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Relaxation amplitude analysis of thiocyanate and formate binding to human aquomethemoglobin A

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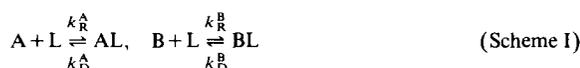
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The kinetics of the reaction of thiocyanate and formate ions with aquomethemoglobin can be adequately accounted for by a scheme in which the ligand-binding step in both the alpha and beta subunits is preceded by a fast transition of the iron atom from high to low spin (Okonjo, K.O. (1980) *Eur. J. Biochem.* **105**, 329–334). Amplitude expressions derived from this scheme are used to analyse the relaxation amplitude data for alpha and beta subunits within the methemoglobin tetramer. The mean of the reaction enthalpies for ligand binding by the subunits within the tetramer is in good agreement with the reaction enthalpy for ligand binding by the methemoglobin tetramer obtained from a Van't Hoff plot of equilibrium titration data.

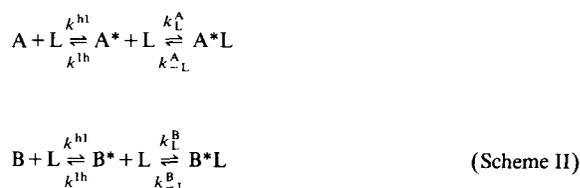
Introduction

Ferrous hemoglobin is capable of existing in one of two spin states. Deoxyhemoglobin, which has the T quaternary structure, is high-spin, whereas liganded hemoglobin, which exists in the R quaternary state, is low spin. Considerable controversy [1–6] surrounds Perutz's suggestion [7] that the T → R allosteric transition in ferrohemo-globin is triggered by a change in the spin state of the iron atoms from high to low spin. Nevertheless, Perutz has demonstrated [8] that ligand binding to one subunit produces a spin change in an adjacent non-liganded subunit. In a recent communication [9] we showed that the spin transition plays an important role in the binding of formate ion to methemoglobin.

A reaction scheme which fulfills the minimal requirements for ligand binding to methemoglobin consists of two bimolecular reactions, that is, binding to the α and β subunits:



In this scheme, A and B represent α and β chains, respectively. We have shown that Scheme I does not adequately account for formate binding to methemoglobin because of an internal inconsistency between binding constants determined kinetically and thermodynamically [9]. A scheme that, for formate, results in a consistency between kinetic and thermodynamic data is one in which a fast iron spin transition precedes the ligand-binding step [9]:



In this scheme, A (B) represents the α (β) met chain in the high-spin form; A* (B*) is the corresponding low-spin species; L is the ligand. Under conditions in which the ligand is in large excess, the reciprocal relaxation times for the ligand bind-

ing steps of Scheme II are [9]:

$$1/\tau_A = k_L^A C_L + k_{-L}^A (1 + k^{1h}/k^{h1}) \quad (1)$$

$$1/\tau_B = k_L^B C_L + k_{-L}^B (1 + k^{1h}/k^{h1}) \quad (2)$$

where C_L is the total ligand concentration. The rate constants of Scheme I are therefore seen to be merely apparent rate constants and are related to those of Scheme II as follows ($i = A, B$):

$$k_R^i = k_L^i;$$

$$k_D^i = k_{-L}^i (1 + k^{1h}/k^{h1})$$

It has been reported [10] that the kinetics of thiocyanate binding to methemoglobin are complex and that there is no consistency between the kinetic and thermodynamic results obtained under the same experimental conditions. Since thiocyanate closely resembles formate, whose kinetics are adequately accounted for by Scheme II, it seemed reasonable to reinvestigate the binding of thiocyanate to methemoglobin. Moreover, thiocyanate and formate, by their very nature, seemed very attractive for an attempt at a relaxation amplitude analysis of the kinetic data, because of the relatively simple amplitude expressions that can be derived for them. Such an analysis would throw more light on the adequacy of Scheme II.

Our results show that the kinetics of the binding of thiocyanate to methemoglobin can be adequately accounted for by Scheme II. Furthermore, for both thiocyanate and formate, the values of the binding enthalpies obtained from amplitude analyses based on Scheme II are in good agreement with those obtained from Van't Hoff plots of equilibrium binding data.

