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The co-ordination of ligands by iron porphyrins: a comparison of ligand binding by myoglobin from sperm whale and the haem undecapeptide from cytochrome *c*

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The effect of the protein on the ligand binding properties of a ferric porphyrin was investigated by determining spectrophotometrically the temperature dependence of the binding constants, log K_{eq}, and hence the thermodynamic parameters ΔH and ΔS, for coordination of F^-, N_3^-, SCN^-, CN^- and imidazole by the haemundecapeptide from cytochrome *c*, known as *N*-acetyl microperoxidase-11 (NAcMP11), and ferric myoglobin from sperm whale. The axial binding site of the iron porphyrin in NAcMP11 is relatively open to the solvent, but in metMb it is buried in the protein and surrounded by tightly-packed distal amino acid residues. Whereas the N-donor ligands ethanolamine, glycine, pyridine, ammonia and imidazole are readily coordinated by NAcMP11, only the latter is coordinated by metMb, although the affinity of the haemoprotein for imidazole is nearly two orders of magnitude smaller than for NAcMP11 because of a much less favourable reaction enthalpy. Examination of the crystal structures of metMb and its imidazole complex suggests that steric factors are largely responsible. Of all the halides, only F^- is coordinated by metMb. In contrast with imidazole, the anionic ligands N_3^-, SCN^-, F^- and CN^- are coordinated more strongly by metMb than by NAcMP11. In the case of the first two, the difference in affinity is enthalpically driven, although there is a compensating entropic effect; this is attributed to the difference in the polarity of the environment of the porphyrin, with the relatively apolar environment of the haemoprotein stabilising the complex due to an electrostatic interaction between the anionic ligand and the residual positive charge at the metal centre. The effect is not generally true for all anionic ligands. Thus, although both F^- and CN^- are coordinated more strongly by metMb than by NAcMP11, the difference in affinity now stems largely from an entropic effect. Hence, whereas very significant differences in affinity for an exogenous ligand exist between the relatively exposed ferric porphyrin of the haempeptide and the buried ferric porphyrin of the haemoprotein, such differences cannot be attributed to a single factor only.

1 Introduction

The proteolytic digestion of cytochrome *c* affords a range of haempeptides that retain the proximal His-18 ligand of Fe(III) (Fig. 1).[1,2] These haempeptides have proved to be useful models for the peroxidases[3–8] and cytochrome P450.[9] The retention of the proximal ligand in mildly acidic, neutral and alkaline solutions[10,11] also makes them potentially useful models for haemoproteins such as haemoglobin and myoglobin that contain a single His residue as an axial ligand for iron. However, physical and spectroscopic studies of the haempeptides are complicated by their propensity to aggregate in aqueous solution.[12–15] This can be minimised by use of surfactants[16,17] or alcohol–water mixtures,[10,14,18] but more satisfactory is the blocking of the N-terminal amino group of the polypeptide and (in the case of MP11) the terminal amino group of the side chain of Lys-13 by acetylation,[19–23] which leads to species that are sufficiently monomeric in aqueous solution for study by conventional spectroscopic methods. The solution chemistry of both *N*-acetylated MP8 (NAcMP8†)[21] and *N*-acetylated MP11 (NAcMP11)[23] has been fully delineated, and a variety of spectroscopic methods have been used to probe the electronic

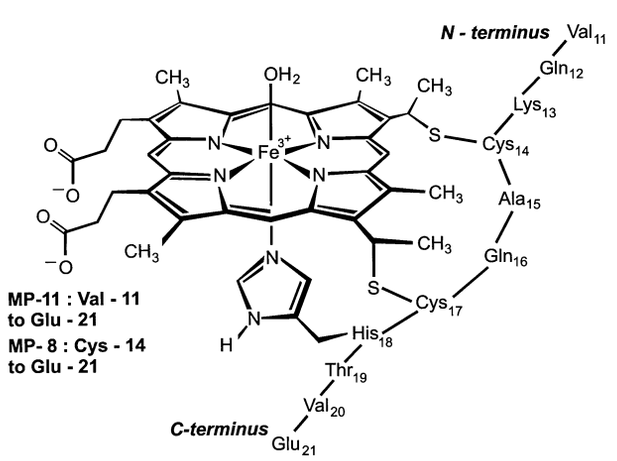

Fig. 1 The haempeptides from cytochrome *c* contain the proximal His-18 ligand (numbering is that of the original protein) and an iron porphyrin covalently linked through two Cys residues to the peptide. The two best studied of these haempeptides are microperoxidase-11 (MP11) and microperoxidase-8 (MP8). The former retains residues 11–21 and the latter residues 14–21 of the original protein.

† Abbreviations used: CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; metHb, ferrihaemoglobin; CHES, 2-[*N*-cyclohexylamino]ethanesulfonic acid; metMb, ferrimyoglobin; MOPS, 3-[*N*-morphilino]-propanesulfonic acid; MP*X*, the haempeptide from cytochrome *c* where *X* is the number of amino acid residues from the parent protein retained in the haempeptide; OEP, octaethylporphyrin; PPIX, protoporphyrin-IX; TMP, 5,10,15,20-tetramesitylporphyrin; TPP, 5,10,15,20-tetraphenylporphyrin; TRIS, tris(hydroxymethyl)aminomethane; X*NN*Y Hp, the mutation of the amino acid X to the amino acid Y in the haemoprotein Hp at position *NN*.

structure of the metal ion.[20,22–24] Since the solution behaviour of NAcMP11 is now as well characterised as that of NAcMP8, the former is the more attractive model because of its greater ease of preparation from the parent protein.[23]

That a common active site can display such disparate roles as dioxygen storage and transport (in haemoglobin and myoglobin[25,26]), the disproportionation and reduction of hydrogen peroxides (the catalases and the peroxidases[27,28]), electron transport (the cytochromes[29,30]), oxygenase and dioxygenase activity,[31,32] and as the terminal oxidase of the mitochondrial electron transport chain[33,34] is testimony to the ability of the protein to manipulate function. The availability of the microperoxidases as well characterised, monomeric ferric porphyrins, affords one the opportunity of comparing and contrasting their properties with those of the haemoproteins and hence probing the effect the structure of the protein has on the intrinsic properties of the iron porphyrin.

