Protein-free models for the proton-coupled reduction of haemoproteins

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Reduction of the bis-pilocarpate–haemin complex at pH $\geqslant$ 10 involves the simultaneous uptake of an electron by the Fe(III) ion and a proton by the pendant alkoxide group of an axial ligand. This provides a protein-free model for reactions such as the proton-coupled reduction of cytochromes which involve cooperative Coulombic interaction between two non-bonded sites.

Hemin *Pilocarpate* *Proton-coupled reduction* *Cooperative interaction* *Coulombic interaction* *Cytochrome*

1. INTRODUCTION

The use of steric effects by the protein to modify and control the activity of the metal in metalloproteins has been elucidated with the help of protein-free models in the case of haemoglobin [1] and the B_{12}-dependent isomerases [2]. Far less is known about the use of Coulombic effects (including hydrogen-bonding), which must be involved in phenomena ranging from the ion-coupled reduction of cytochromes and the activation of H_2O_2 by peroxidases (see [3]) to the ion-dependent ATPases. Protein-free models are needed in which one can study cooperative Coulombic interaction between two non-bonded sites, involving the uptake of a negative charge (electron or anion) at one site and a positive charge (proton or cation) at the other.

We have therefore been studying the formation and reduction of complexes of Fe(III) protoporphyrin IX (haemin), in which both axial sites are occupied by imidazole derivatives (denoted by B, see fig.1) with side-chains possessing various functional groups, and have been examining how the

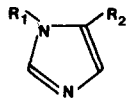

Fig.1. Structure of the imidazole derivatives (I–VI) mentioned in the text; all are denoted by B in formulae and equations.

I	Imidazole	$R_1 = H$	$R_2 = H$
II	*N*-Methylimidazole	Me	H
III	Histamine	H	$-CH_2-CH_2-NH_3^+$
IV	Histidine	H	$-CH_2-CH(CO_2^-)-NH_3^+$
V	Pilocarpine	Me	$-CH_2-CH(-CH_2-O-CO-)-CHEt$
VI	Pilocarpate	Me	$-CH_2-CH(CH_2OH)-CHEt-CO_2^-$

p*K* values of such groups are affected by the proximity of the net charge of +1 on the Fe(III) ion (with two negative charges on the porphyrin) and by subsequent reduction to the Fe(II) state. The Fe(III) complexes of interest (viz. FeB_2, with only the axial ligands designated) are formed in aqueous solution by reaction of the dimeric (concentrations were, however, calculated on the basis of monomeric FeOH) alkaline form of haemin [4–6]

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(here written $(FeOH)_2$) with B according to equilibrium 1 without the intermediate formation of any mononuclear monoimidazole complex [6,7]; the equilibria cannot be studied below pH 8 because haemin forms aggregates. We are therefore interested primarily in functional groups with 'normal' pK values above 8, in particular $-NH_2$ (as in Lys, Arg) and -OH (Ser, Thr, Tyr).

$$(FeOH)_2 + 4B = 2FeB_2 + 2OH^- \quad (1)$$

We have previously reported that the FeB_2 complex with histidine (IV) undergoes proton-coupled reduction and identified the site of proton-uptake as a pendant $-NH_2$ group of IV [3]. We now report that the complex with pilocarpate (VI) also exhibits proton-coupled reduction, which can be ascribed to a change in the pK of a pendant -OH group. This provides an example of the second type of functional group and emphasises the generality of the underlying mechanism of cooperative interactions in proton-coupled reductions.

2. MATERIALS AND METHODS

Solutions of haemin (BDH) were prepared by dissolution in deoxygenated (N_2) 0.1 M NaOH. Solutions of VI were prepared by hydrolysing V (pilocarpinium nitrate, Merck) in 0.1 M NaOH at 30°C for 1 h [8]. II (Merck) was used as supplied. Buffers ($NaHCO_3$ + NaOH, μ = 0.3) were prepared according to [9]. UV-visible spectra were recorded with a JASCO Uvidec-1 spectrophotometer, and pH determined with a Metrohm microelectrode model 9100. Unless otherwise indicated, all experiments were carried out in aqueous solution (pH 8–14) at 25°C.

3. RESULTS

3.1. *Preliminary experiments*

As a background to experiments on the FeB_2 complex with VI we (a) determined the pK values of free VI and (b) studied the complex with the simpler ligand II, whose identity has already been established [5]. (a) Two 40-ml samples of 0.2 M VI in 0.1 M NaOH were titrated with 0.2 M HCl from pH 13 to 1.3; both gave pK values of 7.5 and 3.7 (each corresponding to 1.0 ± 0.1 protons), which can be assigned to protonation of the imidazole (7.5) and carboxylate (3.7) groups, with no detectable ($pK \geqslant 13.5$) ionisation of the hydroxyl. This also confirms that hydrolysis of the lactone is essentially complete. (b) We found no change in the wavelength (411 nm) of this complex over the whole range of pH 8–14; although equilibrium 1 is reversed by high pH, the rate is slow enough for the spectra to be recorded.

