Interaction of Thiocyanate with Horseradish Peroxidase

^{1}H AND ^{15}N NUCLEAR MAGNETIC RESONANCE STUDIES*

Sandeep Modi, Digambar V. Behere, and Samaresh Mitra‡

From the Chemical Physics Group, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Bombay 400005, India

Interaction of thiocyanate with horseradish peroxidase (HRP) was investigated by relaxation rate measurements (at 50.68 MHz) of the ^{15}N resonance of thiocyanate nitrogen and by following the hyperfine shifted ring methyl proton resonances (at 500 MHz) of the heme group of SCN$^-$·HRP solutions. At pH 4.0, the apparent dissociation constant (K_D) for thiocyanate binding to HRP was deduced to be 158 mM from the relaxation rate measurements. Chemical shift changes of 1- and 8-ring methyl proton resonances in the presence of various amounts of thiocyanate at pH 4.0 yielded K_D values of 166 and 136 mM, respectively. From the pH dependence of K_D and the ^{15}N resonance line width, it was observed that thiocyanate binds to HRP only under acidic conditions (pH < 6). The binding was found to be facilitated by protonation of an acid group on the enzyme with pK_a 4.0. The pH dependence of the ^{15}N line width as well as the apparent dissociation constant were quantitatively analyzed on the basis of a reaction scheme in which thiocyanate in deprotonated ionic form binds to the enzyme in protonated acidic form. The K_D for thiocyanate binding to HRP was also evaluated in the presence of an excess of exogenous substrates such as resorcinol, cyanide, and iodide ions. It was found that the presence of cyanide (which binds to heme iron at the sixth coordination position) and resorcinol did not have any effect on the binding of thiocyanate, indicating that the binding site of the thiocyanate ion is located away from the ferric center as well as from the aromatic donor binding site. The K_D in the presence of iodide, however, showed that iodide competes with thiocyanate for binding at the same site. The distance of the bound thiocyanate ion from the ferric center was deduced from the ^{15}N relaxation time measurements and was found to be a 6.8 Å. From the distance as well as the change in the chemical shifts and line width of 1- and 8-methyl proton resonances, it is suggested that the binding site of thiocyanate may be located near heme, placed symmetrically with respect to 1- and 8-methyl groups of the heme of HRP. Similarity in the modes of binding of iodide and thiocyanate suggests that the oxidation of thiocyanate ion by H_2O_2 may also proceed via the two-electron transfer pathway under acidic conditions, as is the case for iodide.

Horseradish peroxidase (HRP, EC 1.11.1.7, donor, H_2O_2, oxidoreductase) is a plant heme protein enzyme that catalyzes primarily the oxidation of a wide variety of oxidizable organic donor molecules by hydrogen peroxide (1, 2). The oxidation reaction for organic substrates generally proceeds through two distinct intermediates, HRP-I and HRP-II. The mechanism of oxidation involves initial binding of the donor to the enzyme. Several studies have therefore been reported on the binding of oxidizable organic substrates to the native enzyme (2–5). HRP is also known to catalyze oxidation of inorganic substrates such as iodide, thiocyanate, nitrite, and bisulfite ions (2, 6). Among the oxidizable inorganic substrates, the interaction of iodide ion with HRP has been studied to further understanding of the mechanism of thyroid hormone biosynthesis catalyzed by thyroid peroxidase and lactoperoxidase (7, 8). Kinetic (9), fluorometric (10), and NMR (11) studies have suggested that iodide ion forms a 1:1 complex with HRP and binds near the heme group. The kinetic studies (9, 12) have suggested further that the oxidation of iodide with hydrogen peroxide catalyzed by HRP occurs via two-electron transfer under acidic conditions. In this process, HRP-I is converted to native enzyme directly by two-electron oxidation of the substrate without formation of HRP-II.

Besides iodide, thiocyanate ion is also an attractive inorganic substrate because thiocyanate is classified as a pseudo-halide and bears many resemblances to iodide in its chemical behavior. SCN$^-$·H_2O_2·lactoperoxidase provides a potent non-specific bacteriostatic or bacteriocidal system (13, 14). Magnusson et al. (15) have studied the catalytic activity of lactoperoxidase using iodide and thiocyanate ions and have suggested that the oxidation of iodide and thiocyanate with hydrogen peroxide catalyzed by lactoperoxidase and thyroid peroxidase may also occur via two-electron transfer. We have recently studied the interaction of thiocyanate with lactoperoxidase using ^{15}N and ^{1}H NMR (16). Thiocyanate and iodide ions have been shown to bind to lactoperoxidase at a distal site of heme at the histidyl residue with an apparent dissociation constant of 90 and 38 mM, respectively at pH 6.1 (16, 17). The interaction of thiocyanate with HRP has, however, not been studied in any detail. The only study appears to be that of Lukat and Goff (18), who investigated the interaction by optical spectroscopy and reported that the interaction of thiocyanate with HRP is optically inoperable even at a high concentration of thiocyanate. Thus, in spite of the fact that the oxidation of thiocyanate ion by H_2O_2 is catalyzed by HRP like that of iodide ion, it is still not known where thiocyanate

---

^{1} The abbreviations used are: HRP, horseradish peroxidase; SCN$^-$, deprotonated thiocyanate; $\Delta\nu_{1/2(obs)}$, observed line width; K_{SH}, protolytic dissociation constant of substrate; T_1, relaxation time; T_{1b}, T_1 of the HRP-substrate complex; T_{1f}, T_1 of substrate in the absence of enzyme; T_{1d}, diamagnetic contribution to T_{1b}; T_m, paramagnetic contribution to T_1; τ_c, autocorrelation time; τ_m, lifetime of the enzyme-substrate complex.

ion binds and how electrons are transferred from thiocyanate to the heme iron of HRP-I.

In the present study, the interaction of thiocyanate with HRP was investigated using ^{15}N and ^{1}H NMR spectroscopy. From the measurements of relaxation times of $(SC^{15}N)^-$ in the presence and absence of HRP, the apparent dissociation constant (K_D) was evaluated, and the distance of the ^{15}N of SCN^- from the ferric ion of HRP was estimated. The line width measurements on ^{15}N resonance were utilized to deduce the autocorrelation time (τ_c). The pH dependence of the line width gave a pK_a of the ionizable group in the heme crevice, which is responsible for the binding of thiocyanate. The K_D was also estimated from the chemical shift changes of the heme methyl resonances of HRP with varying concentrations of thiocyanate. The results of these studies have been used to determine the strength and site of the binding of thiocyanate to HRP and are compared with the results on the interaction of iodide with HRP (11) and the interaction of thiocyanate with lactoperoxidase (16).

MATERIALS AND METHODS

Horseradish peroxidase was purified from crude HRP (Sigma, Rz $= A_{403}/A_{280} = 0.8$) by DEAE and CM-cellulose column chromatography (19). The B and C HRP isoenzymes were collected (Rz = 3.2) after elution with acetate buffer (100 mM, pH 4.4). The concentration of the enzyme was determined spectrophotometrically using a molar extinction coefficient of 1.02×10^5 cm^{-1} M^{-1} at 403 nm for HRP (20). Deuterium oxide (>99.85%) was purchased from Aldrich. Enriched ^{15}N sodium thiocyanate (NaSC^{15}N, atom % of ^{15}N > 99) was purchased from MSD Isotopes. All other reagents were of analytical grade.

