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An NMR and circular dichroism study of the interaction of thiocyanate with human and cross-linked hemoglobin: identification of Lys-α-99 as a possible dissociation linked binding site

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Abstract

The interaction of thiocyanate with human native and cross-linked oxyhemoglobin (oxyHb), and methemoglobin (metHb) has been investigated by optical spectroscopy, circular dichroism (CD) and nuclear spin lattice relaxation rate measurements. The interaction of thiocyanate anion with human hemoglobin has been investigated by NMR measurements of the nuclear spin lattice relaxation rate of N^{15} labeled thiocyanate in the presence of cyanomethemoglobin and cross-linked cyanomethemoglobin. Results show that thiocyanate is located approximately 8.9 and 6.2 Å away from the heme group in cyanomethemoglobin and cross-linked cyanomethemoblobin, respectively. These results are consistent with the binding of SCN^- at the lys-α-99 in the unmodified hemoglobin. Since this site is blocked in the cross-linked hemoglobin, the binding site is different. Results show that one mole of SCN^- is binding to one mole of oxyhemoglobin suggesting that binding at the lys-α-99 is linked to dissociation of the hemoglobin tetramer into dimers due to its location at the $\alpha_1\beta_2$ interface. Circular dichroism studies show that the interaction of thiocyanate with oxyHb decreases the optical rotation at 240 nm indicating a conformational change of the protein, which influences the electronic transitions of a number of peptide bonds or (and) a few aromatic side chains.

Keywords: Hemoglobin; Dissociation; Thiocyanate binding site

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1. Introduction

Hemoglobin is the main component of erythrocytes and ensures oxygen transport to all organs and parts of the body. It is known to exist in at least two different molecular conformations, which corresponds to the absence and presence of oxygen as sixth ligand of the heme iron. The conformational change occurring upon ligand binding provides the most reasonable explanation for the co-operative interactions between the hemes groups in hemoglobin molecule [1,2]. The interaction of small molecules with hemoglobin is important in view of understanding the mechanism of regulation of oxygen affinity and co-operativity.

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Hemoglobin consists of two alpha and two beta subunits. The contacts between like subunits are relatively small. The contacts between unlike subunits are of two types, $\alpha_1\beta_1$ and $\alpha_1\beta_2$. The contacts between residues are more extensive in the $\alpha_1\beta_1$ dimer [1]. Thus, when hemoglobin dissociates, it is the $\alpha_1\beta_1$ dimer, which forms:

$$\alpha_2\beta_2 \rightleftharpoons 2\ \alpha\beta$$

In human hemoglobin the binding of anions is known to affect the stability of the tetrameric structure [1,3,4]. The effect of chloride ion on the oxygen affinity of hemoglobin has been shown to be due to preferential binding to, and the resultant stabilization of the deoxy conformation [2]. The effect on subunit assembly is oxygen-linked and can be ascribed to stronger binding of the anion to the liganded derivative [5]. In the oxy-conformation hemoglobin dissociates to a much greater extent than in the deoxy-conformation [1,3]. Thus, at neutral pH, chloride ions favor dissociation of oxyhemoglobin at low concentrations (≥ 0.01 M) but do not appreciably dissociate deoxyhemoglobin even at a concentration of 2 M [3,4].

Many studies have indicated that the effect of anions on the functional properties of hemoglobin is the result of binding of the anions to specific sites. Several studies have been devoted to the identification of the oxygen-linked chloride binding sites. Evidence has accumulated that points to the existence of two such sites: (i) One located at the N-termini of the α chains near the arg-α-141 [6] and (ii) another at the lysine β-82 in the organophosphate binding site [7–10]. However, information on the dissociation-linked binding sites is lacking. In a previous study, we presented evidence indicating that the dissociation-linked and the oxygen-linked binding sites are different [11].

