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^{13}C Nuclear magnetic resonance studies of binding of thiocyanate to lactoperoxidase and horseradish peroxidase heme enzymes

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Interaction of thiocyanate with lactoperoxidase (LPO) and horseradish peroxidase (HRP) has been investigated by relaxation rate measurements (at 125.77 MHz) of ^{13}C resonance of thiocyanate carbon. The apparent dissociation constant (K_D) for thiocyanate binding to LPO at pH = 6.1 and to HRP at pH = 4.0 has been deduced to be 85 mM and 160 mM respectively from the relaxation rate measurements. The pH dependence of K_D and ^{13}C resonance line-width of thiocyanate has been used to calculate pK_a value of amino acid residue on these enzymes where the thiocyanate is shown to be binding. From the pH dependence of K_D and ^{13}C resonance line-width, it is observed that thiocyanate binds to LPO and HRP only under acidic conditions (pH < 6.1 for LPO and pH < 4.0 for HRP). The binding is facilitated by protonation of an acid group on the enzyme with pK_a = 6.1 for LPO and 4.0 for HRP. The pH dependence of ^{13}C resonance line-width of thiocyanate as well as K_D have been quantitatively analysed on the basis of a reaction scheme in which thiocyanate in deprotonated ionic form binds to the enzyme in protonated acidic form. K_D for thiocyanate binding to the enzyme has also been evaluated in the presence of excess of exogenous substrates such as resorcinol, cyanide and iodide. The presence of cyanide (which binds to heme iron of enzyme at sixth coordination position) does not have any effect on the binding of thiocyanate, indicating that binding site of thiocyanate ion is located away from the ferric centre of these enzymes. The presence of resorcinol, has significant effect on K_D for binding of thiocyanate to LPO but it has no effect on thiocyanate binding to HRP. The K_D in the presence of iodide however shows that iodide competes with thiocyanate for binding at the same site in both the cases. Distance of the carbon atom of bound thiocyanate ion from ferric centre has been deduced from the ^{13}C-T_1 measurements and is found to be 8.4 Å and 8.0 Å for LPO and HRP respectively. This distance remains unchanged by the presence of cyanide ion at the sixth coordination site of the heme iron of these enzymes. Similarity in the mode of binding of iodide and thiocyanate suggests that the oxidation of thiocyanate ion by H_2O_2, like that of I^- by H_2O_2 may also proceed via two-electron transfer pathway under acidic conditions.

Lactoperoxidase (LPO, EC 1.11.1.7, donor-H_2O_2 oxidoreductase) and horseradish peroxidase (HRP, EC 1.11.1.7, donor-H_2O_2 oxidoreductase) are heme protein enzymes which catalyse primarily the oxidation of a wide variety of organic and inorganic substrates by hydrogen peroxide[1,2]. The native enzyme contains a single prosthetic group with high-spin ferric heme iron[3-6]. In HRP the prosthetic group is a protoheme IX while in LPO it is an iron porphyrin thiol[7-10]. The mechanism of oxidation of organic and inorganic substrates by hydrogen peroxide involves initial binding of the donor to the enzyme[1]. Several studies have therefore been reported on the binding of oxidizable organic and inorganic substrates to the native enzymes[11-20]. Among the oxidizable inorganic substrates, the interaction of iodide ion with LPO[19] and HRP[20] has been studied to understand the mechanism of thyroid hormone biosynthesis catalysed by thyroid peroxidase and lactoperoxidase. Beside iodide, thiocyanate is also an attractive inorganic substrate because the thiocyanate ion/H_2O_2/LPO provides a potent non-specific bacteriostatic or bacteriocidal system[21-24]. It has been suggested that the oxidation of thiocyanate with hydrogen peroxide catalysed by LPO or HRP occurs via two-electron transfer in contrast to one-electron transfer for usual aromatic donor molecules[25]. The studies on the interaction of thiocyanate with these enzymes are important in understanding the mechanism of the two-electron transfer process.

The interaction of SCN^- with LPO and HRP has been shown to produce only a slight change in the electronic absorption spectrum of these enzymes at low pH[26,27]. Recently we have studied interaction of thiocyanate with LPO and HRP using ^{15}N NMR of SCN^- and heme peripheral ^1H

NMR[28,29]. The studies have shown that the thiocyanate ion binds to LPO at a distal site of heme at histidyl residue at a distance of 7.2 Å from the iron centre with apparent dissociation constant, $K_D = 90 \pm 5$ mM at pH 6.1[28]. For HRP, the exact binding site of thiocyanate has not been identified but it is shown to be located at a distance of 6.8 Å from the iron centre, and placed between 1- and 8-methyl groups of the heme[29]. However, in these studies the ionic state of the enzyme and the substrate upon binding has not been determined. Since in these studies only ^{15}N NMR of thiocyanate was utilised, orientation of the thiocyanate with respect to the heme iron could not be decided. Further, distinction between specific binding of thiocyanate to the enzymes, and random diffusion of thiocyanate about the heme iron of these enzymes could not be established.

Carbon-13 NMR study of SCN^- binding to LPO and HRP is likely to provide some answer to these questions, and together with previous ^{15}N NMR study, would uniquely determine the orientation of SCN^- in the heme cavity, and also help to differentiate between the specific binding and random diffusion models. Further, ^{13}C NMR study is expected to provide an independent determination of the dissociation constant and structural parameters, and a check on the reliability of these parameters. It is also considered useful to carry out studies on the binding of a particular inorganic substrate to the two structurally similar heme enzymes and utilise the data in terms of a comparative study of their heme crevice structure and binding affinites.

These considerations have led us to study the interaction of thiocyanate ion with LPO and HRP by ^{13}C NMR. From the measurements of relaxation times of $(S^{13}CN)^-$ in the presence and absence of LPO and HRP, the K_D values have been evaluated, as well as the distance of the ^{13}C of SCN^- from the heme iron of LPO and HRP has been determined. The pH dependence of ^{13}C linewidth has given pK_a value of the amino acid residue in the heme crevice, where the thiocyanate is shown to be binding. The pH dependence of ^{13}C line-width and K_D was quantitatively analysed on the basis of a reaction scheme in which thiocyanate in deprotonated ionic form binds to the enzyme in protonated acidic form. From the temperature dependence of K_D, thermodynamic parameters have been evaluated. The results of LPO and HRP have been compared among themselves and used to examine the two-electron transfer mechanism.

