Hemes and Hemoproteins. Part 7.[1] Co-ordination of Ammonia, Aniline and Pyridine by the Iron(III) Porphyrin Microperoxidase-8†

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The equilibrium constant for the substitution of co-ordinated H_2O by NH_3 in the monomeric iron(III) haem octapeptide microperoxidase-8 (MP-8) has been determined by UV/VIS spectrophotometry in 20% aqueous MeOH (I = 0.1 mol dm^{-3}) at 25 °C from the pH dependence of the apparent binding constant over the range pH 7–10 as (2.21 ± 0.15) × 10^3 dm^3 mol^{-1} ($\log_{10} K$ 3.34 ± 0.03); this appears to be the first such equilibrium constant for the co-ordination of NH_3 reported for any iron(III) porphyrin or haemoprotein. Variation of the spectrum of the NH_3 complex in aqueous solution over the range pH 9–13 revealed a second equilibrium with pK 11.9 involving one proton, which is ascribed to ionisation of the imidazole ring of the *trans*-histidine ligand to give the co-ordinated imidazolate. The octapeptide binds pyridine and aniline in 20% aqueous MeOH at 25 °C with equilibrium constants of $\log_{10} K$ = 2.73 ± 0.02 and 2.67 ± 0.01 respectively to give products which also exhibit further pH-dependent equilibria (pK at 11.2 and 11.8 respectively) and show spectra very similar to those of the NH_3 complexes; this indicates that they both bind through co-ordination to the metal and not π–π interaction with the porphyrin ring.

There is an urgent need to develop the co-ordination chemistry of water-soluble iron porphyrins, especially those possessing a single imidazole (or His itself) as axial ligand, which could serve directly (*a*) as protein-free models or 'co-factors' for several important haemoproteins such as haemoglobin, myoglobin, HRP and cytochrome a_3, which all contain His as one axial ligand with the second axial site either vacant or occupied by H_2O, and, after co-ordination of a second different axial ligand, (*b*) as models for cyt c, where the axial ligands have been established as His + Met (*i.e.* RSMe),[2] cyt f and the alkaline form of cyt c, where the axial ligands are probably His + Lys (*i.e.* RNH_2).[3,4]

We[5–8] and others (see, for example, the more recent refs. 9–11) have therefore been studying the properties of the haem octapeptide or microperoxidase-8 (MP-8) which is obtained by proteolysis of cyt c, sometimes as Ac-MP-8 in which the terminal Cys-NH_2 has been acetylated.[12] Other haem oligopeptides, such as MP-6, -9 and -11, have also been studied; see, for example, refs. 13 and 14. The octapeptide MP-8 possesses a substituted porphyrin c ligand in which the two cysteine (Cys) residues of the octapeptide (Cys-Ala-Gln-Cys-His-Thr-Val-Glu) have added to the two vinyl substituents of the proto-porphyrin IX ligand (present in haemoglobin, *etc.*) to form thioether links; the peptide side-chain also contains His which is co-ordinated to the Fe at pH ≥ 5 (see below). The substituents on the porphyrin ring are therefore different in the above-mentioned haemoproteins and in protein-free MP-8, but it appears that changes in these substituents have relatively minor effects on equilibrium constants; log K values of 4.42, 4.52 and 4.29 were reported for the binding of N_3^- and 4.50, 4.48 and 4.42 for the binding of CN^- by myoglobins reconstituted with proto-, deutero- and meso-porphyrin respectively.[15,16] Since the electron-withdrawing effect of the substituents in porphyrin c is expected to be intermediate between that in proto- and meso-porphyrins,[17] we conclude that the use of MP-8 as a protein-free model for the above-mentioned haemoproteins is not vitiated by differences in the side-chains.

Conditions have been described under which MP-8 is monomeric at sufficiently high concentration for study by conventional UV/VIS spectrophotometry using the intense Soret band at *ca.* 400 nm, *viz.* 50% aqueous ethylene glycol, as used by Kassner and co-workers,[9,18] and 20% aqueous methanol (≈98% monomer with 10^{-6} mol dm^{-3} MP-8 at pH 7, normally used with 4 or 10 cm cells), as used by us.[5,6] The octapeptide also remains monomeric up to > 3 × 10^{-5} mol dm^{-3} at pH 12 even in the absence of MeOH and can be studied in 1 cm cells.[5] The Fe^{III} is axially co-ordinated by the retained proximal His-18 of cytochrome c;[6] the second site is occupied by H_2O and/or is vacant (see Discussion). Spectrophotometric titration of monomeric MP-8 identified three reversible and concentration-independent pK values which we have ascribed to protonation and displacement of His from co-ordination (pK_1 = 4.4), ionisation of co-ordinated H_2O to give the HO^- complex (pK_2 = 8.9) and loss of a proton from His to give the imidazolate complex (pK_3 = 10.5), respectively.[6] There are five other sites of reversible protonation including four carboxylates (two propionate side-chains on the porphyrin ring, and both the C-terminal and side-chain carboxylates of Glu-21) and the N-terminal amine of Cys-14. Cyclic voltammetry of the imidazole complex of MP-8 revealed the existence of groups with pK 10.1 (no detectable effect on the UV/VIS spectrum) and 12.9 (significant effect on the spectrum) in the oxidised form, which

† *Abbreviations employed:* B = any nitrogen-containing base and potential ligand; cyt = cytochrome; HRP = horse radish peroxidase; MP-8 = microperoxidase-8 or haem octapeptide (hence also MP-6, MP-11); NADH = reduced nicotinamide–adenine dinucleotide; py = pyridine; Ala = alanine; Cys = cysteine; Glu = glutamic acid; Gln = glutamine; His = histidine; Lys = lysine; Met = methionine; Phe = phenylalanine; Thr = threonine; Trp = trytophan; Val = valine; proto-, deutero- and meso-porphyrin = 3,7,12,17-tetramethyl-8,13-divinyl-, 3,7,12,17-tetramethyl- and 8,13-diethyl-3,7,12,17-tetramethyl-porphyrin-2,18-dipropanoic acid; Tris = aminotris(hydroxymethyl)methane.

we assigned to proton loss from Cys-NH_3^+ and from the co-ordinated His respectively;[19] we shall be interested here in obtaining evidence for the existence of analogous pK values for complexes with other nitrogen-containing bases (see below). The carboxylates probably all have pK < 6 and will not be discussed further. There is also evidence for the existence of pH-independent equilibria involving changes in spin state and/or co-ordination number[12,18,20] (see Discussion).

