Coordination of N-donor ligands by hematohemin

Helder M. Marques*, Orde Q. Munro and Megan L. Crawcour
Centre for Molecular Design, Department of Chemistry University of the Witwatersrand, P.O. Wits, 2050 Johannesburg (South Africa)

(Received January 23, 1992; revised March 30, 1992)

Abstract

Ferrihematoporphyrin-IX, prepared by insertion of iron into hematoporphyrin-IX, is shown to be high-spin and monomeric in methanol. Equations which describe the variation of absorbance at a fixed wavelength with increasing ligand concentration from which binding constants for the coordination of ligands by the porphyrin can be determined spectroscopically, are derived. It is shown that N-donor ligands, L (L = ethanolamine, imidazole, *N*-acetyl imidazole, and pyridine) bind at 25 °C in a stepwise manner, to form initially the high-spin mono(L) intermediate before a second ligand is bound to form the low-spin bis(L) complex. The binding constants K_1 and K_2 for the two steps can be determined, although the results are dependent on the monitoring wavelength because of the very small spectroscopic changes accompanying the coordination of the first ligand. There is a linear relationship between the magnitude of the binding constant for the first ligand and the pK_a of the ligand, but no such simple relationship is found for binding of the second ligand. Some factors which are likely to control the tendency of the mono(L) intermediate to bind a second ligand are discussed. Only one benzimidazole ligand is coordinated because of steric interaction with the porphyrin.

Introduction

The coordination chemistry of iron porphyrins has attracted considerable interest because of the widespread occurrence of the hemoproteins and studies of their properties have been prompted at least partially by the belief that an understanding of the chemistry of the protein-free prosthetic group will lead to a better understanding of how the protein controls and modifies these in a hemoprotein.

We have been interested in the coordination of imidazoles and their derivatives by iron porphyrins and have reported on the coordination of histidine, histamine and pilocarpate by ferriprotoporphyrin-IX (hemin) [1–3]. In the course of our studies we found that hemin exists in aqueous solution as a dimer. The dimer reacts with one equivalent of the imidazole ligand to form a donor–acceptor complex which, on increasing the ligand concentration, breaks up to form the monomeric bis(imidazole) complex without evidence for the formation of the mono(imidazole) intermediate.

Ferric porphyrins have two axial coordination sites available; provided the first ligand which coordinates is not an exceptionally good π-acceptor such as CO and NO, a second ligand will coordinate in the *trans* position [4]. In principle, axial ligation of an iron porphyrin, M occurs in two steps (eqn. (1)). (Unless otherwise indicated, the departing ligand is assumed to be either solvent or some weak field ligand such as Cl^-, and the charge is neglected for convenience.)

$$M + L \rightleftharpoons ML \quad (K_1)$$
$$ML + L \rightleftharpoons ML_2 \quad (K_2) \qquad (1)$$

There have been many reports in the literature of workers failing to observe the formation of the mono(ligated) intermediate, so that the reaction appears to proceed in a single step from M to ML_2 (eqn. (2)) which implies that $K_1 < K_2$.

$$M + 2L \rightleftharpoons ML_2 \quad (\beta_2) \qquad (2)$$

Scheler appears to have been the first to report that on reaction of hemin in slightly acidified H_2O:DMF solutions with imidazole only the bis(imidazole) complex is formed [5]. This observation was subsequently confirmed in aqueous alkaline solution [6], aqueous ethanol solutions [7, 8], in surfactant micelles in aqueous and non-aqucous solutions [9, 10], and in aqucous, aqueous–organic or organic solutions either with free imidazole [11–14] or with imidazole bound to a polymer [15]. Similar observations have been reported with other ligands, for example, with cyanide [16, 17] and with *cis*- and *trans*-urocanic acids [18].

Evidence for the mono(ligated) complex apparently requires rather special conditions. Harel and Felton [19] found evidence that reaction of imidazole with a

*Author to whom correspondence should be addressed.

0020-1693/92/$5.00

bifacially-hindered ferric porphyrin leads to coordination of one imidazole, and retention of OH^- as *trans* ligand which is hydrogen bonded to a second imidazole. The synthesis of a deuterohemin to which an undecapeptide was attached through one of the carboxylate side-chains of the porphyrin has recently been reported [20]. In this case $\log K_1 > \log K_2$ (3.2 versus 2.3) for binding of imidazole, and it was suggested that this is due to steric hindrance of one face of the porphyrin by the polypeptide. Fe(III)(TPP)(SbF_6) is reported to react differently to other ferric tetraphenylporphyrin salts in allowing a stepwise addition of imidazole [21].

Tohjo and Shibata [22] predicted that the mono(ligated) species of a ferric porphyrin with a ligand of moderate field strength such as imidazole would be high-spin. The bis(ligated) complex of an N-donor is low-spin, so coordination of the second ligand is accompanied by a change in spin state. It seems reasonable to expect that the smaller low-spin ferric ion, without electrons in the e_g^* orbitals will coordinate ligands more strongly than the high-spin analogue [12]. It is therefore this drive towards a change in spin state which is generally accepted to lead to $K_1 < K_2$.

