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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 H_2O_2. 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 (a^N = 15.0 G and $a\beta^H$ = 16.5 G). The inactivation of the enzyme in the presence of SCN^- and H_2O_2 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 H_2O_2. 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.

Horseradish peroxidase (HRP)[1] (EC 1.11.1.7; donor H_2O_2 oxidoreductase) catalyzes the oxidation of a wide variety of organic and inorganic electron donors by H_2O_2 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 $C1H_3$ and $C18H_3$ 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-H_2O_2-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), $(SCN)_2$ (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 H_2O_2 and SCN^-, but CN^- may be formed if the ratio of $[H_2O_2]/[SCN^-]$ exceeds 1 (28). The pathway for SCN^- oxidation has been proposed (23) at equimolar concentrations of H_2O_2 and SCN^- as follows.

$$2\,SCN^- + H_2O_2 + 2\,H^+ \xrightarrow{LPO} (SCN)_2 + 2\,H_2O \qquad \text{(Eq. 1)}$$

$$(SCN)_2 + H_2O \longrightarrow HOSCN + SCN^- + H^+ \qquad \text{(Eq. 2)}$$

$$HOSCN \rightleftharpoons H^+ + OSCN^- \qquad \text{(Eq. 3)}$$

Alternatively, SCN^- may be directly oxidized to $OSCN^-$ as follows.

$$SCN^- + H_2O_2 \xrightarrow{LPO} OSCN^- + H_2O \qquad \text{(Eq. 4)}$$

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

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[1] 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.

of pK_a 6.4, which is presumably distal histidine (36). It binds with the K_d 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. ^1H and ^{15}N NMR studies indicate that SCN^- binds to HRP away from the distal histidine, near the heme methyl $C1H_3$ and $C18H_3$ with the K_d value of 158 ± 19 mM and the binding is facilitated by protonation of an acid group with pK_a 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 KNO_3 were from Merck (India). Other chemicals were of analytical grade. The concentration of HRP and LPO was determined from ϵ_{403} = 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 H_2O_2 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 was stopped by adding 100 nM 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 $FeCl_3$ were added and the absorbance of the $FeSCN^{2+}$ complex was measured at 450 nm (42). The concentration of SCN^- was calculated from a standard curve.

Determination of H_2O_2 Consumption during SCN^- Oxidation—H_2O_2 consumption was assayed by measuring the concentration of H_2O_2 (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 H_2O_2 added last to start the reaction. At different time intervals, a 0.1-ml aliquot was added to 3 ml of 80 mM HCl followed by the addition of 20 μl of 100 mM ferroammonium sulfate and 0.2 ml of 2.5 M KSCN to measure the absorbance at 480 nm (43). The concentration of H_2O_2 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 H_2O_2—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 H_2O_2 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 353 nm after addition of 2.0 mM KI followed by 0.6 mM H_2O_2 to start the reaction (45). The increase in absorbance was followed for 1.5 min at an interval of 10 s, and the activity was calculated from the linear rate of the reaction. HRP-catalyzed I_3^- formation in the presence of varying concentrations of SCN^- without preincubation served as control for the calculation of inactivation of HRP on preincubation with SCN^- and H_2O_2.

Inactivation of HRP by Varying Concentrations of H_2O_2 in the Presence of SCN^-—HRP (2 nM) was incubated with varying concentrations of H_2O_2 (0–0.6 mM) with a fixed concentration of SCN^- (1 mM) in 50 mM sodium acetate buffer, pH 4.5, in a final volume of 2 ml. After 5 min of incubation, 1 ml each of the reaction mixture was used for measurement of enzyme activity by I_3^- assay and for the consumption of H_2O_2 as already described.

Identification of Inactivating Species—HRP (1.0 μM) was incubated for 10 min with 1 mM SCN^- and 1 mM H_2O_2 in 50 mM sodium acetate buffer, pH 4.5, for complete inactivation. Residual H_2O_2 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-H_2O_2-SCN^- and LPO-H_2O_2-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 a standard curve.

