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Binding of Thiocyanate to Lactoperoxidase: ^1H and ^{15}N Nuclear Magnetic Resonance Studies

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ABSTRACT: The binding of thiocyanate to lactoperoxidase (LPO) has been investigated by ^1H and ^{15}N NMR spectroscopy. ^1H NMR of LPO shows that the major broad heme methyl proton resonance at about 61 ppm is shifted upfield by addition of the thiocyanate, indicating binding of the thiocyanate to the enzyme. The pH dependence of line width of ^{15}N resonance of $SC^{15}N^-$ in the presence of the enzyme has revealed that the binding of the thiocyanate to the enzyme is facilitated by protonation of an ionizable group (with pK_a of 6.4), which is presumably distal histidine. Dissociation constants (K_D) of $SC^{15}N^-$/LPO, $SC^{15}N^-$/LPO/I^-, and $SC^{15}N^-$/LPO/CN^- equilibria have been determined by ^{15}N T_1 measurements and found to be 90 ± 5, 173 ± 20, and 83 ± 6 mM, respectively. On the basis of these values of K_D, it is suggested that the iodide ion inhibits the binding of the thiocyanate but cyanide ion does not. The thiocyanate is shown to bind at the same site of LPO as iodide does, but the binding is considerably weaker and is away from the ferric ion. The distance of ^{15}N of the bound thiocyanate ion from the iron is determined to be 7.2 ± 0.2 Å from the ^{15}N T_1 measurements.

Lactoperoxidase (LPO, EC 1.11.1.7, donor:H_2O_2 oxidoreductase) is a heme protein enzyme found in milk, saliva, and tears. In common with other peroxidases, the enzyme catalyzes oxidation of a number of organic and inorganic substrates by hydrogen peroxide and is therefore a component of the biological defense mechanism of mammalians. Of many inorganic substrates, thiocyanate is very attractive because thiocyanate ion/H_2O_2/LPO provides a potent nonspecific bacteriostatic or bacteriocidal system (Reiter et al., 1963, 1976). This system operates in vivo to protect the gut of the calf from enteric pathogens (Reiter et al., 1980; Marshall et al., 1986) and has been used to preserve raw milk without refrigeration (Bjorck et al., 1979). However, the mechanism of the action and oxidation of thiocyanate ion is not yet well understood, although several studies have been reported to elucidate the nature of the active agent(s) in the SCN^-/H_2O_2/LPO system. Chung and Wood (1970) have suggested that the antibacterial action of the system may be due to the cyanide ion produced as one of the oxidation products. Hog and Jago (1970) have, however, proposed from their polarographic studies that cyanosulfurous acid or cyanosulfuric acid may account for its antimicrobial activity. Aune and Thomas (1977) have suggested that $OSCN^-$ may be the relatively stable antimicrobial species that accumulates during peroxidase-catalyzed oxidation of SCN^-. This proposal has been supported by studies of other workers (Hoogendoorn et al., 1977; Marshall & Reiter, 1980). Recently, Magnusson et al. (1984) have studied catalytic activity of LPO using iodide and thiocyanate ions. They have proposed that the oxidation of iodide and thiocyanate with hydrogen peroxide catalyzed by lactoperoxidase and thyroid peroxidase occurs via a two-electron transfer, in contrast to one-electron transfer for more usual aromatic donor molecules, and thus species such as IO^- and $OSCN^-$ may be produced. The mechanism of two-electron transfer is, however, still obscure. To elucidate the mechanism, studies on the interaction of thiocyanate ion and LPO are needed.

In the present study, the interaction of thiocyanate ion with LPO was investigated by use of ^{15}N NMR and ^1H NMR. From the measurements of relaxation times of $(SC^{15}N)^-$ in the presence and absence of LPO, the dissociation constant

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(K_D) was evaluated and the distance of the ^{15}N of SCN^- from the ferric ion was estimated. The line-width measurements on ^{15}N resonance were utilized to deduce autocorrelation time (τ_c). The pH dependence of the line width gave a pK_a value of the amino acid residue in the heme crevice, where the thiocyanate is shown to be binding. The dissociation constant was also estimated by chemical shift changes in the methyl resonance of LPO with varying concentrations of thiocyanate ion. The results have been compared with those on the interaction of iodide ion with LPO, recently reported by Sakurada et al. (1987), and used to examine the two-electron-transfer mechanism.