Situations where the mono(ligated) intermediate has been observed are not without precedent, however. Brault and Rougee [23] obtained direct evidence for the stepwise coordination of imidazole by a ferrous porphyrin in organic solvents, with $\log K_1 \approx 3.5$ and $\log K_2 \approx 4.6$. Walker *et al.* [24] demonstrated the stepwise addition of two ligands to Fe(TPP)Cl in chloroform with the observation of shifts in the isosbestic points of the UV–Vis spectrum. They attempted to determine values of $\log K_1$ and $\log K_2$ from plots of $\log(A - A_0)/(A_\infty - A)$ against log[ligand], but analyses such as this suffer from not knowing the appropriate value of the absorbance for the intermediate at the monitoring wavelength.

It is clear that the reaction of Fe(III) porphyrins with ligands of intermediate field strength such as imidazole tend to form the bis(ligated) complex with identification of the mono(ligated) species a rare occurrence. We have reported on the preparation [25] and characterisation [26] of the Fe(III) hemeoctapeptide, microperoxidase-8 (MP8), obtained from the proteolytic cleavage of cytochrome *c*. MP8 contains a high-spin Fe(III) porphyrin coordinated in one axial coordination site by histidine and in the second by H_2O. A decrease in pH leads to protonation and release of histidine. This system therefore allows for an unequivocal characterisation of the electronic spectrum of an iron porphyrin coordinated by a single intermediate field ligand. The spectra of this Fe(III) porphyrin with, and without, a single histidine in the coordination sphere are quite similar. Release of histidine results in the Soret band shifting from 397.2 to 394.9 nm with a 13% increase in intensity; the only significant change in the visible region is the band at 622 nm which shifts to 619 nm, and increases 20% in intensity. It is only on addition of a second intermediate field ligand such as imidazole [26] that a pronounced change in the appearance of the spectrum occurs as the metal changes spin state.

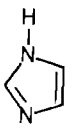
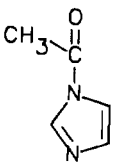
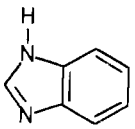
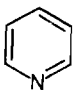
It is therefore possible that workers who are unaware of the very subtle spectral changes accompanying the coordination of a single ligand by a ferric porphyrin might miss the reaction and report direct formation of the bis(ligated) complex. We decided to reexamine the coordination of ligands by a monomeric porphyrin. Five N-donor ligands (ethanolamine, imidazole, *N*-acetyl imidazole, benzimidazole and pyridine) were chosen (Table 1). Since a quantitative study of the reactions of hematohemin with ligands appears not to have been reported, we decided to use this ferric porphyrin (which is very soluble in methanol) for our study. A non-aqueous solvent was chosen to minimise porphyrin aggregation which would complicate the analysis of the results.

Experimental

Materials

Hematohemin dihydrochloride was purchased from Sigma. Methanol (BDH), imidazole, pyridine (Merck)

TABLE 1. Ligands studied in this work, and their acid dissociation constants (in aqueous solution)

Ligand	Structure	pK_a	Reference
Imidazole		7.24	27
N-Acetyl imidazole		4.51	28
Benzimidazole		5.47	29
Pyridine		5.24	30
Ethanolamine	$H_2NCH_2CH_2OH$	9.50	31

and *N*-acetyl imidazole (Aldrich) were of the highest purity available and used as received. Benzimidazole (Merck) was recrystallised from hot toluene; ethanolamine (BDH) was purified by fractional distillation.

Synthesis of hematohemin

Hematohemin was prepared by insertion of iron into hematoporphyrin-IX by an adaptation of a literature procedure [32]. To 100 ml of a stirred pyridine/acetic acid solution (1:50 vol./vol.) under N_2 was added 1 g of hematoporphyrin-IX dihydrochloride. After heating to 80 °C, 1.2 ml saturated $FeSO_4$ was added and stirring continued for 10 min. The solution was opened to the atmosphere and cooled to room temperature. Small volumes of the reaction mixture (*c.* 20 ml) were repeatedly extracted with ether (*c.* 50 ml per extraction), the ether fractions combined, and washed twice with 20% (wt./vol.) HCl. The aqueous layer was bright purple, indicating the presence of substantial amounts of unmetallated porphyrin. The ether layer was washed twice with cold water, then shaken with two volumes of water, and allowed to stand for 1 h. Hematohemin precipitates as the hydroxide complex [32]. The precipitate was collected and dried *in vacuo* over NaOH until a constant mass was recorded (*c.* 3 days).

Analysis of hematoporphyrin-IX and hematohemin

Hematoporphyrin is usually prepared by treatment of protohemin with HBr in acetic acid [33]; it is likely to be contaminated with protoporphyrin and monovinyl-monohydroxyethyl porphyrins. The purity of hematoporphyrin-IX was investigated by HPLC using a Spectra Physics 8800 ternary gradient pump, a Linear 200 UV–Vis detector (400 nm) and a 10 cm×4.6 mm, 5 μm unmodified silica column (Brownlee Laboratories) with a MeOH:toluene gradient at a constant flow rate of 1 ml min^{-1} (10% MeOH to 20% MeOH linearly over 5 min, then linearly to 90% MeOH over the next 10 min).

The purity of hematohemin was determined on the same HPLC system but with detection at 395 nm. A sample (1 mg ml^{-1}) of the product in 5% wt./vol. imidazole in MeOH was eluted on the same silica column using MeOH as mobile phase (1 ml min^{-1}).