Binding Studies by Optical Difference Spectroscopy—For measurements of the difference spectrum of the enzyme-SCN^- complex *versus* enzyme, both the reference and sample cuvettes were filled up with 1 ml of the enzyme solution (5.0 μM) for base-line tracing. This was followed by the addition of a small volume of the ligand to the sample cuvette with concomitant addition of equal volume of solvent into the reference cuvette (46). The apparent equilibrium dissociation constant (K_d) for the complex formation was calculated from

$$\frac{1}{[S]} = \frac{[E]\Delta\varepsilon}{K_d \Delta A} - \frac{1}{K_d} \qquad \text{(Eq. 5)}$$

where [*S*] and [*E*] are the concentrations of substrate and enzyme, Δ*A* is the change in absorbance between the peak and trough of the spectrum, and Δε is the difference in molar absorptivity. K_d was calculated from the plot of 1/Δ*A* *versus* 1/[*S*]. Donors competing for binding to HRP at the same site as that of SCN^- affect the apparent dissociation constant, $K_{d\ obs}$ of SCN^- in the presence of the inhibitor (I^-) and is related to the inhibitor concentration [I^-] by (38, 47, 48)

$$K_{d\,obs} = \frac{K_d\,[I^-]}{K_1} + K_d \qquad \text{(Eq. 6)}$$

where K_i 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 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 HRP, and 2 mM H_2O_2 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 H_2O_2—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 H_2O_2 resulted in concentration and time-dependent inactivation of the enzyme following pseudo-first order kinetics (Fig. 2*A*). 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. 2*A*) when plotted against SCN^- concentrations yielded a straight line (Fig. 2*A*, *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 H_2O_2 concentration in the presence of a fixed concentration of SCN^-. A plot of the percent inhibition against the turnover number ($[H_2O_2]/[HRP]$ ratio) as shown in Fig. 2*B* 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 H_2O_2/pmol of HRP.

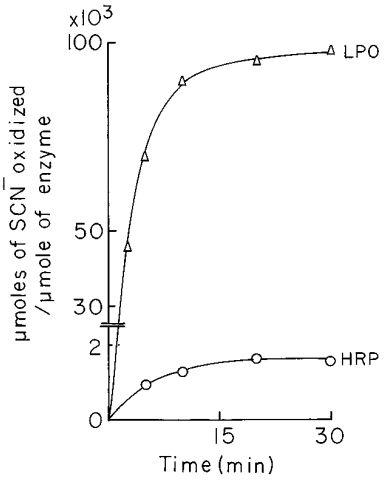


FIG. 1. **Oxidation of SCN^- by HRP and LPO in the presence of H_2O_2.** HRP 0.5 µM (○) or 0.01 µM LPO (△) was incubated with 1 mM SCN^- and 1 mM H_2O_2 in 50 mM sodium acetate buffer, pH 4.5 or 5.6, respectively. After a fixed time, the reaction was stopped by catalase and SCN^- concentration was determined as described under "Materials and Methods."

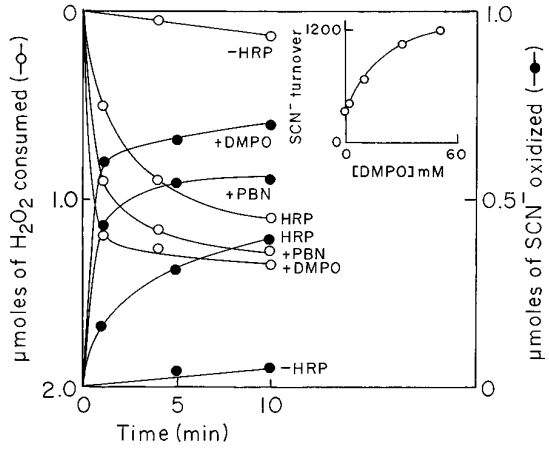


FIG. 3. **HRP-catalyzed SCN^- oxidation and concurrent consumption of H_2O_2 in the presence of PBN or DMPO.** The consumption of H_2O_2 (○) and the oxidation of SCN^- (●) was measured in the presence or absence of 1 mM PBN or 50 mM DMPO in a reaction mixture containing 0.5 µM HRP, 2 mM SCN^-, and 2 mM H_2O_2 in 50 mM acetate buffer, pH 4.5, as described under "Materials and Methods."