Materials and Methods

Lactoperoxidase was isolated from fresh raw unskimmed cow's milk by a procedure similar to that described by Goff et al. (1985). After ion-exchange chromatography on a CM52 column, the LPO fractions were pooled and dialyzed against 5 mM phosphate buffer (pH 6.8). After the dialyzed sample was centrifuged, it was concentrated on an Amicon ultrafiltration cell on PM30 and applied to a Sephadex G-100 column. Fractions with RZ (A_{412}/A_{280}) = 0.85–0.91 were concentrated and lyophilized. Concentration of the enzyme was determined spectrophotometrically by using a molar extinction coefficient of 1.12×10^5 cm^{-1} M^{-1} at 412 nm for lactoperoxidase (Carlstrom, 1969). Deuterium oxide (>99.85%) was purchased from Aldrich. Enriched ^{15}N-labeled sodium thiocyanate ($NaSC^{15}N$, atomic % 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 Bruker WM 500-MHz FT NMR spectrometer at 23 °C. The samples were lyophilized directly inside 5-mm NMR tubes with an excess of D_2O, and final solution was in 0.1 M phosphate buffer at pH 6.1 (volume = 0.4 mL). The NMR spectra were obtained by accumulation of ca. 40 000 transients at 8K data points in quadrature mode. Proton chemical shifts were referred to the proton signal of trace HDO. Quoted pHs are meter readings, uncorrected for isotope effects.

The ^{15}N NMR measurements were made on a Bruker FT NMR spectrometer at 50.68 MHz in a 10-mm NMR tube with D_2O for frequency lock. The spectra were obtained by accumulation of 400–1000 transients at 16K data points. For relaxation time measurements lactoperoxidase was treated with Chelex 100 (Bio-Rad) to remove any traces of free metal ions (Willard et al., 1969). Deionized double-distilled water was used to prepare 0.1 M phosphate buffer (pH 6.1). Filtrates were lyophilized and redissolved in D_2O for NMR studies. Titrations were carried out in the enzyme concentration range 75 μM–8 mM and that of substrate in the range 50–600 mM. To obtain the longitudinal relaxation time (T_{1obs}), inversion recovery method with 180°–τ–90° pulse was used. The T_{1obs} was calculated from

$$M_Z = M_0 [1 - \rho \exp(-\tau/T_{1obs})] \quad (1)$$

by using a three-parameter nonlinear least-squares-fit method, where τ is the interval between 180° and 90° pulses, M_Z is the z component of the magnetization (represented by the intensity of the peak), M_0 is the z component of magnetization when the interval is infinite, and ρ is a parameter that becomes 2.0 at an exact 180° pulse. Line-width data were obtained from the spectra by fitting the thiocyanate ^{15}N resonance to Lorentzian line shape using a Bruker program.

Theory of Paramagnetic Relaxation. The longitudinal (T_{1m}) and transverse (T_{2m}) relaxation times of the bound substrate resonances can be represented by Solomon–Bloembergen equations (Solomon, 1955; Bloembergen, 1957)

$$\frac{1}{T_{1m}} = \frac{2\gamma_I^2 g^2 S(S+1)\beta^2}{15r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right] \quad (2)$$

$$\frac{1}{T_{2m}} = \frac{\gamma_I^2 g^2 S(S+1)\beta^2}{15r^6}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_S^2\tau_c^2}\right] \quad (3)$$

where γ_I is the nuclear gyromagnetic ratio, g is the isotropic splitting factor, β is the Bohr magneton, S is the total spin of the ground state of the paramagnetic ion, r is the distance between the metal and the bound substrate (nucleus under observation), and ω_I and ω_S are the nuclear and electronic Larmor precession frequencies, respectively. In eq 2 and 3 we have included only those terms that arise out of dipole–dipole interaction between electron spin S and nuclear spin I characterized by a correlation time τ_c that modulates this interaction. The scalar interaction terms are omitted because this interaction is expected to be negligible in our case (Schejter et al., 1976; Sakurada et al., 1986; also see Discussion).

T_{1obs}^{-1} can be considered a sum of the relaxation rate of the bound substrate fraction and that of the fraction in the bulk of the solution, and it is related to K_D, T_{1b}, and T_{1f} according to

$$\frac{nE_0}{S_0}\left[\frac{1}{T_{1obs}} - \frac{1}{T_{1f}}\right]^{-1} = \frac{K_D}{S_0^n}\left[\frac{1}{T_{1b}} - \frac{1}{T_{1f}}\right]^{-1} + \left[\frac{1}{T_{1b}} - \frac{1}{T_{1f}}\right]^{-1} \quad (4)$$