NMR Measurements—Proton NMR measurements were carried out on a Bruker AM 500-MHz FT NMR spectrometer at 23 °C. The samples were lyophilized directly inside 5-mm NMR tubes with an excess of D$_2$O, and the final solution was prepared in 0.1 M phosphate buffer at pH 4.0 (volume, 0.4 ml). Proton NMR spectra of HRP were obtained by accumulation of about 40,000 transients at 8K data points in quadrature mode. Proton chemical shifts were referred to a proton signal of trace HDO as a secondary reference at 4.75 ppm. Quoted pH values are meter readings uncorrected for isotope effects.

The ^{15}N NMR measurements were made on a Bruker FT NMR spectrometer operating at 50.68 MHz in a 10-mm NMR tube with D$_2$O for frequency lock. The spectra were obtained by accumulation of 400–1,000 transients at 16K data points. Measurements at different temperatures were done using an automated temperature controller that maintained temperatures within ±0.5 °C.

Line Width Measurements—The line width data were obtained from the spectra by fitting the substrate ^{15}N resonances to Lorentzian line shape. The observed line width $(\Delta\nu_{1/2(\text{obs})})$ of the ^{15}N resonance of the $SC^{15}N^-$ in the presence of the enzyme is considered to be the sum of the line widths due to the enzyme-bound fraction and due to the unbound fraction of the substrate ^{15}N resonance, assuming the chemical shift difference to be negligible (see Fig. 1). Enzyme-substrate interaction is considered to take place between the protonated form of the enzyme and the deprotonated ionic form of the substrate, as the HSCN is strongly acidic with $pK_{SH} = -1.9$ (21) and would be mostly in the ionic form in the pH range of the present study. The scheme for the binding of thiocyanate to HRP is as follows

$$H^+ + E\text{H-S}^- \xrightleftharpoons{K} E\text{H} + S^- + H^+$$
$$\Updownarrow K_a \qquad \Updownarrow K_{SH}$$
$$E + H^+ \qquad \text{SH}$$
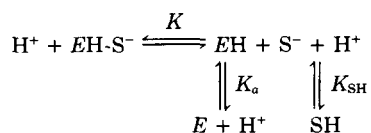
SCHEME 1

where K is the complex dissociation constant for the binding of deprotonated thiocyanate (SCN^-) to protonated enzyme form $(E\text{H})$. K_a and K_{SH} are protolytic dissociation constants of enzyme and substrate, respectively.

The pH dependence of K_D can be explained by Scheme 1, and it is related to K by Equation 1 (22, 23)

$$K_D = K(1 + K_a/\text{H}^+)(1 + \text{H}^+/K_{SH}) \tag{1}$$

The variation of the observed line width as a function of pH is given by Equation 2 (23)

$$\Delta\nu_{1/2(\text{obs})} = \frac{E_0(\Delta\nu_{1/2}^B - \Delta\nu_{1/2}^F)}{S_0 + K(1 + K_a/\text{H}^+)(1 + \text{H}^+/K_{SH})} + \Delta\nu_{1/2}^F \tag{2}$$

where $\Delta\nu_{1/2}^B$ and $\Delta\nu_{1/2}^F$ denote the line widths of the enzyme-bound and unbound substrate ^{15}N resonances, respectively. E_0 and S_0 represent the initial enzyme and substrate concentrations, respectively.

Relaxation Rate Measurements—Since the optimum pH for the binding of SCN^- to HRP is between 3.0 and 5.0 (see "Results"), the relaxation rate measurements were done at pH 4.0. For the relaxation time measurements, HRP was treated with Chelex 100 (Bio-Rad) to remove any traces of free metal ions (24). Deionized double-distilled water was used to prepare 0.1 M phosphate buffer (pH 4.0). Filtrates were lyophilized and redissolved in D$_2$O for NMR studies. Titrations were carried out in the enzyme concentration range of 100 μM–4 mM, and titrations of the substrate were in the range of 20–470 mM. To obtain the longitudinal relaxation time $(T_{1(\text{obs})})$, the inversion recovery method with a $180°$-τ-$90°$ pulse sequence was used (16, 23, 25).

Determination of the Apparent Dissociation Constant of Thiocyanate Binding to HRP Using ^{15}N-T_1 Measurements—$T_{1(\text{obs})}$ can be considered as the sum of the relaxation rates of the bound and free substrate fractions and is related to K_D, T_{1b}, and T_{1f} through Equation 3 for the binding of one molecule of thiocyanate to one protonated molecule of HRP (16, 23)

$$E_0\left[\frac{1}{T_{1(\text{obs})}} - \frac{1}{T_{1f}}\right]^{-1} = K_D\left[\frac{1}{T_{1b}} - \frac{1}{T_{1f}}\right]^{-1} + S_0\left[\frac{1}{T_{1b}} - \frac{1}{T_{1f}}\right]^{-1} \tag{3}$$

where T_{1b} is the T_1 of the HRP-substrate complex, and T_{1f} is the T_1 of the substrate in the absence of the enzyme. K_D and T_{1b} for thiocyanate binding to HRP can be obtained by least squares fit of the data to Equation 3.

Determination of the Apparent Dissociation Constant of Thiocyanate Binding to HRP from Chemical Shifts of HRP Using ^{1}H NMR—K_D was also obtained (at pH 4.0) from the ^{1}H chemical shift of HRP using the following expression (16)

$$K_D = \frac{[\delta_{(\text{HRP·SCN})} - \delta_{\text{obs}}]\left[S_0 - \frac{(\delta_{\text{obs}} - \delta_{\text{HRP}})E_0}{(\delta_{(\text{HRP·SCN})} - \delta_{\text{HRP}})}\right]}{[\delta_{\text{obs}} - \delta_{\text{HRP}}]} \tag{4}$$

where δ_{HRP}, $\delta_{\text{HRP·SCN}}$, and δ_{obs} are the observed heme proton chemical shifts of free HRP, HRP with bound thiocyanate, and at intermediate concentrations of the enzyme and thiocyanate, respectively. The concentrations of the substrate and the enzyme can be varied and K_D evaluated by fitting the data to a nonlinear fitting program (16, 26).

Competitive Binding of Thiocyanate to HRP in the Presence of Iodide—Donors competing for binding to the native HRP at the same site as that of thiocyanate affect the apparent dissociation constant of the latter. The observed apparent dissociation constant, $K_{D(\text{obs})}$, of the thiocyanate in the presence of the inhibitor is related to the inhibitor concentration $[I]$ by the following expression (23, 27, 28)

$$K_{D(\text{obs})} = K_D + \frac{K_D[I]}{K_i} \tag{5}$$

where K_i is the apparent dissociation constant of the inhibitor in the absence of thiocyanate, and K_D, as defined earlier, is the apparent dissociation constant for the binding of thiocyanate to HRP in the absence of inhibitor. The value of K_i of the iodide ion was deduced from intercept and slope of the straight line plot of $K_{D(\text{obs})}$ *versus* $[I]$ (see Equation 5).