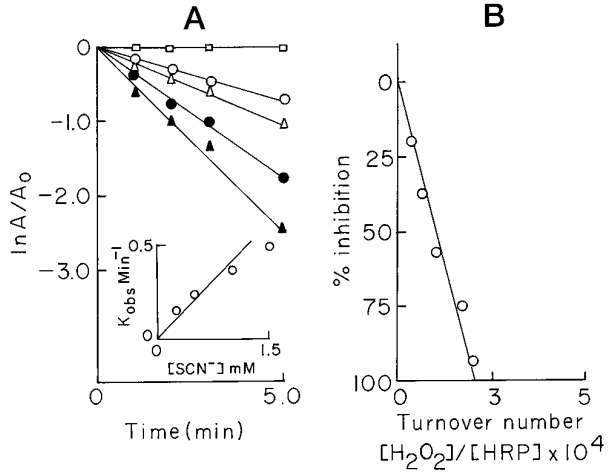


FIG. 2. **Kinetics of the inactivation of HRP on preincubation with SCN^- and H_2O_2.** *A,* HRP (2 nM) was preincubated with different concentrations of SCN^- in the presence of 0.6 mM H_2O_2 in 50 mM sodium acetate buffer, pH 4.5. At different time intervals, the residual activity was measured by I_3^- assay. The concentrations of SCN^- used were 0 mM (□), 0.25 mM (○), 0.50 mM (△), 1.00 mM (●), and 1.5 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_2O_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_2O_2 consumption as described under "Materials and Methods." The turnover number was calculated from nanomoles of H_2O_2 consumed per nanomole of HRP.

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 H_2O_2 consumption were evident in the absence of HRP. However, the initial rate of SCN^- oxidation or H_2O_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_2O_2 system is shown in Fig. 4*A*. Addition of a 5-fold excess of H_2O_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 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 H_2O_2 (Fig. 4*B, trace b*) with the visible peak at 540 nm (Fig. 4*B, 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 H_2O_2 (Fig. 4*C*), 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. 4*D* shows the effect of varying concentrations of H_2O_2 on the formation of the inactive enzyme at 421 nm. Addition of single equivalent of H_2O_2 to native HRP (*trace a*) does not cause the formation of the inactive enzyme (*trace b*). However, a gradual increase in H_2O_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_2O_2 concentration at a fixed concentration of SCN^-. As most of the studies were carried out with an excess of H_2O_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^-

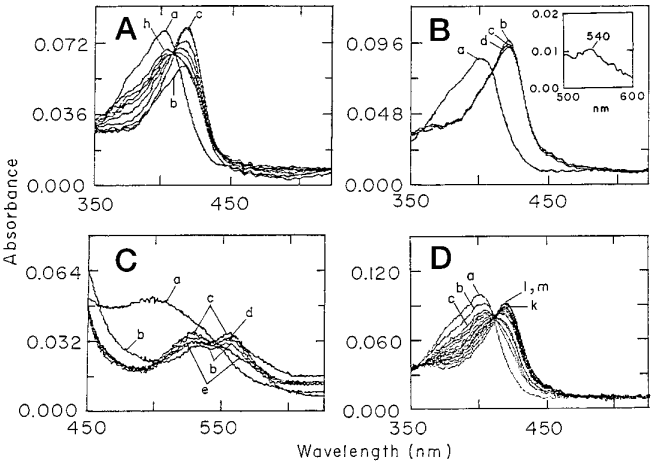


FIG. 4. **Spectral changes in HRP-catalyzed SCN^- oxidation by H_2O_2.** *A:* Soret spectrum of 0.8 μM HRP in 50 mM sodium acetate buffer, pH 4.5 (*trace a*); spectrum of a mixture of compound I and II (*trace b*) obtained immediately after addition of a 5-fold excess of H_2O_2 to native HRP; *trace c*, the complete compound II formation 30 s after the addition of 4 μM 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 μM H_2O_2; *trace c*, *b* + 100 μM I^-; *trace d*, *c* + 10 μM guaiacol. *C: trace a,* the visible spectrum of 2 μM mixture of compound I and compound II in presence of 10 μM H_2O_2; *traces b–e* were recorded at a 1-min interval after the addition of 50 μM 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 H_2O_2 (1 μM); *traces c–k*, after further additions of 1 μM H_2O_2 for each trace until the final concentration of 10 μM is reached (*trace k*). *Traces l* and *m* were obtained at final H_2O_2 concentration of 15 and 20 μM, respectively.