The effect of thiocyanate on the solution properties of hemoglobin has been extensively studied [12]. The effect has often been rationalized from the position of thiocyanate in the Hofmeister series, which is originally based on the effectiveness of salts on the precipitation of serum globulins [12]. In this series, thiocyanate has been classified as an anion that 'salts in' or increases the solubility of hydrophobic proteins. Herskovitz et al. investigated the effect of a series of anions on the dissociation of hemoglobin into dimers and found the effectiveness of the anions varied as in the sequence: $F^- < Cl^- < Br^- < ClO_4^- < SCN^-$, I^- [13]. This series is similar to the Hofmeister lyotropic series.

In this paper, we have sought to determine whether the dissociation of hemoglobin by thiocyanate is due to binding at a specific site or due to non-specific solvent effects by measuring the nuclear spin relaxation rate (T_1) of N^{15} labeled thiocyanate in the presence of cyanomethemoglobin or α,α-cross-linked cyanomethemoglobin and measuring the circular dichroism of oxyhemoglobin in the presence of thiocyanate.

2. Materials and methods

Human hemoglobin was a gift from Prof. Alberto Bertollini and was prepared according to Rossi Fanelli et al. [14]. Human hemoglobin cross-linked between the lys- 99 residues of the alpha chains by treatment of deoxyhemoglobin with bis(3,5-dibromosalicyl)fumarate was provided by the Blood Research Detachment, Walter Reed Army Institute of Research, Washington, DC [15]. Methemoglobin was prepared by oxidation of oxyhemoglobin with sodium nitrite. The reaction was carried out in the dark at 4 °C for approximately 30 min with occasional mixing. The molar ratio of sodium nitrite to oxyhemoglobin was 1.5:1. Excess oxidising agent was removed by dialysis against water. Cyanomethemoglobin and cross-linked cyanomethemoglobin were prepared by adding an excess of KCN to the corresponding methemoglobin. The reaction mixture was kept at 4 °C for 30 min with occasional mixing. Excess KCN was removed by extensive dialysis against water. Potassium thiocyanate, analytical grade was obtained from Glaxo, India. N^{15} labeled thiocyanate was obtained from Isotech, Inc (Miamisburg, Ohio, USA). Potassium cyanide and sodium nitrite were obtained from Glaxo, India.

Spectrophotometric experiments were performed on a Shimadzu UV-2100 spectrophotometer. The CD spectra were recorded on a Jasco-600 spectropolarimeter.

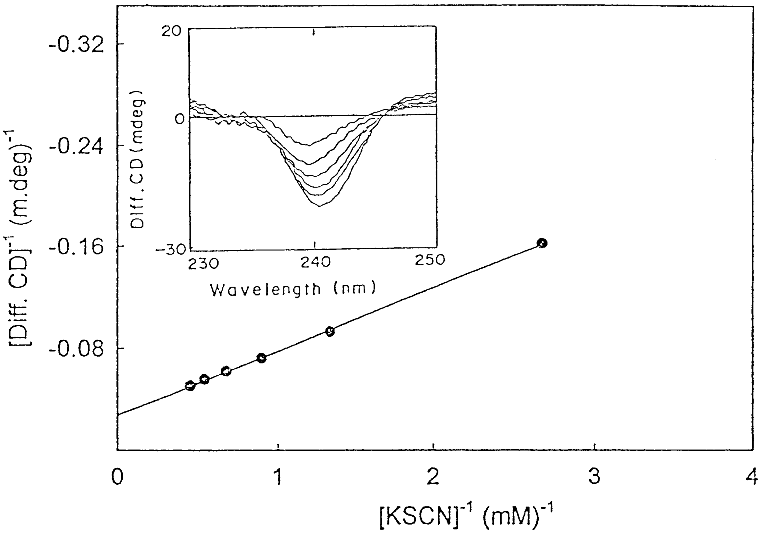

Fig. 1. The plot of the inverse of difference CD at 240 nm for human hemoglobin against $[SCN^-]^{-1}$ at pH 7.3. The inset shows the difference spectra of hemoglobin with increasing concentration of SCN^-. The concentration of SCN^- increases from the top to bottom.