Materials and Methods

Kinetic and equilibrium studies were carried out on mixtures of methemoglobin and thiocyanate or formate as previously described for formate [11]. For thiocyanate the working wavelength was 427 nm and the methemoglobin concentration was 58 μM heme. 7°C temperature jumps were performed on thiocyanate/methemoglobin mixtures preequilibrated at 20°C. The data were analysed

on an IBM 370 computer of the University of Ibadan by a Fourier method of analysis of exponential curves [12–14], using a computer program kindly provided by Dr. Steve Provencher. All linear plots reported were fitted with a linear least-squares computer program. Changes in extinction coefficient, $\Delta\epsilon_{iL}$, arising from ligand binding were determined for formate as in Ref. 11 and for thiocyanate as in Ref. 15.

Results

Kinetics

Klapper and Uchida [10] reported that, of the two kinetic phases observed in their study of thiocyanate binding to methemoglobin, the faster phase appeared not to follow simple second-order kinetics. We have shown that this behavior may be attributed to a rate-limiting iron-spin transition [9].

Fig. 1 shows that the reciprocal relaxation time varies linearly with thiocyanate concentration, up to 150 mM, for the two phases observed by us. This behavior is in accord with Eqns. 1 and 2. As pointed out previously [9], the slopes of the plots in Fig. 1 give the ligand association rate constants, k_L , of Scheme II, while the intercepts give the apparent ligand dissociation rate constants,

$$k_D = k_{-L} (1 + k^{1h}/k^{h1})$$

from which k_{-L} , the ligand dissociation rate constant, can be calculated for each subunit reacting. The value of k^{1h}/k^{h1} used in this calculation was obtained from the magnetic susceptibility data of Anusiem [16], as previously reported [9]. The binding constants calculated from the kinetic data are related to that obtained from equilibrium titration by the equation [9,11]:

$$(K_A K_B)^{1/2} = K_{\text{equ}}$$

where

$$K_A = k_L^A/k_{-L}^A; \quad K_B = k_L^B/k_{-L}^B$$

Values of k_L^i , k_{-L}^i and K_{equ} for thiocyanate binding are collected in Table I. It is seen that $(K_A K_B)^{1/2} = 231 \pm 17 \text{ M}^{-1}$ at 27°C is in good

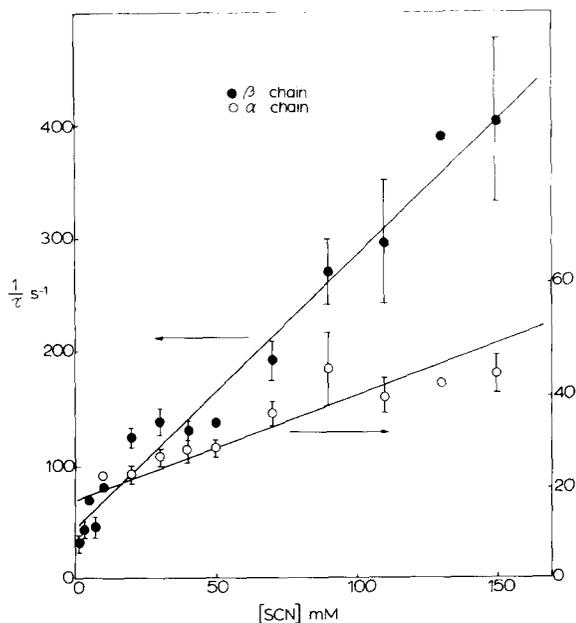


Fig. 1. Dependence of the reciprocal relaxation time, $1/\tau$, on thiocyanate concentration at 27°C. Conditions: 20 mM Tris-maleate buffer (pH 6), ionic strength 0.25 M (added salt, NaCl); methemoglobin concentration, 58 μ M heme. Error bars indicate standard errors from at least three temperature jumps. Left-hand ordinate: fast (or β) chain; right hand ordinate: slow (or α) chain. Observation wavelength, 427 nm. Compare with Eqn. 1 and 2. The lines through the points were calculated by a least-squares method.

agreement with $K_{\text{equ}} = 252 \pm 4 \text{ M}^{-1}$. Clearly, Scheme II adequately accounts for the kinetics of thiocyanate binding to methemoglobin, just as it does for formate binding [9]. By contrast, the value of $(K_A K_B)^{1/2}$ calculated from Scheme I is 29 M^{-1} . Scheme I is therefore inadequate to account for thiocyanate binding.

