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The Kinetics of Thiocyanate Binding to Aquocobalamin*

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ABSTRACT: The apparent binding and dissociation rate constants for the thiocyanate–aquocobalamin reaction are determined over the pH range 2.7–10.0. The rate of thiocyanate binding to hydroxocobalamin is found to be negligible compared to the rate of binding to aquocobalamin.

The kinetic data are interpreted in terms of two mechanisms corresponding to the SN2 and SN1 mechanisms considered for simpler Co(III) complexes. Both mechanisms can fit the kinetic data.

Several features of the structure of aquocobalamin make a study of the kinetics of ligand binding to the Co(III) atom in this molecule of particular interest. (1) The three-dimensional structure of aquocobalamin is known. The vitamin B_{12} structure has been determined (Hodgkin, 1958), and aquocobalamin presumably differs only in having a water molecule instead of a cyanide ion coordinated to the Co(III) atom (Kaczka *et al.*, 1951). (2) The corrin ring–Co(III) complex in aquocobalamin is not surrounded by other portions of a larger molecule as is the porphyrin ring–Fe(III) complex in metmyoglobin and methemoglobin. (3) The planar, tetradentate corrin ring prevents any *cis–trans* isomerization of the remaining two ligands. (4) The benzimidazole group bound to the fifth site on the Co(III) atom prevents any mechanism involving backside attack. (5) Many ligands displace the water molecule on the sixth coordination site (George *et al.*, 1960). The reactions are rapid and easily followed spectrophotometrically. (6) A comparison can be made with the kinetics of binding of various anions to simpler Co(III) complexes.

Hydroxocobalamin is formed by the ionization of the coordinated water molecule of aquocobalamin (Buhs *et al.*, 1951). This transition of aquocobalamin to hydroxocobalamin will introduce a pH dependence

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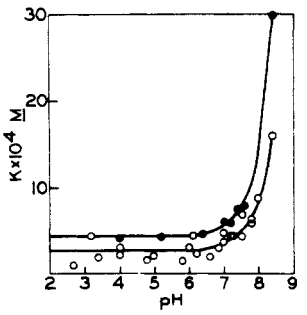

FIGURE 1: Plot of observed dissociation constants *vs.* pH. ●, calculated from spectrophotometric titrations; ○, calculated from the ratio of apparent rate constants, k_{-1app}/k_{+1app}. Solid lines are calculated from the best fit to eq 11 for the two sets of data.

into some of the rate parameters and observed equilibrium constants. The ligand used in this paper was thiocyanate ion which exists in one ionized form over the pH range used, *viz.*, 2.7–10.0 (Morgan *et al.*, 1965).

Experimental Section

Below pH 8.5, the kinetics were investigated by means of a temperature-jump apparatus previously described (Diven *et al.*, 1965). Above pH 8.5, a simple stopped-flow apparatus using spectrophotometric detection was employed. Equilibrium studies were conducted with the use of a Cary Model 14 recording spectrophotometer.

All solutions were prepared with conductivity water. Reagent grade potassium thiocyanate was used without further purification. The buffer systems employed at various pH ranges were: glycine–HCl below pH 4, acetate between pH 4 and 5, phosphate between pH 6 and 7.8, barbital–barbital sodium between pH 8 and 9, and carbonate–bicarbonate at pH 10.

Most of the work reported in this paper was done using commercially available hydroxocobalamin solutions sold under the trade name "alpha-Redisol" by Merck Sharp and Dohme. These solutions contain 1 mg/ml of cobalamin (7.3×10^{-4} M), 0.82% NaCl, 0.02% NaAc, and as preservatives, 0.15% methylparaben and 0.02% propylparaben. Pure hydroxocobalamin, obtained through the courtesy of Walter B. Gall at Merck Sharp and Dohme, was used to check the results obtained with the commercial samples.

The kinetics investigation was conducted with solutions containing a buffer contributing 0.01 to the ionic strength out of a total ionic strength of 0.054. The final temperature of all solutions was 25°.

Equilibrium constants were determined by use of a spectrophotometric titration procedure previously described (Goldsack *et al.*, 1966). A similar titration procedure was employed when using the temperature-jump apparatus. In this procedure, a measured volume of a solution of buffer, aquocobalamin, and enough potassium nitrate to adjust the ionic strength was placed in the temperature-jump cell. A small measured volume of a solution containing a high thiocyanate concentration, but the same aquocobalamin and buffer concentrations and at the same pH and ionic strength as the original solution, was then added. The characteristic relaxation time for the reaction in this solution was then measured. Additional volumes of the second solution were then added and more relaxation times recorded. In this way, the thiocyanate concentration was varied from the lowest to the highest desired values while using a minimum amount of aquocobalamin.

