope of the line as shown in the inset. B, a fixed concentration of HRP (2 nM) was preincubated with 1 mM SCN$^{-}$ in the presence of varying concentrations (0–0.6 mM) of H$_2$O$_2$ in 50 mM sodium acetate buffer, pH 4.5, in a final volume of 2 ml. After 5 min of incubation, 1 ml of the reaction mixture was used for the measurement of the residual activity and H$_2$O$_2$ consumption as described under "Materials and Methods." The turnover number was calculated from nanomoles of H$_2$O$_2$ consumed per nanomole of HRP.

Effect of Spin Trap on the Catalytic Activity of HRP on SCN$^{-}$ Oxidation—To study the mechanism of SCN$^{-}$ oxidation and H$_2$O$_2$ consumption were evident in the absence of HRP. However, the initial rate of SCN$^{-}$ oxidation or H$_2$O$_2$ 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.

Spectral Evidence for SCN$^{-}$ Oxidation and Enzyme Inactivation—The spectral evidence for the oxidation of SCN$^{-}$ by the HRP-H$_2$O$_2$ system is shown in Fig. 4A. Addition of a 5-fold excess of H$_2$O$_2$ to native HRP (trace a) produces a mixture of compound I and compound II as shown in trace b. Low concentrations of SCN$^{-}$ (4 $\mu$M) 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 $\mu$M), a new Soret peak appears at 421 nm after the addition of H$_2$O$_2$ (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 $\mu$M) is added to a mixture of compound I and compound II produced by a 5-fold molar equivalent of H$_2$O$_2$ (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 (49). 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 H$_2$O$_2$ on the formation of the inactive enzyme at 421 nm. Addition of single equivalent of H$_2$O$_2$ to native HRP (trace a) does not cause the formation of the inactive enzyme (trace b). However, a gradual increase in H$_2$O$_2$ 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 H$_2$O$_2$ concentration at a fixed concentration of SCN$^{-}$. As most of the studies were carried out with an excess of H$_2$O$_2$, the results may be interpreted as the observation under steady-state conditions at the particular time.

Spectral Evidence that Inactivation Proceeds through One-electron Oxidation of SCN$^{-}$—To study the mechanism of SCN$^{-}$...
induced inactivation of HRP in the presence of H$_2$O$_2$, spectral studies were carried out in the presence of the spin trap, DMPO. When SCN$^-$ was added to the reaction mixture containing HRP and H$_2$O$_2$ in the presence of DMPO, it immediately reduced compound II to the native state absorbing at 402 nm (Fig. 5A). Only DMPO is ineffective in reducing compound II under this condition. Thus, in the presence of DMPO, the stable Soret and visible bands at 421 and 540 nm, respectively, for the inactivated enzyme, did not appear. This indicates that SCN$^-$ is oxidized by one-electron oxidation to the thiocyanate radical and that either this radical or a product derived from it is involved in the inactivation. When the radical is scavenged by DMPO, the enzyme is protected and the catalytic cycle continues. In contrast, LPO compound II absorbing at 431 nm is immediately reduced by SCN$^-$ to form native LPO absorbing at 412 nm (Fig. 5B) instead of formation of any stable complex. The inactive HRP which absorbs at 412 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 1, 2, 10, and 100 mM iodide, H$_2$O$_2$ 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/A versus 1/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.
The stable oxidation product of SCN$^-$ is recovered from the reaction mixture after filtration when mixed with the native HRP, native LPO, or ferrous LPO. 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 filtrate or CN$^-$ to HRP is also exactly similar to the spectrum (Fig. 4B) obtained during inactivation of HRP in the presence of SCN$^-$ and H$_2$O$_2$.

Quantitation of CN$^-$ production during SCN$^-$ oxidation by the HRP or LPO System—Table III shows that CN$^-$ production in the HRP-H$_2$O$_2$-SCN$^-$ system is $98 \pm 10$ mM, which is significantly inhibited by PBN, indicating its generation from thiocyanate radical. In contrast, LPO produces only $10 \pm 5$ mM 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-H$_2$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
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The presence of SCN⁻ and H₂O₂ 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 thio-cyanate 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. Modì 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 C1H₃ and C18H₃ groups, having a pKₐ 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 pKₐ 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 Kₐ of 60 μM (57), which is much higher than the concentration of CN⁻ (10 μM) formed in the reaction mixture. In contrast, HRP binds CN⁻ with very high affinity of Kₐ of 2.3 μM (58), which is much lower than the concentration of CN⁻ (98 μM) 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 thio-cyanate 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 thio-cyanate 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: Quantitative estimation of CN⁻ in HRP or LPO-catalyzed SCN⁻ oxidation system

| Incubation conditions | CN⁻ production μM |
|-----------------------|-------------------|
| HRP + SCN⁻ | 98 ± 10 |
| HRP + SCN⁻ + H₂O₂ | 15 ± 5 |
| LPO + SCN⁻ | 0 |
| LPO + SCN⁻ + H₂O₂ | 10 ± 5 |

**Scheme 1: Proposed mechanism for the inactivation of HRP by SCN⁻ and H₂O₂.**
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 H\(_2\)O\(_2\). In other words, 3 mol of H\(_2\)O\(_2\) 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 H\(_2\)O\(_2\) concentration at fixed enzyme and SCN\(^-\) concentrations, which is evident from the kinetic and spectral studies. As \(2 \times 10^4\) turnovers are required for complete inactivation of the enzyme (Fig. 2B), 40 nmol of H\(_2\)O\(_2\) 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 \(\mu\)m of CN\(^-\) could be formed in the system, which is compatible with the dissociation constant of the enzyme-CN\(^-\) complex formation. Thus, 13.3 \(\mu\)m of CN\(^-\) over the \(k_d\) value might be explained as due to its competition with the H\(_2\)O\(_2\) 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 iodide or the aromatic electron donor guaiacol. Although iodide 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 iodide protects the enzyme by competing with SCN\(^-\) for binding at the same site. This is consistent with the earlier findings that both iodide 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), which is away from the iodide 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 iodide, guaiacol, and ascorbate, being better substrates (high turnover) than SCN\(^-\), can consume H\(_2\)O\(_2\) 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 H\(_2\)O\(_2\), especially in the acid compartments such as vacuoles and apoplastic space (68).
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