3.2. *Equilibrium between haemin and pilocarpate (VI); ionisation of the ligand*

Reacting haemin (Soret band at 385 nm) with VI revealed two successive and partly overlapping equilibria. The first involves minor changes in spectrum (decrease in intensity without significant shift in the wavelength of the Soret band). Titration of 9.05×10^{-5} M haemin solutions at pH 8.5, 10.0 and 11.0 with VI (up to $\sim 2 \times 10^{-2}$ M) and analysis of the changes in A_{590} (isosbestic point of the second equilibrium) agreed with the pH-independent formation of an adduct according to equilibrium 2 (cf. the analogous adduct with IV [3]) and $\log K_2 = 2.0 \pm 0.2$

$$(FeOH)_2 + B = (FeOH)_2 \cdot B \quad (2)$$

$$(FeOH)_2 \cdot B + 3B = 2FeB_2 + 2OH^- \quad (3)$$

$$\log ([FeB_2]/[(FeOH)_2B]) = \log K_3 - 2\log [OH^-] + 3\log [B] \quad (4)$$

$$\alpha \log [Fe_T] = 0.5(1 - \alpha) \log [Fe_T] \quad (5)$$

The second equilibrium is characterised by the appearance of a new Soret band in the 411–413 nm region, typical of a FeB_2 complex [5]. Two tests of the expected equilibrium 3 can be made. Firstly, the equilibrium constant K_3 can be rearranged to give eq.4. Titrating a solution of 7.46×10^{-6} M haemin with up to $\sim 5 \times 10^{-2}$ M at pH 10 and plotting the first against the last term in eq.4 gives a slope of 3, as expected, and an apparent value of $\log K_3 = -8.3$. We next keep [B] constant but vary $[Fe_T]$ and apply eq.5 (the dilution test [8]), where α is the fraction of the total iron present as FeB_2; a plot of the first against the last term should give a slope of 0.5. Dilution over the range of ~ 0.1–3×10^{-5} M Fe gave slopes of 0.4–0.5 at all pH values, but the values of $\log K_3$ varied with pH as follows: −9.5, −9.5 and −9.7 (separate experiments) at pH 8.5 with 0.005 M B; −9.0 at pH 9.0 with 0.005 M B; −8.4 at pH 10.0 with 0.025 M B; −7.4, −7.5 and −7.3 at pH 11.0 with 0.05 M B. This indicates that the FeB_2 com-

plex can lose one proton with pK = 10.0, as shown by the virtually pH-independent values of −9.6, −9.1, −9.0 and −9.5 obtained for log K_3 at pH 8.5, 9, 10 and 11 respectively after correcting the observed values for the additional formation of the deprotonated complex at higher pH. In agreement with this, the Soret band shows a small shift from 411 nm below, to 413 nm above, pH 10.

3.3. *Reduction of the bis-pilocarpate-Fe(II) complex*

Reducing the complex with $Na_2S_2O_4$ produces a spectrum (maxima at 422, 529 and 556 nm) similar to that of the Fe(II) form of cytochrome b_5 (422.5, 525 and 556 nm [10]), where both axial ligands are known to be His, i.e., the FeB_2 core remains unchanged. The reversibility and pH-dependence of reduction were studied (in 9 separate experiments) by cyclic voltammetry under N_2 from pH 8 to 11 using a Pt working electrode with solutions containing 0.5 × 10^{-3} M haemin in 0.2 M VI and 0.1 M $NaHCO_3$, raising the pH by adding 6 M NaOH, and using a scan rate of ⩾100 mV/s (cf. [3]). As shown for one experiment in fig.2, $E_{½}$ is virtually pH-independent below pH 9 ($E_{½}$ = −0.197 ± 0.004 V *vs* SHE at pH 8) but approaches a slope of 59 mV per pH unit (i.e., one proton per electron) at pH ⩾ 10.5; the inflection corresponds to pK_{III} = 9.7 ± 0.1 in the Fe(III) complex (strictly speaking at the electrode surface, but clearly related to the pK of 10.0 observed in solution) and pK_{II} > 13 in the Fe(II) complex. Further experiments showed that the pH-dependence was reversible and not significantly affected by changing the working electrode (Pt or hanging mercury drop) or the supporting electrolyte (Li/Na/KNO_3, NaCl).

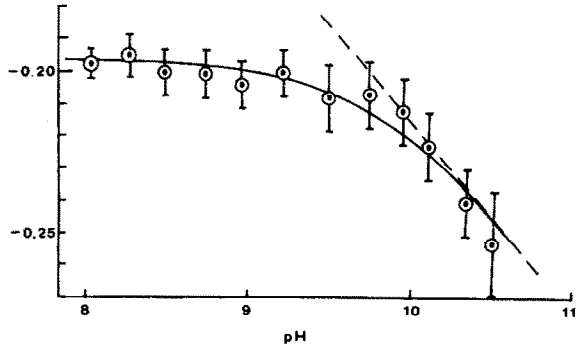

Fig.2. Plot of $E_{½}$ (in V *vs* SHE) against pH for reduction of the bis-pilocarpate–haemin complex. Curve calculated with $E_{½}$ = −0.197 V at pH 8, pK_{III} = 9.7 and pK_{II} > 13 (——), limiting slope of 59 mV/pH (– – –).