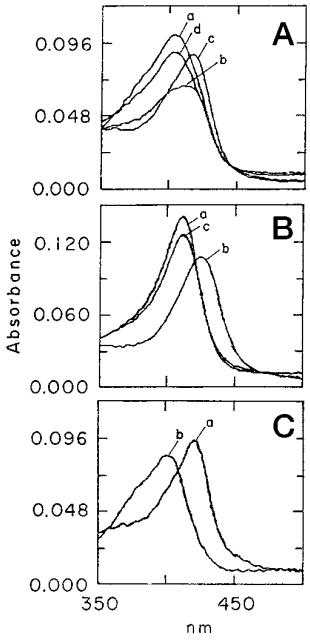


FIG. 5. **Protection of HRP by DMPO against inactivation by SCN^--H_2O_2 system (*A*), spectral evidence for the oxidation of SCN^- by LPO compound II (*B*), and spectral evidence for the reversibility of the inactivated HRP (*C*).** *A: trace a*, the Soret spectrum of 0.8 μM HRP in 50 mM acetate buffer, pH 4.5; *trace b*, *a* + H_2O_2 (10 μM); *trace c*, *b* + 50 μM DMPO; *trace d*, *c* + 25 μM SCN^-. *B:* LPO (1.4 μM) in 50 mM acetate buffer, pH 5.6 (*trace a*); a +10-fold excess of H_2O_2 (*trace b*); *b* + 50 μM SCN^- (*trace c*). *C:* Soret spectrum of 0.9 μM inactivated HRP (*trace a*). *Trace b* was recorded after passing the inactivated enzyme through the Sephadex G-25 column.

induced inactivation of HRP in the presence of H_2O_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_2O_2 in the presence of DMPO, it immediately reduced compound II to the native state absorbing at 402 nm (Fig. 5*A*). 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. 5*B*) instead of formation of any stable complex. The inactive HRP which absorbs at 421 nm (Fig. 5*C*) 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

TABLE I

Spectral evidence for the protection of HRP by electron donors against inactivation by SCN^--H_2O_2 system

Electron donors were added before the addition of H_2O_2 (15 μM) in the preincubation system containing HRP (1.5 μM) and SCN^- (25 μM) in 50 mM sodium acetate buffer, pH 4.5, in a final volume of 1 ml. In each case the spectrum of the preincubated mixture was taken 3 min after the addition of H_2O_2. The concentration of the electron donor used was 1 μM guaiacol, or 10 μM iodide, 50 μM ascorbate, or 50 μM indoleacetic acid.

Incubation conditions	Soret peak	Visible peak
	nm	*nm*
HRP	402	500, 650
HRP + SCN^-	402	500, 650
HRP + H_2O_2	418	527, 556
HRP + SCN^- + H_2O_2	421	540
HRP + I^- + SCN^- + H_2O_2	402	500, 650
HRP + guaiacol + SCN^- + H_2O_2	402	500, 650
HRP + ascorbate + SCN^- + H_2O_2	402	500, 650
HRP + IAA + SCN^-	410	555, 670

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. 6*A*). The equilibrium dissociation constant, K_d, for the HRP-SCN^- complex as calculated from the plot of $1/\Delta A$ *versus* $1/[SCN^-]$ was 125 mM. SCN^- binding was also studied in the presence of varying concentrations of iodide. The plot (Fig. 6*B*) 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.