Materials and Methods

Lactoperoxidase (LPO) was isolated from fresh raw unskimmed cow's milk by a procedure similar to that described earlier[15,24,28,30]. After ion-exchange chromatography on CM52 column, the LPO fractions were pooled and dialysed against 5 mM phosphate buffer (pH = 6.8). The dialysed sample was centrifuged and concentrated on Amicon ultrafiltration cell on PM30 and applied to Sephadex G-100 column. Fractions with Rz ($= A_{412}/A_{280}$) = 0.85-0.91 were concentrated and lyophilised. Concentration of the enzyme was determined spectrophotometrically using molar extinction coefficient of 1.12×10^5 cm^{-1} M^{-1} at 412 nm for LPO[31]. Horseradish peroxidase (HRP) was purified from crude HRP (Sigma, $Rz = A_{403}/A_{280} = 0.8$) by DEAE and CM-cellulose column chromatography[7]. The HRP isoenzymes B and C were collected (Rz = 3.2) after elution with acetate buffer (100 mM, pH = 4.4). Concentration of HRP was determined spectrophotometrically using molar extinction coefficient of 1.02×10^5 cm^{-1} M^{-1} at 403 nm for HRP[7,29]. Deuterium oxide (> 99.85%) was purchased from Aldrich. Enriched ^{13}C sodium thiocyanate ($NaS^{13}CN$, atomic % of $^{13}C > 90$) was purchased from MSD Isotopes. All other reagents were of analytical grade.

Optical difference spectroscopy for binding of resorcinol to enzyme

Optical difference spectra (enzyme-substrate versus enzyme) were obtained using a Varian Cary 17D spectrophotometer with quartz cells (10 mm pathlength). Titrations were carried out at room temperature (23°C) by adding aliquots (10-500 μl) of the substrates (200-250 mM) to enzyme (10 μM) solution (1 ml, 0.1 M phosphate buffer, pH = 6.1) in sample cell and by diluting the enzyme solution by same amount of buffer in reference side. Dissociation constants were calculated using expression[12,15] (1),

$$\frac{1}{\Delta A} = \left[\frac{K_D}{\Delta A_\infty}\right]\frac{1}{S_0} + \frac{1}{\Delta A_\infty} \qquad \ldots (1)$$

where ΔA and ΔA_∞ are the changes in absorbance at the observation wavelength at given and saturating substrate concentrations respectively; S_0 is the initial substrate concentration; K_D is the dissociation constant of the enzyme-substrate complex. K_D and ΔA_∞ can be evaluated from the slope and intercept of $1/\Delta A$ versus $1/S_0$ plot.

NMR measurements

The ^{13}C NMR measurements were made on a Bruker FT NMR spectrometer at 125.77 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. Sweep-width was adjusted to give approximately 0.12 Hz/pt digital resolution. Measurements at different temperatures were carried out using an automated temperature controller (accuracy ± 0.5°C). To remove any traces of free metal ions enzymes were treated with Chelex-100 (BioRad)[32] and deionized doubly distilled water was used to prepare 0.1 *M* phosphate buffer (*p*H = 6.1). Filtrates were lyophilized and redissolved in D_2O. Quoted *p*H's are meter readings, uncorrected for isotope effects. Titrations were carried out in the enzyme concentration range of 100 μM to 5 m*M* and that of substrate in the range of 40 to 500 m*M*. The longitudinal relaxation time ($T_{1\,obs}$) was measured by an inversion-recovery sequence (180°-τ-90°) on Bruker WM 500 operating at carbon-13 frequency of 125.77 MHz. Relaxation delays of at least $5T_1$ values were used for the inversion recovery method. Typically, 20 to 30 tau values (τ) were used ranging from $0.001 \times T_1$ to $10 \times T_1$. The T_{1obs} was calculated from non-linear least square fit method[5,15,28,29,33] using Eq. (2):

$$M_z = M_0[1 - \rho \exp.(-\tau/T_{1\,obs.})] \quad \ldots (2)$$

where τ is the interval between 180° and 90° pulse, M_z is the z-component of the magnetization (represented by the intensity of the peak) and ρ is a parameter which becomes 2.0 at exact 180° pulse[33].

Theory of paramagnetic relaxation

The longitudinal (T_{1m}) and transverse (T_{2m}) paramagnetic relaxation times of bound substrate resonances can be represented by Eqs (3) and (4) respectively[34,35]:

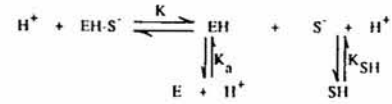
$$\frac{1}{T_{1m}} = \frac{2\gamma_I^2 g^2 S(S+1)\beta^2}{15\,r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right] \quad \ldots (3)$$

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

where γ_I, g, β and S have their usual meanings; r is the distance of the metal atom from the substrate (observed nucleus), ω_I and ω_S are the nuclear and electronic Larmor precession frequencies respectively. In Eqs (3) and (4) we have only included terms arising out of dipole-dipole interaction between electron spin (S) and nuclear spin (I) which is characterized by a correlation time τ_c that modulates this interaction. Since the substrate is not bound to the Fe(III) here, only the dipolar terms have been considered and the scalar interaction terms have been neglected[9,12,14,15,28,29].

Line width measurements

The line-width data were obtained from the spectra by fitting ^{13}C resonances of the substrate to Lorentzian line shape. 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 $pK_{SH} = -1.9$[36] and would be mostly in the ionic form in the *p*H range of 3.0 to 9.0. Scheme 1 shows the binding of thiocyanate to the enzyme (HRP and LPO)[9,29].



Scheme 1

where K is a dissociation constant for binding of deprotonated thiocyanate ($S^{13}CN^-$) to protonated enzyme form (EH); and K_a and K_{SH} are protolytic dissociation constants of enzyme and substrate respectively. The *p*H dependence of K_D can be explained by Scheme 1 and it is related to K by Eq. (5)[9,29]:

$$K_D = K(1 + K_a/H^+)(1 + H^+/K_{SH}) \quad \ldots (5)$$

Variation of the observed line-width as a function of *p*H is given by Eq. (6)[9,29]:

$$\Delta\nu_{1/2}(\text{obs}) = \frac{E_0(\Delta\nu_{1/2}^B - \Delta\nu_{1/2}^F)}{S_0 + K(1 + K_a/H^+)(1 + H^+/K_{SH})} + \Delta\nu_{1/2}^F \quad \ldots (6)$$

where $\Delta\nu_{1/2}^B$ and $\Delta\nu_{1/2}^F$ denote the line-widths of ^{13}C resonance of enzyme-bound and unbound substrate respectively; and E_0 and S_0 represent the initial enzyme and substrate concentrations, respctively.