This sort of approach enabled Harbury and co-workers[35] to suggest that the spectroscopic properties of cytochrome *c* were better explained by assuming that His and Met were the axial ligands, rather than His and His, the prevailing view at the time. The microperoxidases, like methaemoglobin (metHb) and metmyoglobin (metMb),[25] bind ligands such as CN^- as the anion,[36] whereas the peroxidases require the mandatory uptake of a proton as well,[37] a feature of their chemistry related to the activation of H_2O_2 at physiological pH.[3,4,38] Byfield and Pratt[39] studied the coordination of a number of amino acids by MP8 (in 20% MeOH–H_2O to minimise aggregation) and showed that the presence of an aromatic side chain significantly increases log K_{eq} (for example, from 2.89 and 3.46 for Ala and Gly, respectively, to 4.76 and 5.64 for Phe and Trp). This was ascribed to donor–acceptor interactions between the aromatic moiety of the amino acid side chain and the porphyrin. A consequence of such an interaction would be to increase the polarisability of the porphyrin system and would explain the presence of an invariant distal Trp in the peroxidases to stabilise the Fe^{4+} and porphyrin radical cation that occur during enzyme turnover. We recently examined the effect of the axial ligands on the reduction potential of NAcMP8[40] and showed that varying the axial ligand field modulates the reduction potential only by a small amount; this emphasised that other factors, amongst them the charges and dipoles of the amino acid residues surrounding the metal, the solvent accessibility of the metal-binding cavity, and the non-polar nature of the protein matrix,[41,42] play a major role in controlling the reduction potential.

We have continued our comparison of the properties of the microperoxidases and the haemoproteins by determining spectrophotometrically the temperature-dependence of the substitution of the axial H_2O ligand of Fe^{3+} (eqn. 1; {P} =

$$His\text{–}Fe^{3+}\{P\}\text{–}H_2O + L \rightleftharpoons His\text{–}Fe^{3+}\{P\}\text{–}L + H_2O\ (K_{eq}) \quad (1)$$

porphyrin; only axial ligands are shown; charges omitted for convenience) in NAcMP11 and, where data are not available, in sperm whale metMb, by a variety of ligands.

MetMb was chosen for these purposes as it is arguably the best characterised of all the haemoproteins. From the temperature-dependence of log K_{eq}, we have determined ΔH and ΔS values, and compare these for the two porphyrin systems in this paper.

2 Experimental

2.1 Materials and instrumentation

UV-visible spectra were recorded on a Cary 1E or a Cary 3E spectrophotometer; the cell compartment was thermostatted with a water-circulating bath at the required temperature. Water was purified with a Millipore Milli-Q system (18MΩcm). The pH of solutions was determined using a Metrohm 713 pH meter and combination glass electrode, calibrated against standard buffer solutions, and adjusted with either NaOH or HCl solutions, as appropriate. All reagents used were of the highest purity available, and used as received, unless otherwise indicated. Sodium cyanide, glycine, imidazole and TRIS were from Merck; MOPS, CHES and CAPS were from Sigma; sodium perchlorate was from Fluka; potassium fluoride, ammonium chloride, magnesium sulphate and 30% NH_3 were from SAARchem. Potassium thiocyanate, from Riedel de Haën, was recrystallised from water. Pyridine (Merck) and ethanolamine (BDH) were distilled before use. NAcMP11 was prepared as previously described.[23] Sperm whale ferrimyoglobin (metMb) was from Sigma. The concentrations of metMb and NAcMP11 were determined spectrophotometrically (ε = 1.57 × 10^5 M^{-1} cm^{-1},[43] and 1.48 × 10^5 M^{-1} cm^{-1},[23] respectively, at the Soret maximum).

2.2 Ligand binding studies

The coordination of all ligands by metMb and NAcMP11 was studied by UV-visible spectrophotometry. The coordination of imidazole and SCN^- by metMb was conducted at pH 8.5 (0.1 mol dm^{-3} TRIS buffer), whilst coordination by F^- was conducted at pH 7.0 (0.1 mol dm^{-3} MOPS buffer), with ionic strength adjusted to 0.1 mol dm^{-3} (KCl) between 10 and 35 °C in a 1 cm pathlength cuvette; absorbance changes were monitored at 409 nm.

The coordination of cyanide by NAcMP11 was studied between 10 and 35 °C in 0.1 mol dm^{-3} CAPS buffer, at pH 10.0, and with ionic strength adjusted to 0.1 mol dm^{-3} (KCl). The absorbance of the Soret band (397.2 nm) was monitored after successive additions of the ligand solution, until no further changes in the absorbance occurred. All absorbance readings were corrected for dilution. A similar approach was used to study the coordination of imidazole in 0.1 mol dm^{-3} TRIS buffer, pH 8.5, with ionic strength adjusted to 0.1 mol dm^{-3} (KCl).