ESR spectroscopy

The ESR spectrum of hematohemin-IX was recorded on 3.3 mg of solid hematohemin in a 3 mm diameter quartz tube immersed in liquid N_2 in a Dewar flask constructed to fit the cavity of a Varian E-Line Century Series X-band spectrometer coupled to an HP 9121 Data Station.

UV–Vis spectroscopy

All UV–Vis spectra were recorded in 1 cm pathlength cuvettes fitted into the thermostatted cell holder of either a Cary 2300 or a Cary 219 recording spectrophotometer.

Determination of binding constants

The binding constants were determined at 25 °C by successive addition of aliquots of a stock solution of the appropriate ligand dissolved in MeOH to a cuvette containing *c.* 10 μmol dm^{-3} hematohemin in MeOH. Between 20 and 30 ligand additions were made and the change in absorbance in either the Soret region or at around 600 nm was monitored as a function of ligand concentration. The absorbance readings were corrected for dilution effects. The binding constants were determined by curve fitting using standard non-linear least-squares methods (employing a Newton–Raphson procedure and Marquardt's alogorithm) the curve of absorbance against ligand concentration described by the binding isotherms derived below.

Results and discussion

Synthesis of hematohemin

Under the preparative conditions used, hematohemin precipitates as the hydroxide complex [32]. Various attempts at chromatographic separations both on silica and C-18 columns were attempted. Generally, good analyses of the starting porphyrin could not be obtained because of peak tailing on the column and analysis on silica using the gradient elution profile described above was found to be the most successful. Despite the poor quality of the separation we were nevertheless able to observe at least four impurities (*c.* 30% of total area counts) in the material. No attempt was made to purify the starting material before metallation. Significant amounts of the starting material failed to react and were discarded in the acid wash steps, so that overall yield was only 40%.

The HPLC analysis of hematohemin was only successful when imidazole was added to coordinate the metal ion (Fig. 1). Without it very pronounced peak tailing occurred. Two small peaks (5% of signal) due to unreacted hematoporphyrin eluted before the main peak. Since the unmetallated porphyrin is unlikely to have any effect on the ligand binding behaviour of hematohemin, no further purification was undertaken. It would appear that despite the substantial impurities in the starting material metallation of hematoporphyrin-IX was selective and yielded a single product; however the possibility of a number of porphyrin species co-eluting under the chromatographic conditions cannot be ruled out.

Fig. 1. HPLC of ferrihematoporphyrin-IX (1 mg ml^{-1} with 5% wt./vol. imidazole in MeOH) eluted on a silica column with MeOH (1 ml min^{-1}). The small leading peaks are unreacted hematoporphyrin.

TABLE 2. Absorption coefficients for hematohemin in methanol

Wavelength (nm)	Absorption coefficient (dm^3 mol^{-1} cm^{-1})
345(sh)	6.4×10^4
392.7	1.67×10^5
490	1.27×10^4
525(sh)	2.1×10^4
593	8.18×10^3

Properties of hematohemin

The ESR spectrum of hematohemin is typical of a high-spin Fe(III) porphyrin [34] with an intense band at 1080 G ($g=6.0$) and a small signal at 3280 G ($g=2.0$); there was no evidence of a signal at around $g=3.5$, which would be indicative of a low-spin component in the sample. Hydroxide on its own as an axial field ligand has insufficient ligand field strength to impart any measureable low-spin character to the metal ion.

The high-spin nature of hematohemin was confirmed by UV–Vis spectroscopy. In MeOH (in which hematohemin is very soluble) there is a Soret band at 392.7 nm with the N band as a shoulder at 345 nm, and visible bands at 593, 325 (sh) and 490 nm; this is typical of a high-spin Fe(III) porphyrin [35]. The molar absorption coefficients at various wavelengths determined on a standardised solution (see below) are given in Table 2.

Porphyrins are notoriously prone to dimerisation, especially in aqueous solution although not usually in non-aqueous solvents [36]; this is particularly problematic for any study of their ligand binding properties as it is likely to make interpretation of the experimental data very difficult. To verify that hematohemin is monomeric in the concentration range usually used for spectrophotometric studies (5–50 μmol dm^{-3}), a Beer's law study at 490 nm (the Q_v band) was undertaken. A stock solution (2.55×10^{-3} mol dm^{-3}) of hematohemin in methanol was prepared and standardised by the pyridine hemochromogen method [37] using $\epsilon_{551}=29.1\times10^{-3}$ dm^3 mol^{-1} cm^{-1}. Aliquots of this solution were successively added to a spectrophotometer cuvette thermostatted at 25 °C and the absorbance values corrected for dilution. A plot of absorbance against concentration was a straight line through the origin for concentrations up to 75 μmol dm^{-3}, suggesting that the species is monomeric over this concentration range.

Ligand binding: preliminary observations

Addition of a ligand such as *N*-acetyl imidazole to a solution of hematohemin in methanol results in conversion of the iron porphyrin from a high-spin complex (Fig. 2). Well-defined isosbestic points were not observed during the titrations and the reaction appears to involve the formation of an intermediate whose spectrum is not very different to that of the starting material, viz.