The stopped-flow investigations involved mixing together a buffered solution containing a cobalamin concentration of 1.5×10^{-4} M and an ionic strength of 0.054 with solutions containing no cobalamin, a high thiocyanate concentration (5×10^{-3}–2.5×10^{-2} M), and the same ionic strength and pH as the first solution.

Results

At any pH, a plot of the reciprocal of the observed relaxation time for the thiocyanate–cobalamin reaction *vs.* the total thiocyanate concentration was linear within experimental error. The slopes and intercepts of these plots, corrected in a manner which will be given in the Discussion, are presented in Table I.

Dissociation constants for the binding of thiocyanate ion to cobalamin were calculated from equilibrium spectrophotometric titrations and from rate constants calculated from the kinetics experiments. These data are presented in Figure 1 and Table I.

Discussion

A simple mechanism for the binding of thiocyanate ion to cobalamin is

$$CBM^1 + SCN^- = CBM\text{-}SCN \qquad (1)$$

This mechanism corresponds to the SN2(lim) mechanism which has been considered for anions binding to inorganic Co(III) complexes (Basolo and Pearson, 1958). For this mechanism, the relaxation time and rate constants are related by

$$1/\tau = k_{+1app}[\overline{(CMB)} + \overline{(SCN^-)}] + k_{-1app} \qquad (2)$$

where $\overline{(CBM)}$ and $\overline{(SCN^-)}$ are the equilibrium concentrations of CBM and SCN^-, respectively. In the temperature-jump experiments reported here, the total thiocyanate concentration was always considerably greater than the total cobalamin concentration. Therefore $\overline{(CBM)}$ is negligible in eq 2 and $\overline{(SCN^-)}$ may be

[1] Abbreviations used: CBM, cobalamin in the aquo and hydroxo forms; CBM-OH_2, aquocobalamin; CBM-OH, hydroxocobalamin; CBM^-, the hypothetical intermediate containing five bound groups; τ, the relaxation time for the ligand binding reaction.

TABLE I: Rate Constants and Dissociation Constants for the Thiocyanate–Cobalamin Reaction.

pH	Exptl Rate Constants with Std Dev		Dissociation Constants with Std Dev	
	$k_{+1app} \times 10^{-3}$ M^{-1} sec^{-1}	k_{-1app} sec^{-1}	Kinetic $K^a \times 10^4$ M	Equil $K^b \times 10^4$ M
2.70	7.8 ± 0.6	0.8 ± 0.6	1 ± 1.6	
3.20	5.8 ± 0.6	2.7 ± 0.8	4.5 ± 1.9	
3.40	7.6 ± 0.4	1.5 ± 0.5	2.0 ± 0.8	
4.00	6.1 ± 0.5	1.9 ± 0.7	3.0 ± 1.4	4.1 ± 0.2
4.00	8.3 ± 0.5	1.9 ± 0.7	2.3 ± 1.0	
4.80	7.9 ± 0.7	1.3 ± 0.7	1.6 ± 1.0	
5.00	7.1 ± 1.0	1.8 ± 1.0	2.0 ± 1.8	
5.20				4.3 ± 0.2
5.80	7.0 ± 0.4	1.0 ± 0.6	1.4 ± 0.9	
6.00	7.4 ± 0.2	2.3 ± 0.3	3.1 ± 0.5	
6.10	6.0 ± 0.6	2.7 ± 0.8	4.5 ± 2	
6.20	6.6 ± 0.4	1.5 ± 0.5	2.3 ± 0.9	
6.40				4.6 ± 0.1
6.60	5.9 ± 0.1	1.1 ± 0.2	1.9 ± 0.4	
6.84	6.3 ± 0.3	1.9 ± 0.5	3.0 ± 0.9	
6.95	5.4 ± 0.2	2.0 ± 0.3	3.7 ± 0.7	
7.00	5.3 ± 0.5	2.6 ± 0.8	5 ± 2	6.1 ± 0.3
7.20	5.0 ± 0.2	2.2 ± 0.3	4.4 ± 0.6	5.9 ± 0.2
7.40				7.6 ± 0.2
7.50	3.4 ± 0.2	2.4 ± 0.3	7 ± 1	
7.50	3.5 ± 0.2	1.5 ± 0.3	4.3 ± 1.3	
7.60				8.0 ± 0.6
7.80	2.2 ± 0.2	1.4 ± 0.5	6.0 ± 0.9	
7.80	2.9 ± 0.1	1.8 ± 0.2	6.2 ± 1	
7.97	2.2 ± 0.1	1.9 ± 0.3	9 ± 1	
8.40	1.1 ± 0.1	1.7 ± 0.2	16 ± 3	30 ± 3
9.00	0.12 ± 0.01	1.2 ± 0.1	100 ± 20	
10.00	0.03 ± 0.003	0.9 ± 0.05	300 ± 40	