The NMR experiments were carried out on Bruker AM 500 MHz FTNMR spectrometer. Samples were prepared in 100 mM Hepes buffer in D_2O solution. For relaxation rate measurements, both hemoglobin and ^{15}N labeled SCN^- were treated with Chelex-100 to remove any traces of free paramagnetic metal ions. To determine the spin-lattice relaxation time ($T_{1,obs}$), NMR spectra were obtained using the inversion recovery method with 180°-τ-90° pulse sequence. Here, τ is the interval between 180 and 90° pulse. $T_{1,obs}$ was obtained from the experimental data following the reported procedure [16].

Both CD and NMR measurements were carried out at 24 °C.

3. Results

3.1. Circular dichroism study

To determine the nature of the interaction of thiocyanate (SCN^-) with hemoglobin, we have utilized both optical and circular dichroism (CD) spectroscopy. The optical spectrum of hemoglobin does not show any changes in the presence of SCN^- ion. However, the CD spectrum of tetrameric hemoglobin is found to show a minimum at 240 nm in the presence of SCN^- (Fig. 1). Brunori et al. [17] and Hanisch et al. [18] have studied the interaction of small molecules such as O_2, CO, NO and ethyl-isocyanide with hemoglobin by optical rotatory dispersion, and have observed a decrease in the optical rotation at these wavelengths. The decrease in the optical rotation was ascribed to the conformational change of the protein, which influences the electronic transitions of a number of peptide bonds or (and) a few aromatic side chains. We have measured the CD of oxyhemoglobin as a function of thiocyanate concentration and have observed similar results indicating a conformational change of the protein in the presence of SCN^- ions. To determine the dissociation constant (K_d) due to the interaction of SCN^- with hemoglobin, we measured the difference CD by subtracting the CD spectra of the hemoglobin from the thiocyanate treated hemoglobin. The difference spectrum of thiocyanate treated tetrameric hemoglobin shows a minimum around 240 nm. The interaction of thiocyanate with hemoglobin did not show any change in the CD band around 425 nm, which is characteristic of heme, indicating that thiocyanate is not interacting at the heme region.

We have monitored the equilibrium formation of thiocyanate treated hemoglobin at 240 nm as a function of thiocyanate concentration. The dissociation constant (K_d) for the binding of thiocyanate to hemoglobin was determined from Eq. (1):

$$\frac{1}{\Delta\mathrm{CD}}=\frac{K_d}{\Delta\mathrm{CD}_\infty}\frac{1}{[\mathrm{SCN}^-]}+\frac{1}{\Delta\mathrm{CD}_\infty} \tag{1}$$

where ΔCD is the difference CD intensity of hemoglobin at various concentrations of thiocyanate and $\Delta\mathrm{CD}_\infty$ is the difference CD intensity at saturating concentration of thiocyanate. The plot of $\Delta\mathrm{CD}^{-1}$ vs. $[\mathrm{SCN}^-]^{-1}$ in Fig. 1 shows linearity of the plot and indicates that one molecule of thiocyanate binds to one molecule of hemoglobin. K_d was determined using Eq. (1) from the data in Fig. 1, and was found to be 1.8 mM at pH 7.3. The dissociation constant was found to be 1.7 mM for tetrameric hemoglobin at pH 8.5 indicating that pH does not have significant role on the interaction of thiocyanate with hemoglobin. In order to compare the effect of other cations and anions of the Hofmeister series with that of thiocyanate, CD measurements of hemoglobin in the presence of guanidinum, iodide and perchlorate ions were carried out. The CD spectra did not show any change, indicating that these CD observations were unique to SCN^-. The measurements of the interaction of cross-linked hemoglobin with these anions gave results similar to that obtained for human hemoglobin.