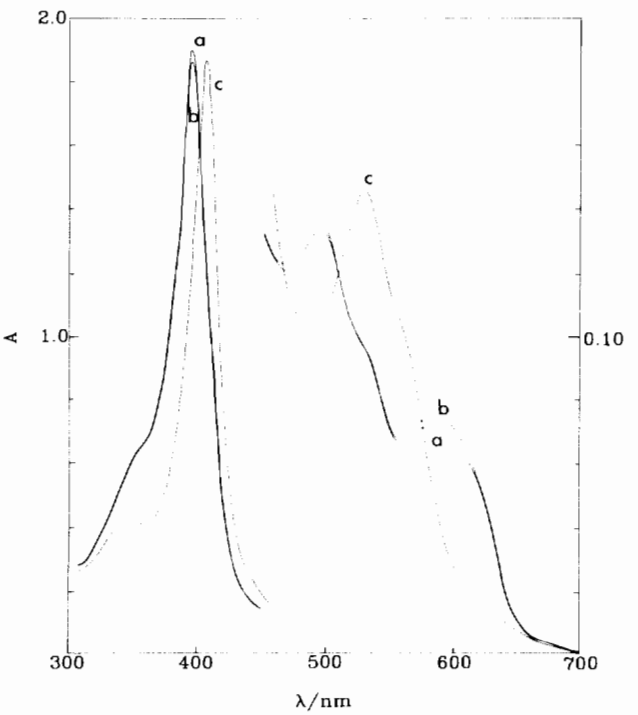


Fig. 2. Titration of hematohemin (11.5 μmol dm^{-3}) with *N*-acetyl imidazole: a, 0; b, 4×10^{-4}; c, 1.5×10^{-2} mol dm^{-3}.

there is a small increase in intensity of the band at around 600 nm, and a small decrease in intensity of the Soret band. The major spectral changes in the reaction are associated with conversion of this intermediate to the low-spin complex which has a prominent band at around 530 nm and the Soret band around 403 nm. Similar results were obtained with the other ligands studied, except for benzimidazole. Addition of this ligand to a solution of hematohemin resulted in small changes in the spectrum suggesting that coordination of the ligand took placc; but even at a concentration of 0.5 mol dm^{-3} in ligand the spectrum retained all the characteristics of a high-spin species.

In methanol solutions it is uncertain whether the axial ligand on Fe(III) is OH^- or methoxide; either, in the absence of a 6th ligand, would produce a high-spin complex.

Ligand binding: the binding isotherms

There are various ways of deriving binding constants from spectrophotometric titrations [38], the most common probably being a plot of $\log(A-A_0)/(A_\infty-A)$ against log[ligand], the intercept of which gives log K and the slope the number of ligands. Plots such as this are only useful, however, if (i) a single ligand coordinates; or (ii) two ligands bind apparently simultaneously; or (iii) $K_1>K_2$ so that A_∞ for the first step, which is also A_0 for the second step, is well-defined. Even small errors in these parameters will lead to significant errors in K values.

We decided therefore to derive expressions (binding isotherms) which describe the behaviour of the absorbance at some wavelength as a function of ligand concentration, and to determine the values of the binding constants by fitting the experimental data to these.

Three situations need to be considered. In the following, M is used to represent the metalloporphyrin and L a ligand. The ligand departing from the coordination sphere of the porphyrin (either OH^- or solvent) is omitted for convenience. The subscript T stands for total.

Case 1. A single ligand coordinates to the iron porphyrin. The reaction is given by eqn. (3) and the binding constant by eqn. (4).

$$M+L \rightleftharpoons ML \quad (K) \tag{3}$$

$$K=\frac{[ML]}{[M][L]_{free}} \tag{4}$$

It therefore follows that the equilibrium concentrations of M and ML are given by eqn. (5).

$$[M]=\frac{[M]_T}{1+K[L]_{free}} \quad [ML]=\frac{[M]_T K[L]_{free}}{1+K[L]_{free}} \tag{5}$$

Defining f_i as the fraction of the ith species at equilibrium, the observed absorbance, A, at any given wavelength will vary as a function of [L] and is given by eqn. (6), where A_0 and A_∞ are the absorbance due to 100% M and ML, respectively.

$$A=\frac{A_0+A_\infty K[L]_{free}}{1+K[L]_{free}} \tag{6}$$

The free ligand concentration, $[L]_{free}$, which will only approximately equal the total ligand concentration, [L], if K is small, is given by eqn. (7).

$$[L]_{free}=\frac{[L]}{1+K[M]_T} \tag{7}$$

Provided $A_0 \neq A_\infty$, the observed experimentally determined absorbance values can be fitted as a function of [L] to eqn. (6) by non-linear least-squares methods with A_0, A_∞ and K as variables.