We have hitherto been interested primarily in using MP-8 to model reactions of the haemoproteins such as (*i*) peroxidase activity (catalysing the reduction of H_2O_2 by various donors),[8,21] (*ii*) the reduction of co-ordinated O_2 with H-atom donors,[1] which occurs with haemoglobin–dioxygen and may also be relevant to the mechanism of action of cyt a_3, and (*iii*) the catalysis of the hydroxylation of aniline by NADH and O_2.[22] In addition, various microperoxidases (including MP-8) catalyse the autoxidation of reduced cytochrome c,[23] while an unspecified microperoxidase (probably MP-11) catalyses the autoxidation of thiols.[24] The octapeptide can, therefore, provide an extremely useful, versatile and simple model for many of the reactions of haemoproteins which contain a single His in the co-ordination sphere of Fe. We are now using MP-8 to study metal–ligand bonding in the iron porphyrins and, as explained in the following paper,[25] have initiated a systematic study of the co-ordination of nitrogen-containing bases including alkylamines (in which the N approximates to tetrahedral sp^3 hybridisation in both the free and co-ordinated base), pyridines and imidazoles (trigonal sp^2 hybridisation in both free and co-ordinated base). In this paper we report our studies on the co-ordination of NH_3, aniline and py by the iron(III)-containing MP-8 over a wide pH range (6–12), together with a few points on the co-ordination of imidazole in addition to those previously reported.[6,19] The bases NH_3, py and imidazole are of interest as the parent compounds of the three named series; aniline represents an intermediate case where the N changes its hybridisation from approximately trigonal sp^2 in the free base to approximately tetrahedral sp^3 when co-ordinated (see Discussion in Part 8).[25] In the following paper[25] we focus on non-heterocyclic amines and amino acids; heterocyclic ligands will be dealt with in subsequent papers.

Although NH_3 is the prototype nitrogen-containing base, surprisingly little work has been reported on the co-ordination of such an important ligand by iron porphyrins. Amongst protein-free iron(III) porphyrins the co-ordination of NH_3 by MP-8 was reported in 1960[26] [apparently the first example for any iron(III) porphyrin], by haematin in 1973[27] and subsequently by two other iron(III) porphyrins in non-aqueous solvents,[28,29] but no equilibrium constants were determined. The co-ordination of NH_3[30] (also NH_2Me, *etc.*)[31] by the iron(III) methaemoglobin has long been known and equilibrium constants determined; the equilibrium appears, however, to involve the pH-independent substitution of coordinated HO^- by NH_3, which has never been satisfactorily explained (see Discussion).

Pyridine and aniline are also of interest because they may, in principle, interact with an iron porphyrin either by co-ordination to the Fe as a ligand or by forming an adduct through π–π interaction (involving some charge-transfer or donor–acceptor as well as hydrophobic interaction) with some part of the conjugated porphyrin system in a manner analogous to that established for compounds such as benzene or indole and their derivatives. Theoretical calculations suggest that in iron porphyrins such as haemin there is reduced π-electron density on the C atoms of the methene bridge and the C_α atoms of the pyrrole rings but an increased π-electron density on the C_β atoms, *i.e.* they can act as both π acceptors and donors.[32] The potential balance between co-ordination and adduct formation should be displaced in predictable ways; since pyridines are π-deficient compounds,[33] adduct formation should be promoted by increasing the donor power of the iron porphyrin either by the presence of a strong donor ligand on the Fe^{III} or by reduction to Fe^{II}. Relevant evidence can be summarised as follows. Crystal structure determinations have established the occurrence of π–π interactions between several metalloporphyrins and even the simple benzene, toluene and xylene (for summary see ref. 34) and between the indole ring of Trp and the porphyrin ring in, for example, cytochrome c peroxidases;[34] there appear to be no data on analogous adducts with pyridines or anilines. Nuclear magnetic resonance studies have, however, shown that the relaxation times of benzene, toluene, pyridine, aniline and xylidine (2,6-dimethylaniline) are all similarly affected by the presence of Hb (also by myoglobin and P-450 in the case of xylidine) in a way which indicates that these compounds bind 'in proximity to the Fe' (*i.e.* probably by interaction with the porphyrin ring) whether the Fe is co-ordinated (by CN^-, F^-) or not;[36–39] xylidine (where co-ordination is likely to be severely inhibited, even if not totally excluded, by steric hindrance from the methyl substituents) shows a binding constant as high as 2.5×10^3 $dm^3\ mol^{-1}$ towards P-450.[37] The fact that the hydroxylation of aniline and xylidine by NADH and O_2 is catalysed by haemoglobin, myoglobin and P-450,[40,41] where the Fe must be co-ordinated by some ligand derived from O_2, also requires the binding of these substrates close to the Fe through adduct formation.