Determination of Distance Using ^{15}N-T_1 Measurements—T_{1b} obtained from Equation 3 is related to T_{1m} through

$$T_{1b}^{-1} - T_{1d}^{-1} = [(T_{1m} + \tau_m)^{-1}]$$

where τ_m is the lifetime of the enzyme-substrate complex. Since the fast exchange limit, $T_{1m} \gg \tau_m$, and since the diamagnetic contribution (T_{1d}^{-1}) is negligibly small, T_{1b}^{-1} can be taken to be the same as T_{1m}^{-1} (4, 5, 16, 23, 29–33). Using Solomon (34) and Bloembergen (35) equations, the distance of the nitrogen of the bound thiocyanate from the metal center in HRP (r) can be determined, provided the value of τ_c is known. For Fe^{3+} $(S = 5/2)$, the metal-^{15}N distance in HRP·SCN$^-$ is given by (16).

$$r(cms) = \left\{ 2.958 \times 10^{-33} \, T_{1m} \left[\frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2\tau_c^2} \right] \right\}^{1/6} \quad (6)$$

The value of τ_c was estimated from the ratio of T_{2m} to T_{1m} (16, 23, 29, 36).

RESULTS

Interaction of SC^{15}N$^-$ with HRP Probed by ^{15}N NMR—Fig. 1 shows the ^{15}N NMR spectra of thiocyanate in the absence and presence of HRP. The sharp ^{15}N signal is broadened from 2.5 to 9.0 Hz by the addition of 50 μM HRP at pH 4.0. Fig. 2 shows that the line width of ^{15}N resonance of SC^{15}N$^-$ increases monotonically by sequential addition of HRP, suggesting the
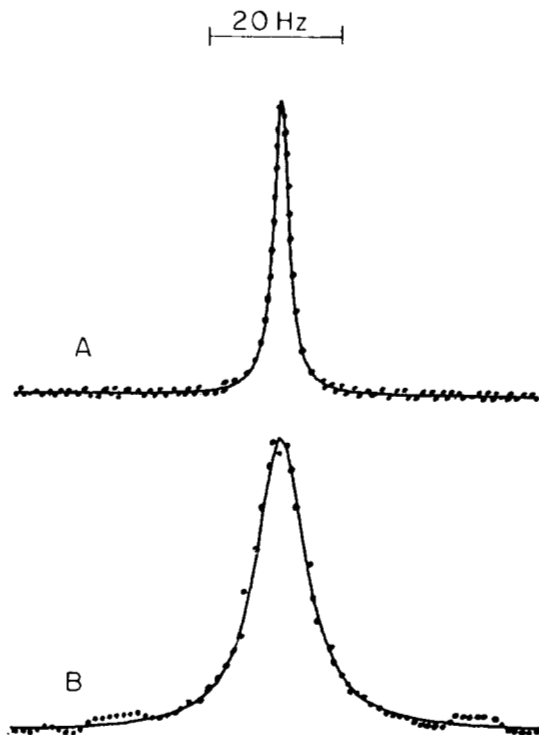

FIG. 1. **^{15}N NMR spectrum of thiocyanate (20.5 mM) in 0.1 M phosphate buffer (pH 4.0) in the absence (*A*) and presence (*B*) of HRP (50 μM).** The *dotted lines* are resonance traces; the *solid lines* are fitted once to the Lorentzian line shape function. Sweep widths of 1 kHz over 16,000 data points were used to ensure an instrumental resolution of 0.12 Hz.
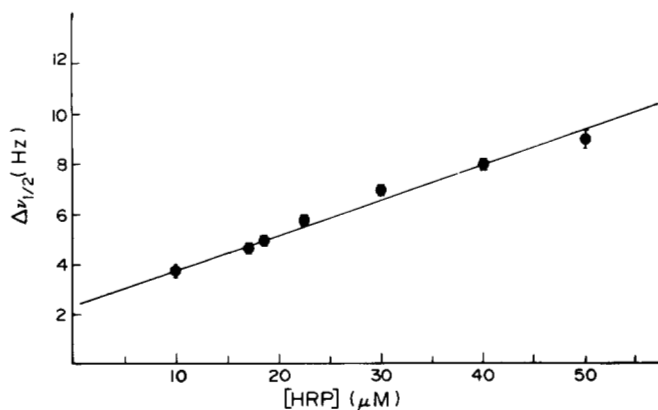

FIG. 2. **Variation of the ^{15}N line width of thiocyanate (20.5 mM) as a function of HRP concentration.** The height of the *vertical bars* is equal to twice the standard deviation of the Lorentzian line shape fit.

increase in the substrate-bound enzyme fraction.

The effect of pH on the line width of the HRP·SCN$^-$ system was studied in the pH range of 3.0–6.7. No data could be taken below pH 3.0, as the linkage of heme to the apoenzyme is then broken. Fig. 3 shows that pH has a pronounced effect on the line width of ^{15}N resonance in the presence of HRP and hence on the binding of SCN$^-$ to the enzyme. In the presence of the enzyme, the line width of the resonance increases from 2.5 Hz at pH 6.7 to 9.5 Hz at pH 3.0. No pH effect on the line width of ^{15}N resonance of thiocyanate was observed in the absence of the enzyme (see Fig. 3). Above pH 6.0, the line width of the resonances in the absence or presence of the enzyme is the same (2.5 Hz), indicating that thiocyanate binds to HRP only below pH 6.0. The variation of the line width below pH 6.0 clearly reflects titration of an ionizable group. The pH dependence data were least squares fitted to Equation 2, which yielded a pK_a of 4.0 and a K of 80 mM. Thus, protonation of an ionizable group with pK_a = 4.0 enhances the binding of thiocyanate to HRP. The good fit of the data to Equation 2 confirms the involvement of one molecule of thiocyanate binding/one-proton transfer to the enzyme as assumed in Scheme 1. As the optimum pH for the thiocyanate ion binding is around pH 4.0, all the proton and ^{15}N NMR data refer to pH 4.0 unless otherwise specified.

^{15}N-T_1 Measurements—The spin lattice relaxation time (T_1) measurements at 50.68 MHz for SC^{15}N$^-$ were done at different enzyme and substrate concentrations by the inversion recovery method. A typical set of inversion recovery spectra is shown in Fig. 4A. $T_{1(obs)}$ at various enzyme and substrate concentrations and T_{1f}, the spin lattice relaxation time of SC^{15}N$^-$ in the absence of HRP, were used to calculate T_{1b} and K_D by least squares fit of the data to Equation 3. Fig. 4B shows that the plot of $E_0(1/T_{1(obs)} - 1/T_{1f})^{-1}$ *versus* S_0 is a straight line, which is consistent with the 1:1 stoichiometry of the HRP·SCN$^-$ complex (16). $K_D = 158 \pm 19$ mM and $T_{1b}^{-1} = 8.3 \pm 0.4$ s^{-1} were obtained from the least squares fit of the data to Equation 3. Similarly, ^{15}N-T_1 measurements were carried out in the presence of cyanide ion (0.1 M). Cyanide
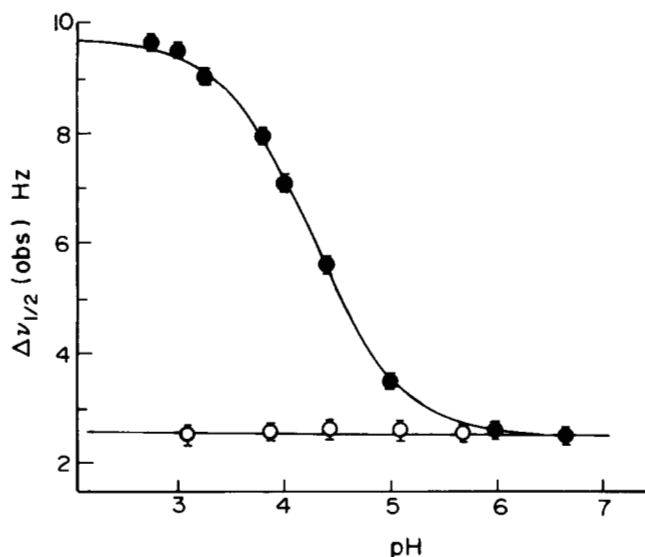