Since K_{eq} values for coordination of fluoride, azide and thiocyanate by NAcMP11 are low, high ligand concentrations are required. There is therefore an inevitable increase in ionic strength. However, the haempeptides are susceptible to aggregation, characterised by a marked decrease in absorbance in the Soret region;[21] for example, the related haempeptide NAcMP8 is only 80% monomeric at an ionic strength of 2 mol dm^{-3}, and dimerisation increases markedly at higher ionic strengths. Difference spectroscopy techniques were therefore used for determination of K_{eq} for these ligands. Equal amounts of NAcMP11 were placed in 0.1 mol dm^{-3} MOPS solutions, pH 7.0, in the sample and reference cuvettes housed in the thermostatted compartment of the spectrophotometer. Aliquots of a ligand solution were injected into the sample cuvette, and similar aliquots of an $NaClO_4$ (for titrations with N_3^- and SCN^-) or $MgSO_4$ (titrations with F^-) solution of the same ionic strength as the ligand solution were injected into the reference cuvette. The decrease in absorbance due to aggregation is therefore compensated for, and any changes in absorbance are a consequence of co-ordination of the ligand, provided the salts injected into the sample and reference cuvettes cause the same aggregation behaviour in the haempeptide (see Discussion). Coordination of thiocyanate, azide and fluoride by NAcMP11 was studied at 410, 408 and 402 nm, respectively.

The absorbance at the monitoring wavelength, A_λ, was fitted to eqn. 2 as objective function with A_0 (the initial absorbance in

$$A_\lambda = \frac{A_0 + A_1 K_{obs}[L]_{free}}{1 + K_{obs}[L]_{free}} \quad (2)$$

absence of any complex formation), A_1 (the absorbance of the complex between the ligand and the iron porphyrin) and K_{obs} as variables, using standard non-linear least-squares methods.

When K_{obs} is small, the concentration of the free, unbound ligand $[L]_{free} \approx [L]_T$, the total concentration of added ligand. In the case of imidazole and cyanide, however, K_{eq} is large, eqn. 2 is inappropriate, and eqn. 3 applies. ($[M]_T$ is the total metal ion

$$K_{obs}[L]^2_{free} + (1 + K_{obs}([M]_T - [L]_T))[L]_{free} - [L]_T = 0 \quad (3)$$

concentration.) This equation can be solved iteratively using Newton's method (eqn. 4) until the difference between successive iterations is small (we used 10^{-7}), where a_i is the appropriate coefficient of $[L]_{free}$ from eqn. 3.

$$[L]_{free,k+1} = [L]_{free,k} - \left[\frac{a_1[L]_{free}^2 + a_2[L]_{free} + a_3}{2a_1[L]_{free} + a_2}\right] \quad (4)$$

However, we have found that if K_{eq} is very large ($\log K_{eq} > 6$, as for CN^- binding to NAcMP11), this fitting procedure does not produce reliable and consistent results, despite apparently good experimental data. In these cases, we used an analytical rather than a numerical solution. Eqn. 5 readily follows, where

$$\frac{A_\lambda - A_0}{A_1 - A_\lambda} = K_{eq}([L]_T - [P]) = K_{eq}\left([L]_T - \frac{A_0 - A_\lambda}{A_0 - A_1}[M]_T\right) \quad (5)$$

P is the product of the reaction. If we define, $a = [M]_T/A_0 - A_1$, then expansion of eqn. 5 leads to a quadratic $RA_\lambda^2 + SA_\lambda + T = 0$, where $R = K_{eq}a$, $S = 1 + K_{eq}\{[L]_T - a(A_0 + A_1)\}$, and $T = K_{eq}aA_0A_1 - A_0 - K_{eq}[L]_TA_1$, only one root of which has physical significance.

Where necessary, the observed equilibrium constants (K_{obs}) obtained by fitting the titration data to eqn. 2 were corrected for the fraction of unbound, protonated ligand (HL), and the fraction of the hydroxo-NAcMP11 or hydroxo-metMb in solution by using eqn. 6, where pH is the average pH recorded

$$K_{eq} = \frac{K_{obs}([H^+] + K_L)([H^+] + K_{Fe})}{K_L[H^+]} \quad (6)$$

before and after the titration, pK_{Fe} is the ionisation constant for ionisation of Fe(III)-bound H_2O and pK_L is the ionisation constant for the ligand. If no such correction was required (*i.e.*, when the pH at which the titration was conducted was far from both pK_{Fe} and pK_L), then clearly $K_{eq} \approx K_{obs}$.

2.3 The temperature dependence of the ionisation of bound H_2O in NAcMP11

We have previously reported that pK_{Fe} for NAcMP11 in aqueous solution at 25 °C is 9.56 ± 0.01.[23] In order to correct K_{eq} data for pH (eqn. 6), the temperature-dependence of K_{Fe} is required. This was determined spectrophotometrically by titrating 25.00 cm^3 of a 2–4 μmol dm^{-3} solution of the haempeptide in 0.01 mol dm^{-3} of both TRIS and CHES with ionic strength maintained at 0.5 mol dm^{-3} ($NaClO_4$) in a thermostatted cell external to the spectrophotometer. The pH was adjusted from pH ≈ 7.5 to pH ≈ 11 by diffusion of microscopic aliquots of concentrated NaOH solution into the solution from a glass capillary. At each pH value, 5 cm^3 of solution was transferred with a peristaltic pump into a thermostatted 2.0 cm pathlength quartz cuvette housed in the spectrometer turret, and the absorbance at the Soret band (397.2 nm) was measured, before returning the sample to the reaction mixture.

Table 1 The dependence of pK_{Fe}, the acidic dissociation constant for coordinated water in NAcMP11, as a function of temperature

T/°C	5.0	10.0	15.0	20.0	25.0	30.0	35.0
pK_{Fe}	10.02	9.92	9.82	9.70	9.61	9.50	9.42

3 Results

3.1 The temperature dependence of the ionisation of bound H_2O in NAcMP11

Fig. 2 shows a typical titration of NAcMP11 through the ionisation constant of coordinated H_2O, pK_{Fe}. The results are summarised in Table 1. From the temperature dependence of pK_{Fe} we find that ΔH = 33.9 ± 0.6 kJ mol^{-1} and ΔS = −70 ± 2 J K^{-1} mol^{-1}.