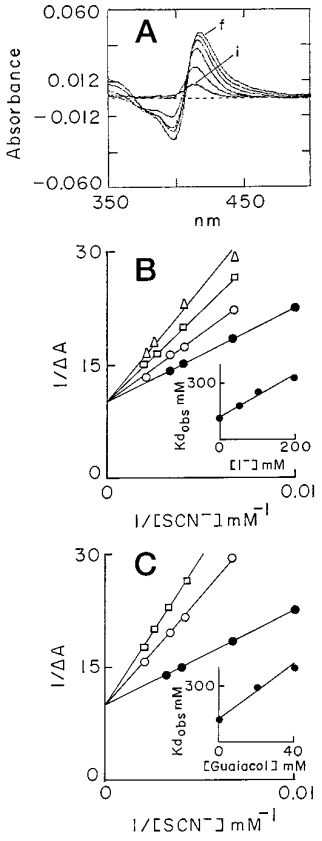


FIG. 6. **SCN^- binding to HRP by optical difference spectroscopy.** *A*, difference spectra of HRP-SCN^- complex *versus* HRP in 50 mM sodium acetate buffer, pH 4.5, were obtained with 5 μM HRP using SCN^- concentrations from 20 mM (*i*) to 300 mM (*f*). *B* and *C*, plots of $1/\Delta A$ *versus* $1/[SCN^-]$ used for calculating K_d of SCN^- in the presence or absence of iodide or guaiacol, respectively. The apparent K_d of iodide or guaiacol was deduced from the corresponding plot shown in the *inset*. The curves were best fit by least square analysis. The concentrations of I^- used were 0 mM (●), 50 mM (○), 100 mM (□), and 200 mM (△). The concentrations of guaiacol used were 0 mM (●), 20 mM (○), and 40 mM (□).

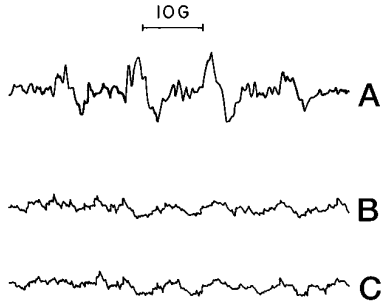


FIG. 7. **ESR spectra of DMPO-thiocyanate radical adduct in the HRP-H_2O_2-SCN^- system.** The incubation mixture for the detection of the DMPO adduct of thiocyanate radical (*A*) has been described under "Materials and Methods." The spectrometer settings were as follows: scan range, 100 G; modulation amplitude, 100 kHz; time constant, 1.0 s; gain, 6.3×10^4; microwave power, 10 mW; scan time, 2 min. *B*, -HRP; *C*, -SCN^-.

TABLE II
Optical spectra of HRP and LPO in presence of the oxidation product of HRP-SCN^--H_2O_2 system

Preparation of the filtrate from HRP-$SCN^{-\delta 1}$-H_2O_2 system and the addition of enzyme to it for measurement of optical spectrum were described under "Materials and Methods." For obtaining the cyanide spectrum, 10 or 100 μM KCN was added to 1 μM HRP or LPO, respectively. Native LPO was reduced to ferrous form by the addition of a few crystals of sodium dithionite.

Incubation conditions	Soret peak	Visible peak
	nm	*nm*
HRP	402	500, 650
HRP + filtrate	421	540
HRP + CN^-	421	540
LPO	412	500, 640
LPO + filtrate	430	555
LPO + CN^-	430	555
LPO (ferrous) + filtrate	434	570
LPO (ferrous) + CN^-	434	570

Similarly, guaiacol also competitively inhibits SCN^- binding (Fig. 6*C*) with the K_d value of 16 mM at or near this site (Fig. 6*C, inset*).

Formation of Thiocyanate Radical in HRP-catalyzed SCN^- Oxidation—From the kinetic and spectral studies, it is evident that DMPO or PBN prevents inactivation of HRP during SCN^- oxidation, suggesting involvement of a free radical species in the inactivation process. Fig. 7*A* shows the ESR spectrum obtained when HRP was incubated with SCN^- and H_2O_2 in the presence of DMPO as a spin trap. The ESR spectrum was due to the formation of spin-trapped sulfur-centered thiocyanate radical with the hyperfine splitting constants of a^N = 15.0 G and $a\beta^H$ = 16.5 G, which are consistent with the splitting constants of the known DMPO-sulfur centered radicals (50, 51). In the absence of H_2O_2 (Fig. 6*B*) or HRP (Fig. 6*C*), no significant ESR signal was detected. The signal characteristic of $O_2^{\cdot-}$ or $OH^{\cdot}$ (52) was not observed. This result indicates the formation of sulfur-centered thiocyanate radical through one-electron oxidation of SCN^- by the catalytic intermediates of HRP.

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 filtrate or CN^- to HRP is also exactly similar to the spectrum (Fig. 4*B*) obtained during inactivation of HRP in the presence of SCN^- and H_2O_2.

Quantitation of CN^- production during SCN^- oxidation by the HRP or LPO System—Table III shows that CN^- production in the HRP-H_2O_2-SCN^- system is 98 ± 10 μM, which is significantly inhibited by PBN, indicating its generation from thiocyanate radical. In contrast, LPO produces only 10 ± 5 μM CN^-, which is 10-fold lower than the HRP system.