Calculation of K_D and distance using ^{13}C-T_1 measurements

T_{1obs} can be considered as sum of relaxation

rates of the bound substrate fraction and that of fraction in the bulk of solution, and it is related to K_D, T_{1b} and T_{1f} by Eq. (7):

$$\frac{nE_0}{S_0}\left[\frac{1}{T_{1obs.}}-\frac{1}{T_{1f}}\right]^{-1}=\frac{K_D}{S_0^n}\left[\frac{1}{T_{1b}}-\frac{1}{T_{1f}}\right]^{-1}+\left[\frac{1}{T_{1b}}-\frac{1}{T_{1f}}\right]^{-1} \quad \ldots(7)$$

where T_{1b} is the T_1 of the enzyme-substrate complex and T_{1f} is the T_1 of the free substrate. Here n is the number of substrate molecules associating per molecule of the enzyme. For n = 1, Eq. (7) reduces to Eq. (8)[9,15,28,29]:

$$E_0\left[\frac{1}{T_{1obs.}}-\frac{1}{T_{1f}}\right]^{-1}=K_D\left[\frac{1}{T_{1b}}-\frac{1}{T_{1f}}\right]^{-1}+S_0\left[\frac{1}{T_{1b}}-\frac{1}{T_{1f}}\right]^{-1} \quad \ldots(8)$$

K_D and T_{1b} for thiocyanate binding to LPO can be obtained by least square fit of the plot of $[E_0(1/T_{1obs.}-1/T_{1f})^{-1}]$ versus S_0[9,15,28,29]. T_{1b} consists of paramagnetic component T_{1m} and diamagnetic component T_{1d} and is related to T_{1m} via Eq. (9)[15,29]:

$$T_{1b}^{-1}-T_{1d}^{-1}=\frac{1}{T_{1m}+\tau_m} \quad \ldots(9)$$

When the chemical exchange is fast as compared to the relaxation rate ($1/T_{1m}$), then τ_m (the lifetime of the enzyme-substrate complex) in Eq. (9) can be neglected[15,29,37,38]. Since T_{1d}^{-1} has been shown to be negligibly small as compared to T_{1m}^{-1}[19,29,39], T_{1b}^{-1} obtained from Eq. (8) may be regarded as T_{1m}^{-1} (also see Results). The distance (r) between the carbon atom of the bound thiocyanate and heme iron can then be determined from Eq. (3) using $T_{1b}=T_{1m}$, provided the value of correlation time τ_c is known. For $Fe^{+3}(S=5/2)$ this distance (in Å) is given by Eq. (10)[9,28,29]:

$$r(Å)=379\left\{T_{1m}\left\{\frac{3\,\tau_c}{1+\omega_I^2\tau_c^2}+\frac{7\,\tau_c}{1+\omega_S^2\tau_c^2}\right\}\right\}^{1/6} \quad \ldots(10)$$

The value of τ_c estimated from the ratio of T_{2m} to T_{1m}[9,15,28,29,38,40]:

$$F(\tau_c)=\frac{T_{2m}}{T_{1m}}=\frac{6+14\,\kappa}{(7+4\,\omega_I^2\tau_c^2+13\,\kappa)} \quad \ldots(11)$$

where $\kappa=\dfrac{1+\omega_I^2\tau_c^2}{1+\omega_S^2\tau_c^2}$

The ^{13}C transverse relaxation time, T_{2b} of the enzyme bound thiocyanate was evaluated from the line-width of ^{13}C resonances of thiocyanate assuming the lines to be Lorentzian at different enzyme and substrate concentrations and the chemical shift difference between ^{13}C resonances of bound and free substrate to be negligible[12,15,28]. T_{2b} was calculated using an equation similar to Eq. (8); T_{2b} becomes T_{2m} if the diamagnetic contribution is neglected.

Competitive binding of substrates in presence of inhibitor

Donors competing for binding to the native enzyme at the same site as that of thiocyanate affect K_D of the latter. The observed K_D of thiocyanate is related to the competitive inhibitor concentration [I] by expression[9,15,29,41](12):

$$K_D(obs)=K_D+\frac{K_D[I]}{K_i} \quad \ldots(12)$$

where K_D(obs) is the observed apparent dissociation constant of the thiocyanate binding to native enzyme in the presence of inhibitor (iodide, or resorcinol); and K_i is the apparent dissociation constant of inhibitor in the absence of the thiocyanate, and K_D, as defined earlier, is the apparent dissociation constant for binding of thiocyanate to enzyme in the absence of inhibitor. The values of K_i of iodide ion and resorcinol were deduced from intercept and slope of the linear plot of K_D(obs) versus [I] (vide Eq. 12).

Thermodynamic parameters

The change in free energy of substrates upon binding to LPO was obtained Eq. (13):

$$\Delta G=RT\ln K_D \quad \ldots(13)$$

For calculating change in enthalpy (ΔH), K_D at different temperatures was obtained from Eq. (8) by carrying out T_1 measurements ($T_{1obs.}$ and T_{1f}) at different temperatures and ΔH evaluated from the slope of the Arrehenius plot given by Eq. (14):

$$\ln(1/K_D)=-\frac{\Delta H}{R}(1/T)+\text{constant} \quad \ldots(14)$$

Change in entropy (ΔS) was calculated from Eq. (15), assuming ΔC_p constant.

$$\Delta G=\Delta H-T\Delta S \quad \ldots(15)$$

Results

The line-width of sharp ^{13}C resonance of free thiocyanate increases with the increase in the concentration of LPO and HRP. The sharp ^{13}C resonance is broadened from 1.9 Hz to 8.8 Hz and from 1.9 Hz to 7.0 Hz by the addition of 32 μM of LPO and HRP respectively (Fig. 1). Figure 2 shows that the line-width of ^{13}C resonance of thiocyanate increases monotonically by sequential addition of LPO or HRP, suggesting increase in the enzyme-bound substrate fraction. The *p*H-dependence of line-widths of $S^{13}CN^-$/LPO and $S^{13}CN^-$/HRP systems was studied in the *p*H range of 3.0 to 9.0 (Fig. 3)†. As shown in Fig. 3, *p*H has a pronounced effect on line-widths of ^{13}C resonance of thiocyanate in presence of LPO and HRP. Deprotonation of an ionisable group in the case of LPO reduced the line-width from 7.4 Hz (at *p*H = 5.0) to a constant value of 1.9 Hz at *p*H > 8.5. Similarly in the case of HRP deprotonation of ionizing group reduced the line-width from 7.8 Hz (at *p*H = 3.0) to a constant value of 1.9 Hz at *p*H > 7.0. No dependence of line-width on *p*H is observed in the absence of LPO and HRP (Fig. 3). These results suggest that binding of thiocyanate is very specific and increases with decrease in the *p*H. The *p*H dependence of line-width was least square fitted to Eq. (6), which yielded $pK_a = 6.1$ and $K = 45$ mM for LPO, and $pK_a = 4.0$ and $K = 83$ mM for HRP. Thus, protonation of an ionisable group with $pK_a = 6.1$ and 4.0 for LPO and HRP, respectively enhance the binding of thiocyanate to the corresponding enzyme. As the optimum *p*H for thiocyanate binding is around 6.1 (for LPO) and 4.0 (for HRP), the relaxation time measurements were carried out at *p*H = 6.1 and 4.0 for LPO and HRP respectively.