Here K_D is the dissociation constant of the LPO/substrate complex. Since HSCN is a moderately strong acid with a protolytic dissociation constant of 0.125 (Prabhananda et al., 1987), the substrate thiocyanate will therefore predominantly be in dissociated ionic form as SCN^- in the pH range 5–9. T_{1b} is the T_1 of the LPO/substrate complex, and T_{1f} is the T_1 of the free substrate (SCN^-). E_0 and S_0 are the initial concentrations of enzyme and substrate, respectively. n is the number of substrate molecules associating per molecule of the enzyme. For $n = 1$ eq 4 reduces to eq 5, which is the same as one described by Sakurada et al. (1986).

$$E_0\left[\frac{1}{T_{1obs}} - \frac{1}{T_{1f}}\right]^{-1} = K_D\left[\frac{1}{T_{1b}} - \frac{1}{T_{1f}}\right]^{-1} + S_0\left[\frac{1}{T_{1b}} - \frac{1}{T_{1f}}\right]^{-1} \quad (5)$$

Involvement of one ligand ($n = 1$) was confirmed by the straight line obtained from the plot of $E_0[(1/T_{1obs}) - (1/T_{1f})]^{-1}$ vs S_0 (see later). The K_D values can be obtained by linear least-squares fit of the data to the above equation for SCN^- binding to LPO (Table I).

T_{1b} consists of a paramagnetic component T_{1m} and a diamagnetic component T_{1d}. Since the longitudinal electronic relaxation time of low-spin ferric ion is very short ($T_{1e} = 2 \times 10^{-12}$ s; Wüthrich, 1970, 1976), T_{1b} in a solution of LPO/cyanide may be considered as being very close to the diamagnetic contribution of the LPO to the relaxation of the bound substrate. This contribution is expected to be negligibly small as compared to T_{1m}, and therefore T_{1b} of LPO-bound SCN^- obtained from eq 5 may be regarded as T_{1m}. Equation 2 can be used to estimate the distance of the nitrogen of the

Table I: Effect of Iodide, Nitrate, and Cyanide Ions on the Binding of Thiocyanate Ion to LPO[a]

I^-	CN^-	NO_3^-	K_D (mM)	method used for determination of K_D
−	−	−	85 ± 5	^{15}N T_{2m} measurements
−	−	−	87 ± 9	^1H chemical shift variation
−	−	−	90 ± 5	^{15}N T_{1m} measurements
+	−	−	173 ± 20	^{15}N T_{1m} measurements
−	−	+	105 ± 13	^{15}N T_{1m} measurements
−	+	−	83 ± 6	^{15}N T_{1m} measurements

[a] + shows presence (100 mM) of a particular ion. − shows absence of a particular ion.

bound SCN^- from the metal ion in LPO, provided the value of the autocorrelation time τ_c is known. For Fe^{3+} ($S = {}^5/_2$) the metal–N distance in SCN^-/LPO is given by

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

The value of τ_c can be estimated from the ratio T_{2m}/T_{1m} (Dwek et al., 1974)

$$F(\tau_c) = \frac{T_{2m}}{T_{1m}} = \frac{6 + 14K}{(7 + 4\omega_I^2\tau_c^2 + 13K)} \quad (7)$$

where

$$K = \frac{1 + \omega_I^2\tau_c^2}{1 + \omega_S^2\tau_c^2}$$

T_{2m} has been evaluated by calculating the line width of SCN^- resonance, assuming it to be Lorentzian at all enzyme and substrate concentrations (Schejter et al., 1976). Assuming the chemical shift difference of the ^{15}N NMR resonance between bound and free SCN^- to be negligible, the T_{2b} (hence T_{2m}) was estimated by using an equation similar to eq 5.

K_D from Chemical Shifts of LPO Using ^1H NMR. The K_D value was calculated by using (Slejko et al., 1972)

$$K_D = \frac{[\delta_{LPO/SCN} - \delta_{obs}]\left[S_0 - \frac{(\delta_{obs} - \delta_{LPO})E_0}{(\delta_{LPO/SCN} - \delta_{LPO})}\right]}{[\delta_{obs} - \delta_{LPO}]} \quad (8)$$

where E_0 and S_0 are the initial concentrations of the enzyme and SCN^-, respectively. δ_{obs} is the observed chemical shift of LPO at a particular concentration of the enzyme and SCN^-, δ_{LPO} is the chemical shift of free LPO, and $\delta_{LPO/SCN}$ is the chemical shift of LPO with bound thiocyanate. The concentrations of the substrate and the enzyme can be varied and K_D evaluated by fitting the data to a nonlinear fitting program (Sakurada et al., 1987).

Results

Interaction of SCN^- with LPO Probed by ^{15}N NMR. Parts A and B of Figure 1 show ^{15}N NMR spectra of $SC^{15}N^-$ in the absence and presence of LPO, respectively. The sharp ^{15}N signal is broadened from 2.4 to 13.4 Hz by addition of LPO. Figure 2 shows that the line width of the ^{15}N signal linearly increases in this range with sequential addition of LPO.

