A spectrophotometric determination of the binding constants of cyanide by hemin in aqueous ethanol solutions

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Abstract

Binding constants for the coordination of cyanide by monomeric hemin in aqueous ethanol solution were determined in the pH range 8–12 by fitting spectrophotometric data to a binding isotherm using a non-linear least-squares method. Two cyanide ligands are coordinated by Fe(III) without evidence for the intermediate mono-ligated species. The binding constants are strongly pH-dependent because of protonation of the ligand and competition for the metal ion by hydroxide. From the pH dependence of the observed binding constants, a pH-independent value of log $K = 17.62 \pm 0.03$ for the binding of two CN^- ligands by monomeric hemin in alkaline solution at 25 °C, $\mu = 0.100$ M, is obtained.

Introduction

Hemin (ferriprotoporphyrin IX) undergoes aggregation in aqueous solution [1, 2], making the study of the kinetics and thermodynamics of ligand substitution reactions — information which is of considerable interest due to the widespread occurrence of iron porphyrins in nature — difficult and the results unreliable [3–6]. Detergents have been used to try to circumvent these effects [7–9], but the nature and concentration of the detergent itself appears to affect the values obtained [10]. An alternative method is to use mixed solvent systems to suppress aggregation effects [11–14].

As demonstrated with the heme octapeptide from cytochrome *c* microperoxidase-8 (MP-8) [15, 16], which contains a single His residue in the coordination sphere (the other axial coordination site is occupied by H_2O/OH^-), Fe(III) coordinated in its equatorial plane by a porphyrin and in its axial positions by a single strong-field ligand, is high spin. When strong-field ligands such as pyridines [17], imidazoles [18, 19] and cyanide [11, 17] are added to a solution of a free Fe(III) porphyrin such as hemin, two ligands appear to be coordinated simultaneously without formation of the intermediate mono-ligand complex. This means that K_2 (for the binding of the second ligand to the mono-ligated species) $> K_1$ (for the binding of the first ligand) and is probably a consequence of the change in spin state which accompanies the coordination of the second axial ligand. Mixed-ligand complexes, where both ligands are strong-field ligands, such as imidazole and cyanide, can be prepared [20].

In aqueous solution above pH 8, hemin exists as a dimer [21, 22] but is monomeric in 44% (vol./vol.) ethanol–water mixtures [11]. Two water molecules are coordinated by Fe(III) and their acid dissociation constants are 6.5–6.6 [11, 23] and >13 [11]. Hence, under mildly alkaline conditions in this solvent system, hemin exists as the aqua–hydroxo complex, and OH^- necessarily has to be displaced from the coordination sphere on binding a strong-field ligand. The apparent binding constant, K_{obs}, measured for the binding of a ligand, L (eqn. (1), in which only the axial ligands are shown and the overall charge is ignored for convenience), is expected to be strongly dependent on pH. Binding constants for cyanide to monomeric hemin are available; Burger *et al.* [17] studied the reaction in 1:1 to 4:1 methanol–water solutions, finding that the percentage of non-aqueous solvent had no effect on their results. However, the reactions appear to have been done in unbuffered solutions and because of the strong pH-dependence of K_{obs} their results have limited utility. Davies [11] studied the reactions in 44% ethanol as a function of pH and demonstrated the expected pH-dependence in the pH range 12–15. From these data (Fig. 7 of ref. 11) we estimate that the pH-independent formation constant for binding of two CN^- ligands to hemin at 25 °C, log $K = 17.7 \pm 1.1$.

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$$H_2O \cdot Fe^{III} \cdot OH^- + 2L \rightleftharpoons FeL_2 + OH^- \quad (1)$$

The spectrophotometric determination of the binding of ligands to hemin is often done by linear regression methods; this requires a knowledge of the starting and, in particular, the equilibrium absorbance (or coefficients of absorbance) of all species at the monitoring wavelength and any uncertainty in these two values will have a profound effect on the value determined. Further, it is often assumed that the fraction of bound ligand is insignificant compared to the total ligand concentration in solution. In this report, these problems are circumvented by deriving a binding isotherm to which the experimental data are fitted by a non-linear least-squares method. By determining the binding constants as a function of pH over a wide pH range which encompasses the pK_a of cyanide, it is demonstrated that only CN^- and not HCN is coordinated by hemin and, from a fit of the K_{obs}–pH curve, a pH-independent binding constant for the coordination of two CN^- ligands by hemin is determined.