Pyridine has been reported, probably correctly, to co-ordinate as ligand to the iron(III) form of both a derivative of MP-11 (there is, unfortunately, insufficient data to convert the K_{obs} into the simple ligand binding K)[26] and MP-8 itself (no K determined).[9,42] As might be expected, the situation appears more complex with other $Fe^{III}(OH^-)$ and iron(II) porphyrins. A comparison of the binding by the iron(III) haematin dimer in alkaline solution of various substituted pyridines and quinolines, including the 1- (or *N*-)methylated cations, showed that they all cause similar changes in the UV/VIS spectrum and that the binding constants increase in the order pyridine < 2,6-dimethylpyridine (sterically prevented from co-ordinating to Fe) < 4-cyanopyridine < 1-methylpyridinium cation < 1,2-dimethylpyridinium < 1,2-dimethylquinolinium and were dominated by entropy changes.[33] The authors pointed out that their results demonstrated the importance of hydrophobic and donor–acceptor interactions in the formation of such compounds, and contradicted the generally accepted view that the typical changes in spectrum observed with py itself can only indicate co-ordination. They concluded that in their system even the parent py itself interacts with the iron porphyrin mainly through such π–π interactions but 'whether there is also an interaction of py with the Fe atom cannot be excluded by our results'. Their results show that, even for py itself, co-ordination to the Fe and π–π adduct formation may show comparable energies and be in competition. The possibility of such adduct formation, which seems to have been ignored, could account in part at least for the anomalous orders of binding constants found for substituted pyridines with iron(II) porphyrins.[43,44] The binding of *o*-, *m*- or *p*-fluoroaniline by MP-8,[45] myoglobin and P-450[46] has been reported and the effects of temperature and pH studied with myoglobin and P-450; the results are difficult to interpret. For all three isomers with both proteins the values of log K (the binding constant) increase almost linearly over the range pH 6.9–7.5 (*i.e.* above the pK for protonation of the aniline). The authors suggested some change in an amino acid side-chain or deprotonation of the aryl NH_2 group as possible explanations and considered the latter the more likely,[46] but ignored the possibility of adduct formation. No comparable pH studies were, unfortunately, carried out with MP-8 and no details were given of the UV/VIS spectra of the products.[45] It is therefore essential to try to establish whether py and aniline bind to MP-8 through co-ordination to the Fe or through π–π adduct formation, especially in alkaline solution.

We report here the results of our studies on the binding of NH_3, py and aniline in neutral and alkaline solution with three aims: to establish whether py and aniline bind primarily

Table 1 The pH dependence of the co-ordination of NH_3 by MP-8

pH	K_{obs}/dm^3 mol^{-1}	log K_{obs}
6.88	10.56 ± 0.27	1.02 ± 0.01
6.97	17.68 ± 0.08	1.25 ± 0.01
7.26	32.72 ± 0.08	1.51 ± 0.01
7.95	130 ± 11	2.11 ± 0.03
8.31	275 ± 22	2.44 ± 0.03
8.51	356 ± 19	2.55 ± 0.02
9.07	452 ± 26	2.66 ± 0.02
9.58	313 ± 10	2.50 ± 0.01
9.97	123 ± 1.6	2.09 ± 0.01

K_{obs} is defined by equation (5) and the values given all represent the means of three separate determinations. For experimental details see text.

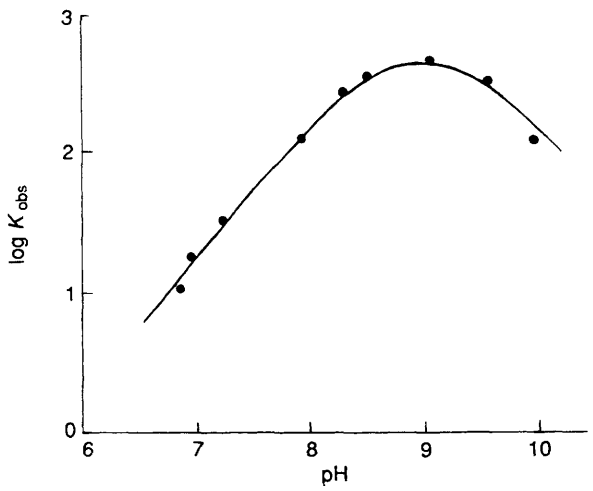

Fig. 1 The pH dependence of log K_{obs} for the co-ordination of NH_3 by MP-8. Data from Table 1

through co-ordination (our conclusion) or π–π adduct formation; to determine values of the equilibrium constant K = [Fe–B]/[Fe–OH_2][B] for substitution of co-ordinated H_2O in MP-8 by the three bases NH_3, py and aniline (here denoted by B) according to the generalised equation (1); and to provide

$$Fe\text{–}OH_2 + B \rightleftharpoons Fe\text{–}B + H_2O \quad (1)$$

$$HHis\text{–}Fe\text{–}B \rightleftharpoons His^-\text{–}Fe\text{–}B + H^+ \quad (2)$$

further information on related equilibria involving proton loss from the co-ordinated His according to (2), where HHis and His^- denote the imidazole and imidazolate forms respectively, and from the terminal Cys-NH_3^+. The results will provide the foundation (pH range available for direct determinations of K with other B, values of K for the parent compounds) for further work on the co-ordination of amines and heterocyclic bases.

Experimental

Materials.—The octapeptide MP-8 was prepared as previously described.[22,47] Ammonium chloride (BDH, AnalaR or Holpro Chemicals, Analytical Grade), pyridine, aniline (both BDH, AnalaR), NH_2Et, NH_2Bu and cyclohexylamine (all BDH) were used without further purification. Benzylamine (Aldrich, 99%) was redistilled.