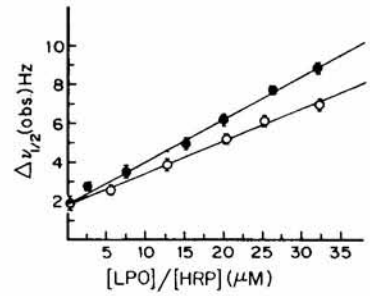


Fig. 2 – Variation of ^{13}C line-width of thiocyanate (21 mM) as a function of HRP (open circle) and LPO (closed circle) concentration. [*p*H of the solution was 4.0 for HRP and 6.0 for LPO. Height of the vertical bars is equal to twice the standard deviation of the Lorentzian line shape fit]

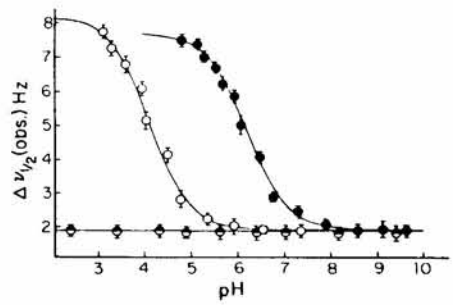


Fig. 3 – ^{13}C-NMR line-width of thiocyanate (21 mM) as a function of *p*H in the range of 3.0 to 9.0 in 0.1 M phosphate buffer, in absence (⊖) and presence of HRP (○) (20 μM) and LPO (●) (20 μM). Height of the vertical bars denotes twice of the standard deviation of the Lorentzian line-shape fit. The solid line is the least square fit to Eq. (6)

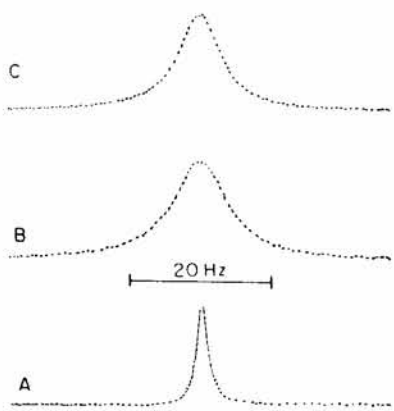


Fig. 1 – ^{13}C-NMR spectrum of thiocyanate (21 mM) in 0.1M phosphate buffer in absence of enzyme (A), presence of LPO (B) (32 μM, *p*H = 6.0), and presence of HRP (C) (32 μM, *p*H = 4.0). [The dotted lines are resonance traces, while the solid lines are fitted once to Lorentzian line shape function. Sweep width of 1 kHz over 16K data points were used to ensure an instrumental resolution of 0.12 Hz]

$T_{1\,obs}$ was calculated from 180°-τ-90° pulse for different enzyme and substrate concentrations at ^{13}C frequency of 125.77 MHz. Figure 4 shows plots of $[E_0(1/T_{1obs} - 1/T_{1f})^{-1}]$ versus S_0 for thiocyanate binding to these enzymes, which are linear. The value of $K_D = 85 \pm 6$ mM and $T_{1b}^{-1} = 250$ s^{-1} for LPO (Fig. 4A), and $K_D = 160 \pm 21$ mM and $T_{1b}^{-1} = 20.4$ s^{-1} for HRP (Fig. 4B) were obtained by least square fit to Eq. (8). These K_D values of thiocyanate binding to LPO and HRP obtained from ^{13}C-T_1 measurements are consistent with those obtained from ^{15}N-T_1 measurements[28,29] (Table 1). Similarly ^{13}C-T_1 measurements were carried out in the presence of cyanide

†No data were taken: (i) below *p*H = 5.0 for LPO, because LPO shows tendency to aggregate below *p*H 5.0[19,28]; and (ii) below *p*H = 3.0 for HRP since below *p*H 3 heme to apoenzyme linkage in HRP breaks[9,29]

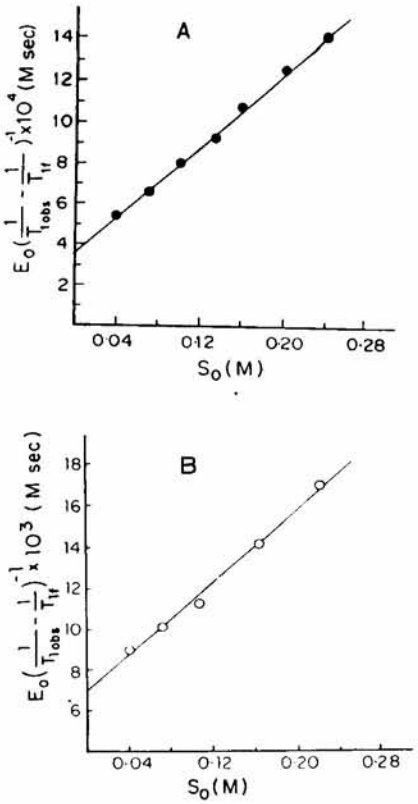


Fig. 4 – Plot of $[E_0(1/T_{1obs} - 1/T_{1f})^{-1}]$ versus S_0 for binding of thiocyanate to LPO (A) and HRP (B). Thiocyanate concentration was varied from 40 m*M* to 240 m*M*. [Linear plot confirmed the binding of one thiocyanate ion to the enzymes]

ion (0.1 *M*). Cyanide ion binds to the heme iron of LPO and HRP at the sixth position to form low-spin species[6,42,43]. $K_D = 87 \pm 4$ m*M* and $T_{1b}^{-1} = 0.91$ s^{-1} for LPO-CN; and $K_D = 154 \pm 16$ m*M* and $T_{1b}^{-1} = 0.023$ s^{-1} for HRP-CN were obtained from the least square fit of the data to Eq. (8). T_{1d}^{-1} values are expected to be even smaller compared to those of T_{1b}^{-1} for the low-spin LPO-CN and HRP-CN, and hence these become negligibly small compared to T_{1b}^{-1} of native LPO and HRP. The K_D of $S^{13}CN^-$ binding to LPO-CN (87 ± 4 m*M*) compares well with that (85 ± 6 m*M*) estimated for $S^{13}CN^-$ binding to native LPO in the absence of cyanide. Similar is the case for HRP (Table 1). 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 HRP, and that binding site of SCN^- is away from the iron centre of LPO and HRP.