[a] Determined from the rate constants by means of eq 4. [b] Determined from equilibrium spectrophotometric titrations.

replaced by the total thiocyanate concentration.

$$\overline{(SCN^-)} + \overline{(CBM)} = (SCN^-)_0 \quad (3)$$

A first approximation of the k_{+1app} value at any given pH value may be obtained from the slope of the $1/\tau$ *vs.* $(SCN^-)_0$ plots. Likewise, the intercept may be calculated to give a first approximation to the k_{-1app} value. The dissociation constant can then be computed from

$$K = k_{-1app}/k_{+1app} \quad (4)$$

From the calculated dissociation constant and the known values of $(SCN^-)_0$ and $(CBM)_0$ values of $\overline{(SCN^-)} + \overline{(CBM)}$ may be calculated. A second approximation to k_{+1app} and k_{-1app} may then be obtained from the slope and intercept of the $1/\tau$ *vs.* $\overline{(SCN^-)} + \overline{(CBM)}$ plot. This procedure is repeated until the K, k_{+1app}, and k_{-1app} values converge. In practice, these values converge rapidly with only small changes in the slope and intercept values.

The stopped-flow experiments were conducted with a thiocyanate concentration so much greater than the cobalamin concentration that pseudo-first-order conditions were obtained. In this case, k_{+1app} and k_{-1app} values are obtained directly from the slope and intercept of the $1/\tau$ *vs.* $(SCN^-)_0$ plots.

Table I contains the values of k_{+1app} and k_{-1app} obtained at various pH values. It can be seen that the k_{+1app} values vary with pH while the k_{-1app} values show a considerable amount of experimental error with no significant pH dependence. The average value of k_{-1app} is 1.8 ± 0.6 sec^{-1}.

The dependence of k_{+1app} on pH means that a more complex mechanism than 1 must be considered. The next step would be to include in the reaction mechanism the ionization of aquocobalamin to form hydroxocobalamin.

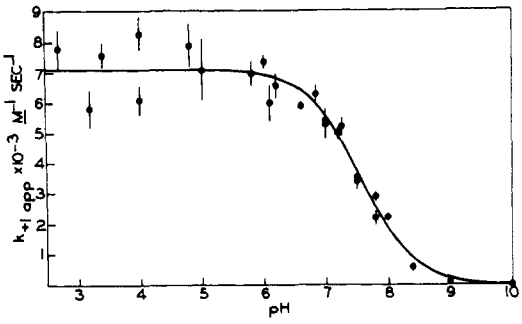

FIGURE 2: Plot of k_{+1app} *vs.* pH for the binding of thiocyanate anion to aquocobalamin. The vertical straight lines indicate the standard deviation associated with each point. The solid line was computed from eq 10 using $k_{+1} = 7.1 \times 10^3\ M^{-1}\ sec^{-1}$ and $pK_0 = 7.55$.

$$\begin{array}{ccc} SCN^- + CBMOH_2 & \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} & CBM\text{-}SCN \\ \downarrow\uparrow K_0 & & \\ SCN^- + CBMOH + H^+ & \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} & CBM\text{-}SCN + OH^- \end{array} \qquad (5)$$

Provided that the protolytic step is adjusted rapidly and the solution is buffered the relation between $1/\tau$ and the equilibrium concentrations of SCN^- and CBM is again given by eq 2. In this case, however, k_{+1app} and k_{-1app} are pH dependent.

$$k_{+1app} = \frac{k_{+1}}{1 + K_0/(H^+)} + \frac{k_{+2}}{1 + (H^+)/K_0} \qquad (6)$$

Here,

$$K_0 = \overline{(CBMOH)}\ \overline{(H^+)}/\overline{(CBMOH_2)} \qquad (7)$$

$$k_{-1app} = k_{-1} + \frac{k_{-2}K_w}{(H^+)} \qquad (8)$$

K_w is the ion product for water. The dissociation rate constant, k_{-1}, has been written as a first-order rate constant since the water concentration does not change during the reaction. The values of k_{+1app} are plotted *vs.* pH in Figure 2. According to eq 6, the k_{+2app} values should approach k_{+1} at low pH and k_{+2} at high pH. From Figure 2 and Table I, it can be seen that k_{+2} must be effectively zero compared to k_{+1}. Likewise, $k_{-2}K_w/(H^+)$ must be effectively zero compared to k_{-1}. Thus mechanism 5 may be simplified to

$$\begin{array}{ccc} CBMOH_2 + SCN^- & \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} & CBM\text{-}SCN \\ \downarrow\uparrow K_0 & & \\ CBMOH + H^+ & & \end{array} \qquad (9)$$

For this mechanism

$$k_{+1app} = \frac{k_{+1}}{1 + K_0/(H^+)} \qquad (10)$$

The results obtained from the best fit of the measured values of k_{+1app} to eq 10 are $k_{+1} = 7.1 \pm 0.3 \times 10\ M^{-1}\ sec^{-1}$ and $pK_0 = 7.55 \pm 0.2$. The value of pK_0 obtained from the rate data at ionic strength 0.054 thus agrees reasonably well with the value 7.72 reported for an ionic strength of 0.042 at 25° (Hanania and Irvine, 1964) and with the value 7.8 reported for a solution containing 0.05 M phosphate buffer at unspecified temperature (Hayward *et al.*, 1965).