Techniques.—The UV/VIS spectra were recorded on a Cary 2300 or Philips Analytical SP8100 spectrophotometer. The pH was measured with a Metrohm 605 or Hanna 8417 pH meter and appropriate glass electrode.

Determination of Equilibrium Constants.—Equilibrium constants corresponding to equation (1) and pK values corresponding to equation (2) were determined by UV/VIS spectrophotometric titration (10–15 additions for the former, up to 40 for the latter). In all cases, checks were made for complete equilibration before readings were taken, the full spectrum (300–700 nm) was scanned after every 3–5 additions to check for the occurrence of isosbestic points and to locate the band positions of the product, and values of the absorbance A at a fixed wavelength (usually the Soret band of the initial or final species) were recorded (after correction for any dilution) for analysis. Equilibrium constants for equation (1) were calculated as follows, where A_0 and A_∞ are the absorbances at the selected wavelength corresponding to 0 and 100% formation of the adduct respectively and A is the absorbance at some ligand concentration, [Z]. Since values of A_∞ could not be observed directly because of the relatively low values of K, they were determined by plotting $1/(A_0 - A)$ *vs.* $1/[Z]$ (linearity confirming $n = 1$) in one of the related equations (3) or (4) to

$$\frac{1}{(A_0 - A)} - \frac{1}{(A_0 - A_\infty)} = \frac{1}{K(A_0 - A_\infty)[Z]^n} \quad (3)$$

$$\frac{K(A_0 - A_\infty)}{(A_0 - A)} - K = \frac{1}{[Z]^n} \quad (4)$$

give values of A_∞ and K from the slopes and intercepts. In the case of the pK values, A_∞ was estimated by graphical extrapolation (see Results).

Results

Equilibrium Constant for the Binding of NH_3 *by* MP-8 *in* 20% *Aqueous* MeOH, pH 7–10.—The pK of NH_3 in 20% (v/v) aqueous MeOH was determined potentiometrically at 25 °C by titrating 25 cm^3 of 0.025 18 mol dm^{-3} NH_4Cl in KCl (I = 0.1 mol dm^{-3}) with 1 mol dm^{-3} NaOH to give (duplicate experiments) pK_a = 9.089 ± 0.003, *i.e.* 9.09.

The pH dependence of K_{obs}, as defined in equation (5), was

$$K_{obs} = \frac{[Fe\text{–}NH_3]}{([Fe\text{–}OH_2] + [Fe\text{–}OH])([NH_4^+] + [NH_3])} \quad (5)$$

determined with 7.3 × 10^{-7} mol dm^{-3} solutions of MP-8 in 20% aqueous MeOH from pH 6.88 to 9.97 (KH_2PO_4–K_2HPO_4 buffers, pH 6.9–7.5; Tris–HCl, pH 7.5–9.0; $NaHCO_3$–NaOH, pH 9.0–10) and (initially) I = 0.1 mol dm^{-3} at 25 °C. Portions (20 cm^3) of the MP-8 solution were placed in a 10 cm pathlength cell and titrated (usually 10–15 additions) with 1–3.5 mol dm^{-3} NH_4Cl solutions of the same buffer and solvent composition, and the pH checked at the end of each titration. Three independent values of K_{obs} were obtained at each pH by analysis (see Experimental section) of the change in absorbance at 397.2 nm. Isosbestic points (at 328, 402, 472, 508 and 590 nm below pH 9) and stoichiometries of n = 1.00 ± 0.01 were observed at all pH values; all changes in spectra were instantaneous. The product showed absorption bands at 350, 403.5 (Soret), 526, 555 (sh) and *ca.* 615 nm (weak); *cf.* Fig. 2 below. The values of K_{obs} are given in Table 1 and plotted against pH in Fig. 1.

Assuming the occurrence of the three equilibria (6)–(8) and the three associated equilibrium constants in equations (9)–(11),

$$Fe\text{–}OH_2 + NH_3 \rightleftharpoons Fe\text{–}NH_3 + H_2O \quad (6)$$

$$Fe\text{–}OH_2 \rightleftharpoons Fe\text{–}OH + H^+ \quad (7)$$

$$NH_4^+ \rightleftharpoons NH_3 + H^+ \quad (8)$$

$$K = [Fe\text{–}NH_3]/[Fe\text{–}OH_2][NH_3] \quad (9)$$

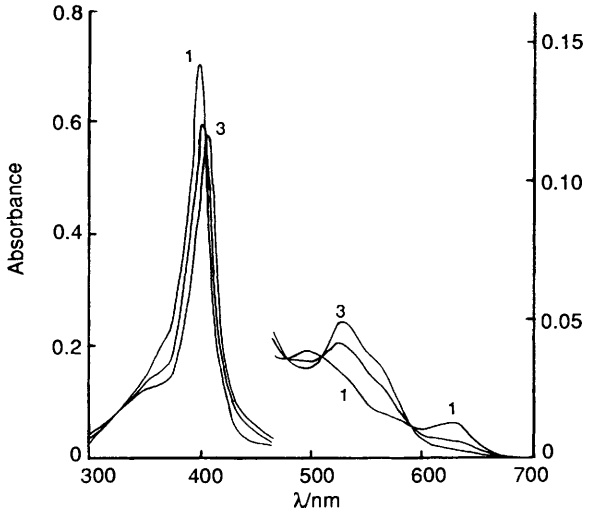

Fig. 2 Changes in spectra observed during the titration of MP-8 with py at pH 7.5 (left-hand scale for 300–470 nm, right-hand scale for 470–700 nm). Spectra 1 and 3 represent the H_2O and py complexes