In previous reports [9,11], the data on formate binding were restricted to concentrations above 10 mM formate. We present in Fig. 2 kinetic data between 2 mM and 14 mM formate, the range over which the amplitude analysis for formate binding was carried out. It is seen that, within experimental error, the reciprocal relaxation time, $1/\tau$, is independent of formate concentration for both relaxation phases. This result, which at first sight would appear surprising, can be explained in terms of Scheme II and Eqns. 1 and 2 using the rate constants reported previously for formate binding under the experimental conditions of Fig.

TABLE I

COMPARISON OF KINETIC AND EQUILIBRIUM CONSTANTS FOR BINDING OF THIOCYANATE TO SUB-UNITS OF HUMAN AQUOMETHEMOGLOBIN WITHIN THE TETRAMER AT 27°C

Compare with Eqns. 1 and 2 and Scheme II. The binding constant determined from equilibrium titration is $K_{\text{equ}} = 252 \pm 4$. Conditions: 20 mM Tris-maleate, ionic strength 0.25 M (added salt NaCl) (pH 6.0). Methemoglobin concentration = 58 μ M heme.

Sub-unit	k_L^i ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{-L}^i (s^{-1})	k_L^i/k_{-L}^i (M^{-1})	$(K_A K_B)^{1/2}$ (M^{-1})
α	225 ± 7	2	107 ± 8	231 ± 17
β	2487 ± 31	5	495 ± 56	

2 [9,11]. These rate constants are $k_L^A = 74 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{-L}^A = 2.56 \text{ s}^{-1}$, $k_L^B = 618 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{-L}^B = 9.89 \text{ s}^{-1}$, $k^{1h}/k^{h1} = 4.46$.

Amplitude analysis

In order to test Scheme II more rigorously, we have undertaken an amplitude analysis of the data for thiocyanate and formate, whose kinetics are reported in Figs. 1 and 2, respectively.

For Scheme II, the total relaxation amplitude is given by:

$$\delta F_{\text{tot}} = \delta E_A + \delta E_B$$

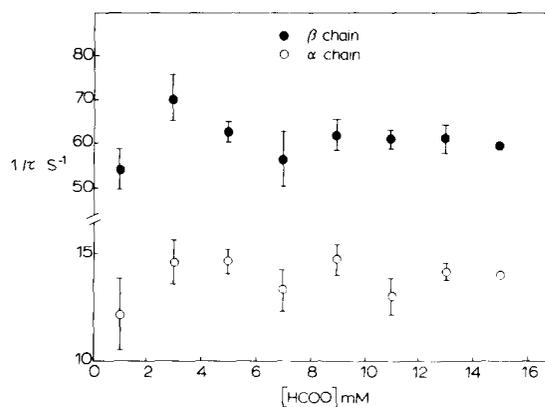


Fig. 2. Dependence of the reciprocal relaxation time on formate concentration (low saturation range) at 27°C. Conditions: 20 mM Tris-maleate buffer (pH 5.5), ionic strength 0.25 M (added salt, NaCl); methemoglobin concentration, 128 μ M heme. Error bars indicate standard errors from at least three temperature jumps. Observation wavelength, 620 nm.