DISCUSSION

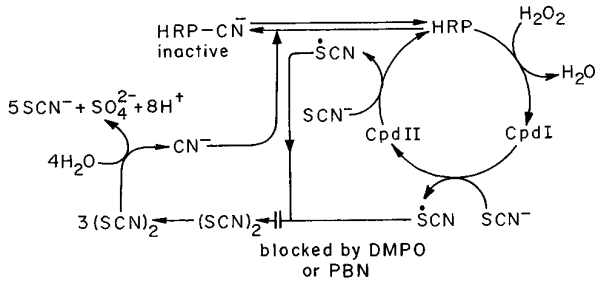
The results of this study indicate the following. (*a*) HRP-catalyzed SCN^- oxidation occurs at a significantly lower rate than LPO due to concurrent inactivation. (*b*) HRP catalyzes SCN^- oxidation through a one-electron transfer mechanism forming sulfur-centered thiocyanate radicals which finally 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_2O_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

TABLE III
Quantitative estimation of CN^- in HRP or LPO-catalyzed SCN^- oxidation system

HRP (0.5 μM) or LPO (0.01 μM) was incubated with 1 mM SCN^- and 0.6 mM H_2O_2 for 30 min at their optimum pH- of 4.5 or 5.6, respectively, using 50 mM acetate buffer. CN^- concentration in the reaction mixture was measured as described under "Materials and Methods."

Incubation conditions	CN^- production
	μM
HRP + SCN^-	0
HRP + SCN^- + H_2O_2	98 ± 10
HRP + SCN^- + PBN + H_2O_2	15 ± 5
LPO + SCN^-	0
LPO + SCN^- + H_2O_2	10 ± 5



SCHEME 1. **Proposed mechanism for the inactivation of HRP by SCN^- and H_2O_2.**

(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_2O_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.

$$\text{HRP} + H_2O_2 \rightarrow \text{compound I} \qquad \text{(Eq. 7)}$$

$$\text{compound I} + SCN^- \rightarrow \text{compound II} + \dot{S}CN \qquad \text{(Eq. 8)}$$

$$\text{compound II} + SCN^- \rightarrow \text{HRP} + \dot{S}CN \qquad \text{(Eq. 9)}$$

$$\dot{S}CN + \dot{S}CN \rightarrow (SCN)_2 \qquad \text{(Eq. 10)}$$

$$3\,(SCN)_2 + 4\,H_2O \rightarrow CN^- + 5\,SCN^- + 8\,H^+ + SO_4^{2-} \qquad \text{(Eq. 11)}$$

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_2O_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 $C1H_3$ and $C18H_3$ 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_d 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 K_d 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 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

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_2O_2. In other words, 3 mol of H_2O_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_2O_2 concentration at fixed enzyme and SCN^- concentrations, which is evident from the kinetic and spectral studies. As 2×10^4 turnovers are required for complete inactivation of the enzyme (Fig. 2*B*), 40 nmol of H_2O_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 μM CN^- could be formed in the system, which is compatible with the dissociation constant of the enzyme-CN^- complex formation (K_d = 2.3 μM) (58) for inactivation. This somewhat higher concentration of CN^- (13.3 μM) over the K_d value might be explained as due to its competition with the H_2O_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 C18H_3 group (12, 18), 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 comprehensible. 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_2O_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_2O_2, especially in the acid compartments such as vacuoles and apoplastic space (68).