Table 2 Soret bands of amine derivatives of MP-8

	λ/nm	
Ligand	HHis–Fe–B*	His^-–Fe–B*
NH_3	403.5	406
NH_2Et	403.5	
NH_2Bu	403.5	
$NH_2(C_6H_{11})$	404	
py	403.5	406
Imidazole	404	
Aniline	406	408.5
$NH_2(CH_2Ph)$	405.5	409
HO^-	*ca.* 400	401

* B represents the amine (or HO^-), HHis the co-ordinated imidazole ring of His and His^- the corresponding imidazolate.

$$K_0 = [\text{Fe–OH}][H^+]/[\text{Fe–OH}_2] \quad (10)$$

$$K_N = [NH_3][H^+]/[NH_4^+] \quad (11)$$

$$K_{obs} = \frac{K}{(1 + K_0[H^+]^{-1})\{1 + ([H^+]/K_N)\}} \quad (12)$$

K_{obs} can be expressed as in equation (12). The values of K_{obs} (see Table 1) were then fitted by equation (12), containing the experimentally determined value of $pK_N = 9.09$ (see above), by using a non-linear least-squares method and allowing both K_0 and K to be variables to give $K = 2205 \pm 147$ dm^3 mol^{-1}, *i.e.* $\log_{10} K = 3.34 \pm 0.03$, and $pK_0 = 8.89 \pm 0.06$ (in excellent agreement with the previously reported value of 8.9). The theoretical curve (Fig. 1) shows excellent agreement with the experimental points.

Equilibrium Constants for the Binding of py and Aniline by MP-8 *in* 20% *Aqueous* MeOH *at* pH 7.5.—Since the pK values of py and aniline (5.2 and 4.6 respectively in aqueous solution)[6] are both well below that of MP-8 for forming the hydroxo complex (8.9 in 20% aqueous MeOH), the main species present at pH 7.5 will be the neutral base and the aqua complex together with the products of the reaction. Equilibrium constants for the binding of py and aniline were therefore determined spectrophotometrically at a single pH using a *ca.* 2×10^{-6} mol dm^{-3} solution of MP-8 in 20% aqueous MeOH buffered at pH 7.5 with 0.08 mol dm^{-3} phosphate. Portions (25 cm^3) of the MP-8

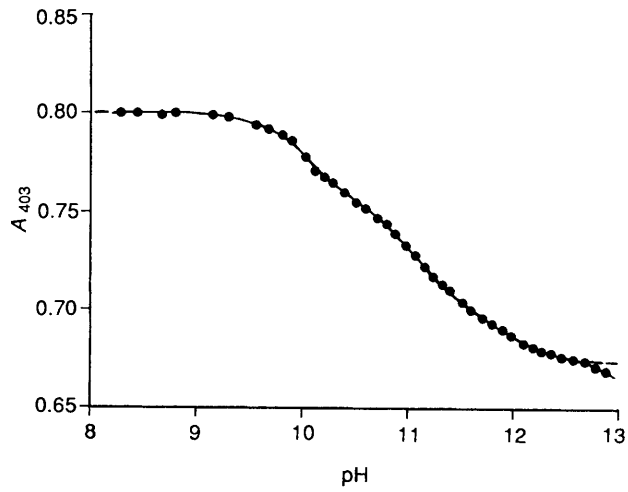

Fig. 3 pH Titration of the py complex of MP-8, showing the change in absorbance *vs.* pH and extrapolation (— —) to obtain the initial and final values of A_{403} for the main p$K \approx 11.3$

solution were placed in a 1 cm spectrophotometer cell thermostatted at 25 °C and titrated with 0.25–5 μl aliquots of the pure organic amine (*ca.* 10 additions overall). Up to 1–2 min were required for mixing of the added amine and for the complete disappearance of turbidity; all changes in the spectra had occurred within this time interval. Duplicate experiments were carried out for both py and aniline. All four titrations gave reasonable isosbestic points, at 326, 401, 473, 505 and 585 nm in the case of py and at 336, 401, 478, 504 and 590 nm in the case of aniline. The products had slightly different spectra with maxima at 403.5, 524 and 556 (sh) nm in the case of py (see Fig. 2) and at 406, 524 and 560 (sh) nm in the case of aniline. Analysis of the change in absorbance at 397 nm (Soret band of the aqua complex) with ligand concentration (see Experimental section) gave the following values of K (the equilibrium constant): py, 520 and 560, average 540 dm^3 mol^{-1}, $\log_{10} K$ 2.73 ± 0.02; aniline, 470 and 480, average 475 dm^3 mol^{-1}, $\log_{10} K$ 2.67 ± 0.01. The linearity of the plots shows that the equilibrium involves the binding of one molecule of base per molecule of MP-8.

pH-*Dependent Equilibria of Derivatives of* MP-8 *with* NH_3, *py, Aniline and Imidazole.*—Initial experiments in alkaline solution on the binding of py and aniline by the hydroxo- (as distinct from the aqua-) form of MP-8 revealed an unexpected increase in the binding constant and a slight change in the spectrum; a similar change in spectrum was then observed with the NH_3 complex. These further equilibria were investigated quantitatively by titrating 250 cm^3 of *ca.* 2×10^{-6} mol dm^{-3} solutions of MP-8 in 20% aqueous MeOH containing 0.1 mol dm^{-3} $NaClO_4$ and 0.5 mol dm^{-3} NH_4Cl, py or NH_2, contained in a conical flask and thermostatted at 25 °C, with aqueous NaOH solutions of varying strength from pH *ca.* 8 to *ca.* 13; a single experiment was carried out with aniline, duplicate experiments with py and NH_3. After each addition of NaOH (*ca.* 40 in all) the pH was measured and an aliquot of solution transferred to a 4 cm spectrophotometer cell. All changes occurred within the time of mixing, stabilisation of the pH and transfer to the cell (*ca.* 3 min). Absorbance changes were recorded at 404 (NH_3), 403 (py) and 406 nm (aniline).