K_D and T_{1b} were calculated at different temperatures using Eq. (8) for the thiocyanate binding to LPO and HRP. The activation energy of the process was calculated from Arrhenius plot[15,29,37] of

Table 1 – Effect of cyanide, iodide, nitrate ions and recorcinol on binding of thiocyanate to the enzymes

Enzyme	CN^-	Resorcinol	I^-	NO_3^-	*p*H	K_D (m*M*)	Measurement used for determination of K_D
LPO	–	–	–	–	6.1	85 ± 6	^{13}C-T_{1m}
LPO	–	–	–	–	6.1	91 ± 7	^{13}C-T_{2m}
LPO	+	–	–	–	6.1	87 ± 4	^{13}C-T_{1m}
LPO	–	+	–	–	6.1	200 ± 22	^{13}C-T_{1m}
LPO	–	–	+	–	6.1	326 ± 36	^{13}C-T_{1m}
LPO	–	–	–	+	6.1	83 ± 9	^{13}C-T_{1m}
HRP	–	–	–	–	4.0	160 ± 21	^{13}C-T_{1m}
HRP	–	–	–	–	4.0	170 ± 18	^{13}C-T_{2m}
HRP	+	–	–	–	4.0	154 ± 16	^{13}C-T_{1m}
HRP	–	+	–	–	4.0	176 ± 19	^{13}C-T_{1m}
HRP	–	–	+	–	4.0	298 ± 31	^{13}C-T_{1m}
HRP	–	–	–	+	4.0	170 ± 21	^{13}C-T_{1m}

(–) and (+) signs show absence and presence (0.1 *M*) of particular substrate respectively.

$\log(T_{1b})^{-1}$ versus inverse of temperature. The relaxation rate is observed to decrease with increase in temperature suggesting presence of fast exchange ($T_{1m} \gg \tau_m$)[9,15,29,38,40]. The activation energy of this process calculated from the slope of Arrhenius plot (Fig. 5) was found to be 3.0 kcal/mol for LPO/SCN^- and 2.8 kcal/mol for HRP/SCN^-, which are in range (1-3 kcal/mol) expected for τ_m independent process[29,40]. Existence of fast exchange between free and bound thiocyanate is consistent with weak binding of thiocyanate to the enzyme ($K_D = 88$ m*M* for LPO and 165 m*M* for HRP). A knowledge of ΔH and ΔS gives additional information about the nature of enzyme-substrate interaction. ΔH values for $S^{13}CN^-$/LPO and $S^{13}CN^-$/HRP were obtained from the slope of Arrhenius plots of $\ln(1/K_D)$ versus inverse of temperature (Fig. 5), and were found to be -23 kJ/mol and -20 kJ/mol respectively. The thermodynamic parameters obtained from ^{13}C relaxation rate measurements of thiocyanate in the presence of LPO and HRP are listed in Table 2. Also included in Table 2 are the values of these parameters obtained from our independent ^{15}N NMR measurements of $SC^{15}N^-$/LPO and $SC^{15}N^-$/HRP interactions.

T_{2m} was calculated in a manner similar to T_{1m} and found to be 0.018 for LPO (also see Materials and Methods). The procedure gave $K_D = 91 \pm 7$ m*M* for thiocyanate binding to LPO,

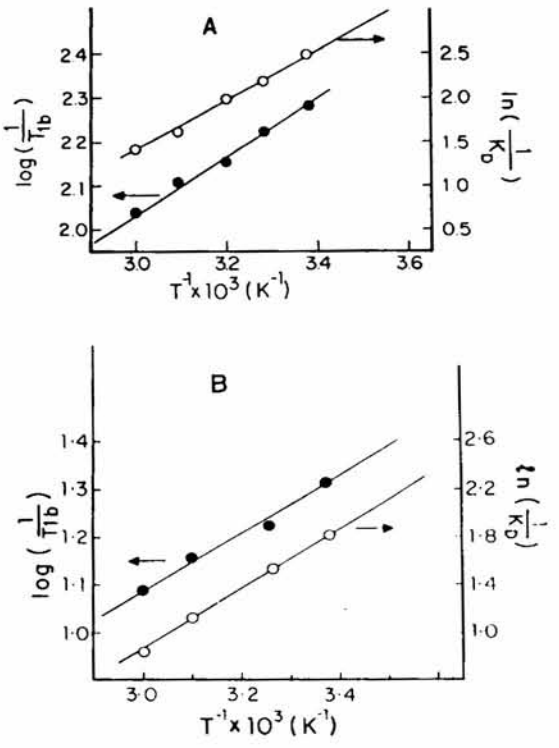


Fig. 5 – Temperature dependence of logarithmic variation of relxation rate ($1/T_{1b}$) of ^{15}N resonance of bound thiocyanate (closed circles) and association constant ($1/K_D$) for binding of thiocyanate (open circles) to LPO (A) and HRP (B) at 50.68 MHz. [Solution conditions were 0.1 *M* phosphate buffer at *p*H = 6.0 for LPO and *p*H = 4.0 for HRP]

which agrees well with that estimated from T_{1m} measurements (Table 1). Using these values of T_{1m} and T_{2m}, the ratio ($T_{2m}/T_{1m} = 0.43$) was utilized to estimate τ_c (see Materials and Methods) and was found to be 1.6×10^{-9} s. The value agrees with the value 2.2×10^{-9} s estimated from ^{15}N-T_{1m} and T_{2m} of bound thiocyanate measurements[28] but differs from $\tau_c = 5.5 \times 10^{-10}$ s estimated from frequency dependence of the water proton relaxivity[43]. For native HRP the value of τ_c (9.8×10^{-11} s) calculated from ratio of T_{2m} to T_{1m} agrees with that reported earlier[12,18] (5×10^{-11} s) (see Table 3). Similarly τ_c values for LPO-CN and HRP-CN were calculated (Table 3). Fortunately these differing τ_c-values do not greatly affect the calculated distance between the iron and the substrates (Table 3). The distances of the carbon atom of thiocyanate from ferric centres of the enzymes are listed in Table 3. The τ_c values deduced from ^{15}N NMR studies are also included in Table 3 for comparison.