REFERENCES

1. Dunford, H. B., and Stillman, J. S. (1976) *Coord. Chem. Rev.* **19,** 187–251
2. Dunford, H. B. (1982) *Adv. Inorg. Biochem.* **4,** 41–68
3. Dunford, H. B. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., and Gresham, M. B., eds.), Vol. 2, pp. 1–24, CRC Press, Boca Raton, FL
4. Ortiz de Montellano, P. R. (1992) *Annu. Rev. Pharmacol. Toxicol.* **32,** 89–107
5. Metodiewa, D., de Melo, M. P., Escobar, J. A., Cilento, G., and Dunford, H. B. (1992) *Arch. Biochem. Biophys.* **296,** 27–33
6. Mehlhorn, H., Lelandais, M., Korth, H. G., and Foyer, C. H. (1996) *FEBS Lett.* **378,** 203–206
7. Bjorkstein, F. (1970) *Biochim. Biophys. Acta* **212,** 396–406
8. Roman, R., and Dunford, H. B. (1972) *Biochemistry* **11,** 2076–2082
9. Modi, S., Behere, D. V., and Mitra, S. (1991) *Biochim. Biophys. Acta* **1080,** 45–50
10. Morrison, M., and Schonbaum, G. R. (1976) *Annu. Rev. Biochem.* **45,** 861–888
11. Magnusson, R. P., Taurog, A., and Dorris, M. L. (1984) *J. Biol. Chem.* **259,** 197–205
12. Sakurada, J., Takahashi, S., and Hosoya, T. (1986) *J. Biol. Chem.* **261,** 9657–9662
13. Sakurada, J., Takahashi, S., and Hosoya, T. (1987) *J. Biol. Chem.* **262,** 4007–4010
14. Ortiz de Montellano, P. R. (1987) *Acc. Chem. Res.* **20,** 289–294
15. Veitch, N. C., and Williams, R. J. P. (1990) *Eur. J. Biochem.* **189,** 351–352
16. Veitch, N. C., Williams, R. J. P., Bray, R. C., Burke, J. F., Sanders, S. A., Thorneley, R. N. F., and Smith, A. T. (1992) *Eur. J. Biochem.* **207,** 521–531
17. La Mar, G. N., Hernandez, G., and De Ropp, J. S. (1992) *Biochemistry* **31,** 9158–9168
18. Veitch, N. C. (1995) *Biochem. Soc. Trans.* **23,** 232–240
19. Veitch, N. C., Williams, R. J. P., Bone, N. M., Burke, J. F., and Smith, A. T. (1995) *Eur. J. Biochem.* **233,** 650–658
20. Loew, G. H., Du, P., and Smith, A. T. (1995) *Biochem. Soc. Trans.* **23,** 250–256
21. Rodriguez-Lopez, J. N., Smith, A. T., and Thorneley, R. N. F. (1996) *J. Biol. Chem.* **271,** 4023–4030
22. Adak, S., Mazumdar, A., and Banerjee, R. K. (1996) *Biochem. J.* **314,** 985–991
23. Aune, T. M., and Thomas, E. L. (1977) *Eur. J. Biochem.* **80,** 209–214
24. Tenovuo, J. O. (1985) in *The Lactoperoxidase System: Chemistry and Biological Significance* (Pruitt, K. M., and Tenovuo, J. O. eds.), pp. 101–122, Marcel Dekker, New York
25. Slungaard, A., and Mahoney, J. R., Jr. (1991) *J. Biol. Chem.* **266,** 4903–4910
26. Das, D., De, P. K., and Banerjee, R. K. (1995) *Biochem. J.* **305,** 59–64
27. Mazumdar, A., Chatterjee, R., Adak, S., Ghosh, A., Mondal, C., and Banerjee, R. K. (1996) *Biochem. J.* **314,** 413–419
28. Modi, S., Deodhar, S. S., Behere, D. V., and Mitra, S. (1991) *Biochemistry* **30,** 118–124
29. Jago, G. R., and Morrison, M. (1962) *Proc. Soc. Exp. Biol. Med.* **111,** 585–590
30. Reiter, B., Marshall, N. M., Bjorck, L., and Rosen, C. G. (1976) *Infect. Immun.* **13,** 800–807
31. Aune, T. M., and Thomas, E. L. (1978) *Biochemistry* **17,** 1005–1010
32. Chung, J., and Wood, J. I. (1970) *Arch. Biochem. Biophys.* **141,** 73–78
33. Hog, D. M., and Jago, G. R. (1970) *Biochem. J.* **117,** 779–790
34. Hughes, M. N. (1975) in *Chemistry and Biochemistry of Thiocyanic Acid and Its Derivatives* (Newman, A. A. ed.), pp. 1–67, Academic Press, New York