For all three adducts the plot of A *vs.* pH showed one major inflection corresponding to a pK in the region 11–12, together with further anomalies at pH *ca.* 10 and above 12.5. Over the whole range of pH the wavelength of the Soret band (see Table 2) indicated that hydroxo complexes formed at most only a minor component of the species present. The anomaly at pH *ca.* 10 is most noticeable with py (see Fig. 3), but barely detectable with NH_3 and aniline, and is assigned primarily to the effect of

deprotonation of the terminal amine in the side-chain (see Discussion), although additional effects due to partial displacement of the base by HO^- cannot be excluded. At pH > 12.5 the py and NH_3 adducts show a further fall in absorbance, which may represent aggregation, while the aniline adduct shows an abrupt levelling off in absorbance. All these anomalies have been discounted in analysing the data for the main inflection by estimating the true end-points through graphical extrapolation, as shown in Fig. 3 for the py adduct.

All five experiments gave good linear plots of log([A^-]/[HA]) against pH (where HA and A^- represent the conjugate acid and base respectively), with a slope close to unity (indicating loss of a single proton) and the following values of the pK: NH_3, 11.8, 11.92, average 11.9; py, 11.2, 11.2, average 11.2; aniline, 11.8 (see Fig. 4).

In the case of the imidazole complex a *ca.* 1×10^{-6} mol dm^{-3} solution of MP-8 in 20% aqueous MeOH containing 0.1 mol dm^{-3} imidazole was titrated from pH *ca.* 7 to 14.3 with *ca.* 10 mol dm^{-3} KOH or, at higher pH, by adding solid KOH to the solution. A plot of A_{404} *vs.* pH started to fall at pH *ca.* 11 and the changes were reversible and instantaneously established up to pH *ca.* 14, above which irreversible bleaching of the spectrum occurred. The Soret band moved from 404.1 nm at pH 8 to 410.1 nm at pH 14 which strongly suggests (see Discussion) the successive loss of two protons (from the simple imidazole as well as the imidazole ring of His) due to two overlapping equilibria but, because of the irreversible fall in A at high pH, it proved impossible to obtain a realistic final value of A and to establish by analysis the number of protons involved. The data indicate a minimum of pK > 12.0 for the lowest pK or pK = 13.0 ± 0.1 if the two pK values are identical.

Spectra of Amine Derivatives of MP-8.—The species below and above the observed pK values will be identified (see Discussion) as the HHis–Fe–B and His^-–Fe–B forms of equation (2). The spectra of the HHis–Fe–B forms with several amines were determined by treating 2 cm^3 of a *ca.* 1×10^{-6} mol dm^{-3} solution of MP-8, pH 8.75, in a spectrophotometer cell with aliquots (50 μl) of a 10% (v/v) aqueous solution of the amine at pH 8.75 until no further change in the wavelength of the Soret band was observed; the spectra of the species were determined similarly at pH *ca.* 13. The wavelengths of the Soret band are listed in Table 2.

Discussion

Analysis of the data of Table 1 and Fig. 1 shows that over the range pH 7–10 the co-ordination of NH_3 by MP-8 is consistent with the simple equilibrium (6) with $\log_{10} K = 3.34$ in 20% aqueous MeOH at 25 °C, together with the optimised value of pK = 8.89 (*cf.* previously reported pK 8.90)[6] for ionisation of the co-ordinated H_2O in MP-8 according to equation (7) and the experimentally determined pK = 9.09 for NH_3 in this mixed solvent. The variation in pH dependence of log K_{obs} (see Fig. 1) from pH 7 to 10 represents the succession of equilibria (13)–(15).

$$Fe\text{–}OH_2 + NH_4^+ \rightleftharpoons Fe\text{–}NH_3 + H_3O^+ \quad (13)$$

$$Fe\text{–}OH^- + NH_4^+ \rightleftharpoons Fe\text{–}NH_3 + H_2O \quad (14)$$

$$Fe\text{–}OH^- + NH_3 \rightleftharpoons Fe\text{–}NH_3 + HO^- \quad (15)$$

This appears (see Introduction) to be the first equilibrium constant reported for the simple substitution of co-ordinated H_2O by NH_3 in any iron(III) porphyrin with or without a protein. It opens up the study of amine and amino acid complexes of MP-8; the former also provide models for cyt f and the alkaline form of cyt c where the axial ligands are probably His + Lys (amine).[3,4] Models for the usual form of cyt c, where the axial ligands are now known to be His + Met (RSMe),[2] have been known since 1965, when Harbury *et al.*[48] used the formation of low-spin complexes of MP-8 with thioethers

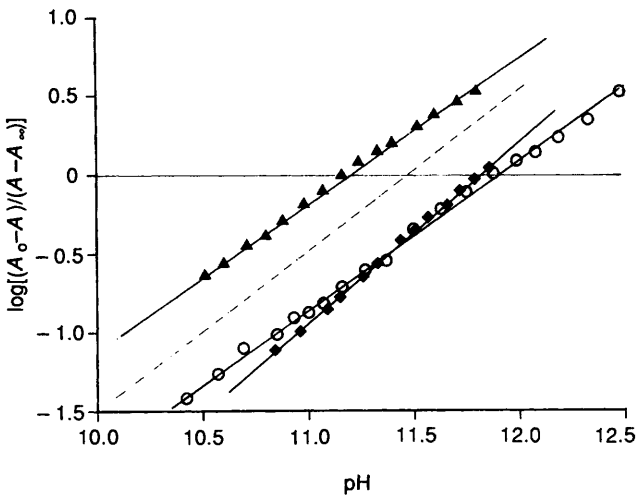

Fig. 4 Analysis of the pH titrations of MP-8 with py (▲), NH_3 (○) and NH_2Ph (◆) to establish the involvement of one proton (theoretical slope for n = 1.0 given by – – –) and the pK value (= pH at y = 0)

(including *N*-Ac-Met) as support for their proposal of Met as a ligand in cyt c.