To understand the mechanism of the interaction of SCN^- with hemoglobin, it is important to locate the binding site of thiocyanate. Nuclear magnetic resonance spectroscopy has proved to be a very convenient and effective technique for such studies on paramagnetic heme proteins. The nuclear spin lattice relaxation time (T_1) is related to the distances of various nuclei from the paramagnetic centre in a protein–ligand complex and hence can be used for their determination [19,20]. In oxyhemoglobin iron is diamagentic. In order to make it paramagnetic, we have prepared high spin methemoglobin (Fe^{3+}) from oxyhemoglobin by oxidation with sodium nitrite. In methemoglobin the sixth coordination position is occupied by water molecules. It is known that thiocyanate binds to the heme Fe^{3+}. In order to avoid the binding of thiocyanate to the methemoglobin heme, measurements were done on the $S=1/2$ low spin (Fe^{3+}) cyano methemoglobin by adding CN^- to methemoglobin. It may be noted that the conformations of oxyhemoglobin and paramagnetic cyano methemoglobin are shown to be similar [1]. Our CD measurements of the interaction of thiocyanate with human and cross-linked cyano methemoglobin (Fe^{3+}) show that SCN^- decreases the optical rotation of cyano methemoglobin at 240 nm, and the magnitude of K_d is similar to that observed for oxyhemoglobin.

3.2. ^{15}N-T_1 measurement

To determine the binding site of SCN^- to human and cross-linked cyano methemoglobin, the nuclear spin-lattice relaxation time (T_1) of N^{15} labeled SCN^- was measured. The observed relaxation rate of SCN^- is the sum of the relaxation rates of the free anion and the paramagnetic relaxation enhancement [21]:

$$\frac{1}{T_{1\mathrm{obs}}}=\frac{1}{T_{1f}}+\frac{1}{T_{1p}} \tag{2}$$

where $T_{1\mathrm{obs}}$ is the observed relaxation time, T_{1f} is the relaxation time in the absence of a paramagnetic contribution and T_{1p} is the paramagnetic contribution to relaxation time. T_{1p} is related to the relaxation time of the bound species ($T_{1\mathrm{M}}$) by the relation [21]:

$$\text{Thus, } \frac{1}{T_{1p}}=\frac{1}{T_{1p'}}+\sum_k\frac{1}{T_{1pk}}$$

$$\frac{1}{T_{1p}}=\frac{1}{T_{1p'}}+p_m q\sum_k\left[\frac{1}{T_{1\mathrm{Mk}}+\tau_{\mathrm{Mk}}}\right] \tag{3}$$

where $T_{1p'}$ is the paramagnetic relaxation time due to the effect of bulk susceptibility of the paramagnetic solute. $p_m q$ is the total fraction of nuclei at the bound site and τ_M is the lifetime of a ligand nucleus in the bound site. In the fast exchange limit, i.e. $1/\tau_{\mathrm{M}} \gg 1/T_{1\mathrm{M}}$ for systems with a single binding site. Eq. (3) can be rewritten as [21]:

$$\frac{1}{T_{1p}} = \frac{E_0}{(K_d + S_0)} \sum_k \frac{1}{T_{1\mathrm{Mk}}} \tag{4}$$

$$T_{1p}.E_0 = \frac{(K_d + S_0)}{\sum_k \frac{1}{T_{1\mathrm{Mk}}}} \tag{5}$$

where E_0 is the concentration of cyano methemoglobin and S_0 is the total concentration of SCN^-. T_{1M} can be determined from the plot of $T_{1p}E_0$ vs. S_0 in Eq. (5) [21]. The spin-lattice relaxation time (T_1) measurements were carried out at different concentrations of SCN^- with low spin ($S = 1/2$) cyano methemoglobins. The concentrations of SCN^- were in the range 215–370 mM. The plots $T_{1p}E_0$ vs. S_0 for human cyano methemoglobin ($E_0 = 0.48$ mM) and cross-linked cyano methemoglobin ($E_0 = 0.25$ mM) at different concentrations of SCN^- are shown in Fig. 2.