with $\delta E_i = \delta E_{\text{spin}} + \delta E_{iL}$. δE_i is the contribution of subunit i to the total amplitude, δE_{spin} is the contribution of the fast spin transition and δE_{iL} is the contribution of subunit i arising from the ligand-binding step. From temperature-jump studies at 405 nm and 427 nm (Ref. 17 and our unpublished observations, respectively) the spin contribution to the amplitude is small and may, to a first approximation, be ignored at 27°C, our working temperature. Thus,

$$\delta E_i \approx \delta E_{iL}$$

and therefore

$$\delta E_{\text{tot}} \approx \delta E_{\text{AL}} + \delta E_{\text{BL}}$$

The two ligand-binding steps of Scheme II are coupled through the common ligand, L, and the total amplitude is given by [18]:

$$\begin{aligned} \delta E_{\text{tot}} \approx \delta E_{\text{AL}} + \delta E_{\text{BL}} \\ = \frac{\Gamma_{\text{AL}}}{1 - \Gamma_{\text{AL}}\Gamma_{\text{BL}}/C_L^2} (\Delta \epsilon_{\text{AL}} - \Gamma_{\text{BL}}\Delta \epsilon_{\text{BL}}/C_L) \delta \ln K_A \\ + \frac{\Gamma_{\text{BL}}}{1 - \Gamma_{\text{AL}}\Gamma_{\text{BL}}/C_L^2} (\Delta \epsilon_{\text{BL}} - \Gamma_{\text{AL}}\Delta \epsilon_{\text{AL}}/C_L) \delta \ln K_B \end{aligned} \quad (3)$$

In this equation,

$$\Gamma_{iL} (i = \text{A,B}) = \left(\frac{1}{C_{i^*}} + \frac{1}{C_L} + \frac{1}{C_{i^*L}} \right)^{-1} \quad [18,19]$$

In the case of ligand buffering, such as obtains for thiocyanate and formate, the two ligand-binding steps in Scheme II are effectively decoupled, i.e., $\Gamma_{\text{AL}}\Gamma_{\text{BL}}/C_L^2 \rightarrow 0$, and Eqn. 3 simplifies to:

$$\delta E_{\text{tot}} = \Delta \epsilon_{\text{AL}}\Gamma_{\text{AL}}\delta \ln K_A + \Delta \epsilon_{\text{BL}}\Gamma_{\text{BL}}\delta \ln K_B \quad (4)$$

In Eqns. 3 and 4, $\Delta \epsilon_{iL}$ is the change in the extinction coefficient of the absorbing species on ligand binding, Γ_{iL} is the amplitude factor for the ligand binding step, and $\delta \ln K_i$ is the change in the equilibrium constant of the ligand binding step of subunit i arising from a temperature jump.

Since the ligand-binding steps of Scheme II are effectively decoupled, it is possible to write down

independent expressions for Γ_{AL} and Γ_{BL} of Eqn. 4. It can be readily shown that, for $C_L \gg \bar{C}_A$, \bar{C}_B and $1/K_A \gg \bar{C}_A$, $1/K_B \gg \bar{C}_B$ (conditions that hold true for thiocyanate and formate):

$$\Gamma_{\text{AL}} = \frac{K_A C_L C_{\text{Hb}}}{2(1 + K_A C_L)^2} \cdot \frac{1}{1 + K_{\text{spin}}} \quad (5)$$

$$\Gamma_{\text{BL}} = \frac{K_B C_L C_{\text{Hb}}}{2(1 + K_B C_L)^2} \cdot \frac{1}{1 + K_{\text{spin}}} \quad (6)$$

where K_{spin} (i.e., k^{1h}/k^{h1}) is the spin equilibrium constant and C_{Hb} is the total methemoglobin concentration (heme basis).

We have employed Eqn. 4 for the analysis of the amplitude data, with the amplitude factor Γ expressed as in Eqns. 5 and 6. In using these expressions it was necessary to assume that $\Delta \epsilon_{\text{AL}} = \Delta \epsilon_{\text{BL}} = \Delta \epsilon_{\text{Hb}}$, where $\Delta \epsilon_{\text{Hb}}$ is the change in extinction coefficient (per mole heme) when methemoglobin is saturated with ligand. The results of Gibson and his co-workers [19], which show that the azide-aquo difference spectra of the two subunits are similar, provide a justification for this assumption.