We have shown that at higher pH the NH_3 complex is associated with a second reversible equilibrium with pK 11.9 involving the loss of one H^+ (see Fig. 4) to give a product (Soret band at 406 nm) which is not the hydroxo complex (Soret band at 401 nm). By analogy with similar equilibria previously reported for other MP-8 derivatives where the axial ligand is HO^- (pK 10.5)[6] or CN^- (pK *ca.* 13.5),[7] we ascribe this equilibrium to the loss of a proton from the imidazole ring of His according to equilibrium (2), where B = NH_3. We therefore have available a set of equilibria and spectra (see Table 2) involving NH_3 as a ligand against which to compare the behaviour of other amines and heterocyclic bases as potential ligands, in particular to establish whether py and aniline interact with MP-8 by co-ordination to the metal or by adduct formation with the porphyrin ring (see Introduction). Two pieces of evidence support co-ordination rather than adduct formation. (*a*) The products of the reactions with both py and aniline, like that with NH_3, show a pH-dependent equilibrium involving one proton with pK 11.2 for py and 11.8 for aniline (see Fig. 4). (*b*) There are obvious similarities in the spectra (see Table 2) between the products with NH_3, py and aniline both above and below this pK; by contrast, the 'adduct' of MP-8 at pH 12 with 1-methylpyridinium (which cannot act as a ligand) shows a Soret band at 394 nm, *i.e.* has a totally different spectrum.[49] We conclude that in the pH region studied, py and aniline both act mainly as ligands to the Fe^{III} in MP-8, though the formation of lower concentrations of some π–π adducts cannot be excluded and could explain the deviation from the stoichiometry of n = 1.0 for aniline in Fig. 4. Further results on adduct formation [with 1-methylpyridinium and caffeine (3,7-dihydro-1,3,7-trimethyl-1*H*-purine-2,6-dione)] will be reported later.

Comparison of the wavelengths of the Soret bands listed in Table 2 (*cf.* also ref. 50) shows that: (*i*) MP-8 complexes possessing any of the bases studied *except* aniline and $NH_2(CH_2Ph)$ (*i.e.* including NH_3, aliphatic amines, py and imidazole) as the axial ligand *trans* to undissociated His exhibit a Soret band at virtually the same wavelength of 403.5–404 nm; (*ii*) the aromatic amines aniline and $NH_2(CH_2Ph)$ (also Phe and Trp) produce a shift to longer wavelength (406, 405.5 nm respectively) of *ca.* 2 nm, which we have ascribed[50] to overlap between the benzene and porphyrin π electrons; and (*iii*) with both NH_3 and py as the axial ligands loss of the proton from the co-ordinated His moves the Soret band from 403.5 to 406 nm and with aniline from 406 to 408.5 nm, *i.e.* a comparable shift of 2.5 nm. The magnitude of this shift provides evidence for

the nature of the pH-dependent equilibrium exhibited by the imidazole complex of MP-8 at pH 12–14 (see Results), which has already been detected by cyclic voltammetry.[18] We previously reported[50,51] that the monomeric bis(imidazole) complex of haemin in 44% aqueous EtOH shows two pH-dependent equilibria with pK 12.7 (corresponding to approximately one H^+) and *ca.* 14.5 and associated with an overall shift in the Soret band of 5 nm from 412 to 417 nm for the two protons, which is very comparable to the shift of 2.5 nm for one H^+ observed here, while the bis(*N*-methylimidazole) complex (which cannot lose a proton from the ligand) shows no change in spectrum from pH 8 to 14.2. We now find that the imidazole complex of MP-8 also shows a reversible shift in the Soret band from 404 nm at pH 11 to 410 nm at pH 14, *i.e.* an overall shift of 6 nm; it proved impossible to establish the number of equilibria and the number of protons involved but, in view of the above results, it seems reasonable to conclude that the observed changes represent overlapping equilibria due to the loss of two protons (from His and the added imidazole) with pK in the region 12–13.

The pK for ionisation of the co-ordinated His in MP-8 therefore increases with the nature of the *trans* ligand in the order: HO^-, 10.5; py, 11.2; aniline, 11.8; NH_3, 11.9; imidazole, 12–13; CN^-, 13.5 (*cf.* free imidazole pK 14.4); this is probably mainly an order of increasing σ-donor power (from HO^- to CN^-). One would expect H_2O to come below HO^- in any ligand order and the pK for loss of a proton from His *trans* to H_2O to be below 10, *i.e.* close to the pK (8.9) for loss of a proton from the co-ordinated H_2O itself. This would predict the potential existence of the pH-independent equilibria (16), and

$$HHis\text{–}Fe\text{–}OH \rightleftharpoons His^-\text{–}Fe\text{–}OH_2 \rightleftharpoons His^-\text{–}Fe + H_2O \quad (16)$$

has been strikingly confirmed by recent results on Ac-MP-8. Van Wart and co-workers[12] have used a combination of ESR, resonance-Raman and MCD as well as UV/VIS spectroscopy to characterise the co-ordination numbers and spin states of the forms of Ac-MP-8 observed over the range pH 2–12. Spectrophotometric titration of Ac-MP-8 established a pK of 9.0 (*i.e.* close to the 8.9 of MP-8), which they assigned to conversion of the HHis–Fe–OH_2 form (present as a mixture of high- and intermediate-spin six-co-ordinate species) into a predominantly five-co-ordinate high-spin His^-–Fe complex. The proportion of this species in equilibria (16) may be different in the case of MP-8 (see below) and remains to be determined experimentally.