35. Thomas, E. L. (1981) *Biochemistry* **20,** 3273–3280
36. Modi, S., Behere, D. V., and Mitra, S. (1989) *Biochemistry* **28,** 4689–4694
37. Pruitt, K. M., and Tenovuo, J. (1982) *Biochim. Biophys. Acta* **704,** 204–214
38. Modi, S., Behere, D. V., and Mitra, S. (1989) *J. Biol. Chem.* **264,** 19677–19684
39. Modi, S., Behere, D. V., and Mitra, S. (1990) *Indian J. Chem.* **29A,** 301–311
40. Aibara, S., Yamashita, H., Mori, E., Kato, M., and Morita, Y. (1982) *J. Biochem. (Tokyo)* **92,** 531–539
41. Morrison, M., and Bayse, G. S. (1970) *Biochemistry* **9,** 2995–3000
42. Betts, R. H., and Dainton, F. S. (1953) *J. Am. Chem. Soc.* **75,** 5721–5727
43. Hildebrandt, A. G., and Roots, I. (1975) *Arch. Biochem. Biophys.* **171,** 385–397
44. Adak, S., Bhattacharyya, D. K., Mazumdar, A., Bandyopadhyay, U., and Banerjee, R. K. (1995) *Biochemistry* **34,** 12998–13006
45. Bhattacharyya, D. Kr., Bandyopadhyay, U., and Banerjee, R. K. (1992) *J. Biol. Chem.* **267,** 9800–9805
46. Harris, R. Z., Newmyer, S. L., and Ortiz de Montellano, P. R. (1993) *J. Biol. Chem.* **268,** 1637–1645
47. Dunford, H. B., and Cotton, M. L. (1985) *J. Biol. Chem.* **250,** 2920–2932
48. Bhattacharyya, D. K., Adak, S., Bandyopadhyay, U., and Banerjee, R. K. (1994) *Biochem. J.* **298,** 281–288
49. Keilin, D., and Hartree, E. F. (1951) *Biochem. J.* **49,** 88–104
50. Mottley, C., Mason, R. P., Chignell, C. F., Sivarajah, K., and Eling, T. E. (1982) *J. Biol. Chem.* **257,** 5050–5055
51. Mottley, C., and Mason, R. P. (1988) *Arch. Biochem. Biophys.* **267,** 681–689
52. Finkelstein, E., Rosen, G. M., and Rauckman, E. J. (1980) *Arch. Biochem. Biophys.* **200,** 1–16
53. Yamazaki, I. (1977) in *Free Radicals in Biology* (Pryor, W. A., ed.), Vol. 3, pp. 183–210, Academic Press, New York
54. Thomas, E. L. (1985) in *The Lactoperoxidase System: Chemistry and Biological Significance* (Pruitt, K. M., and Tenovuo, J. O. eds.), pp. 31–53, Marcel Dekker, New York
55. Ator, M. A., and Ortiz de Montellano, P. R. (1987) *J. Biol. Chem.* **262,** 1542–1551
56. Bandyopadhyay, U., Bhattacharyya, D. K., Chatterjee, R., and Banerjee, R. K. (1995) *Biochem. J.* **306,** 751–757
57. Carlström, A. (1969) *Acta. Chem. Scand.* **23,** 203–213
58. Dolman, D., Dunford, H. B., Chowdhury, D. M., and Morrison, M. (1968) *Biochemistry* **7,** 3991–3996
59. Chance, B. (1952) *Arch. Biochem. Biophys.* **41,** 416–424
60. Yamazaki, I., Mason, H. S., and Piette, L. (1960) *J. Biol. Chem.* **235,**

2444–2449
61. Araiso, T., Miyoshi, K., and Yamazaki, I. (1976) *Biochemistry* **15,** 3059–3063
62. Harman, L. S., Mottley, C., and Mason, R. P. (1984) *J. Biol. Chem.* **259,** 5606–5611
63. Mottley, C., Toy, K., and Mason, R. P. (1987) *Mol. Pharmacol.* **31,** 417–421
64. Mottley, C., and Mason, R. P. (1989) in *Biological Magnetic Resonance* (Berliner, L. J., and Reuben, J. eds.), Vol. 8, pp. 502–507, Plenum Publishing Corp., New York
65. Saunders, B. C., Holmes-Siedle, A. G., and Stark, B. P. (1964) in *Peroxidase* (Saunders, B. C., ed.), pp. 214–252, Butterworths, London
66. Olea, F., and Parras, P. (1992) *J. Anal. Toxicol.* **16,** 258–260
67. Riordan, J. F., McElvany, K. D., and Borders, C. I., Jr. (1977) *Science* **195,** 884–885
68. Chance, B. (1951) *Science* **109,** 204–208