The amplitude contributions of the two types of methemoglobin subunit, δE_{AL} and δE_{BL} , were read from computer printouts of the analyses of the relaxation data [12–14]. Fig 3 shows the dependence of the amplitude contribution of each subunit within the tetramer on Γ for thiocyanate binding. Linear plots are obtained for each relaxation phase. The changes in the equilibrium constants, $\delta \ln K_i$, were determined from the slopes using a predetermined value of $\Delta \epsilon_{iL}$. From the Van't Hoff relation $\delta \ln K_i = (\Delta H_{iL}/RT^2)\delta T$, the enthalpy of reaction (for the ligand binding step) was calculated for each subunit i . These values are shown in Table II. The ΔH_{iL} value for each subunit was then added to the value of ΔH_{spin} reported previously [9], that is, 24.8 kJ/mol heme, and the average ΔH_i for the two subunits was calculated. These values are also reported in Table II. A comparison of the ΔH_i calculated from the amplitude analysis according to Scheme II and that obtained from Van't Hoff plots of our equilibrium titration data (Fig. 4) shows that the two values are in reasonably good agreement. Thus, Scheme II is adequate for analysing the amplitude

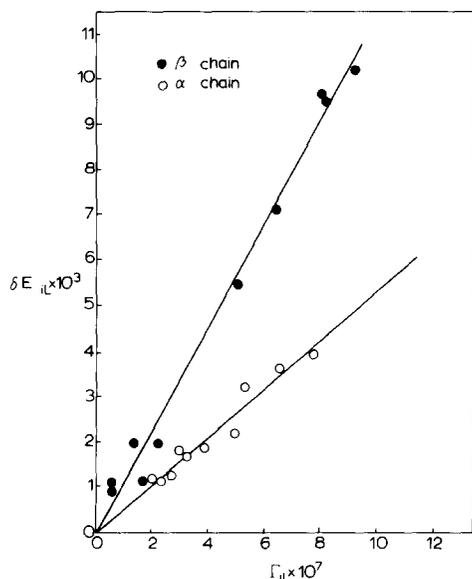


Fig. 3. Relaxation amplitude, δE_{iL} , as a function of the amplitude factor Γ_{iL} for the binding of thiocyanate ion by the α and β subunits within the methemoglobin molecule. Conditions as in Fig. 1. Values of Γ_{iL} were calculated from Eqns. 5 and 6 with $K_{\text{spin}} = 6.929$ (see Ref. 9) and $K_A = 107.3 \text{ M}^{-1}$, $K_B = 495.2 \text{ M}^{-1}$ (compare with Table I). The lines through the points were calculated by a least-squares method and ΔH_{iL} values from the slopes with $\Delta \epsilon_{iL} = 34656 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ($\lambda = 427 \text{ nm}$).

data for thiocyanate binding.

The variation of the relaxation amplitude with the amplitude factor is shown in Fig. 5 for binding of formate to the methemoglobin subunits within