As mentioned in the Introduction, cyclic voltammetry revealed a further pK at 10.1 for the imidazole complex of MP-8 which had no detectable effect on the UV/VIS spectrum (confirmed here) and was assigned to the terminal Cys-NH_2.[19] We have now found that detectable anomalies in plots of A *vs.* pH are shown at pH *ca.* 10 by the complexes with NH_3, aniline (both only just detectable) and py (more noticeable). The relatively small effect of this pK on the spectrum and of the nature of the ligand on the pK value are both consistent with a 'neighbouring group effect' of the terminal Cys-NH_2. A more pronounced effect might be expected with anionic ligands due to Coulombic interaction; one might, for example, expect the terminal NH_3^+ in MP-8 to stabilise the HHis–Fe–OH^- form over the other forms compared to the NHAc group in Ac-MP-8. Regardless of any differing ratios in MP-8 and Ac-MP-8, the observed pK of 8.9 and 9.0 respectively can be treated as analogous and, for the purposes of ligand-binding studies, as equivalent to the ionisation of co-ordinated H_2O.

The above equilibria involving the ready deprotonation of the co-ordinated imidazole ring of His provide a parallel for the alkaline isomerisation of cyt c, suggest an explanation for the anomalous pH dependence of the co-ordination of NH_3 by haemoglobin and, when coupled with electron transfer, further suggest a possible role for co-ordinated His as H-atom donor which may be relevant to the mechanism of action of cytochrome oxidase. The neutral iron(III) form of cyt c undergoes conversion into the alkaline form by a relatively slow process involving a conformation change; this appears to proceed *via* the initial loss of a proton (pK 11.0) to form the His^-–Fe–Met species (*cf.* the analogous equilibria and similar pK values discussed above) to give the five-co-ordinate His^-–Fe which then picks up both Lys and a H^+ to give HHis–Fe–Lys, with pK 9.3 for the slowly established overall equilibrium (17).[4]

$$HHis\text{–}Fe\text{–}Met + HLys^+ \rightleftharpoons HHis\text{–}Fe\text{–}Lys + Met + H^+ \quad (17)$$

It has also been shown that the alkaline form is a far more powerful reducing agent (by 440–460 mV) than the neutral form,[52] but whether this change of ligand and redox potential plays any role in the mechanism of action of cytochrome oxidase has yet to be established. In studying the binding of NH_3 by the Fe^{III} in haemoglobin at pH 10.6 (*i.e.* above the pK of both NH_3 and the Fe–OH_2 complex), Coryell and Stitt[30] obtained qualitative evidence that the expected equilibrium (18)

$$HHis\text{–}Fe\text{–}OH^- + NH_3 \rightleftharpoons HHis\text{–}Fe\text{–}NH_3 + HO^- \quad (18)$$

was, in fact, pH independent. Scheler[31] subsequently found that NH_3, NH_2Me, NH_2Et and $NHMe_2$ all reacted with haemoglobin at pH *ca.* 9 to give similar changes in spectra (Soret band at 410.5–411.5 nm) and similar values of log K; he studied the binding of NH_2Me in more detail over the range pH 7–11 and a plot of his results (from Table 3 of ref. 31) again shows an apparent pH-independent equilibrium at pH > 10. As explanations for the observed pH independence of the apparent substitution of HO^- by NH_3, Coryell and Stitt[30] suggested either the formation of a seven-co-ordinate complex (retaining both NH_3 and HO^- as ligands) or a coupled reduction in the pK of some neighbouring functional group, while Scheler[31] merely commented that the pH independence of the binding of amines by haemoglobin was fundamentally different from that of anions or even of imidazole. A possible explanation is that the lower relative permittivity of the protein environment reduces the pK for ionisation of the co-ordinated His of the more highly charged HHis–Fe–NH_3 below that of His–Fe–OH^-, so that the observed equilibrium actually corresponds to (19), *i.e.* 'the neighbouring functional group' of

$$HHis\text{–}Fe\text{–}OH^- + NH_3 \rightleftharpoons His^-\text{–}Fe\text{–}NH_3 + H_2O \quad (19)$$

Coryell and Stitt is the second axial ligand. The greater *trans* effect of imidazole compared to NH_3 (see above) and/or additional delocalisation of charge could explain why the analogous equilibrium is not observed with imidazole. Finally, it is well established that co-ordinated O_2 [whether to Fe^{II} in haemoglobin Hb[53–55] or MP-8[1] or to a corrinoid or other cobalt(II) complex[56]] is most readily reduced by a H atom (from a quinol, thiol, *etc.*) and we have suggested[1] that the Cu_B situated close to the cyt a_3 may serve to convert a supply of electrons and protons into readily transferable H atoms by the cyclic reaction (20) where HX could be the thiol (RSH) group of

$$X^-\text{–}Cu^{II} \underset{-H^\cdot}{\overset{+e\ +\ H^+}{\rightleftharpoons}} HX\text{–}Cu^{I} \quad (20)$$

co-ordinated Cys. The present results suggest that co-ordinated HHis would be another good candidate for the role of HX. It is interesting that there is no positive evidence for the co-ordination of any Cys residues to the Cu in any of the various haem- and Cu-containing terminal oxidases cyt aa_3, ba_3 or bo, and that recent work on one of these, cyt ba_3, suggests that Cu_B is co-ordinated by four N atoms, probably from four His side-chains.[57]

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