FIG. 3. **^{15}N NMR line width of thiocyanate (20.5 mM) as a function of pH in the range of 3.0–6.7 in 0.1 M phosphate buffer, in the absence (*open circles*) and presence (*closed circles*) of HRP (33 μM).** The height of the *vertical bars* denotes twice of the standard deviation of the Lorentzian line shape fit. The *solid line* is the least squares fit to Equation 2.
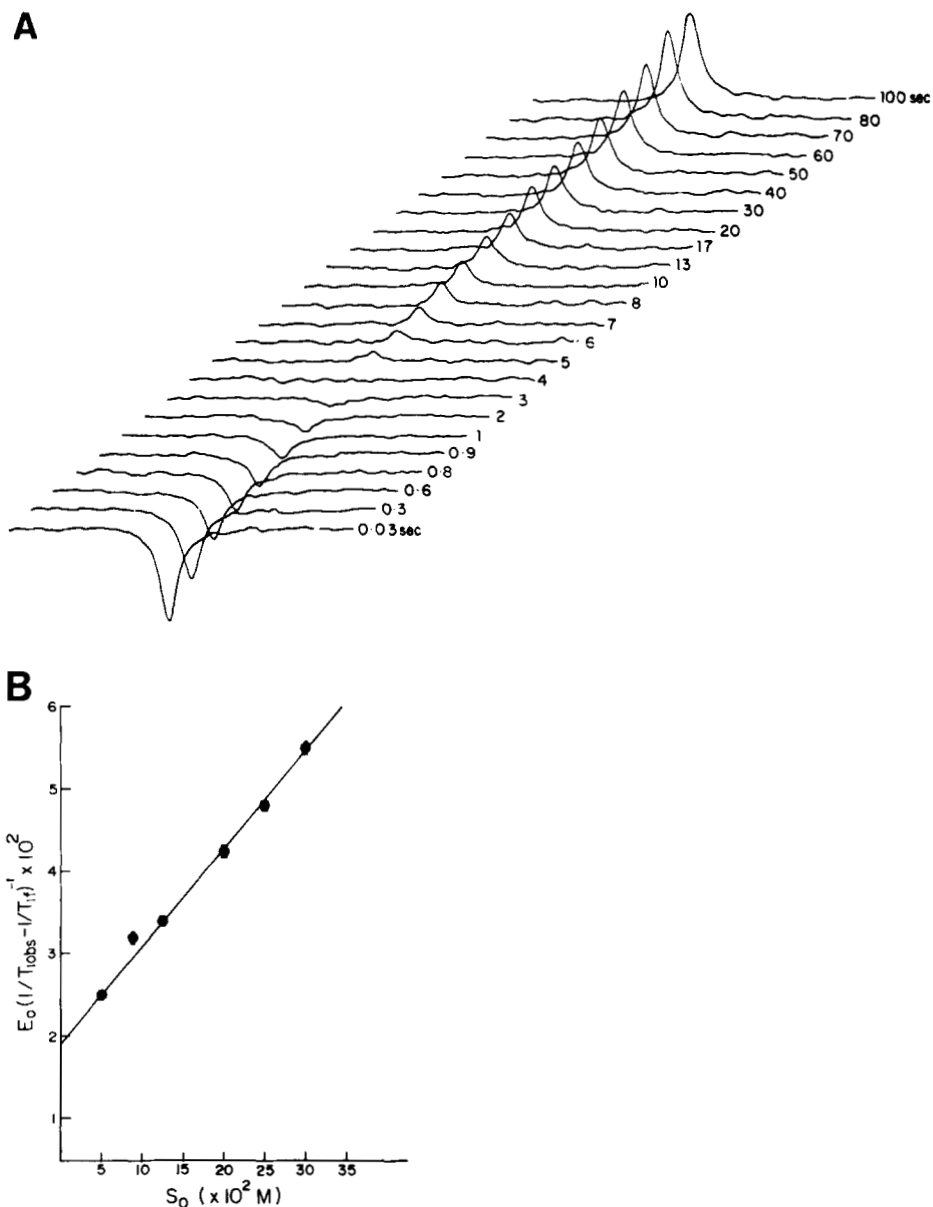
A

FIG. 4. *A*, $180°$-τ-$90°$ pulse ^{15}N NMR spectra (at 50.68 MHz) of thiocyanate (200 mM) in the presence of 2.5 mM HRP (0.1 M phosphate buffer, pH 4.0). τ values were varied from 0.03 ms to 100 s, which are shown in the figure. *B*, plot of $[E_0(1/T_{1(obs)} - 1/T_{1f})^{-1}]$ *versus* S_0, where S_0 was varied from 50 to 300 mM. Observation of the *straight line* confirmed the binding of one thiocyanate ion to HRP.

B

ion binds to ferric ion of the heme of HRP at the sixth position to form low spin species (37). The T_{1b}^{-1} calculated from Equation 3 (data not shown) was found to be $T_{1b}^{-1}(CN^-)$ $= 8 \times 10^{-2}$ s^{-1}. This may be considered as a diamagnetic contribution (T_{1d}^{-1}) to the T_{1b}^{-1} of thiocyanate (4, 5, 16) and is very small as compared with the T_{1b}^{-1} of $SC^{15}N^-$ binding and hence is neglected. The K_D of $SC^{15}N^-$ binding to HRP·CN was estimated to be 134 ± 21 mM, which compares well with that of 158 ± 19 mM estimated for thiocyanate binding to native HRP (Table I). This suggests that the binding of cyanide to the ferric center at the sixth position does not inhibit the binding of thiocyanate to HRP and that the binding site of SCN^- is away from the ferric center.

Spin lattice relaxation rates ($T_{1(obs)}^{-1}$ and T_{1f}^{-1}) for $SC^{15}N^-$· HRP were measured at different temperatures (6–50 °C). T_{1b}^{-1} was calculated at different temperatures using Equation 3. The dependence of T_{1b}^{-1} on temperature is represented as an Arrhenius plot of $\log(T_{1b})^{-1}$ *versus* inverse of the temperature (Fig. 5). In this representation, the slope gives the activation energy of the processes related to variation of τ_c with temperature (29, 36). The relaxation rate decreases with

TABLE I

Effect of cyanide, iodide, nitrate ions, and resorcinol on the binding of thiocyanate to horseradish peroxidase

− and + show the absence and presence (0.1 M), respectively, of particular substrate.