The effect of pH on the line width of SCN^-/LPO solution was studied in the pH range 5.0–9.0. Below pH 5.0 the enzyme showed a tendency to aggregate, as observed by Sakurada et al. (1987) for the LPO/I^- system. As shown in Figure 3 the deprotonation of an ionizing group (with $pK_a = 6.4 \pm 0.1$) reduced the line width from 9 Hz (at pH 5.0) to a constant value of 3 Hz for pH >9. No pH effect on line width was observed in the absence of the enzyme (Figure 3). The results of Figure 3 suggest that below pH 8 the binding of the thiocyanate is very specific and increases with decrease in the pH. Since the optimum pH for the thiocyanate ion binding is between pH 6 and 7, the relaxation times and ^1H NMR measurements were carried out at pH 6.1.

The temperature dependence of the line width of the SCN^-/LPO system and of free SCN^- (in the absence of LPO)

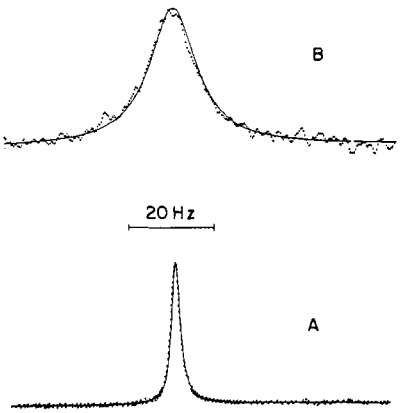

FIGURE 1: ^{15}N NMR spectrum of thiocyanate ion (17.4 mM) in 0.1 M phosphate (pH 6.1) (A) refers to free thiocyanate and (B) to the thiocyanate in the presence of LPO (49.4 μM). The dotted lines are NMR resonance traces, while the solid lines are the fitted ones for Lorentzian line-shape function. Sweep width of 1 kHz over 16K data points was used to ensure an instrumental resolution of 0.12 Hz.

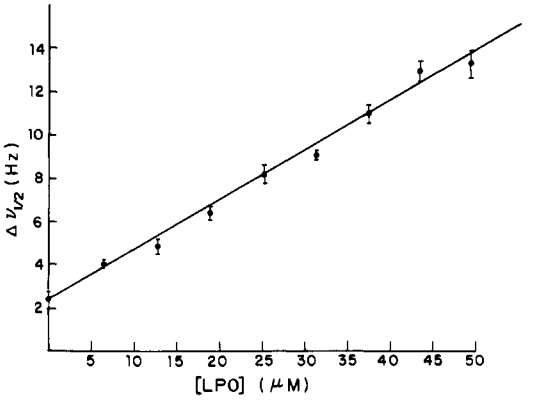

FIGURE 2: Variation of ^{15}N line width of thiocyanate ion (17.4 mM) as a function of LPO concentration (5–50 μM). Height of the vertical bars denotes twice the standard deviation of the Lorentzian line-shape fit.

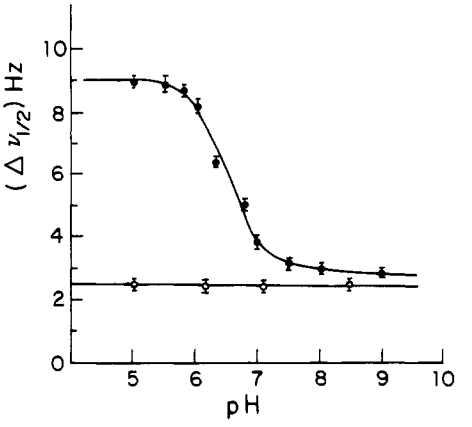

FIGURE 3: ^{15}N NMR line width of thiocyanate ion as a function of pH in the range pH 5–9 in 0.1 M phosphate buffer. Solid circles represent variation of line width of thiocyanate (17.4 mM) in the presence of LPO (22 μM), while the open circles represent that in the absence of LPO. Height of the vertical bars denotes twice the standard deviation of the Lorentzian line-shape fit.