The value of T_{1M} obtained from Eq. (5) can be used to determine the distance of SCN^- from the metal ion using the Solomon and Bloembergen equation [19,22–24].

$$\frac{1}{T_{1M}} = \frac{2\gamma_I^2 g^2 S(S+1)\beta^2}{15r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right) + \frac{2S(S+1)A^2}{3h^2}\left(\frac{\tau_c}{1+\omega_s^2\tau_e^2}\right) \tag{6}$$

where τ_c is the total correlation time, τ_e is the isotropic exchange correlation time, A is the electron nuclear hyperfine coupling constant, γ_I is the magnetogyric ratio of the nucleus, β is the Bohr magnetron, ω_I and ω_s are the nuclear and electronic Larmor precession frequencies, S is the total electron spin on the paramagnetic centre and r is the distance between the nuclear spin I (of SCN^-) and the electron spin on the metal ion. In the present case, the hyperfine coupling interaction term can be neglected, since no hyperfine shift of the ^{15}N resonance in the NMR spectra was observed. Taking spin as $S = 1/2$ for each low spin Fe^{3+} heme of cyano methemoglobin with SCN^-,

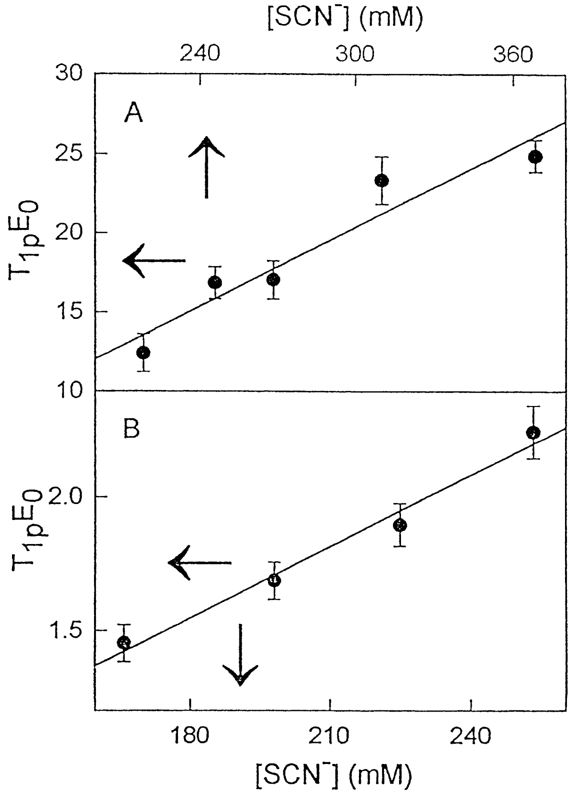

Fig. 2. Plots of $T_{1p}E_0$ vs. $[S_0]$ (concentration of SCN^-) for (a) human native cyanomethemoglobin and (b) cross-linked cyanomethemoglobin.

Eq. (6) can be simplified [21–24] to

$$\frac{1}{T_{1M}} = \sum_k \frac{1}{T_{1Mk}} = \sum_k \frac{C^6}{r_{1/k}^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right) \approx \frac{kC^6}{r_{\mathrm{av}}^6} 10\tau_c \tag{7}$$

where $C = 252$ Å $s^{-1/3}$ for ^{15}N probe nucleus with Fe^{3+} ($S = 1/2$ for each subunit) as the paramagnetic centre and $k = 4$ for both human native and cross-linked Hb. In this case, each heme is separately enhancing relaxation rate of the ^{15}N nucleus of SCN^- bound to the protein. The value of τ_c depends on the metal ion and size of the protein.

For human cyano methemoglobin its value was taken as 6×10^{-10} s [20]. The experimental values of T_{1M} for human and cross-linked cyano methemoglobin were found to be 0.0814 and 0.00896 s from Fig. 2. Assuming a unique binding site near the center of the hemoglobin tetramer, using these values, average distances of the thiocyanate binding region to the iron of human native and cross-linked cyano methemoglobin were found to be 8.9 Å and 6.2 Å, respectively. This suggests that SCN^- binds at a different site in cross-linked Hb than in human native hemoglobin.