Experimental

All work was done in 50% (vol./vol.) absolute ethanol:water solutions. The acid dissociation constant, pK_L, of HCN was determined by glass electrode potentiometry using a 0.100 M KCN solution and titrating with 0.100 M HCl (prepared by diluting a 0.200 M HCl solution 50% with ethanol) using a Metrohm 605 ion/pH meter. Hemin solutions were prepared by dissolving solid hemin (BDH) in 0.050 M NaOH and diluting with a suitable buffer solution (Tris/HCl or carbonate, $\mu = 0.1$ M; at high pH unbuffered NaOH solutions were used). The concentration was determined using $\epsilon = 58.0 \times 10^3\ M^{-1}\ cm^{-1}$ [24]. All pH readings were glass electrode readings uncorrected for the effect of the non-aqueous solvent. Cyanide solutions were freshly prepared just prior to the experiments by dissolving potassium cyanide (Merck) in the appropriate buffer solution. The coordination of cyanide by hemin was determined spectroscopically by following the absorbance change at 426.5 nm on addition of an aliquot of the cyanide solution to 2.50 ml hemin solution (8.45×10^{-6} M) in a 1 cm pathlength cuvette, thermostatted in the cell block of a Cary 2300 UV–Vis spectrometer by means of an external circulating water bath. Between 10 and 15 ligand aliquots were added with an Hamilton microsyringe (to a total volume of <50 μl) and the absorbance corrected for dilution effects. All experimental data were fitted using a non-linear least-squares computer programme utilising a Newton–Raphson procedure.

Results and discussion

The acid dissociation constant, pK_L, for HCN in 50% (vol./vol.) ethanol:water at 25 °C was found to be 9.94 ± 0.01. (Titrations were also performed at 5 and 45 °C; the pK_L at these temperatures was 10.37 ± 0.02 and 9.64 ± 0.02, respectively, from which it can be determined that $\Delta H = 31.7 \pm 1.2$ kJ mol^{-1} and $\Delta S = -84 \pm 4$ J K^{-1} mol^{-1}). Hence, as expected, pK_L increases with the non-aqueous nature of a solution, viz. from 9.04 in pure H_2O [25] to 9.76 in 20% MeOH [26], to 9.94 in 50% ethanol.

For the general ligand substitution reaction of a transition metal ion (eqn. (2)), the equilibrium constant, K, is defined by eqn. (3).

$$M + nL \rightleftharpoons Z \quad (2)$$

$$K = \frac{[Z]}{[M][L]^n} \quad (3)$$

where M represents a complex of a transition metal ion, and L is an incoming ligand. It is readily shown that

$$[Z] = \frac{A - A_0}{(\epsilon_Z - \epsilon_M)b}$$

$$[M] = \frac{A_\infty - A}{(\epsilon_Z - \epsilon_M)b}$$

where A is the equilibrium absorbance at some wavelength observed for a cell pathlength of b cm when [L] is the free ligand concentration in solution, A_0 and A_∞ are the absorbances at the same wavelength corresponding to 0% and 100% formation of the complex Z, respectively, and ϵ_i is the molar absorptivity of the species i. It therefore follows that

$$\frac{A - A_0}{A_\infty - A} = K[L]^n$$

from which the usual linear methods of determining K follow (e.g. a plot of log $[(A - A_0)/(A_\infty - A)]$ versus log[L] is a straight line with slope $= n$ and intercept $=$ log K). If K is not very large, $[L]_{total} \approx [L]_{free}$, and the value of K obtained is reasonable provided A_0 and A_∞ are known with high certainty.

In the case of cyanide, $n = 2$ but the binding isotherm derived below is readily generalised to any value of n. When K is not small such that for any given $[L]_{total}$ a significant fraction of L is coordinated, then

$$[L]_{total} = [L]_{free} + [L]_{bound}$$

$$= [L]_{free} + 2[M]_{total}\left(\frac{A_0 - A}{A_0 - A_\infty}\right)$$

If we define

$$\alpha = \left(\frac{2[M]_{total}}{A_0 - A_\infty}\right)$$

it follows, after expansion and grouping of terms, that

$$A^3 + a_1A^2 + a_2A + a_3 = 0 \qquad (6)$$

where

$$a_1 = \frac{2([L]_{total} - \alpha A_0) - \alpha A_\infty}{\alpha}$$

$$a_2 = \frac{1 + K(\alpha^2 A_0(2A_\infty + 1) - 2[L]_{total}\alpha(A_\infty + A_0) + [L]^2_{total})}{K\alpha^2}$$

$$a_3 = \frac{A_0(K\alpha A_\infty(2[L]_{total} - \alpha A_0) - 1) - K[L]^2_{total}A_\infty}{K\alpha^2}$$

The cubic eqn. (6) is solved iteratively by Newton's method, i.e.

$$A_{k+1} = A_k - \left[\frac{A^3 + a_1A^2 + a_2A + a_3}{3A^2 + 2a_1A + a_2}\right]$$

until, typically, $(A_{k+1} - A_k) < 0.0002$, which is about the noise level in absorbance of the spectrometer used. The experimental data (values of A as a function of $[L]_{total}$) are fitted by non-linear least-squares methods with A_0, A_∞ and K as variables.