4. DISCUSSION

Coordination of the imidazole ring of III by the Co(III) ion in cyanoaquocobinamide (net charge of +1) reduces the pK of the pendant -NH_2 from 9.5 (when free) to 4.5 [11]. Formation of the FeB_2 complex with both III (unpublished) and IV [3] also involves the loss of one proton per Fe due to a lowering of the pK of a pendant -NH_2. Our results show that the FeB_2 complex with VI, which is formed according to eq.1 at pH 8–10, can ionise with pK = 10. This could involve either (a) ionisation of a pendant -OH (expected pK ~ 15 in the free ligand [12]) or (b) formation of an ion-pair with OH^-. The behaviour of coordinated III and IV provides a precedent for (a), while failure to detect any analogous changes with II (see section 3.1) militates against (b). We conclude that the equilibrium involves ionisation of a pendant -OH group.

We reported previously [3] that the pH-dependence of $E_{½}$ for reduction of the bis-histidine–Fe(III) complex corresponds to the simultaneous uptake of one proton per electron over the whole range of pH 8–10, and we ascribed this to the rise in pK of the pendant -NH_2 group as the residual charge on the Fe(III) ion was eliminated by the uptake of an electron. The bis-pilocarpate complex provides an opportunity to test this theory by examining the pH-dependence of $E_{½}$ both above and below the observed pK of the Fe(III) complex. Fig.2 shows that $E_{½}$ is pH-independent below pH 9 but approaches a slope corresponding to one proton taken up per electron above pH 10, in agreement with the proposed mechanism.

The bis-histidine and bis-pilocarpate complexes of haemin appear to be the first reported examples of proton-coupled reduction in protein-free metal complexes where Coulombic interaction provides the main or only interaction between the site of protonation and the metal; they are particularly relevant as models for mitochondrial cytochrome b, which exhibits proton-coupled reduction [13,14], is implicated in proton translocation

[14,15] and probably possesses two His residues as axial ligands [16]. Our results support a mechanism whereby (i) the presence of a residual positive charge on the metal can significantly lower the pK of a neighbouring group by stabilising a negatively charged conjugate base (e.g., $-O^-$) or destabilising a positively charged conjugate acid (e.g., $-NH_3^+$); and (ii) the subsequent uptake of an electron by the metal can be coupled with the simultaneous uptake of a proton by the neighbouring functional group as the Coulombic interaction is reduced and the pK rises towards a more normal value; with a negatively charged core (as in ferredoxins and rubredoxins) the relevant pK would, of course, be more normal when the metal is oxidised and abnormally high when the metal is reduced.

REFERENCES

[1] Perutz, M.F. (1979) Annu. Rev. Biochem. 48, 327.
[2] Pratt, J.M. (1983) Inorg. Chim. Acta 79, 27.
[3] Baldwin, D.A., Campbell, V.M., Carleo, L.A., Marques, H.M. and Pratt, J.M. (1981) J. Am. Chem. Soc. 103, 186.
[4] Hogness, T.R., Zscheile, F.P., Sidwell, A.E. and Barron, E.S.G. (1937) J. Biol. Chem. 118, 1.
[5] Scheler, W., Mohr, P., Pommerening, K. and Behlke, J. (1970) Eur. J. Biochem. 13, 77.
[6] Bednarski, T.M. and Jordan, J. (1967) J. Am. Chem. Soc. 89, 1552.
[7] Adams, P.A., Baldwin, D.A., Hepner, C.E. and Pratt, J.M. (1978) Bioinorg. Chem. 9, 479.
[8] Cowgill, R.W. and Clark, W.M. (1952) J. Biol. Chem. 198, 33.
[9] Long, C. (1971) Biochemists' Handbook, Spon, London.
[10] Berman, M., Adnams, C., Ivanetich, K. and Kench, J. (1976) Biochem. J. 157, 237.
[11] Hanania, G., Irvine, D. and Irvine, M. (1966) J. Chem. Soc. A, 296.
[12] Allinger, N., Cava, M., De Jongh, D., Johnson, C., Lebel, N. and Stevens, C. (1971) Organic Chemistry, Worth, New York.
[13] Straub, J.P. and Colpa-Boonstra, J.P. (1962) Biochim. Biophys. Acta 60, 650.
[14] Von Jagow, G. and Engel, W.D. (1980) FEBS Lett. 111, 1.
[15] Chance, B. (1972) FEBS Lett. 23, 3.
[16] Carter, K.R., Tsai, A. and Palmer, G. (1981) FEBS Lett. 132, 243.