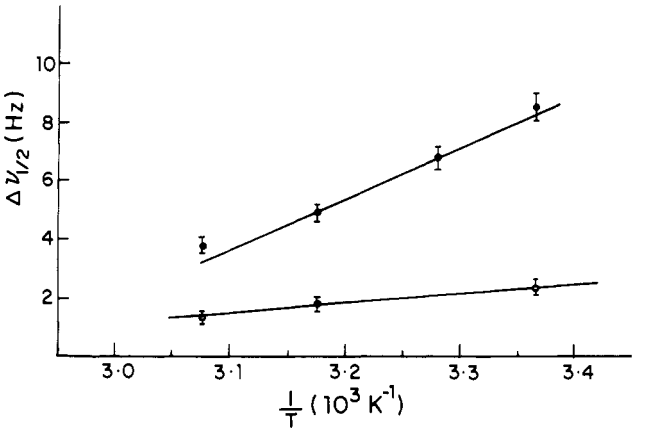

FIGURE 4: Variation of line width of ^{15}N resonance line of thiocyanate ion (17.4 mM, 0.1 M phosphate buffer, pH 6.1) as a function of temperature in the range 25–52 °C. Solid circles represent line width in the presence of and open circles in the absence of LPO (25.5 μM). Height of vertical bars denotes twice the standard deviation of the Lorentzian line-shape fit.

is shown in Figure 4. The SCN^-/LPO line width increases with decrease in temperature. The line width of the ^{15}N resonance of free thiocyanate, which is dominated by chemical shift anisotropy relaxation mechanism (Mason, 1981), shows a very slight increase as temperature is decreased (Figure 4).

Interaction of SCN^- with LPO Probed by ^1H NMR. Proton NMR of LPO in D_2O (Figure 5A) shows a prominent broad peak around 61 ppm that is very similar to that reported previously (Morishima & Ogawa, 1982; Goff et al., 1985; Shiro & Morishima, 1986; Sakurada et al., 1987). The poorly resolved peak has not yet been assigned with certainty due to difficulty in heme reconstitution. However, from comparison with ^1H NMR of HRP or Mb the peak has been tentatively assigned to unresolved heme peripheral methyl proton resonances. On titration with thiocyanate ion at pH 6.1 the broad peak experiences further broadening as well as upfield shift (Figure 5A), suggesting that binding of the thiocyanate ion to LPO is in the vicinity of the ring methyl protons. Figure 5B shows a plot of the variation in the chemical shift of the methyl proton resonance with substrate concentration. The solid line in the figure is a theoretical fit of the data to eq 8, which gives K_D = 87 ± 9 mM.

^{15}N T_1 Measurements. Spin–lattice relaxation times (T_1) at pH 6.1 and 50.68 MHz for $SC^{15}N^-$ were recorded as a function of temperature between 6 and 50 °C (data not shown). The relaxation rate (T_{1b}^{-1}) decreases with the increase in temperature, suggesting fast chemical exchange in this temperature range (Dwek et al., 1974). Figure 6A shows a typical inversion recovery (180°–τ–90°) NMR spectra of $SC^{15}N^-$ (300 mM) in LPO (6.77 mM) at 23 °C. Figure 6B shows a plot of $E_0[(1/T_{1obs}) - (1/T_{1f})]^{-1}$ vs S_0, which is a straight line (see Materials and Methods). The values K_D = 90 ± 5 mM and T_{1b}^{-1} = 95.2 s^{-1} were obtained by least-squares fit to eq 5 (with n = 1). Similarly, ^{15}N T_1 measurements were carried out in the presence of CN^- ion. Cyanide binds to ferric ion of the heme iron of LPO at the sixth position, giving low-spin species (Behere et al., 1985; Shiro & Morishima, 1986). The T_{1b}^{-1} calculated from eq 5 (data not shown) was found to be T_{1b}^{-1} = 8.5 × 10^{-2} s^{-1}. This can be considered to be the diamagnetic contribution (T_{1d}^{-1}) to the T_{1b}^{-1} of the thiocyanate ^{15}N (Schejter et al., 1976; Sakurada et al., 1986). This contribution is expectedly very small as compared to T_{1b}^{-1} of $SC^{15}N^-$ binding to native LPO and hence neglected. The K_D of $SC^{15}N^-$ binding to LPO/CN was estimated to be K_D = 83 ± 6 mM, which compares well with that of 90 ± 5 mM