The binding of thiocyanate to LPO and HRP was also studied in the presence of exogenous substrates such as resorcinol, iodide and nitrate

Table 2 – Thermodynamic parameters for formation of thiocyanate-enzyme complex at 23°C, deduced from ^{13}C NMR studies. [Values in parenthesis refer to those determined by us from ^{15}N NMR studies[28,29]]

Enzyme	K_D (m*M*)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/mol/K)
LPO	88	−6	−23	−57
	(87)	(−6)	(−26)	(−67)
HRP	165	−4	−20	−53
	(158)	(−4)	(−19)	(−49)

Table 3 – Distance of observed nucleus of thiocyanate from iron centre of LPO and HRP obtained using Eq. (5)

CN^-	Nucleus observed	r(Å)	τ_c(s)
		LPO	
–	^{13}C	8.4 ± 0.2	1.6×10^{-9} a
–	^{13}C	7.0 ± 0.2	5.5×10^{-10} b
–	^{15}N	7.2 ± 0.2	2.2×10^{-9} c
–	^{15}N	6.1 ± 0.1	5.5×10^{-10} b
+	^{13}C	8.2 ± 0.3	5.0×10^{-12} a
+	^{15}N	7.3 ± 0.3	1.5×10^{-12} a
		HRP	
–	^{13}C	8.0 ± 0.3	9.8×10^{-11} a
–	^{13}C	7.2 ± 0.2	5.0×10^{-11} d
–	^{15}N	6.8 ± 0.1	9.5×10^{-11} e
–	^{15}N	6.1 ± 0.1	5.0×10^{-11} d
+	^{13}C	8.0 ± 0.2	7.1×10^{-13} a
+	^{15}N	6.6 ± 0.3	7.5×10^{-13} a

Value of τ_c determined in the present work and earlier by others are included above. a = present work, b = Ref. 43, c = Ref. 28, d = Ref. 12,18 and e = Ref. 29.

by ^{13}C-T_1 measurements. As discussed earlier, presence of cyanide does not have any effect on K_D for binding of thiocyanate to HRP and LPO (Table 1). However, the K_D values increase in the presence of iodide. Thus, the binding of thiocyanate to LPO and HRP in the presence of iodide appears to be competitive for both LPO and HRP. To confirm this, the K_D(obs) of thiocyanate binding to HRP and LPO was evaluated as a function of iodide concentration. Figure 6A shows that K_D(obs) increases linearly with iodide concentration as expected from Eq. (12). The apparent dissociation constant (K_i) of iodide ion binding to the enzymes in the absence of thiocyanate was deduced from the intercept and slope of the plot of Eq. (12) (Fig. 6A), and was found to be 35 ± 3 m*M* for LPO which agrees very well with $K_i = 38 \pm 4$ m*M* reported earlier[19] from the chemical shift changes of ring methyl proton re-

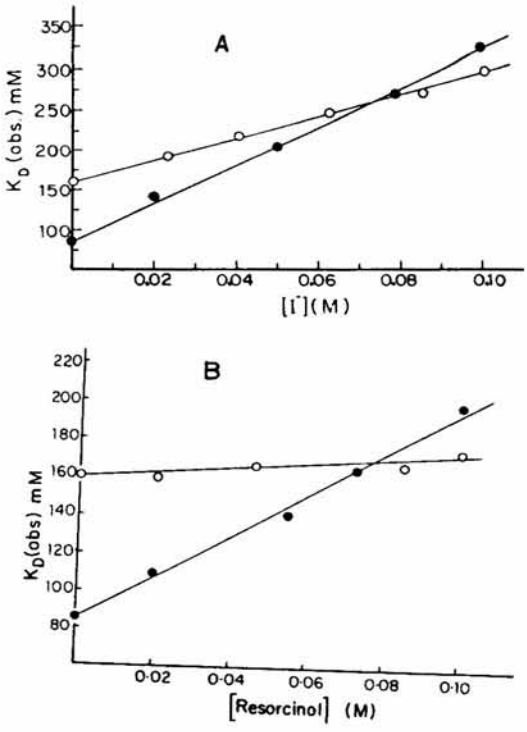


Fig. 6 – Variation of K_D for binding of thiocyanate to LPO (closed circles) and HRP (open circles) as a function of iodide (A), and resorcinol (B) concentrations. [Solution conditions were 0.1 *M* phosphate buffer at *p*H = 6.0 for LPO and *p*H = 4.0 for HRP]

sonances of LPO. Similarly K_i for HRP was calculated to be 123 ± 9 m*M* which also agrees well with the reported[20] value of 112 ± 12 m*M*. This shows that thiocyanate and iodide bind to LPO and HRP at the same site. To confirm that increase in K_D of thiocyanate ion binding to LPO and HRP in the presence of iodide ion was not an ionic strength effect, K_D was also evaluated in the presence of nitrate ion. The presence of nitrate (0.1 *M*) has very little effect on K_D for binding of thiocyanate to LPO ($K_D = 83 \pm 9$ m*M*) and HRP (170 ± 21 m*M*) (Table 1).

The K_D of thiocyanate binding to LPO increases in the presence of an organic donor, such as resorcinol (Table 1). Thus, the binding of thiocyanate to the LPO in the presence of resorcinol appears to be competitive. To confirm this, K_D(obs) of thiocyanate binding to LPO was evaluated by ^{13}C measurements as a function of resorcinol concentration (Fig. 6B), and was found to increase linearly with the concentration of resorcinol, as expected from Eq. (12). The apparent dissociation constant (K_i) of resorcinol binding to LPO in the absence of thiocyanate deduced from the intercept and slope of the plot in Fig. 6B is

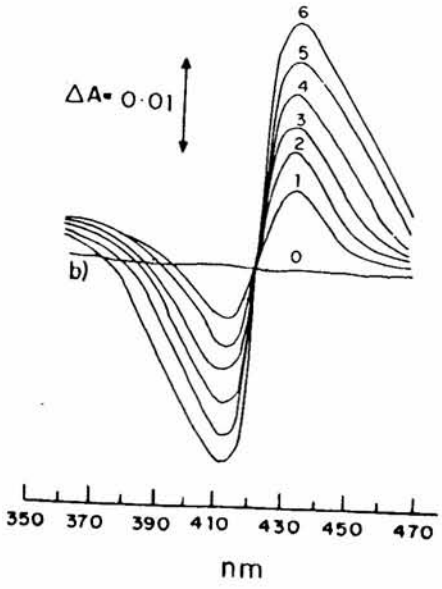


Fig. 7(A) – Optical difference spectra for LPO/resorcinol complex. [Initial condition (0) is 1 ml of LPO (10 μM) in 100 m*M* phosphate buffer (*p*H = 6.0). Titrations were carried out by addition of 75(1), 120(2), 150(3),225(4), 325(5) and 470(6) μl of resorcinol solution (208 m*M*) to the sample cell and equal amount of same buffer to reference cell]