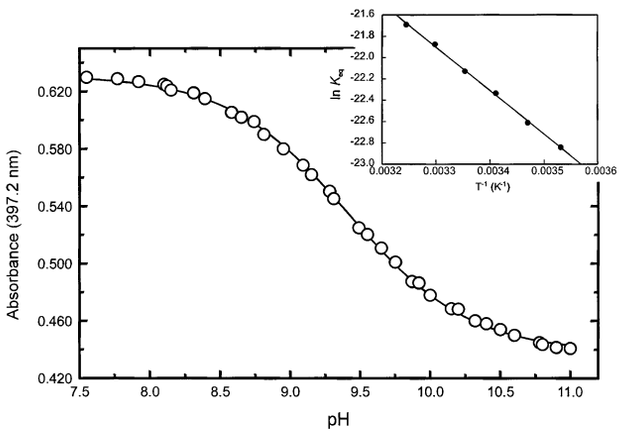

Fig. 2 Spectrophotometric titration of 2.1 μmol dm^{-3} NAcMP11 in a 2.00 cm pathlength cell at 35.0 °C, 0.5 mol dm^{-3} ionic strength ($NaClO_4$). The solid line is a non-linear least squares fit to the experimental data. *Insert*: From the temperature dependence of pK_{Fe}, ΔH = 33.9 ± 0.6 kJ mol^{-1} and ΔS = −70 ± 2 J K^{-1} mol^{-1}.

3.2 Preliminary investigation of the coordination of ligands by NAcMP11 and metMb

The effect of the spin state of the metal ion on the electronic spectra of iron porphyrins has been well documented.[44,45] High spin complexes of Fe(III) porphyrins (usually a mixture of S = 3/2 and 5/2 states[21]) are characterised by a Soret or B band (a composite of transitions to two vibronic components) near 400 nm, an N band near 360 nm, the $Q_v[a_{1u},a_{2u}(\pi) \longrightarrow e_g^*(\pi^*)](1,0)$ vibronic component of the Q–B coupled states near 490 nm, Q_o near 530 nm, and two charge transfer transitions, $a_{2u}'(\pi) \longrightarrow e_g(d\pi)$ and $b_{2u}(\pi) \longrightarrow e_g(d\pi)$ near 570 and 620 nm, respectively. When the metal becomes low spin on increasing the strength of the axial ligand field, the Soret band is red shifted to around 410 nm, the Q_v band to around 530 nm, and the Q_o band to 560 nm, the position of the N band remains virtually unchanged, whilst the charge transfer transitions move to the near infrared. Many complexes are known where the ligand induces a spin equilibrium in Fe(III) between S = 3/2, 5/2 and S = 1/2 states, and they have spectra with features of the two spin states.

Both imidazole and cyanide induce formation of low spin Fe(III) on displacing coordinated H_2O in metMb and in NAcMP11. Whereas N_3^- produces a low spin complex in metMb, its complex with NAcMP11 (as we have previously reported for NAcMP8[40]) is in spin equilibrium. The thiocyanato and fluoro complexes of both metMb and NAcMP11 are predominantly high spin.

We found no evidence for coordination of ethanolamine, glycine, pyridine or ammonia by metMb in the presence of 0.5 mol dm^{-3} of the ligand. This is in marked contrast to the

microperoxidases where all four N-donor ligands form low spin complexes with relatively high K_{eq} values (log K_{eq} = 3.68, 3.44, 2.62 and 3.20, respectively, for coordination by NAcMP8[46]). Of the halides, we found that only fluoride coordinates to metMb (*i.e.*, we observed no significant spectroscopic changes in the presence of 1.0 mol dm^{-3} Cl^-, Br^- or I^-).

3.3 Quantitative determination of the coordination of ligands by metMb and NAcMP11

Values of ΔH and ΔS for coordination of F^- and CN^- by metMb, as well as the temperature dependence of the aqua/hydroxo transition for this haemoprotein, have been reported (see Table 2).[47] We measured the temperature dependence of K_{eq} for the binding of F^- (to confirm the previously-reported value[47]), N_3^-, SCN^- and imidazole by metMb. Fig. 3 shows an

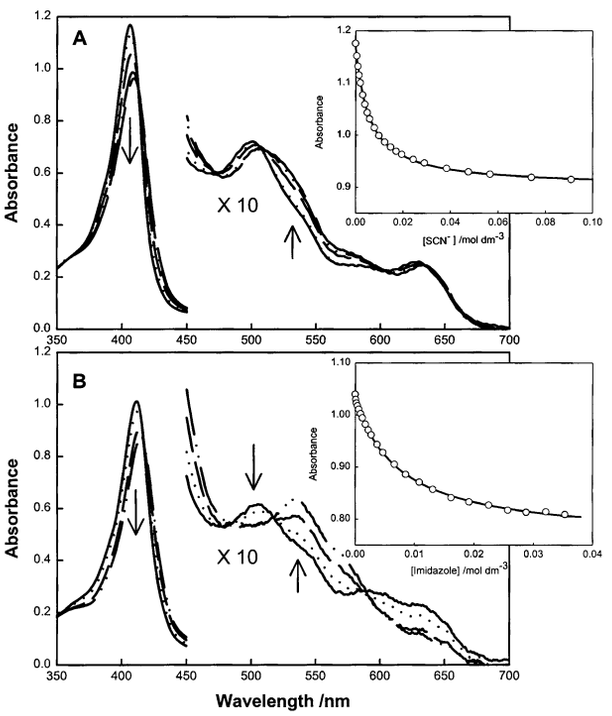

Fig. 3 (A) Titration of 7.6 μmol dm^{-3} sperm whale metMb with SCN^- at 15.0 °C, pH 7.0 (0.1 mol dm^{-3} MOPS), 0.1 mol dm^{-3} ionic strength (KCl) in a 1 cm pathlength cell. The metal ion remains predominantly high spin. **Insert**. The change in the Soret maximum as a function of [SCN^-]. The solid line is a fit to eqn. 2. (B) Titration of 6.6 μmol dm^{-3} sperm whale metMb with imidazole at 25.0 °C, 0.1 mol dm^{-3} ionic strength (KCl). The spectra show the transition from a predominantly high spin electronic configuration for Fe(III) to a predominantly low spin configuration on coordination of imidazole. **Insert**. Fit of eqn. 2 to the absorbance change of the Soret maximum of aquametMb on displacement of H_2O by imidazole.

example of a titration with SCN^- and with imidazole, where coordination causes Fe(III) to remain predominantly high spin, and to switch to low spin, respectively. The van't Hoff plots for these two ligands are shown in Fig. 4.