The relation between the observed equilibrium constants and hydrogen ion concentration is

$$K = K_{dis}(1 + K_0/[H^+]) \qquad (11)$$

where

$$K_{dis} = \frac{\overline{(CBMOH_2)}\ \overline{(SCN^-)}}{\overline{(CBM\text{-}SCN)}} \qquad (12)$$

Equation 11 may be fit to the K values found both by means of the equilibrium spectrophotometric titration procedure and by means of the ratio k_{-1app}/k_{+1app}. When this is done, one obtains $K_{dis} = 4.4 \pm 0.4 \times 10^{-4}$ M and $pK_0 = 7.6 \pm 0.1$ from the equilibrium values and $K_{dis} = 2.8 \pm 0.5 \times 10^{-4}$ M and $pK_0 = 7.7 \pm 0.2$ from the rate data.

It can be seen from Figure 1 that the values of K determined from equilibrium experiments are consistently larger than those determined from kinetics data. The considerable error in k_{-1app} makes it difficult to tell if this difference is significant.

The rate constants for replacement of an anion by a water molecule have been determined for several octahedral Co(III) complexes (Basolo and Pearson, 1958; Ingold *et al.*, 1960). These rate constants are all much smaller, generally by several orders of magnitude, than the k_{-1app} values reported in this paper. The increase in the rate constant for aquation of thiocyanato-cobalamin compared to the simpler Co(III) complexes may be related to the observation (Williams, 1961) that the nitrogen end of the thiocyanate ion is bound to simple Co(III) complexes, but the sulfur end is bound to the cobalamin complex.

Another possible mechanism involves the dissociation of the water molecule coordinated to the Co(III) atom in aquocobalamin to produce a pentacoordinated intermediate. This intermediate species could then react with the thiocyanate anion. Mechanism 13 corresponds to the SN1 mechanism considered for anion replacement reactions in simpler Co(III) complexes

$$\begin{array}{ccc} CBMOH_2 & & \\ {\scriptstyle k_{+2}}\uparrow\downarrow{\scriptstyle k_{-2}} & & \\ CBM- + SCN^- & \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} & CBM\text{-}SCN \\ + & & \\ H_2O & & \end{array} \qquad (13)$$

(Basolo and Pearson, 1958). In general, mechanism 13 would give rise to two relaxations in temperature-jump

studies. When one of the two steps proceeds so rapidly that it remains at equilibrium with respect to the other step, only one relaxation will be observed.

If the release of the water molecule is considered to be much slower than the reaction with the thiocyanate anion, the reciprocal of the relaxation time is given by

$$1/\tau = k_{+2} + \frac{k_{-2}}{1 + (\overline{SCN^-})/K_1} \quad (14)$$

$$K_1 = \frac{(\overline{CBM^-})(\overline{SCN^-})}{(\overline{CBM\text{-}SCN})} \quad (15)$$

Equation 14 indicates that $1/\tau$ should increase with decreasing values of $(\overline{SCN^-})$ and approach a limiting value for $(\overline{SCN^-}) = 0$. This is not observed.

If the release of the water molecule in mechanism 13 is considered to be at equilibrium with respect to the reaction of the pentacoordinated intermediate with the thiocyanate anion, the reciprocal of the relaxation time is given by

$$1/\tau = \frac{k_{+1}}{1 + K_2}[(CBM) + (SCN^-)] + k_{-1} \quad (16)$$

$$K_2 = \frac{(\overline{CBMOH_2})}{(\overline{CBM^-})} \quad (17)$$

This is identical in form with eq 2 with

$$k_{+1app} = k_{+1}/(1 + K_2) \quad (18)$$

When the aquocobalamin–hydroxo–cobalamin transition is introduced

$$\begin{array}{l} CBMOH + H^+ \\ \downarrow\uparrow K_0 \\ CBMOH_2 \\ \downarrow\uparrow \\ CBM- + SCN^- \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} CBM\text{-}SCN \end{array} \quad (19)$$

the resulting pH dependence of k_{+1app} would be

$$k_{+1app} = \frac{k_{+1}}{(1 + K_2[1 + K_0/(H^+)])} \quad (20)$$

For $K_2 \gg 1$, this becomes identical in form with eq 10. Therefore, in this limit, it is not possible to distinguish between the SN2 and the SN1 mechanism.

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