Case 2. The ligands add to the porphyrin in a stepwise manner. The stepwise addition is given by eqn. (8) and the binding constants by eqn. (9). The total metal concentration $[M]_T$, is then given by eqn. (10) or, using fractional abundances, by eqn. (11), where these are defined by eqn. (12).

$$M+L \rightleftharpoons ML \quad (K_1)$$

$$ML+L \rightleftharpoons ML_2 \quad (K_2) \tag{8}$$

$$K_1=\frac{[ML]}{[M][L]} \quad K_2=\frac{[ML_2]}{[ML][L]} \tag{9}$$

$$[M]_T=[M]+[ML]+[ML_2] \tag{10}$$

$$f_{[M]}+f_{[ML]}+f_{[ML_2]}=1 \tag{11}$$

$$f_{[M]}=\frac{1}{1+K_1[L]_{free}+K_1K_2[L]^2{}_{free}}$$

$$f_{[ML]}=\frac{K_1[L]_{free}}{1+K_1[L]_{free}+K_1K_2[L]^2{}_{free}} \tag{12}$$

$$f_{[ML_2]}=\frac{K_1K_2[L]^2{}_{free}}{1+K_1[L]_{free}+K_1K_2[L]^2{}_{free}}$$

The free ligand concentration is related to the total ligand concentration added to the solution by eqn. (13) from which the relationships of eqn. (14) follow. The solution to this equation is found by Newton's method (eqn. (15)) where a_i is the ith coefficient of the cubic equation and α is set at some acceptable value, typically 10^{-7}.

$$[L]_{free}=[L]_T-[ML]-2[ML_2] \tag{13}$$

$$[L]_{free}=[L]_T-\frac{K_1[L]_{free}[M]_T}{1+K_1[L]_{free}+K_1K_2[L]^2{}_{free}}$$

$$-\frac{2K_1K_2[L]^2{}_{free}[M]_T}{1+K_1[L]_{free}+K_1K_2[L]^2{}_{free}}$$

Therefore

$$K_1K_2[L]^3_{free} + (K_1 - K_1K_2[L]_T + 2K_1K_2)[L]^2_{free} + (1 - K_1[L]_T + K_1)[L]_{free} - [L]_T = 0 \quad (14)$$

$$[L]_{free,\,k+1} = [L]_{free,\,k} - \left[\frac{a_1[L]^3_{free} + a_2[L]^2_{free} + a_3[L]_{free} + a_4}{3a_1[L]^2_{free} + 2a_2[L]_{free} + a_3}\right]$$

$$\text{until } |[L]_{free,\,k+1} - [L]_{free,\,k}| < \alpha \quad (15)$$

Case 3. Two ligands bind simultaneously to the porphyrin (eq. (16)); the binding constant is given in eqn. (17)

$$M + 2L \rightleftharpoons ML_2 \quad (\beta_2) \quad (16)$$

$$\beta_2 = \frac{[ML_2]^2}{[M][L]_{free}} \quad (17)$$

The porphyrin is either free or coordinated (eqn. (18)), and the fractional abundances of the two forms are given by eqn. (19).

$$[M]_T = [M] + [ML_2] \quad (18)$$

$$f_{[M]} = \frac{1}{1 + \beta_2[L]^2_{free}}$$

$$f_{[ML_2]} = \frac{\beta_2[L]^2_{free}}{1 + \beta_2[L]^2_{free}} \quad (19)$$

The absorbance is fitted to eqn. (20) with A_0, A_∞ and β_2 as variables, where $[L]_{free}$, given by eqn. (21) is found iteratively using Newton's method (eqn. (22)).

$$A = A_0 f_{[M]} + A_\infty f_{[ML_2]} \quad (20)$$

$$\beta_2[L]^3_{free} + (2[M]_T\beta_2 - \beta_2[L]_T)[L]^2_{free} + [L]_{free} - [L]_T = 0 \quad (21)$$

$$[L]_{free,\,k+1} = [L]_{free,\,k} - \frac{a_1[L]^3_{free} + a_2[L]^2_{free} + a_3[L]_{free} + a_4}{3a_1[L]^2_{free} + 2a_2[L]_{free} + a_3} \quad (22)$$

with $a_1 = \beta_2$, $a_2 = 2[M]_T\beta_2 - \beta_2[L]_T$, $a_3 = 1$, $a_4 = -[L]_T$

Ligand binding: quantitative determination of binding constants

The reaction of hematohemin with imidazole was followed at the Soret band for hematohemin (393 nm) and in the visible region (595 nm). The spectral changes observed are shown in Fig. 3. The absorbance dependence on imidazole concentration was fitted with the three cases considered and the fits obtained are shown in Fig. 3.