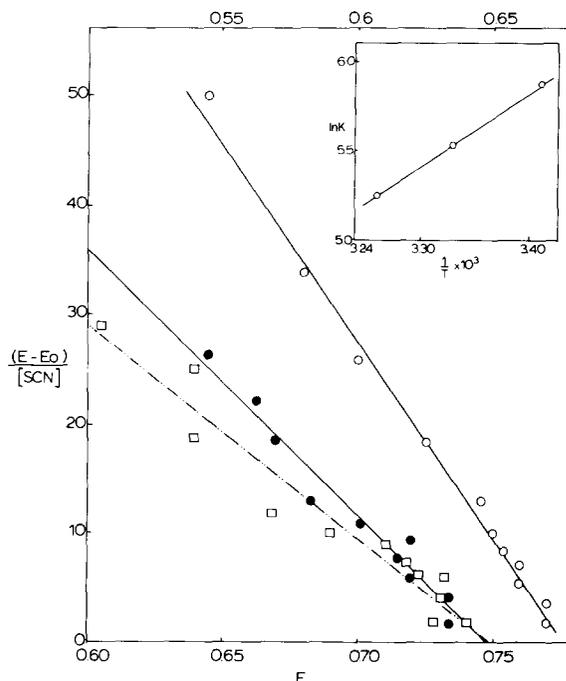


Fig. 4. Plots of $(E - E_0)/[\text{SCN}^-]$ vs. E for binding of thiocyanate to methemoglobin. E and E_0 are the absorbances at finite and zero thiocyanate concentration, respectively. The binding constants, K_{equ} , are obtained from the slopes. The lines through the points are least-squares lines. Conditions: 20 mM Tris-maleate buffer, ionic strength 0.25 M (added salt NaCl) (pH 6.0) 27°C; methemoglobin concentration, 58 μM heme. The working wavelength was 540 nm. \circ , 20°C, upper abscissa; \bullet , 27°C, lower abscissa; \square , 34°C, lower abscissa. The lines through the points were calculated by a least-squares method. Inset. Van't Hoff plot of the slopes of the lines in the main figure as a function of reciprocal temperature. The line was fitted by least squares. The slope of this plot gives $-\Delta H/R$ where ΔH is the enthalpy change for thiocyanate binding in $\text{kJ} \cdot \text{mol}^{-1}$ (heme basis).

TABLE II

VALUES OF THE REACTION ENTHALPIES (FROM AMPLITUDE MEASUREMENTS) FOR BINDING OF THIOCYANATE AND FORMATE TO METHEMOGLOBIN SUBUNITS WITHIN THE TETRAMER

Compare with Eqn. 4 of text. ΔH_{iL} is the enthalpy change for the ligand-binding step of Scheme II and $\Delta H_i = \Delta H_{iL} + \Delta H_{\text{spin}}$. ΔH_{iL} values have been obtained from the slopes in Figs. 3 and 5 with $\Delta \epsilon_{iL} = 34656 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ($\lambda = 427 \text{ nm}$) and $\Delta \epsilon_{iL} = 2200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ($\lambda = 620 \text{ nm}$) for thiocyanate and formate, respectively. All values are quoted in kJ/mol heme.

Ligand	Methemoglobin	$-\Delta H_{iL}$	$-\Delta H_{\text{spin}}$	$-\Delta H_i$	Mean
Thiocyanate	α subunit	15.7 ± 1.1	24.8 ± 1	40.5 ± 2.1	49.4
	β subunit	33.4 ± 1.3	24.8 ± 1	58.2 ± 2.3	
	tetramer	—	—	34.3	
Formate	α subunit	8.3 ± 1	24.8	33.1	32.4
	β subunit	7.0 ± 1	24.8	31.8	
	tetramer	—	—	39.3	

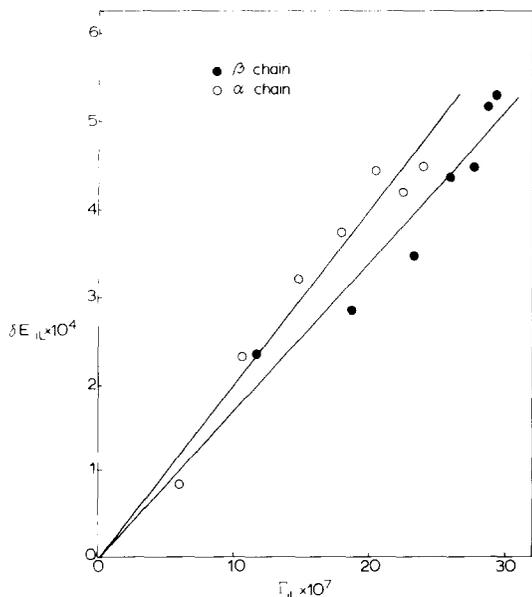


Fig. 5. Relaxation amplitude as a function of amplitude factor for formate binding to methemoglobin subunits within the tetramer. Conditions as in Fig. 2. Values of Γ_{iL} were calculated from Eqns. 5 and 6 with $K_{spin} = 4.46$, $K_A = 28.94 \text{ M}^{-1}$ and $K_B = 62.79 \text{ M}^{-1}$ (values of K_A , K_B were calculated from the data in Ref. 11). The lines through the points are the least-squares lines.

the tetramer. ΔH_{iL} values were calculated from the slopes of the linear plots obtained. These values and the ΔH obtained from a Van't Hoff plot of equilibrium titration data (not shown) are given in Table II.