An examination of the variation of the change in absorbance at 395 nm as a function of hemin concentration (up to 1.2×10^{-4} M) at pH 9 and 12, in 10, 5, 1 and 0.1 cm pathlength cells showed that Beer's law is obeyed ($\epsilon = 8.99 \pm 0.04 \times 10^4$ M^{-1} cm^{-1} at pH 9 and $9.30 \pm 0.03 \times 10^4$ M^{-1} cm^{-1} at pH 12) and hence strongly suggests that hemin, at least up to a concentration of 1.2×10^{-4} M, is monomeric in this solvent system.

A typical set of spectra for a single titration is shown in Fig. 1. Clearly defined isosbestic points are evident at 302, 411, 478, 520, 575 and 653 nm. Figure 2 shows a typical fit of the binding isotherm (eqn. (6)) to the data from a spectrophotometric titration.

Between pH 8 and 12 in 50% ethanol solution, hemin exists as the monomeric aqua–hydroxo complex. The binding of two CN^- ligands by monomeric hemin (eqn. (1), $L = CN^-$) is governed by an equilibrium constant, K; at any given pH, the observed binding constant is K_{obs} (eqn. (7)).

$$K = \frac{[Fe(CN^-)_2]}{[H_2O \cdot Fe \cdot OH^-][CN^-]^2[H^+]}$$

$$K_{obs} = \frac{[Fe(CN^-)_2]}{[H_2O \cdot Fe \cdot OH^-][CN^-]^2_{total}} \qquad (7)$$

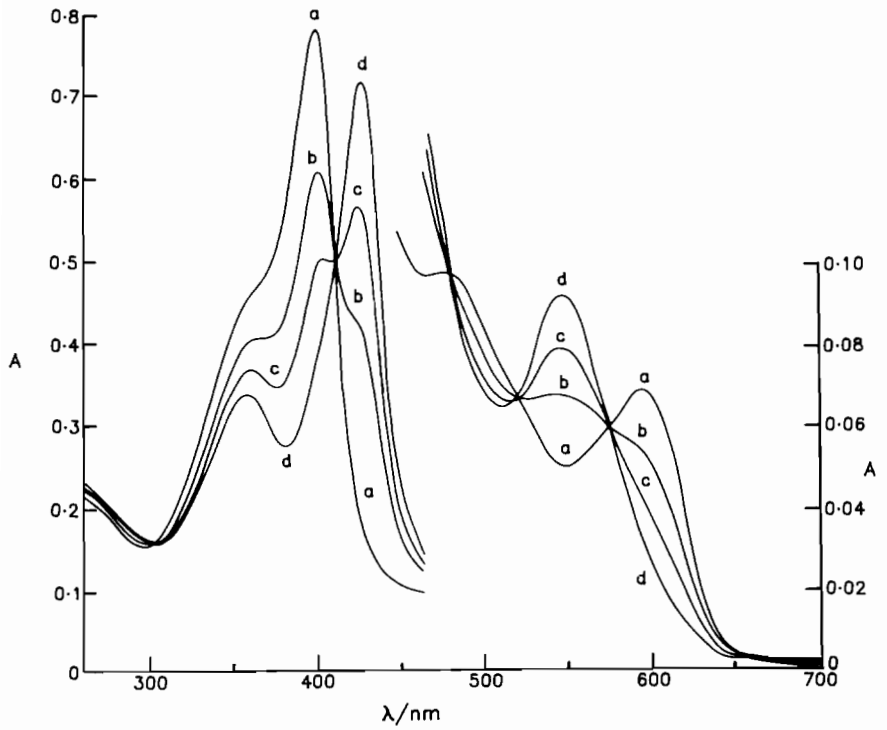

Fig. 1. Spectrophotometric titration of hemin (8.45×10^{-5} M) at pH 12.0 in 50% aqueous ethanol solution with cyanide: a, hemin; b, +1.49 mM CN^-; c, +2.49 mM CN^-; d, +6.44 mM CN^-.