CN^-	Resorcinol	I^-	NO_3^-	K_D	Method used for determination of K_D
				mM	
−	−	−	−	158 ± 19	^{15}N-T_{1m} measurements
−	−	−	−	150 ± 20	^{15}N-T_{2m} measurements
−	−	−	−	166 (1-methyl) 136 (8-methyl)	^1H chemical shifts variation
+	−	−	−	134 ± 21	^{15}N-T_{1m} measurements
−	+	−	−	167 ± 23	^{15}N-T_{1m} measurements
−	−	+	−	292 ± 30	^{15}N-T_{1m} measurements
−	−	−	+	170 ± 21	^{15}N-T_{1m} measurements

an increase in temperature, suggesting the presence of fast exchange ($T_{1m} \gg \tau_m$) (29, 31, 36). This is also consistent with the observed low binding affinity of thiocyanate to HRP (K_D = 158 mM). The activation energy of 2.6 ± 0.7 kcal/mol
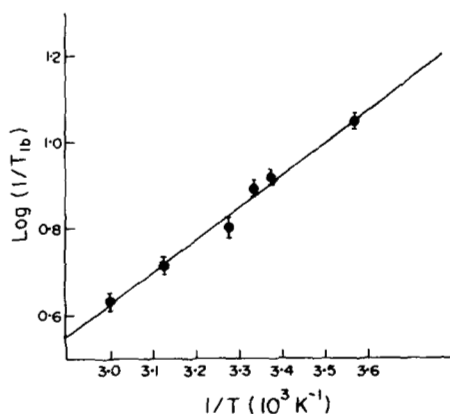
FIG. 5. **Temperature dependence of logarithmic variation of relaxation rate $(1/T_{1b})$ of ^{15}N resonance of bound thiocyanate at 50.68 MHz.**

calculated from the slope of the plot of Fig. 5 is in the range of 1–3 kcal/mol expected for τ_m-independent processes (29, 30, 36). The temperature dependence of the line width of ^{15}N NMR resonance of the $SC^{15}N^-\cdot HRP$ system shows an increase in the line width with a decrease in temperature (data not shown), which is also consistent with the fast chemical exchange (16, 38) of the $HRP\cdot SCN^-$ system.

The positive slopes of the temperature variation of relaxation rate and line width data suggest that the conditions $\omega_I^2\tau_c^2 \ll 1$ and $\omega_S^2\tau_c^2 \gg 1$ prevail in the present system (31, 32). We have shown earlier that $T_{1b}^{-1} = T_{1m}^{-1}$ (see "Materials and Methods"). Equation 6, therefore, simplifies to

$$r(cms) = (8.874 \times 10^{-33} \, T_{1b}\tau_c)^{1/6}$$

Hence, the distance of the ^{15}N of thiocyanate from the paramagnetic ferric ion of the HRP heme can now be calculated using this expression, provided the value of τ_c is known. The value of τ_c was deduced from the ratio of T_{2m} to T_{1m} as discussed earlier (16, 23). T_{2m} was calculated using an equation similar to Equation 3, assuming the chemical shift difference to be negligible. The calculated value of $\tau_c = 9.5 \times 10^{-11}$ s compares with the value of 5.0×10^{-11} s used previously (3–5). Fortunately, the calculated distance does not differ much if either of the two values is used. The distances of the ^{15}N of the thiocyanate from ferric ion ($Fe^{3+} - {}^{15}N$) calculated using the two values of τ_c are 6.8 and 6.1 Å, respectively.

Effect of Exogenous Substrates on the Binding of Thiocyanate Ion to HRP—The binding of thiocyanate ion to HRP in the presence of exogenous substrates such as resorcinol, iodide, and nitrate was also studied by ^{15}N-T_1 measurements. The data in Table I show that the presence of the excess of resorcinol has no appreciable effect on the K_D of thiocyanate ion binding to HRP. Thus, resorcinol does not inhibit the binding of thiocyanate. This was confirmed further by determining the K_D of resorcinol in the presence of the excess of thiocyanate (0.1 M) by optical difference spectroscopy (4, 23). Titrations were carried out at room temperature (23 °C) by adding 10–500-μl aliquots of resorcinol (250 mM) to the enzyme (10 μM) solution (1 ml, 0.1 M phosphate buffer, pH 4.0) in a sample cell and by diluting the enzyme by same amount of buffer in reference side. K_D values were calculated from the slope and intercept of the straight line of the double-reciprocal plot (23). The results in Table II show that thiocyanate does not have a significant effect on the K_D of resorcinol binding to HRP at pH 4.0 and 7.0. The value of $K_D = 20 \pm 4$ mM in the presence of thiocyanate compares very well with that of 22 ± 4 mM in the absence of thiocyanate for the binding of

TABLE II
Effect of thiocyanate on the binding of resorcinol to horseradish peroxidase studied by optical difference spectroscopy
− and + show the absence and presence (0.1 M), respectively, of thiocyanate ion.

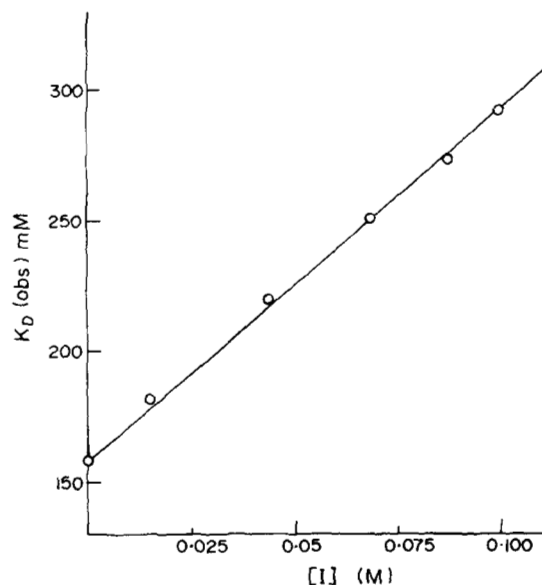| pH	SCN$^-$	K_D
		mM
4.0	−	22 ± 4
	+	20 ± 4
7.0	−	12 ± 2
	+	12 ± 3

FIG. 6. **Variation of K_D as a function of iodide concentration [I].** Solution conditions were 0.1 M phosphate buffer at pH 4.0.

resorcinol to HRP at pH 4.0. It is seen, however, that the K_D of thiocyanate binding increases in the presence of iodide (Table I). Thus, the binding of thiocyanate to HRP in the presence of iodide appears to be competitive. To confirm this, the $K_{D(obs)}$ of thiocyanate binding was evaluated as a function of iodide concentration. Fig. 6 shows that the $K_{D(obs)}$ increases linearly with iodide concentration as expected from Equation 5. The apparent dissociation constant of iodide ion in the absence of thiocyanate (K_i) deduced from the intercept and slope of the plot (Fig. 6) is 115 ± 10 mM, which falls within the range of the values $K_i = 124$ and 99 mM reported earlier (11) from the chemical shift changes of 1- and 8-methyl proton resonances, respectively. This shows that thiocyanate and iodide bind to HRP at the same site, which is located 6.8 Å away from the ferric center, and which is different from the site at which resorcinol binds. Resorcinol has been suggested to bind HRP at tyrosine 185 (5). To confirm that the increase in the apparent dissociation constant of thiocyanate ion binding to HRP in the presence of iodide ion was not an ionic strength effect, K_D was also evaluated in the presence of nitrate ion. Table I shows that the presence of nitrate (0.1 M) has very little effect on K_D for binding of thiocyanate to HRP.