The titration of NAcMP11 with CN^- and imidazole could be carried out using conventional methods, but, because of aggregation effects with increasing ionic strength, difference titrations were used for the other ligands. As an example, Fig. 5 shows the titration of NAcMP11 with fluoride and cyanide at 25 °C.

The log K_{eq} values determined for coordination of F^-, N_3^-, CN^-, SCN^- and imidazole by metMb and NAcMP11, as well as ΔH and ΔS values determined from van't Hoff plots, are listed in Table 2.

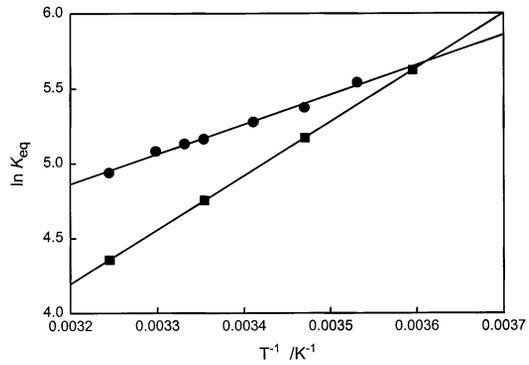

Fig. 4 Plot of ln K_{eq} against T^{-1} for coordination of (●) imidazole and (■) SCN^- by sperm whale metMb.

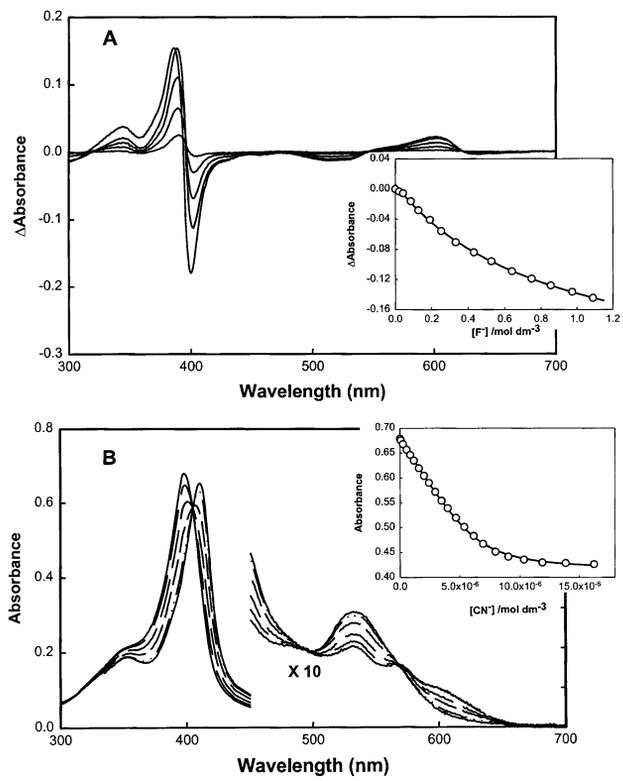

Fig. 5 (A) Difference titration of 6.0 μmol dm^{-3} NAcMP11 with fluoride at 25 °C, pH 7.0 (0.1 mol dm^{-3} MOPS) in a 1 cm pathlength cell. **Insert**. The change in absorbance at 402 nm as a function of [F^-]. The solid line is a fit to eqn. 2. (B) Titration of 5.0 μmol dm^{-3} NAcMP11 with cyanide at 25 °C, pH 10.0 (0.1 mol dm^{-3} CAPS), 0.1 mol dm^{-3} ionic strength (KCl) in a 1 cm pathlength cell. **Insert**. The change in absorbance at 397.2 nm as a function of [CN^-]; the solid line is a fit to eqn. 5.

4 Discussion

4.1 Effect of ionic strength on NAcMP11 aggregation

We have previously reported[21] that in aqueous solution the related haemoctapeptide, NAcMP8, forms π-stacked dimers as the ionic strength of the solution is increased. In that study we found that a model, based on charge neutralisation by specific binding of Na^+ ions to form, firstly, a more weakly associated dimer (on binding of *ca.* 2 Na^+ ions per dimer) and, secondly, a more tightly associated dimer on binding of a further 4 Na^+ ions per dimer, adequately described the behaviour of NAcMP8 in solutions up to an ionic strength of 6 mol dm^{-3}. The present results show that, at least with NAcMP11, the situation may be somewhat more complex and that the identity of the anion also plays a role in determining the nature of the aggregation of this haempeptide in aqueous solution. Thus, we

Table 2 Equilibrium constants and thermodynamic data for coordination of ligands by the ferric ion in sperm whale metMb and in the haempeptide from cytochrome *c*, NAcMP11