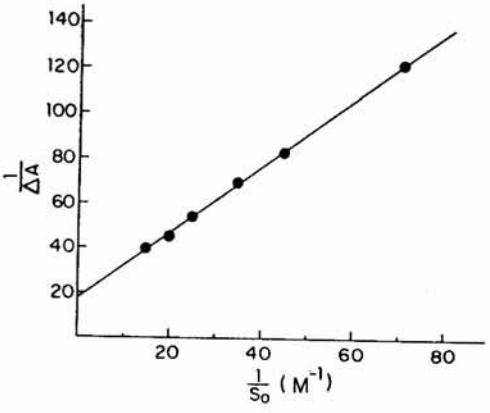


Fig. 7(B) – Variation of change in absorbance (ΔA) at 430 nm with resorcinol concentration (S_0)

80 ± 9 m*M* which agrees well with $K_i = 78 \pm 9$ m*M* reported earlier[15]. The resorcinol does inhibit the binding of thiocyanate for LPO. This was further confirmed by studying binding of resorcinol to LPO in the presence of 0.1 *M* of thiocyanate ion employing optical difference spectroscopy. Figure 7A shows typical optical difference spectra between LPO and LPO/resorcinol in the 350 to 470 nm range at different degrees of saturation. Figure 7B shows the double reciprocal plot of $1/\Delta A$ versus $1/S_0$ for resorcinol. The linear plot shows binding of one mole of donor per mole of LPO ($n = 1$)[15]. Apparent dissociation constants

Table 4 – Effect of thiocyanate on the binding of resorcinol to HRP and LPO studied by optical difference spectroscopy

Enzyme	*p*H	SCN^-	K_D (m*M*)
HRP	4.0	–	22 ± 4
		+	20 ± 4
		–	78 ± 9
LPO	6.0	+	154 ± 20

(–) and (+) signs show absence and presence (0.1 *M*) of thiocyanate ion.

(K_D) were evaluated by least square fit of the data using Eq. (1) for resorcinol binding in the absence and presence of thiocyanate, and are listed in Table 4. The presence of thiocyanate on K_D of resorcinol binding to LPO appears very significant (Table 4), unlike that with HRP. Table 1 shows that K_D for thiocyanate binding determined by ^{13}C-T_1 measurement at *p*H = 4.0 is nearly same in the presence and absence of resorcinol. Thus, binding of thiocyanate to HRP is not competitive with binding of resorcinol to HRP. This was further confirmed by studying binding of resorcinol to HRP in the absence and presence of 0.1 *M* thiocyanate employing optical difference spectroscopy at *p*H = 4.0. The data in Table 4 show that thiocyanate does not have significant effect on the K_D of resorcinol binding to HRP at *p*H = 4.0. The value of $K_D = 20 \pm 4$ m*M* in the presence of thiocyanate compares very well with that of 22 ± 4 m*M* in its absence. These results confirm that in HRP the binding sites of thiocyanate and resorcinol are, unlike LPO, not the same.

Scheme 1 shows different equilibria involved in solution of thiocyanate and the enzyme at any given *p*H. The *p*H dependence of apparent dissociation constant (K_D) can be explained by Eq. (5). In Fig. 8 the K_D's of thiocyanate binding to LPO and HRP determined by ^{13}C-T_1 measurements at different *p*H (using Eq. 8) are plotted against *p*H. The solid line is the least square fit to Eq. (5) with $K = 43$ m*M* for LPO and 85 m*M* for HRP. The good fit of the data to Eq. (5) supports our assumptions that the ionic form of thiocyanate preferentially binds to protonated form of the enzyme, as expected from the *p*H variation of line-width of ^{13}C resonance of thiocyanate in the presence of LPO and HRP. ($K = 45$ m*M* for LPO and $K = 83$ m*M* for HRP, obtained from *p*H dependence of line-width).

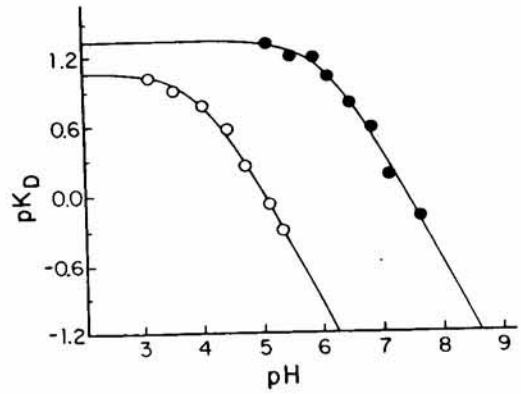


Fig. 8 – *p*H variation of pK_D for binding of thiocyanate to LPO (0.1 *M* phosphate buffer, *p*H = 6.0; closed circles)and HRP (0.1 *M* phosphate buffer, *p*H = 4.0; open circles). [Open and closed circles are experimental points and solid line is a least square fit to Eq. (5).

Discussion

The interaction of thiocyanate with LPO and HRP is optically inoperable because absorption spectra of LPO and HRP at *p*H = 6.1 and 4.0 respectively do not show any change except for small changes in the Soret region, in the presence of even large excess of thiocyanate (> 1000 fold). Linear increase in ^{13}C NMR resonance line-width of thiocyanate with LPO and HRP concentration (Fig. 2) clearly indicates binding of thiocyanate to LPO and HRP. No chemical shift is observed in ^{13}C NMR resonance for thiocyanate in the absence and presence of LPO and HRP (Fig. 1). The binding of thiocyanate to these enzymes is, however, very specific as shown by *p*H dependence of line-width (Fig. 3). Further, random diffusion of thiocyanate, if any, appears negligible as the line-width of ^{13}C resonance of $S^{13}CN^-$/enzyme at high *p*H is exactly the same as that of free thiocyanate, both being 1.9 Hz. The interaction of thiocyanate is strengthened by protonation of an amino acid residue with $pK_a = 6.1$ for LPO. The observed pK_a of 6.1 is very close to that of histidyl imidazole group ($pK = 6.1$)[15,19,28]. The existence of a histidyl imidazole group in the distal site of the heme crevice has been suggested on the basis of binding of iodide ion[19] and aromatic substrates[15] to LPO. The binding of thiocyanate to HRP is found to be facilitated by protonation of an acid residue on HRP with $pK_a = 4.0$ (Fig. 3). Kinetic study of cyanide and fluoride binding to HRP has identified the presence of ionisable acid residues on the enzyme with $pK_a = 4.1$, 6.4 and 10.8[ref. 44]. Recent proton NMR and fluorometric studies[20,45] 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 identified as the propionic acid