Interaction of SCN$^-$ with HRP Probed by ^1H NMR—Fig. 7A shows proton NMR spectra of HRP at pH 4.0 in the absence and presence (150 and 800 mM) of thiocyanate ion. The spectrum in the absence of the donor is the same as that reported earlier (39). The downfield-shifted 4-ring methyl peaks were assigned to the 5-, 1-, 8-, and 3-ring methyl protons of the heme periphery as indicated in Fig. 7A (39). It is observed that the addition of thiocyanate induces pronounced
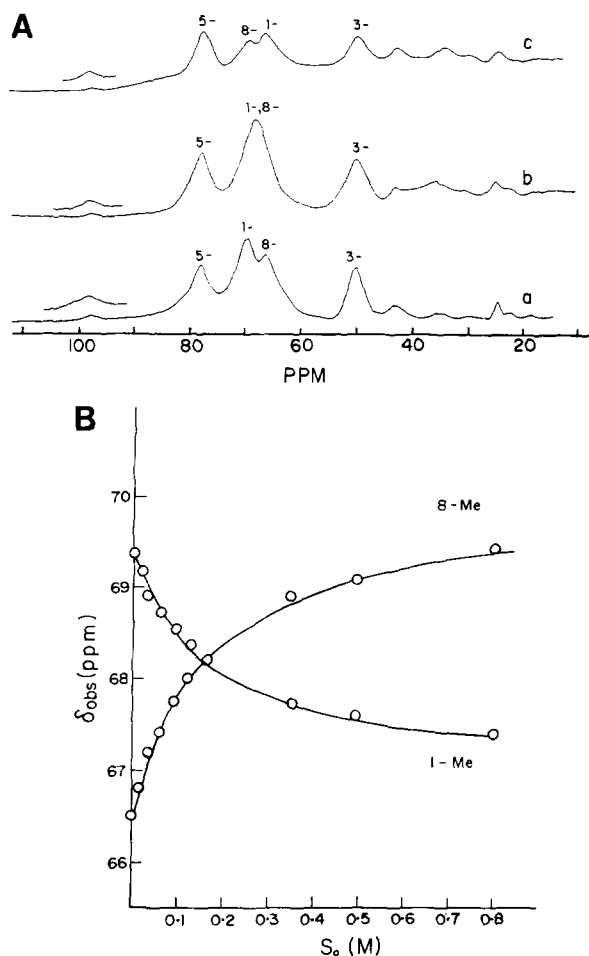
Interaction of Thiocyanate with Horseradish Peroxidase

A

B

FIG. 7. *A*, ^{1}H hyperfine shifted NMR spectra of HRP (2.3 mM, 0.1 M phosphate buffer, pH 4.0 at 23 °C) in the presence of 0 mM (*a*), 150 mM (*b*), and 800 mM (*c*) thiocyanate. The chemical shifts refer to trace HDO as a secondary standard. Only downfield regions are shown in the figure. *B*, variation in the chemical shift of the 1- and 8-ring methyl (*1-Me* and *8-Me*, respectively) proton resonances of the HRP (2.3 mM) prosthetic group as a function of thiocyanate concentration. The *open circles* are the experimental points, and the *solid lines* are calculated from the least squares fit of the data to Equation 4.

changes in both chemical shift and line width of only 1- and 8-ring methyl proton resonances. At 150 mM thiocyanate, the resonances due to 1- and 8-methyl protons merge together, and at a higher concentration (800 mM), they again appear separately. Thiocyanate appears to induce an upfield shift for 1-methyl resonance and a downfield shift for the 8-methyl resonance. Fig. 7*B* shows the variation in chemical shifts of the 1- and 8-methyl proton resonances with thiocyanate concentration. The apparent dissociation constants of thiocyanate binding to HRP at pH 4.0 were calculated by least squares fit of the data of Fig. 7*B* to Equation 4 and were found to be 166 and 136 mM, respectively. This is consistent with $K_D = 158 \pm 19$ mM obtained from the T_1 measurements. It is observed (data not shown) that there is no effect on the chemical shifts or line width of ring methyl proton resonances of HRP at pH 7.0 even at a very high concentration of thiocyanate (800 mM), suggesting that thiocyanate does not bind HRP at neutral pH. This is consistent with our earlier observation that the line width of ^{15}N of SC[15]N[-] above pH 6.0 remains unaltered at 2.5 Hz in the absence or presence of HRP (Fig. 3).

La Mar and de Ropp (40) have shown that the ^{1}H resonance

peak observed at about 100 ppm in native HRP arises from the exchangeable N-H proton of the proximal histidine imidazole. Fig. 7*A* shows that the broad signal at 98 ppm is not appreciably affected by the addition of even 800 mM thiocyanate at pH 4.0. This suggests that the binding site of thiocyanate may not be near the proximal histidine.

Gonzalez-Vergara *et al.* (41) have shown that a broad upfield signal at about −50 ppm in HRP is attributable to ring meso protons of the heme, and the upfield position of this signal is suggested to be diagnostic of five-coordinate Fe(III) structure in the native HRP. Fig. 8 shows that the broad meso-H resonance at about −50 ppm in the absence of thiocyanate (at pH 4.0) remains upfield and undergoes further broadening in the presence of a large excess (800 mM) of thiocyanate. This suggests that the binding of thiocyanate does not take place at the ferric site, which remains essentially a five-coordinate ferric heme. This is consistent with our earlier observation that the binding of thiocyanate to HRP is not affected by the presence of cyanide ion. The further broadening of meso signal suggests that the thiocyanate-binding site may be closer to the heme edge, which agrees with the Fe-^{15}N distance of 6.8 Å deduced from ^{15}N-T_1 measurements.