Ligand	Compound	pK_{Fe} (25 °C)	T/°C	K_{eq}[a]/dm^3 mol^{-1}	ΔH/kJ mol^{-1}	ΔS/J K^{-1} mol^{-1}	log K_{eq}[b] (25 °C)	Ref.
OH^-	metMb	8.9			26	−83		47
	NAcMP11	9.59			33.9(6)	−70(2)		This work
	NAcMP8	9.56			48(1)	−22(3)		21
F^-	metMb				−6	4.5	1.29	47
					−8.4(3)	2(1)	1.58	This work
	NAcMP11		10.0	1.33(8)	−7.0(3)	−22(1)	0.08	This work
			15.0	1.26(8)				
			25.0	1.16(6)				
			35.0	1.04(8)				
N_3^-	metMb		5.0	$4.33(13) \times 10^4$	−41.0(7)	−59(2)	4.10	This work
			15.0	$2.44(5) \times 10^4$				
			25.0	$1.33(3) \times 10^4$				
			35.0	$7.76(8) \times 10^3$				
	NAcMP11		5.0	43(1)	−19(1)	−38(5)	1.33	This work
			7.5	33.4(5)				
			10.0	31.7(4)				
			15.0	28.1(2)				
			20.0	26(4)				
			25.0	23.3(2)				
			30.0	19.9(3)				
			32.5	18.4(1)				
			35.0	17.0(1)				
SCN^-	metMb		5.0	277(9)	−30.1(1)	−61.4(2)	2.07	This work
			15.0	176(3)				
			25.0	115(2)				
			35.0	78(1)				
	NAcMP11		10.0	0.769(14)	−5.9(5)	−23(2)	−0.17	This work
			15.0	0.750(12)				
			20.0	0.722(10)				
			25.0	0.695(22)				
			35.0	0.629(8)				
CN^-	metMb				−78	−100	8.45	47
	NAcMP11		5.0	$4.40(5) \times 10^7$	−75(5)	−123(22)	6.55	This work
			10.0	$2.5(1) \times 10^7$				
			15.0	$1.1(5) \times 10^7$				
			25.0	$3.6(23) \times 10^6$				
			35.0	$2.1(3) \times 10^6$				
Imidazole	metMb		10.0	255(3)	−16.6(7)	−13(2)	2.23	This work
			15.5	216(3)				
			20.0	196(2)				
			23.0	175(3)				
			25.0	169(7)				
			30.0	161(3)				
			35.0	139(4)				
	NAcMP11		10.0	$2.55(8) \times 10^4$	−29.4(8)	−19(3)	4.16	This work
			15.0	$1.98(10) \times 10^4$				
			20.0	$1.59(3) \times 10^4$				
			25.0	$1.36(20) \times 10^4$				
			27.0	$1.27(3) \times 10^4$				
			30.0	$1.11(3) \times 10^4$				
			35.0	$8.96(4) \times 10^4$				

[a] Standard error in parenthesis. [b] From ΔH and ΔS values.

have found during the course of the current investigation that the binding of N_3^- and SCN^- by NAcMP11 can be determined very adequately by difference titration when $NaClO_4$ is used to induce in the reference cell the aggregation produced in the sample cell by addition of NaN_3 and NaSCN. However, when $NaClO_4$ was used during titrations with NaF, systematic deviations of the fits were found, with the experimental data consistently above the best fitting curve at lower [F^-] and below the curve at higher [F^-]. If $MgSO_4$ was used instead, good fits were obtained (insert, Fig. 5A), with no systematic deviations. This suggests that fluoride, in addition to coordinating Fe(III), has a different influence to other anions (ClO_4^-, N_3^-, SCN^-) on the nature of the NAcMP11 aggregates formed, or perturbs the position of the equilibria between monomeric NAcMP11 and the dimers previously identified. We report this as an empirical observation at this stage, and intend to investigate more fully the effect of different salts on the aggregation of these haempeptides; our results will be reported elsewhere.

4.2 The ionisation of Fe(III)-bound H_2O in NAcMP11

A plot of ln K_{Fe} against T^{-1} for the ionisation of coordinated H_2O in NAcMP11 gives a straight line (Fig. 2, insert) from which it can be calculated that $\Delta H = 33.9 \pm 0.6$ kJ mol^{-1} and $\Delta S = -70 \pm 2$ J K^{-1} mol^{-1}. For the related haemoctapeptide NAcMP8 we found[21] that $\Delta H = 48 \pm 1$ kJ mol^{-1} and $\Delta S = -22 \pm 3$ J K^{1-} mol^{-1}. For sperm whale metMb, George[47] reported that for the reaction $Fe(H_2O) + OH^- \longrightarrow Fe(OH^-) + H_2O$, $\Delta H = -32$ kJ mol^{-1} and $\Delta S = -11$ J K^{-1} mol^{-1}. From the temperature dependence of the ionisation of water between 0 and 35 °C,[48] we find $\Delta H = 58.4 \pm 0.7$ kJ mol^{-1} and $\Delta S = -72 \pm 2$ J K^{-1} mol^{-1}; hence for the pK_{Fe} of coordinated water in sperm whale metMb, $\Delta H = 26$ kJ mol^{-1} and $\Delta S = -83$ J K^{-1} mol^{-1}. This gives pK_{Fe} = 8.9 at 25 °C, in good agreement with the value of 8.99 reported subsequently.[49]

As shown in Table 2, there is a decrease in ΔH for ionisation of coordinated H_2O from 48 kJ mol^{-1} for NAcMP8 to 33.9 kJ

mol^{-1} for NAcMP11 as the length of the peptide chain is increased; this decreases further to 26 kJ mol^{-1} in metMb. There is a concomitant decrease in ΔS (from -22 to -70 to -83 J K^{-1} mol^{-1} for NAcMP8, NAcMP11 and metMb, respectively) that compensates for the more facile enthalpic contribution to the ionisation of Fe(III)-bound H_2O (the values of $-T\Delta S$ are, respectively, 6.5, 20.9 and 24.7 kJ mol^{-1}), so that overall the value of pK_{Fe} does not change very significantly (ΔG_{298} is, respectively, 54.6, 54.8 and 50.7 kJ mol^{-1}). Ionisation of bound H_2O will produce a neutral porphyrin moiety (the charge on the porphyrin dianion and coordinated OH^- is balanced by the +3 charge on the metal); we suggest that the decrease in ΔH values for pK_{Fe} is a result of the decrease in the permitivity of the environment in the vicinity of metal site as the length of the peptide chain increases.