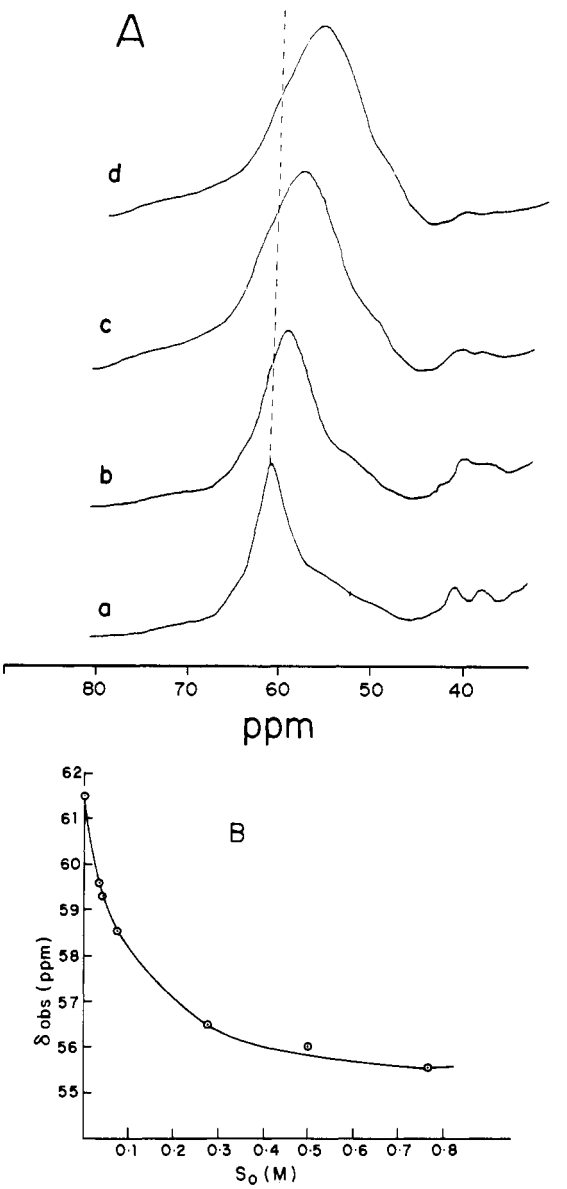

FIGURE 5: (A) ^1H hyperfine shifted NMR spectra of LPO (2 mM, 0.1 M phosphate buffer, pH 6.1) in the presence of (a) 0, (b) 38, (c) 76.9, and (d) 770 mM thiocyanate. The spectra were recorded at 23 °C and referred to the trace of HDO. (B) Variation of hyperfine shift of LPO (2 mM) as a function of substrate concentration. Open circles are experimental data, and the solid line is a theoretical fit to eq 8.

estimated for $SC^{15}N^-$ binding to native LPO. This suggests that the binding of CN^- to the ferric ion at the sixth position does not inhibit the binding of thiocyanate to LPO and that the binding site of SCN^- is away from the ferric center.

Binding of $SC^{15}N^-$ to LPO was also studied in the presence of iodide and nitrate ions by using ^{15}N T_1 measurements, and K_D values were calculated. The data are listed in Table I. The results show a marked increase in K_D of thiocyanate binding to LPO in the presence of external iodide. Iodide is known to bind LPO with K_D = 38 mM (Sakurada et al., 1987). Thus, iodide effectively inhibits the binding of thiocyanate ion, suggesting that the binding site of thiocyanate to LPO may be close to that of the iodide. To confirm that the inhibition of the thiocyanate binding by iodide was not due to ionic strength effect, the binding of $SC^{15}N^-$ was studied in the presence of the same concentration of nitrate. The K_D of $SC^{15}N^-$ in the presence of NO_3^- was found to compare very well with that in its absence (Table I).