Variation of K_D as a Function of pH—Scheme 1 shows different equilibria involved in a solution of thiocyanate and
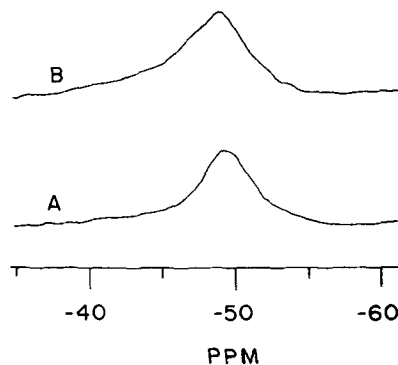

FIG. 8. **Upfield meso-proton NMR spectra of ferric native HRP (2.3 mM, 0.1 M phosphate buffer, pH 4.0 at 23 °C, HDO as secondary reference) in the presence of 0 mM (*A*) and 800 mM (*B*) thiocyanate.**
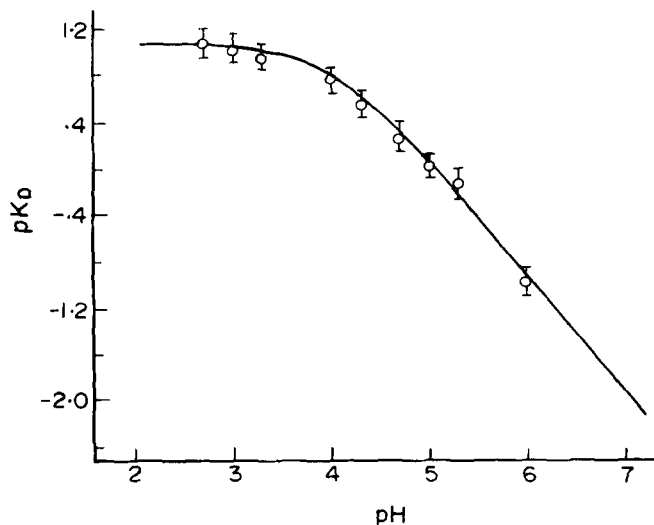

FIG. 9. **pH variation of pK_D for binding of thiocyanate to HRP.** The *open circles* are experimental points, and the *solid line* is a least squares fit to Equation 1. The height of the *vertical bars* represents twice the standard deviation in the calculation of K_D using Equation 3.

HRP at any given pH. K_D is related to K for the interaction between the protonated form of the enzyme and the ionic form of thiocyanate by Equation 1. In Fig. 9, the K_D values of thiocyanate binding to HRP determined by ^{15}N-T_1 measurements at different pH values (using Equation 3) are plotted in the pH range of 3-6. The *solid line* is the least squares fit to Equation 1 with $K = 83$ mM. The good fit of the data to Equation 1 supports our assumption that the ionic form of thiocyanate preferentially binds to the protonated form of the enzyme, as expected from the pH variation of the line width of ^{15}N resonance. $K = 80$ mM obtained from the pH dependence of the line width agrees very well with the $K = 83$ mM from the pH variation of K_D.

DISCUSSION

The ^{15}N and ^{1}H NMR studies presented here clearly demonstrate that thiocyanate binds to HRP under acidic conditions (pH < 6). The pH dependence of the ^{15}N NMR line width and K_D further shows that thiocyanate, predominantly in the ionic form, binds to the protonated form of the enzyme (Scheme 1). This behavior of thiocyanate ion binding contrasts with the binding of other anionic ligands to HRP. Inorganic anions such as CN^-, F^-, and N_3^- are known to bind to the heme proteins such as ferrimyoglobin and ferrihemoglobin at the ferric center in deprotonated anionic form. The distinguishing feature of peroxidase enzymes has been suggested to be that these anions bind at the ferric center predominantly in the protonated form (2, 42). Recent proton NMR studies have shown that the binding of cyanide anion involves a concomitant transfer of proton to distal histidyl imidazole (42). Our results show that the nature of thiocyanate ion binding to HRP is different compared with that of the above anions binding to the enzyme. It is well known (43) that CN^-, F^-, and N_3^- anions inhibit the peroxidative activity of HRP. Thiocyanate ion is, however, an oxidizable substrate (2, 6). From the ^{15}N relaxation measurements presented here, it is seen that the binding site of SCN^- is 6.8 Å away from the ferric center. Since no significant change in the proximal histidine N-H exchangeable proton resonance is observed with the addition of thiocyanate, we suggest that thiocyanate ion does not bind to HRP at the proximal site because binding at the proximal histidine would have led to a substantial change in the unpaired electron spin density distribution on the heme, causing a drastic change in the chemical shift of all methyl peaks (11, 44). Therefore, the binding site of thiocyanate to HRP is at a distal site of heme. It is to be noted that thiocyanate ion binds to the ferric center in ferrimyoglobin (45), but it binds to HRP away from the ferric center. This points to the differences in the heme crevice structures between the ferrimyoglobin and HRP.

It is also observed that the binding of thiocyanate to HRP with $K_D = 158 \pm 19$ mM is comparable to that of iodide binding, with K_D lying between 99 and 124 mM (11). However, it is considerably weaker compared with cyanide and fluoride ions binding to HRP with $K_D = 2.9 \times 10^{-6}$ M and 1.3×10^{-2} M, respectively (46). The binding of thiocyanate is found to be facilitated by protonation of an acid residue on HRP with $pK_a = 4.0$. Kinetic study of cyanide and fluoride binding to HRP has identified the presence of ionizable acid residues on the enzyme with $pK_a = 4.1$, 6.4, and 10.8 (46). Recent proton NMR and fluorometric studies (10, 11) on iodide ion binding to HRP have also suggested that binding of iodide ion is facilitated by protonation of an acid residue with $pK_a = 4.0$. This residue has been attributed to the propionic acid group of the heme moiety (11, 44). The same value of pK_a obtained in the case of iodide and thiocyanate ions binding to HRP

suggests that their modes of binding may be similar.

The measurements of the apparent dissociation constant of thiocyanate binding in the presence of iodide (see Fig. 6) show that iodide and thiocyanate ions compete for binding at the same site. Thus, the present results support the suggestion of Ugarova *et al.* (10) that the protonation of propionic acid residue helps to break the salt bridge, giving easy access for the entry of the anion near heme. The distance of 6.8 Å suggests that the binding site is located close to the heme edge. Aromatic donors such as *p*-cresol and resorcinol have been suggested to bind HRP by hydrogen bonding and hydrophobic interaction with tyrosine 185 (5). Since it is observed (Table I) that the binding of resorcinol does not affect the binding of thiocyanate to HRP, the binding site near tyrosine 185 for thiocyanate may be ruled out. In this respect, the chemical shift change of the 1- and 8-methyl group appears significant. Thus, the binding of these anions close to both 1- and 8-methyl substituents of the heme may bring about conformation change in the vicinity of 1- and 8-methyl moieties and cause the chemical shift changes. Since 1- and 8-methyl groups are about 5.9 Å away from the center of the porphyrin moiety (47), the binding site of thiocyanate and iodide ions may be placed symmetrically with respect to 1- and 8-methyl moieties and at the edge of the heme group. Considerations of Van der Waals contact radii of methyl group proton, thiocyanate, and iodide ions suggest that these ions are placed at about 4 Å or more from both the methyl groups.

It may be mentioned here that both lactoperoxidase and horseradish peroxidase bind to iodide and thiocyanate ions. The apparent dissociation constants for lactoperoxidase are 38 mM (17) and 90 mM (16), respectively, and for HRP, 100 mM (11) and 158 mM (Table I), respectively. From the weaker binding of these anions to HRP as compared with lactoperoxidase, it seems likely that the structure of lactoperoxidase may be more favorable than HRP for the binding of these anions.