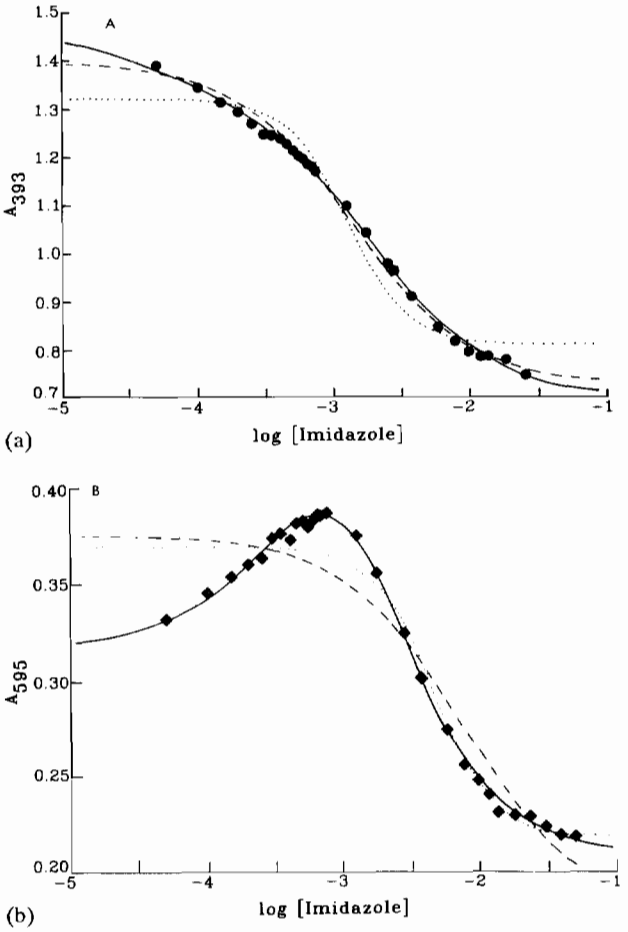


Fig. 3. Dependence of absorbance at (a) 393 and (b) 595 nm on the concentration of imidazole on its addition to (a) 8.6 μmol dm^{-3} and (b) 39 μmol dm^{-3} hematohemin in methanol. The curves are the least-squares fits to the experimental data based on models assuming (– – –) the binding of a single ligand (eqn. (3) of text); (· · · · ·) the simultaneous binding of two ligands (eqn. (16)); and (——) the stepwise binding of two ligands (eqn. (8)).

Clearly the greater spectral change occurs in the Sorct region, and this would appear to be the logical choice for following these reactions. The present results highlight the problem of using only this wavelength. The fit based on a model involving the binding of a single ligand to the iron porphyrin yields an acceptable (albeit not the best) fit to the experimental data. Furthermore, the usual plot of log $[(A_0 - A)/(A - A_\infty)]$ against log[imidazole] (Fig. 4), is a reasonably good straight line with slope $= 0.79 \pm 0.02$ which could be taken to be consistent with the binding of a single ligand. Such an interpretation could be dismissed on the grounds that binding of a single ligand of intermediate field strength such as used in this study would be unlikely to produce a low-spin complex, the spectral characteristics of which are readily recognisable.

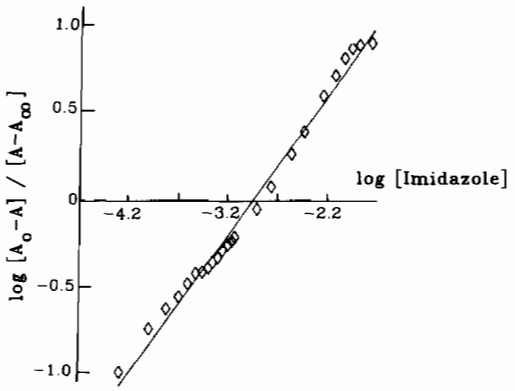


Fig. 4. Plot of $\log[(A_0-A)/(A-A_\infty)]$ against log[imidazole] for the addition of imidazole to hematohemin in MeOH at 25.0 °C monitored at 393 nm. The slope of the line is 0.79 ± 0.02 and the intercept (the log of the apparent binding constant) is 2.33 ± 0.06.

However, the absorbance at 595 nm shows an initial increase, followed by a decrease, which is only consistent with a two step reaction. We attribute the first step to the binding of a single ligand to form a high-spin mono(imidazole) complex, with the departure of either OH^- or MeO^-, which then in the second, subsequent step, binds a second ligand, and the metal ion undergoes a change in spin state. Therefore, given the data at 595 nm, and the somewhat better fit of a model assuming sequential binding of two ligands to the data at 393 nm, it is reasonable to conclude that two imidazole ligands are coordinated by hematoporphyrin in a stepwise manner via a high-spin mono(imidazole) intermediate.

It is difficult, however, to obtain reliable values for the binding constants associated with the two steps. Both at 393 and 595 nm, the absorbance due to the monoimidazole intermediate is experimentally impossible to determine. (Accepting for the purposes of discussion the values of K_1 and K_2 obtained at 595 nm, viz. $\log K_1=3.1$ and $\log K_2=2.9$, see below, a species distribution diagram — not shown — indicates that the maximum concentration of the intermediate monoimidazole complex never exceeds 40%.) The particularly large uncertainty in the value of K_1 stems in part from the rather small spectroscopic changes which accompany the binding of the first ligand, and in part from the strong correlation between this value and the (unknown) absorbance of the mono(imidazole) intermediate (Table 3). Therefore, although the data provide evidence for the stepwise addition of two imidazole ligands to hematohemin, the value of the binding constants (and in particular that of K_1) can only be regarded as estimates.

Similar results were obtained with the other ligands studied. However, in the case of benzimidazole, despite using concentrations up to 0.5 mol dm^{-3}, evidence was found for the binding of only one ligand with the spectrum of the final product retaining all the characteristics of a high-spin complex. The average values of the stability constants obtained from fits to the data in the Soret and in the visible region are summarised in Table 4.