The decrease in ΔS values that accompanies decreases in ΔH may be related to the density of states in an enthalpic well as explained by Williams and co-workers.[50,51] If an associated state lies in a deep enthalpic well (*i.e.*, ΔH is large and negative), then the density of states is low and there is little motion compared with the unassociated state (ΔS is small); as the enthalpic well becomes more shallow (ΔH becomes less negative), the larger amplitude vibrations close to the lip of the well (where the density of states is larger) become accessible, and ΔS increases.

4.3 Coordination of neutral ligands by Fe(III)

We have previously reported on the coordination of 31 N-donor ligands (pyridines, imidazoles, primary and secondary amines) by NAcMP8.[46] In particular, log K_{eq} values determined were 3.68 for ethanolamine, 3.44 for glycine, 2.62 for pyridine, 3.20 for ammonia, and 4.08 for imidazole; by contrast, we found no evidence for coordination of these representative N-donor ligands by metMb in the presence of 0.5 mol dm^{-3} of the ligand. We note that Chen *et al.*[52] used two-dimensional chemical exchange ^1H NMR spectroscopy to investigate the complexation between pyridine and horse metMb. From the temperature-dependence of the haem methyl shifts, they postulated the existence of a rapid equilibrium between $S = 1/2$ and $S = 5/2$ states. They determined both the on and off rate constants (k_{on} = 873 M^{-1} s^{-1} and k_{off} = 154 s^{-1}) from the peak amplitude in the 2D EXSY spectrum, and found K_{obs} = 5.68 M^{-1} at pH 7.5. Since pK_a of pyridine = 5.19[53] and that of horse metMb = 8.93,[54] correcting for pH gives a pH-independent equilibrium constant K_{eq} = 5.9 (log K_{eq} = 0.77). Thus, if the metMb values from the two sources behave similarly, we would have expected approximately 75% binding of pyridine by sperm whale metMb in the presence of 0.5 mol dm^{-3}. Even though K_{eq} is small, we have determined K_{eq} values of comparable magnitude with other ligands; moreover, the spectral changes should have been readily apparent since the putative pyridine complex is low spin.[52] We presently have no adequate explanation for the difference between our observations and those of Chen and co-workers.[52]

Imidazole is coordinated nearly two orders of magnitude more strongly by NAcMP11 than by metMb (log K_{eq} = 4.16 and 2.23 at 25 °C, respectively). The difference stems largely from the enthalpic contribution ($\Delta H = -29.4 \pm 0.8$ and -16.6 ± 0.7 kJ mol^{-1}, respectively), since the entropic contribution is marginally less favourable for coordination by the haempeptide than by metMb ($-T\Delta S = 6 \pm 1$ kJ mol^{-1} and 3.9 ± 0.6 kJ mol^{-1} at 25 °C, respectively). As shown in Table 3, the much less favourable value of ΔH for coordination of imidazole by metMb causes K_{eq} to decrease by over two orders of magnitude, whereas the somewhat more favourable entropy factor merely doubles K_{eq} (*i.e.*, $\Delta(\log K_{eq}) = 0.31$).

The crystal structure of metMb[55] shows that the ligand-binding site of Mb is buried in the tightly packed interior of the protein with no permanently open channels to the protein surface. When a bulky ligand such as imidazole,[56] phenyl[57] or ethyl isocyanide[58] is coordinated by the metal ion, major structural rearrangements occur in the porphyrin's distal environment (Fig. 6). The distal histidine, His64, rotates outwards towards the solvent and Arg45 to disrupt hydrogen bonding between the latter and a haem propionate, leaving an open channel to the protein surface. Yet, despite this very significant reorganisation, there is only a small difference in the entropy for binding imidazole to metMb and to NAcMP11. What is quite

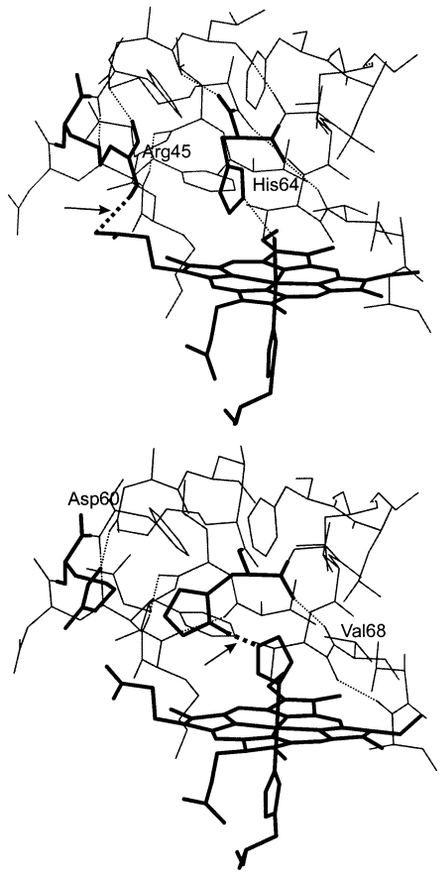

Fig. 6 The immediate distal environment of (top) sperm whale metMb and (bottom) its imidazole complex. Coordination of imidazole causes His64 to swing outwards, clashing with Arg45, and disrupting the hydrogen bonding between Arg45 and a haem propionate (arrow, top). His64 is hydrogen bonded to coordinated imidazole (arrow, bottom), and Arg45 forms a hydrogen bond with Asp60. This movement opens a ligand channel from the protein surface to the metal ion. The crystal structure of metMb is by Takano[55] (the Brookhaven Protein Databank, PDB, access code is 4MBN) and that of the imidazole complex is by Lionetti *et al.*[56] (PDB access code 1MBI).