The line width of the ^{15}N resonance of SCN^- in the presence

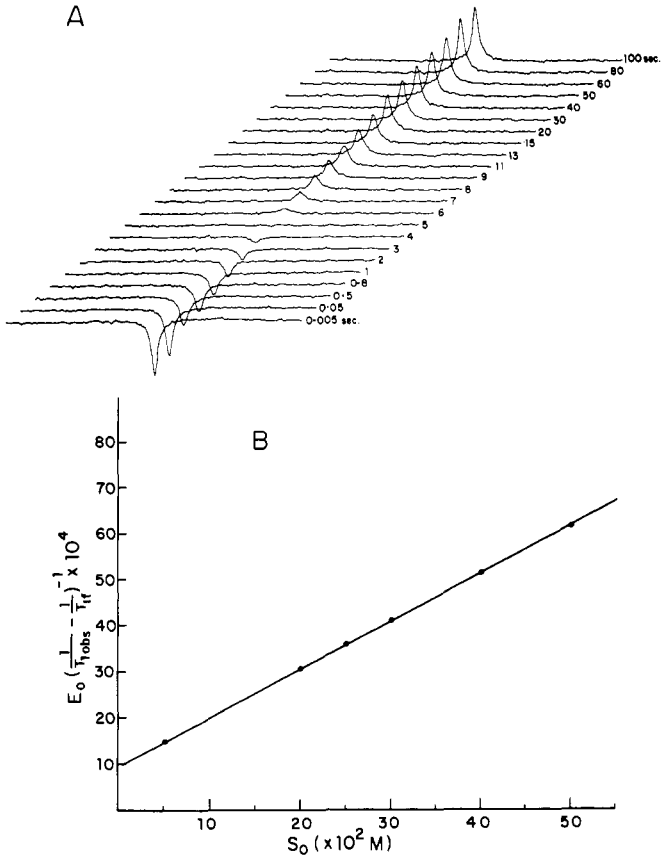

FIGURE 6: (A) 180°–τ–90° pulse ^{15}N NMR spectra (at 50.68 MHz) of thiocyanate ion (300 mM) in the presence of 6.77 mM LPO in 0.1 M phosphate buffer (pH 6.1). τ values were varied from 0.5 ms to 100 s, which are shown in the figure. (B) Plot of $E_0[(1/T_{1obs}) - (1/T_{1f})]^{-1}$ vs S_0, where S_0 was varied from 50 to 500 mM. Observation of straight line confirmed the binding of one thiocyanate ion.

of varying amounts of LPO and thiocyanate was calculated by fitting the resonance to a Lorentzian line shape using a Bruker program. The chemical shift difference between bound and free thiocyanate was neglected, and T_{2m} was calculated in a manner similar to T_{1m} and found to be (7.1 ± 0.2) × 10^{-3} s. The procedure gave K_D = 85 ± 5 mM, which agrees well with that estimated from T_{1m} measurements (Table I).

By use of the above values of T_{1m} and T_{2m}, the ratio (T_{2m}/T_{1m}) was calculated to be 0.67 ± 0.03. This was then utilized to estimate τ_c (Dwek et al., 1974). Figure 7 shows a theoretical plot of $F(\tau_c) = T_{2m}/T_{1m}$ as a function of $X = 1/(1 + \omega_I^2\tau_c^2)$ assuming $\omega_S \gg \omega_I$. The value of X was varied between 0.01 and 1.0. For a value of $F(\tau_c)$ = 0.67 ± 0.03, the value of X was found to be 0.67 ± 0.04 (see Figure 7). This gives a value of τ_c = (2.2 ± 0.2) × 10^{-9} s. The value differs from that estimated by Shiro and Morishima (1986; τ_c = 5.5 × 10^{-10} s) from frequency dependence of the water proton relaxivity. There is, however, no general agreement on the value of τ_c, and several values have been used [see Sakurada et al. (1986)]. Fortunately, these differing values of τ_c do not greatly affect the calculated distance between the iron and the substrates. When τ_c = 2.2 × 10^{-9} s was used, the distance of the ^{15}N of the thiocyanate from the ferric ion was estimated to be 7.2 ± 0.2 Å. When the value of τ_c of Shiro and Morishima (1986) is used, this distance is calculated to be 6.1 ± 0.2 Å.

Discussion

Although UV–visible spectroscopy of LPO in the presence of even large excess of thiocyanate ion (>1000-fold) shows

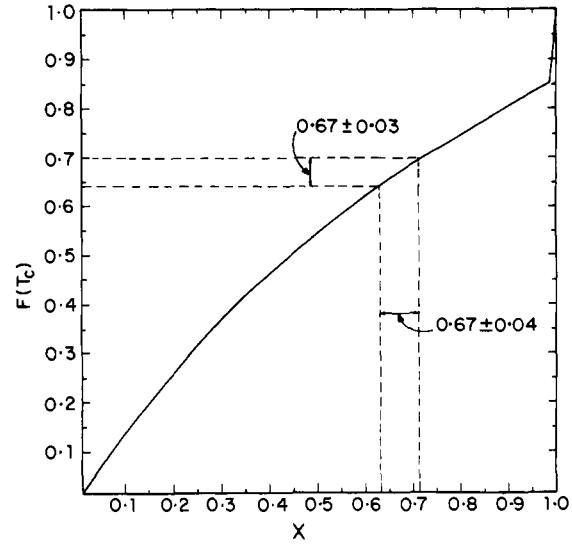

FIGURE 7: Theoretical plot of $F(\tau_c) = (T_{2m}/T_{1m})$ vs $X = 1/(1 + \omega_I^2\tau_c^2)$; X was varied from 0.01 to 1.0. The plot assumes $\omega_S \gg \omega_I$. The calculated ratio of $F(\tau_c)$ = 0.67 ± 0.03 gives X = 0.67 ± 0.04.

no change except for a very small change in the Soret region, ^{15}N NMR resonance line width of thiocyanate in the presence of LPO clearly indicates the binding of thiocyanate to LPO (Figure 1). The line width of the ^{15}N resonance correlates linearly with LPO with no apparent chemical shift (Figure 2). This is consistent with a weak binding and fast chemical exchange between the free and bound thiocyanate. The binding is, however, very specific as shown by the pH dependence of the line width (Figure 3). The interaction of the SCN^- is strengthened by protonation of amino acid residue in the enzyme. The observed pK_a of 6.4 is very close to that of the histidyl imidazole group (pK_a = 6.1). The iodide ion binding studies of Sakurada et al. (1987) have suggested the existence of a histidyl imidazole group in the distal site of the heme crevice which is responsible for the specific binding by the iodide ion.