group of the heme moiety[17,20,44]. The same value of pK_a obtained in the case of iodide and thiocyanate ions binding to HRP suggests that their mode of binding may be similar. The K_D measurements of thiocyanate binding in the presence of iodide (see Fig. 6) show that iodide and thiocyanate ions compete for the binding at the same site. Thus the present results support the suggestion of Ugarova *et al.*[45] that the protonation of propionic acid residue helps to break the salt bridge giving easy access for the entry of the anion near heme. Aromatic donors such as resorcinol have been suggested to bind HRP via H-bonding and hydrophobic interaction with tyrosine-185[14]. Since it is observed that the binding of resorcinol does not affect the binding of thiocyanate to HRP (Table 1), the binding site near tyrosine-185 for thiocyanate may be ruled out.

The *p*H dependence of K_D for thiocyanate binding to LPO and HRP further shows that thiocyanate, predominantly in the ionic form, binds to the protonated form of the enzymes (Scheme 1, Fig. 8). A distinguishing feature of peroxidase enzymes is that inorganic anions such as CN^-, F^- and N_3^- bind at the ferric centre predominantly in the protonated form[1,46]. Recent proton NMR studies have shown that binding of cyanide anion involves concomitant transfer of proton to distal histidyl imidazole[46]. The nature of thiocyanate ion binding to LPO and HRP is therefore different compared to that of the above anions binding to the ferric centre of the enzymes. It is well known[47] that CN^-, F^- and N_3^- anions inhibit the peroxidative activity of LPO and HRP. Thiocyanate is however an oxidizable substrate[1,24] and binds to the enzymes away from the ferric centre. From ^{13}C relaxation measurements presented here it is seen that binding site of SCN^- in LPO and HRP is at the same distance of ~8.0 Å from the ferric centre. The distance of the C and N atoms of thiocyanate from iron centre of LPO and HRP remains the same in the presence of cyanide (Table 3), which is consistent with our earlier observation[28,29] that cyanide does not inhibit binding of thiocyanate to the enzyme. The distance between carbon atom of bound thiocyanate and iron centre of LPO and HRP is more as compared to that of nitrogen atom of thiocyanate (Table 1), showing that thiocyanate specifically interacts with these enzymes. If the interaction was not specific, but was merely random diffusion about iron, then the average distance of nitrogen and carbon atoms of thiocyanate from the iron centre atom would have been the same.

The addition of thiocyanate broadens the methyl resonances of heme of LPO and induces an upfield shift of 5.9 ppm[28]. This observation suggests that the binding site of thiocyanate to LPO is in the vicinity of the prophyrin 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 of ^{13}C resonance of thiocyanate on protonation of the amino acid residue (with $pK_a = 6.1$, Fig.3). Direct contact of thiocyanate with the porphyrin periphery by hydrophobic interaction is ruled out due to rigid heme crevice structure of LPO[3,4,19]. No significant change in the proximal histidine N-H exchangeable proton resonance (at ~100 ppm) of LPO is observed on the addition of thiocyanate at *p*H = 6:1 (data not shown). This is consistent with the earlier observation that thiocyanate ion does not bind to LPO at proximal site because binding at the proximal histidine would have led to substantial change in the unpaired electron spin density distribution on the heme causing drastic change in the chemical shift of all methyl peaks[17,20]. Therefore, the binding site of thiocyanate to LPO is at distal site of heme. In the case of HRP, addition of thiocyanate causes upfield shift in the 1-methyl heme proton resonance and downfield shift in 8-methyl heme proton resonance of HRP. We also observe that the proximal histidine exchangeable proton resonance of HRP is not affected by the addition of thiocyanate at *p*H = 4.0 (data not shown). These observations clearly show that thiocyanate binds to HRP at a distal site which is close to 1- and 8-methyl protons of the heme prosthetic group and is located at about 7 Å from the heme iron.

Iodide has been shown to bind to LPO at distal histidine with $K_D = 38$ m*M* at *p*H = 6.1[ref. 19]. The K_D of SCN^- binding to LPO increases with iodide concentration (Fig. 6A), the iodide obviously inhibiting binding of thiocyanate. Also, the *p*H dependence of line-width of both $S^{13}CN^-$ as well as iodide indicates that protonation of the same amino acid residue with $pK_a = 6.1$ increses the binding. The chemical shift of LPO ring methyl group resonance is appreciably affected by the presence of both SCN^- and I^-. Recent proton NMR and fluorometric studies[20,45] on iodide ion binding to HRP have also suggested that the binding of iodide ion is facilitated by protonation of an acid residue with $pK_a = 4.0$. The K_D of SCN^- binding to HRP increases with iodide concentration in a way similar to that of SCN^- to LPO (Fig. 6A). These observations suggest that thiocyanate binds to LPO and HRP at the same site as the iodide.

Oxidation of thiocyanate which is believed to

occur via two-electron transport is probably mediated by the histidyl imidazole in the distal site in the case of LPO. The distal histidine may bind with both SCN^- and $Fe^{IV}=O$ of LPO. On the basis of distance of N and C atoms of thiocyanate from the iron centre of LPO it is suggested that N atom of thiocyanate may form H-bond with distal histidine. $Fe^{IV}=O$ of LPO has been shown earlier to associate with NH of the imidazole of distal histidine by hydrogen bonding[48]. Therefore electron transfer from thiocyanate to the heme iron may occur through the route, SCN^-----H^+——N ≡≡C≡≡N——H----O ═ Fe^{IV}. Such a pathway for electron transfer has been suggested in the case of catalysed oxidation of iodide by LPO[19].

Table 2 shows that the derived values of ΔG, ΔH and ΔS are all negative. Negative ΔS and a large negative ΔH are characteristic of these enzymes[13,15,16], suggesting a specific donor-enzyme interaction. The slightly larger values of these parameters in LPO is consistent with the observation that binding of thiocyanate to LPO is strogner than that to HRP. This indicates that structure of LPO may be more favourable for binding of thiocyanate than that of HRP. Similar trend is observed for binding of iodide to HRP ($K_D = 100$ mM at pH = 4.0) and to LPO ($K_D = 38$ mM at pH = 6.1). These results point to the important role of distal histidine in oxidation of iodide and thiocyanate catalysed by lactoperoxidase.

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