Under the conditions of the thiocyanate study (Figs. 1 and 3) ΔH_{spin} is known [9]. However, under those of the formate study (Figs. 2 and 5) ΔH_{spin} is not known. Nevertheless, one may reasonably assume that it does not change appreciably between pH 5.5 and 6, that is, $\Delta H_{spin} = 24.8 \text{ kJ/mol heme}$ [9] at both pH values. Using the ΔH_{iL} values obtained for formate (Table II) we calculate a value of 32 kJ/mol heme for the average ΔH_i of the two subunits. This value is in reasonable agreement with a value of 39 kJ/mol heme obtained from a van't Hoff plot of equilibrium titration data (Table II).

The value of ΔH_{spin} reported in Ref. 9 suggests that the spin transition makes a substantial contribution to the thermodynamics of ligand binding to methemoglobin (see Table II). This is in line with

the findings of Anusiem and Kelleher [20].

The formate binding constants of the α and β subunits within the tetramer are $K_A = 5.3 \text{ M}^{-1}$ and $K_B = 11.5 \text{ M}^{-1}$, respectively [11]. When these values are compared with the corresponding values for thiocyanate binding (Table I), it is seen that binding of thiocyanate to methemoglobin exhibits a greater heterogeneity in the affinities of the two subunits than binding of formate. The relative amplitude of the α chains is given by [11]:

$$\frac{\delta E_{AL}}{\delta E_{tot}} = \frac{\frac{1}{K_B} (1 + K_B C_L)^2 \delta \ln K_A}{\frac{1}{K_A} (1 + K_A C_L)^2 \delta \ln K_B + \frac{1}{K_B} (1 + K_B C_L)^2 \delta \ln K_A} \quad (7)$$

Eqn. 7 predicts that for equal intrinsic binding properties of the methemoglobin subunits, $K_A = K_B$, the relative amplitude should remain invariant throughout the saturation range. For different intrinsic affinities, however, the relative amplitude should increase or decrease with increasing saturation with ligand [11]. We reported previously for formate [11] that the relative amplitude of the α chains remained invariant with saturation. In Fig. 6 we present the behavior of the relative amplitude

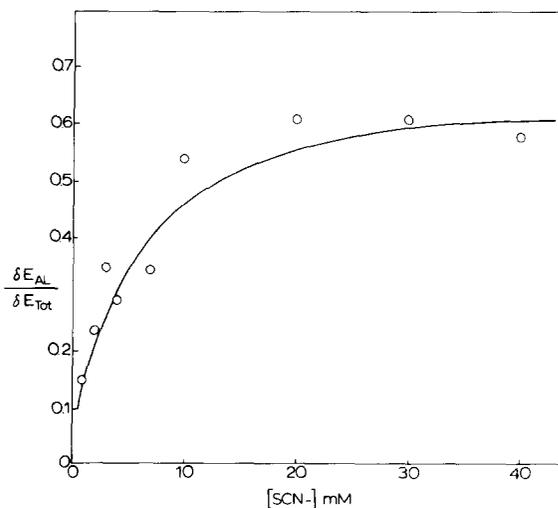


Fig. 6. Relative amplitude of the slow (α chain) relaxation phase as a function of thiocyanate concentration. Conditions as in Fig. 1. The theoretical line was calculated from Eqn. 7. K_A , K_B used for the calculation are tabulated in Table I; $\delta \ln K_A$ and $\delta \ln K_B$ were obtained from the slopes of the plots in Fig. 3.

with thiocyanate as ligand. It is seen that thiocyanate behaves differently from formate. With thiocyanate the relative amplitude increases from 0.1, at the lower saturation range, and levels off around 0.6 at high saturation. Between 10 and 80% saturation of the α subunits, the experimental points follow exactly the trend of the theoretical curve calculated from Eqn. 7. This difference in behavior between thiocyanate and formate is readily explained in terms of the increased heterogeneity of the methemoglobin molecule towards thiocyanate binding as compared to formate binding.