The positive slope in the temperature variation of the line width (Figure 4) suggests that the condition $\omega_I^2\tau_c^2 \ll 1$ or $\omega_S^2\tau_c^2 \gg 1$ is valid here. Hence, the temperature dependence of the line width is dominated by the temperature dependence of the correlation time τ_c. In a multiply bonded small linear molecule such as SCN^- ion the correlation time may be expected to be dominated by chemical shift anisotropy, which might be the dominant mechanism of relaxation at high magnetic fields (Mason, 1981). However, when such ions bind to a large biomolecule such as LPO, the τ_c is expected to be dominated by chemical exchange (Shimizu & Hatano, 1982, 1985). $SC^{15}N^-$/LPO solution in the present case is in the fast exchange region, which is consistent with the low binding ability of the thiocyanate to LPO (K_D = 90 mM). As the temperature increases, the exchange becomes faster, reducing consequently the line width. The temperature variation of the spin–lattice relaxation time is also indicative of fast exchange.

The specific binding of the thiocyanate was further studied by ^{1}H NMR of LPO in the presence of thiocyanate. Figure 5 shows that addition of the thiocyanate broadens the methyl resonances and induces an upfield shift of 5.9 ppm. The K_D = 87 ± 9 mM obtained by least-squares fit of the observed chemical shift to eq 8 is consistent with K_D = 90 mM obtained from the T_1 measurements. The increase in the methyl resonance line width by addition of the thiocyanate is difficult to estimate quantitatively, but the observation does suggest that the binding site of the thiocyanate to LPO is in the vicinity of the porphyrin periphery. The binding of the thiocyanate

to LPO is expected to be dominated by hydrogen-bonding interaction as shown by the increase in the line width on protonation of the amino acid residue (with $pK_a = 6.4$; Figure 3). Direct contact of the thiocyanate with the porphyrin periphery by hydrophobic interaction is therefore ruled out. Such a direct contact may also be unfavorable due to rigid heme crevice structure (Sievers, 1979, 1981; Sakurada et al., 1987). The distance of 7.2 Å of the ^{15}N atom of thiocyanate from the ferric center is much larger compared to the ferric–(β-pyrrole) carbon distance of 4.3 Å observed in many porphyrin model compounds (Hoard, 1973). This also suggests that the thiocyanate ion does not occupy a site that is in direct contact with the ring peripheral group, but it is sufficiently close to affect the chemical shift of the peripheral group. This was further confirmed from the determination of the dissociation constant of thiocyanate binding through ^{15}N T_1 measurements in the presence of CN^- ion. The K_D value of 83 ± 6 mM in the presence of cyanide is very close to the value of 90 ± 5 mM obtained in the absence of cyanide, showing that the occupation of the sixth position by the cyanide does not inhibit binding of the thiocyanate.

Magnusson et al. (1984) have recently shown that the thiocyanate ion is much weaker than the iodide ion in degrading H_2O_2 catalyzed by LPO. This suggests that either the binding sites of the iodide and thiocyanate ions are different or their binding strengths are different. Since the K_D of SCN^- in the presence of the iodide is considerably higher than that in its absence (see Table I), the iodide obviously inhibits the binding of the thiocyanate. Also, the pH dependence of the line width of both $SC^{15}N^-$ and iodide indicates that protonation of the same amino acid residue with $pK_a = 6.4$ increases the binding. The chemical shift of the LPO ring methyl group is appreciably affected by the presence of both SCN^- and I^-. These observations together with those presented earlier suggest that the thiocyanate ion occupies the same site as the iodide ion. The position of the thiocyanate calculated in this study is also consistent with this deduction.

The oxidation of thiocyanate, which is believed to occur through two-electron transport, is probably mediated by the histidyl imidazole in the distal site in the same manner as suggested by Sakurada et al. (1987). The thiocyanate ion thus occupies the same site as iodide at a distance of ca. 7.2 Å away from the ferric ion but in the vicinity of the porphyrin periphery. The weaker binding of the thiocyanate to LPO therefore may account for the lower rate of degradation of H_2O_2 in its presence.

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Registry No. LPO, 9003-99-0; SCN^-, 302-04-5; I^-, 20461-54-5; CN^-, 57-12-5; NO_3^-, 14797-55-8.

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