Our measurements show that one molecule of thiocyanate binds to one molecule of hemoglobin, a result usually seen only for large molecules such as 2,3-diphosphoglycerate.

It has been found that 2,3 DPG (diphospho glycerate) lowers the oxygen affinity of hemoglobin by binding to the deoxyhemoglobin in the ratio of one DPG per hemoglobin tetramer [25]. The presence of one binding site suggested that DPG binds on the symmetry axis of the hemoglobin molecule in the central cavity, where the positively charged amino acid residues of the four subunits are near one another [25]. Using the Biosym program we have determined the distances from the heme group of several positively charged amino acid residues such as arg-α-141 and lys-α-99. These distances have been found to be close to the distance of SCN^- binding site deduced from our nmr measurements. This suggests that SCN^- might be interacting with either of these amino acid residues in the central cavity of the protein. Binding at the arg-α-141 has been shown for chloride ion [6], but such binding apparently does not influence the functional properties. In the cross-linked hemoglobin SCN^- is interacting at a different site compared to the human native hemoglobin due to the modification of lys-α99 residues of the alpha subunits by a fumarate group, which indicates that the binding site of SCN^- in unmodified hemoglobin is at the lys-α-99.

4. Discussion

The decrease in optical rotation at 240 nm for oxyhemoglobin in the presence of thiocyanate was observed previously on conversion of deoxyhemoglobin to the liganded form [18]. Our observation of strong binding of thiocyanate to the oxy-conformation of hemoglobin would suggest that the change brought about by thiocyanate is different although it is still likely to be due to the electronic transitions of a number of peptide bonds or (and) a few aromatic side chains. The observed decrease is unlikely to be due to dissociation into dimers brought about by thiocyanate since Brunori et al. showed that the effect is independent of the concentration of hemoglobin [18]. Thus, the observed changes in optical rotation must be due to the binding of thiocyanate to specific sites in the central cavity of hemoglobin. It is interesting that halothane (2-bromo-2-chloro-1,1,1-trifluroethane) has been reported to bring about a similar reduction in optical rotatory dispersion in oxyhemoglobin [26].

The nuclear spin lattice relaxation time measurements of N^{15} labeled SCN^- in the presence of cyanomethemoglobin showed that the binding site of SCN^- is located approximately 8.9 Å away from the heme group in cyanomethemoglobin, which is close to the distance of arg 141 and lys99 from the heme group in oxyHb. The observation that the binding site was different in the cross-linked hemoglobin between the alpha chains through chemical modification at the lys-α-99 suggests that the binding site is at the latter site. This site is in the central cavity and at the $\alpha_1\beta_2$ interface which suggests the lys-α-99 is the dissociation-linked binding site. That changes in this region may strongly affect the dissociation into dimers is indicated by the much higher degree of dissociation in Hb Turriff, a mutant hemoglobin in which lys G6(99)α residue is glu instead of lys [27]. Perutz et al. has suggested that the allosteric effect of chloride is due to neutralization of the positive charges in the central cavity in deoxyhemoglobin [28]. Our results indicate that dissociation into dimers is triggered by binding to a specific site.

Since thiocyanate is a negatively charged ion with polarisable sulfur at one end and nitrogen at the other end, the binding of thiocyanate with hemoglobin may take place at the positively charged center of the protein or may interact with non-polar residues due to the polarizable sulfur and the fact that the negative charge is distributed over three atoms. It is not known whether lys-α-99 is positively charged in oxyhemoglobin. It would appear to be uncharged in deoxyhemoglobin in order to react with the bis(3,5-dibromosalicyl)fumarate in the cross-linking reaction.

Some interest has been shown in the use of thiocyanate in the treatment of sickle cell anemia [29,30]. The mechanism by which thiocyanate has an anti-sickling effect could be through the binding to the oxyform with an increase in the dissociation into dimers, which would result in an increase in oxygen affinity.

Acknowledgments

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