Despite the large uncertainties in the values of K_1, there is a good linear relationship between the pK_a of the ligand and $\log K_1$ (Fig. 5). The value for benzimidazole is noticeably off the line. This we attribute to steric interaction between the aromatic ring substituent on C8 and the porphyrin ring, which decreases the stability of the complex. There appears to be no cor-

TABLE 3. Parameters from non-linear least-squares fitting to the spectrophotometric titration of the binding of imidazole by hematohemin in methanol at 25 °C

Monitoring wavelength (nm)		A_0	A_i	A_∞	$\log K_1$	$\log K_2$
595		0.316	0.557	0.207	3.1 ± 0.1	2.9 ± 0.1
390		1.461	1.312	0.697	4.3 ± 0.2	$2.7\pm0.0(4)$
		Correlation matrices				
		A_0	A_i	A_∞	$\log K_1$	$\log K_2$
595	A_0	1	0.399	0.202	−0.563	0.377
	A_i		1	0.636	−0.973	0.981
	A_∞			1	−0.579	0.736
	$\log K_1$				1	−0.939
	$\log K_2$					1
390	A_0	1	0.200	0.004	0.367	0.111
	A_i		1	0.609	0.938	0.905
	A_∞			1	0.467	0.830
	$\log K_1$				1	0.763
	$\log K_2$					1

TABLE 4. Binding constants[a] for the binding of N-donor ligands to hematohemin in methanol at 25.0 °C

Ligand, L	log K_1[b]	log K_2[b]
Pyridine	2.6±0.6	1.0±0.2
Ethanolamine[c]	4.4±0.6	0.7±0.2
Imidazole[d]	3.7±0.8	2.8±0.1
N-Acetyl imidazole	2.0±0.4	3.3±0.2
Benzimidazole	0.9±0.1	[e]

[a]The values quoted are the average of the values determined using data from the Soret region and form the visible region. [b]Defined by eqn. (9). [c]The value for log K_2 is known with considerably less certainty than for other ligands because of its low value which meant that sufficient data to obtain a good estimate for A_∞ could not be obtained. [d]See Table 3. [e]Only evidence for coordination of a single ligand.

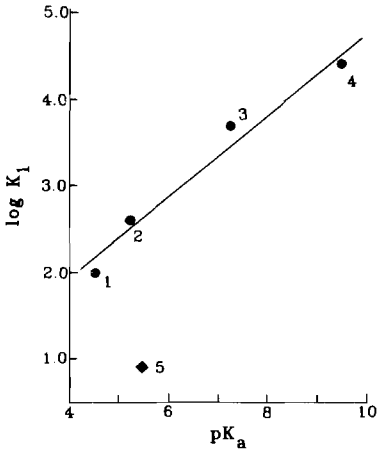


Fig. 5. Dependence of log K_1 on the pK_a of the ligand: 1,*N*-acetyl imidazole; 2, pyridine; 3, imidazole; 4, ethanolamine; 5, benzimidazole. The value for benzimidazole was omitted from the calculation of the best fit straight line (see text).

relation, however, between the ligand pK_a and log K_2.

There are several factors which will affect the drive towards coordination of a second ligand. The mono(ligated) intermediate is expected to have the iron atom displaced from the mean porphyrin plane towards the axial ligand, as is found, for example, in deoxyMb [39] and in Fe(TPP)Cl [40], while in the bis(ligated) species the iron atom will be in, or close to, the mean plane as in, for example, Fe(TPP)(imidazole)$_2^+$ [41]. As mentioned in the introduction to this paper, the gain in ligand field stabilisation energy will favour the low-spin complex and hence favour coordination of a second ligand. This in turn may increase the strength of the bonding between the metal and the axial ligands with the e_g^* orbitals being unoccupied [12]. However, if the initial Fe–axial ligand bond is strong, then the binding of the *trans* ligand will require the iron atom to drop into the porphyrin cavity, hence lengthening the original Fe–axial ligand bond. The tendency to bind a second ligand should, for this effect alone, be inversely dependent on the value of log K_1. This is seen to some extent in the present data, where there is an inverse relationship between K_1 (ethanolamine > imidazole > pyridine > *N*-acetyl imidazole) and K_2 ethanolamine < imidazole < *N*-acetyl imidazole), although pyridine does not fit this trend. (Indeed, the failure of pyridine to conform to the trend seen for the other ligands suggests that the factors which have been considered are likely not to be the only ones which are important.) Given these competing effects it is not surprising that there is apparently no simple relationship between the donor power of the ligand and the tendency to bind a second ligand.

Steric factors are also important. We have shown here with benzimidazole and others [13, 24] have shown with 2-methyl imidazole that only one sterically hindered imidazole will coordinate the metal ion; presumably binding of a second ligand would require the Fe–axial N bond to become very long because of the steric interaction between the porphyrin ring and the substituent on the ligand.

Conclusions

In conclusion we have shown that the determination of binding constants for ligands to iron porphyrins is not a simple matter and the result one apparently arrives at using spectrophotometric techniques will depend on the monitoring wavelength. Care should therefore be taken in such determinations, and when comparing results obtained by different workers. The formation of the bis(ligated) complex of hematohemin with four ligands, viz. imidazole, *N*-acetyl imidazole, ethanolamine and pyridine, proceeds in a stepwise manner through intermediate formation of the mono(ligated) complex. There is a linear relationship between the magnitude of the binding constant for the first ligand and the pK_a of the ligand, but no simple relationship between pK_a and the binding constant for the second ligand (which may be larger or smaller than that for the first), which is likely to depend on a number of factors.

Acknowledgements

We thank Dr J. van Wyk of the Department of Physics, University of the Witwatersrand, for access to the ESR spectrometer. This work was made possible by grants from the Foundation for Research Development, Pretoria, and, through the Centre for Molecular Design, from the University Research Committee of the University of the Witwatersrand.