Although much has been said about two-electron transfer processes in enzymatic oxidation reactions of peroxidase (48, 49), the mechanism is not yet fully understood. It is found to depend on the pH of the solution (50). The oxidation of iodide ion catalyzed by HRP is found to proceed by the two-electron transfer pathway under acidic conditions and is influenced by ionization with $pK_a = 4.6$ (9, 12). Since protonation of the ionizable group with $pK_a = 4.0$ enhances the binding of iodide (11) and thiocyanate ion (present results) and the binding of substrate is a prerequisite for oxidation, it seems likely that the ionizable group in the binding of iodide and thiocyanate ions to HRP is identical to the ionizable group essential for oxidation. From these considerations, it is suggested that under acidic conditions, the oxidation of thiocyanate ion by $H_2O_2 \cdot$HRP may also proceed via the two-electron transfer pathway, as is the case for iodide.

REFERENCES

1. Saunders, B. C. (1973) *Inorganic Biochemistry* (Eichhorn, G. L., ed) Vol. 2, pp. 988–1021, Elsevier Science Publishing Co., Amsterdam
2. Dunford, H. B., and Stillman, J. S. (1976) *Coord. Chem. Rev.* **19**, 187–251
3. Burns, P. S., Williams, R. J. P., and Wright, P. E. (1975) *J. Chem. Soc. Chem. Commun.* 795–796

4. Schejter, A., Lanir, A., and Epstein, N. (1976) *Arch. Biochem. Biophys.* **174**, 36–44
5. Sakurada, J., Takahashi, S., and Hosoya, T. (1986) *J. Biol. Chem.* **261**, 9657–9662
6. Dunford, H. B. (1982) *Adv. Inorg. Biochem.* **4**, 41–68
7. Morrison, M., and Schonbaum, G. R. (1976) *Annu. Rev. Biochem.* **45**, 861–888
8. Nunez, J., and Pommier, J. (1982) *Vitam. Horm.* **39**, 175–229
9. Björkstén, F. (1970) *Biochim. Biophys. Acta* **212**, 396–406
10. Ugarova, N. N., Savitski, A. P., and Berezin, I. V. (1981) *Biochim. Biophys. Acta* **662**, 210–219
11. Sakurada, J., Takahashi, S., and Hosoya, T. (1987) *J. Biol. Chem.* **262**, 4007–4010
12. Roman, R., and Dunford, H. B. (1972) *Biochemistry* **11**, 2076–2082
13. Reiter, B., Pickering, A., Oram, J. D., and Pope, G. S. (1963) *J. Gen. Microbiol.* **33**, xii
14. Reiter, B., Marshall, V. M., Bjorck, L., and Rosen, C. G. (1976) *Infect. Immun.* **13**, 800–807
15. Magnusson, R. P., Taurog, A., and Dorris, M. (1984) *J. Biol. Chem.* **259**, 13783–13790
16. Modi, S., Behere, D. V., and Mitra, S. (1989) *Biochemistry* **28**, 4689–4694
17. Sakurada, J., Takahashi, S., Shimizu, T., Hatano, M., Nakamura, S., and Hosoya, T. (1987) *Biochemistry* **26**, 6478–6483
18. Lukat, G. S., and Goff, H. M. (1986) *J. Biol. Chem.* **261**, 16528–16534
19. Shannon, L. M., Kay, E., and Lew, J. Y. (1966) *J. Biol. Chem.* **241**, 2166–2172
20. Aibara, S., Yamashita, H., Mori, E., Kato, M., and Mortia, Y. (1982) *J. Biochem. (Tokyo)* **92**, 531–539
21. Sono, M., Dawson, J. H., Hall, K., and Hager, L. P. (1986) *Biochemistry* **25**, 347–356
22. Prabhananda, B. S., Rittger, E., and Grell, E. (1987) *Biophys. Chem.* **26**, 217–224
23. Modi, S., Behere, D. V., and Mitra, S. (1989) *Biochim. Biophys. Acta* **996**, 214–225
24. Willard, J. M., Davis, J. J., and Wood, G. H. (1969) *Biochemistry* **8**, 3137–3144
25. Sass, M., and Ziessow, D. (1977) *J. Magn. Reson.* **25**, 263–276
26. Slejko, F. L., Drago, R. S., and Brown, D. G. (1972) *J. Am. Chem. Soc.* **94**, 9210–9216
27. Aviram, I. (1981) *Arch. Biochem. Biophys.* **212**, 483–490
28. Wood, W. B., Wilson, D., Benbow, R. M., and Hood, L. E. (1981) *Biochemistry: A Problems Approach*, pp. 144–150, Benjamin/Cummings Publishing Co., Menlo Park, CA
29. Dwek, R. A. (1973) *NMR in Biochemistry*, pp. 11–142, Oxford University Press, London
30. Dwek, R. A., Williams, R. J. P., and Xavier, A. V. (1974) in *Metal Ions in Biological Systems* (Sigel, H., ed.) Vol. 4, pp. 62–204, Marcel Dekker, New York
31. Jarori, G. K., Ray, B. D., and Rao, B. D. N. (1985) *Biochemistry* **24**, 3487–3494
32. Wuthrich, K. (1970) *Structure and Bonding* **8**, 53–121
33. Wuthrich, K. (1976) in *NMR in Biological Research: Peptides and Proteins*, p. 259, North-Holland Publishing Co., Amsterdam
34. Solomon, I. (1955) *Physiol. Rev.* **99**, 559–565
35. Bloembergen, N. (1957) *J. Chem. Phys.* **27**, 572–573
36. Burton, D. R., Forsen, S., Karlstrom, G., and Dwek, R. A. (1979) *Prog. NMR Spectroscopy* **13**, 1–45
37. Williams, R. J. P., Wright, P. E., Mazza, G., and Ricard, J. R. (1975) *Biochim. Biophys. Acta* **412**, 127–147
38. Shimizu, T., and Hatano, M. (1985) *Inorg. Chem.* **24**, 2003–2009
39. La Mar, G. N., de Ropp, J. S., Smith, K. M., and Langry, K. C. (1980) *J. Biol. Chem.* **255**, 6646–6652
40. La Mar, G. N., and de Ropp, J. S. (1979) *Biochem. Biophys. Res. Commun.* **90**, 36–41
41. Gonzalez-Vergara, E., Meyer, M., and Goff, H. M. (1985) *Biochemistry* **24**, 6561–6567
42. Thanabal, V., de Ropp, J. S., and La Mar, G. N. (1988) *J. Am. Chem. Soc.* **110**, 3027–3035
43. Keilin, D., and Mann, T. (1937) *Proc. R. Soc. Lond. B. Biol. Sci.* **122**, 119–123
44. Morishima, I., and Ogawa, S. (1979) *J. Biol. Chem.* **254**, 2814–2820
45. Morishima, I., Ogawa, S., Inubushi, T., Yonezawa, T., and Iizuka, T. (1977) *Biochemistry* **16**, 5109–5115
46. Ellis, W. D., and Dunford, H. B. (1968) *Biochemistry* **7**, 2054–2062
47. Hoard, J. L. (1973) *Ann. N. Y. Acad. Sci.* **206**, 18–31
48. Yamazaki, I. (1971) *Adv. Biophys.* **2**, 33–76
49. Yamazaki, I. (1977) in *Free Radicals in Biology* (Pryor, W. A., ed) Vol. 3, pp. 183–218, Academic Press, New York
50. Ohtaki, S., Nakagawa, H., Nakamura, M., and Yamazaki, I. (1982) *J. Biol. Chem.* **257**, 13398–13403