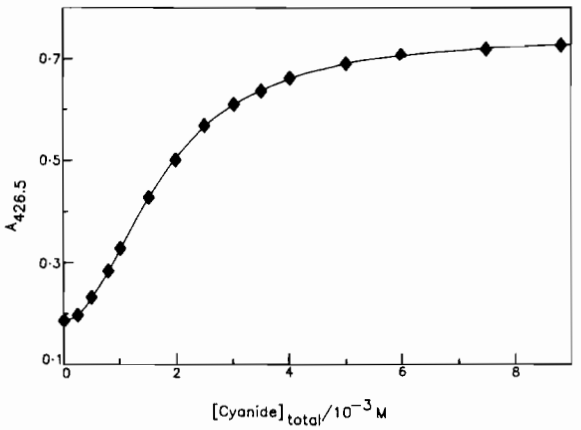

Fig. 2. Variation in $A_{426.5}$ with cyanide concentration at pH 12.0. The solid line is a fit of eqn. (6) of the text to the experimental data.

TABLE 1. Observed binding constants (log K_{obs}) as a function of pH for the binding of two CN^- ligands by hemin in 50% ethanol solution, 25 °C, $\mu = 0.100$ M

pH	log K_{obs}
8.00	5.64 ± 0.01
8.50	6.14 ± 0.01
9.00	7.04 ± 0.03
9.50	7.06 ± 0.01
10.00	6.94 ± 0.01
10.50	6.47 ± 0.01
11.00	6.47 ± 0.01
11.50	6.13 ± 0.01
12.00	5.50 ± 0.01

This pH range encompasses the acid dissociation constant of cyanide (pK_L). If it is assumed that CN^- but not HCN is coordinated by hemin, then the expression for K_{obs} must be modified (eqn. (8)) which leads to a final expression (eqn. (9), or, in logarithmic form, eqn. (10)) for the pH-independent binding constant for the simultaneous coordination of two CN^- ligands by hemin.

$$K_{obs} = \frac{[Fe(CN^-)_2]}{[H_2O \cdot Fe \cdot OH][CN^-]^2\left(1 + \frac{[H^+]}{K_L}\right)^2} \quad (8)$$

$$K = \frac{K_{obs}\left(1 + \frac{[H^+]}{K_L}\right)^2}{[H^+]} \quad (9)$$

$$\log K_{obs} = \log K + \log[H^+] - 2\log\left(1 + \frac{[H^+]}{K_L}\right) \quad (10)$$

The experimental values of K_{obs} as a function of pH are given in Table 1. These were fitted (Fig. 3) to

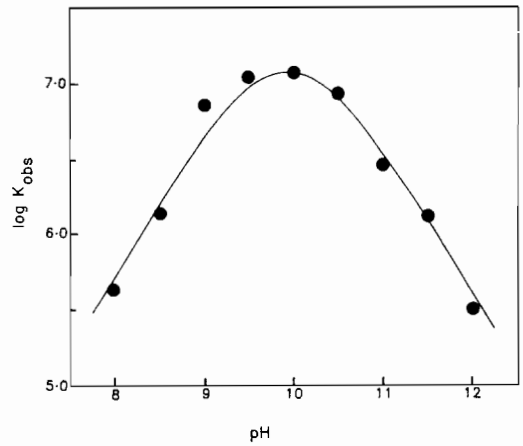

Fig. 3. Dependence of log K_{obs} (eqn. (7) of the text) on pH. The solid line is a fit of eqn. (10) of the text to the data, yielding log $K = 17.62 \pm 0.03$ for the pH-independent binding of two CN^- ligands by monomeric hemin in alkaline aqueous ethanol solution.

eqn. (10) using a non-linear least-squares method with log K as sole variable; from this fit it was found that log $K = 17.62 \pm 0.03$ for the binding of two CN^- to hemin.

The existence of well-defined isosbestic points (Fig. 1) and the very good fits obtained for eqn. (6) which was derived assuming the simultaneous binding of two ligands by Fe(III) (Fig. 2) serves as further evidence that $K_2 > K_1$ when the incoming ligand, L, is a strong-field ligand. This study has confirmed that equilibrium constants for the binding of cyanide by hemin are strongly dependent on pH. K_{obs} is a maximum when $dK/d[H^+] = 0$, i.e. when $pH = pK_L$, and decreases at lower pH values because of protonation of the ligand, and at higher pH values because of competition by OH^- for the metal ion. The generally adequate fit of eqn. (9) to the experimental data (Fig. 3) is proof of the validity of this equation and hence demonstrates that only CN^-, and not HCN, is coordinated by Fe(III). The value obtained for the pH-independent binding constant, log $K = 17.62 \pm 0.03$, is in excellent agreement with the value deduced from the results of Davies (log $K = 17.7 \pm 1.1$) [11] in 44% ethanol solutions of apparently variable ionic strength.

Acknowledgements

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