Discussion

We have demonstrated that the spin transition is an important elementary step in the ligand binding process in methemoglobin. Apart from the kinetic and amplitude analyses presented here that support Scheme II, further evidence for the participation of the spin transition in the ligand-binding process is to be found in our demonstration that the rate of formate binding to methemoglobin β chains, in the presence of inositol hexakisphosphate, is limited by a zero-order process (Fig. 1 of Ref. 9). Klapper and Uchida [10] have also suggested the involvement of a rate-limiting zero-order process in their study of thiocyanate binding to methemoglobin. Furthermore, Arrhenius plots of the kinetic data from three different laboratories [9,10,17] gave straight lines, indicating that the data from the three laboratories, one of which measured directly the kinetics of the spin transition, the others measuring the kinetic

ics of ligand binding, must refer to the same phenomenon, namely, the iron spin transition (Fig. 3 of Ref. 9).

The values of the binding constants for both types of methemoglobin subunit, as determined from Scheme II and Eqns. 1 and 2, are in very good agreement with the overall binding constant determined from equilibrium titration, with thiocyanate as ligand (Table I). This is also true for formate [9]. Furthermore, the values of the subunit-binding enthalpies determined from the amplitude data are in reasonably good agreement with those determined for the tetramer (for both thiocyanate and formate) from Van't Hoff plots of equilibrium titration data (Table II). Since Eqns. 4–6, from which the enthalpies were calculated, are based on Scheme II, this provides additional support for the validity of Scheme II.

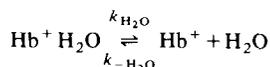
Gibson et al. [21] were the first to demonstrate that the kinetic inequivalence observed in the reaction of methemoglobin with ligands could be attributed to the α and β subunits within the tetramer. Uchida et al. [22] have shown that the affinities for imidazole of the α and β chains within the methemoglobin tetramer differ by a factor of 7. A similar result was obtained by Beetlestone for azide binding [23]. To the best of our knowledge, ours is the first determination of thermodynamic parameters for ligand binding by the subunits within the methemoglobin tetramer (Table III).

We have considered an alternative scheme (Scheme III) to Scheme II. In Scheme III, the rate-limiting unimolecular dissociation of the water molecule attached to the sixth coordination position of the ferric iron atoms of each subunit pre-

TABLE III
THERMODYNAMIC PARAMETERS FOR BINDING OF THIOCYANATE AND FORMATE TO METHEMOGLOBIN SUBUNITS WITHIN THE TETRAMER AT 27°C AND pH 6 AND 5.5, RESPECTIVELY

Ligand	Methemoglobin	$-\Delta H$ (kJ/mol heme)	$-\Delta G$ (kJ/mol heme)	ΔS (J·K ⁻¹ ·mol ⁻¹)
Thiocyanate	α subunit	40.5	11.7	96.0
	β subunit	58.2	15.5	142.3
	tetramer	34.4	13.7	69.0
Formate	α subunit	33.1	10.3	76.0
	β subunit	31.8	8.4	78.0
	tetramer	39.3	9.2	100.3

cedes the binding of ligand:



For this scheme the reciprocal relaxation time, with the ligand in large excess, is given by:

$$1/\tau = \frac{k_{\text{H}_2\text{O}}[\text{H}_2\text{O}]}{1 + [\text{L}]/K_{-\text{L}}} + k_{-\text{H}_2\text{O}} \quad (8)$$

where $K_{-\text{L}} = k_{-\text{L}}/k_{\text{L}}$. This equation predicts that for each subunit, $1/\tau$ should decrease as the ligand concentration increases. It was in fact the search for such behavior that led us to study the formate-binding kinetics at low saturation (Fig. 2). Eqn. 8 and Scheme III are not supported by the results in Figs. 1 and 2, which are easily explained in terms of Scheme II.

The kinetic and amplitude results presented in this report strongly indicate that Scheme II is adequate to account for thiocyanate and formate binding to methemoglobin. It is quite possible that this scheme may not be sufficient to account for the binding of all ligands. Nevertheless, it must be regarded as a scheme whose minimal requirements must be considered basic for the explanation of the binding of any ligand to methemoglobin. Scheme II may be of some relevance to an understanding of the detailed mechanism of binding of ligand to ferrohemeoglobin.

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