References

1 D. A. Baldwin, V. M. Campbell, L. A. Carleo, H. M. Marques and J. M. Pratt, *J. Am. Chem. Soc., 103* (1981) 186.
2 D. A. Baldwin, V. M. Campbell, H. M. Marques and J. M. Pratt, *FEBS Lett., 167* (1984) 339.
3 D. A. Baldwin, H. M. Marques and J. M. Pratt, *S. Afr. J. Chem., 39* (1986) 189.
4 J. W. Owens and C. J. O'Connor, *Coord. Chem. Rev., 84* (1988) 1.
5 W. Scheler, *Biochem. Z., 332* (1960) 542.
6 W. A. Gallagher and W. B. Elliot, *Biochem. J., 108* (1968) 131.
7 B. B. Hasinoff, H. B. Dunford and D. G. Horne, *Can. J. Chem., 47* (1969) 3225.
8 T. H. Davies, *Biochim. Biophys. Acta, 329* (1973) 108.
9 J. Simplicio, K. Schwenzer and F. Maenpa, *J. Am. Chem. Soc., 97* (1975) 7319.
10 W. L. Hinze and J. H. Fendler, *J. Chem. Soc., Dalton Trans.,* (1976) 1469.
11 P. A. Adams, D. A. Baldwin, C. E. Hepner and J. M. Pratt, *Bioinorg. Chem., 9* (1978) 479.
12 C. L. Coyle, P. A. Rafson and E. H. Abbott, *Inorg. Chem., 9* (1973) 2007.
13 P. R. Ciaccio, J. V. Ellis, M. E. Munson, G. L. Kedderis, F. X. McConville and J. M. Duclos, *J. Inorg. Nucl. Chem., 38* (1976) 1885.
14 T. Uno, K. Hatano, T. Nawa, K. Nakamura, Y. Nishimura and Y. Arata, *Inorg. Chem., 30* (1991) 4322.
15 D. A. Baldwin, C. E. Hepner and J. M. Pratt, *J. Inorg. Biochem., 10* (1979) 159.
16 K. Burger, J. Molnar, L. Molnar-Hamvas and F. Gaizer, *Acta Chim. Acad. Sci. Hung., 91* (1976) 403.
17 H. M. Marques, *Inorg. Chim. Acta, 190* (1991) 291.
18 R. Quinn, J. Mercer-Smith, J. N. Burnstyn and J. S. Valentine, *J. Am. Chem. Soc., 106* (1984) 4136.
19 Y. Harel and R. H. Felton, *J. Chem. Soc., Chem. Commun.,* (1984) 206.
20 L. Casella, M. Gullotti, L. De Gioia, E. Monzani and F. Chillemi, *J. Chem. Soc., Dalton Trans.,* (1991) 2945.
21 R. Quinn, M. Nappa and J. S. Valentine, *J. Am. Chem. Soc., 104* (1982) 2588.
22 M. Tohjo and K. Shibata, *Arch. Biochem. Biophys., 103* (1963) 401.
23 D. Brault and M. Rougee, *Biochem. Biophys. Res. Commun., 57* (1974) 654.
24 F. A. Walker, M. Lo and M. T. Ree, *J. Am. Chem. Soc., 98* (1976) 5552.
25 J. Aron, D. A. Baldwin, H. M. Marques, J. M. Pratt and P. A. Adams, *J. Inorg. Biochem., 27* (1986) 227.
26 D. A. Baldwin, H. M. Marques and J. M. Pratt, *J. Inorg. Biochem., 27* (1986) 245.
27 H. M. Marques, T. J. Egan, J. H. Marsh, J. R. Mellor and O. Q. Munro, *Inorg. Chim. Acta, 166* (1989) 249.
28 O. Q. Munro and H. M. Marques, unpublished results.
29 M. J. Balis, O. Enea and G. Berthon, *Thermochim. Acta, 20* (1977) 335.
30 I. R. Bellobono and P. Beltrame, *J. Chem. Soc. B,* (1969) 620.
31 R. F. Jameson, G. Hunter and T. Kiss, *J. Chem. Soc., Perkin Trans. II,* (1980) 1105.
32 S. Brown and H. Hatzikonstantinou, *Biochim. Biophys. Acta, 585* (1979) 143.
33 K. G. Paul, *Acta Chem. Scand., 5* (1951) 389.
34 G. Palmer, in A. B. P. Lever and H. B. Gray (eds.), *Iron Porphyrins,* Part 2, Addison-Wesley, Reading, MA, 1983, pp. 43–88.
35 M. W. Makinen and A. K. Churg, in A. B. P. Lever and H. B. Gray (eds.), *Iron Porphyrins,* Part 1, Addison-Wesley, Reading, MA, 1983, pp. 141–235.
36 H. B. Dunford and J. S. Sillman, *Coord. Chem. Rev., 19* (1976) 187.
37 J. E. Falk, *Porphyrins and Metalloporphyrins,* Elsevier, Amsterdam, 1964.
38 K. A. Connors, *Binding Constants: The Measurement of Molecular Complex Stability,* Wiley, New York, 1987.
39 T. Takano, *J. Mol. Biol., 110* (1977) 569.
40 D. F. Koenig, *Acta Crystallogr., 18* (1965) 663.
41 D. M. Collins, R. Countryman and J. L. Hoard, *J. Am. Chem. Soc., 94* (1972) 2066.