notable (Fig. 6) is that, in response to a close steric contact with Val68, imidazole coordinates in a tilted fashion, with an angle of 16° between the mean imidazole plane and the normal to the mean porphyrin plane. Moreover, the Fe–N bond to co-ordinated imidazole is somewhat long at 2.14 Å (although it has to be appreciated that the resolution of the structure is only 2.0 Å). The Fe–N bond length to the proximal His93 is 2.04 Å. It is considerably shorter in bis(imidazole) low spin Fe(III) porphyrins: 1.966 and 1.988 Å in $[Fe(PPIX)(1\text{-}MeIm_2)_2]$,[59] between 1.957 and 1.991 Å in $[Fe(TPP)(HIm)_2]^+$,[60,61] 1.970 and 1.978 Å in $[Fe(TPP)(1\text{-}MeIm)_2]^+$,[62] and 1.965 and 1.975 Å in $[Fe(TMP)(1\text{-}MeIm)_2]^+$.[63] In all cases, the imidazoles coordinate perpendicular to the mean porphyrin plane in protein-free porphyrins. In response to steric pressure of the methyl group in coordinated 2-methylimidazole, the Fe–N bond can increase considerably (for example, to 2.249 Å in $[Fe(OEP)(2\text{-}MeIm)_2]^+$)[64] provided the porphyrin remains relatively planar; in porphyrins that are severely distorted from planarity, as in $[Fe(TMP)(1,2\text{-}Me_2Im)_2]^+$,[65] the Fe–N bond is shorter (2.004 Å).

Table 3 Thermodynamic factors and their effect on log K_{eq} values for coordination of ligands by a ferric porphyrin in NAcMP11 and metMb[a]

	NAcMP11								metMb			
Ligand	ΔH/kJ mol^{-1}	ΔS/J K^{-1} mol^{-1}	$-T\Delta S$/kJ mol^{-1}	log K_{eq}	log K_{eq} when ΔH = metMb value	Δ(log K_{eq})	log K_{eq} when ΔH and ΔS = metMb value	Δ(log K_{eq})	ΔH/kJ mol^{-1}	ΔS/J K^{-1} mol^{-1}	$-T\Delta S$/ kJ mol^{-1}	
SCN^-	−5.9	−23	6.9	−0.17	4.07	4.24	2.07	−2.01	−30.1	−61.4	18.3	
F^-	−7	−22	6.6	0.08	0.32	0.25	1.58	1.25	−8.4	2	−0.6	
N_3^-	−19	−38	11.3	1.33	5.20	3.86	4.10	−1.10	−41	−59	17.6	
Imidazole	−29.4	−19	5.7	4.16	1.92	−2.24	2.23	0.31	−16.6	−13	3.9	
CN^-	−75	−123	36.7	6.55	7.25	0.53	8.45	1.20	−78	−100	29.8	

[a] The values of ΔH and ΔS, and $-T\Delta S$ at 298 K, for coordination of the ligand in column 1 by NAcMP11 are given in columns 2–4, and log K_{eq} values are listed in column 5. In column 6, the effect of differences in ΔH values for ligand binding between the haempeptide and the hemoprotein are assessed by calculating what log K_{eq} would have been had the values for ΔH for coordination by NAcMP11 been the same as for coordination by metMb; the difference in log K_{eq} is given in column 7. Column 8 lists log K_{eq} values if both ΔH and ΔS were the same for the two ferric porphyrin systems. The change brought about by the change in ΔS values is given in column 9.

As the porphyrin in the imidazole complex of metMb is relatively planar, it is clear that, in response to a crowded environment, the Fe–N bond elongates and the ligand is tilted off-axis. We presume these are the main contributors to the difference in ΔH values for imidazole coordination to a relatively open Fe(III) porphyrin (in NAcMP11) and one buried in a protein matrix (metMb). The hydrogen bonding between coordinated imidazole and His64, clearly present in the imidazole complex of metMb, and which cannot be present in NAcMP11, is evidently insufficient to compensate for the steric effects.

Hydrogen bonding effects in the distal pocket of metMb are known to play an important role in stabilising complexes between exogenous ligands and the haemoprotein (as, for example, in oxyMb[66]). The Mb from the mollusc *Aplysia limacina* lacks the distal His (it is replaced by Val), yet there is evidence that the side-chain of Arg66 folds into the haem pocket on ligand binding, and initiates a complex set of polar interactions involving the ligand itself, ordered water molecules nearby, a haem propionate, and amino acid residues from the region of the corner of helix C and helix D.[67–72] At pH 6 the distal coordination site of Fe(III) in *Aplysia* Mb is vacant and Arg66 is well removed from the heme pocket;[73] on coordination of cyanide, the amino acid residue undergoes a major conformational change, enters the haem pocket and is H-bonded to bound cyanide. The electron density map for the thiocyanato derivative was not as clear, but it is likely that Arg66 also H-bonds to this ligand.[73] Even on coordination of the bulky imidazole, which causes significant structural perturbations in the distal cavity, with residues 43 to 49 in the CD region displaced from the vicinity of the haem pocket, Arg66 faces and is H-bonded to the coordinated ligand. Perhaps the inability of even a sterically undemanding ligand like NH_3 to hydrogen bond to the distal His, accounts for its apparent inability to bind to Fe(III) in metMb. Pyridine, which is bulkier than imidazole and cannot hydrogen bond to His66, fails to coordinate (at least, in our experience—see above). Although both glycine and ethanolamine could indeed form a hydrogen bond to His66, they are presumably too bulky to be adequately accommodated in the protein's distal cavity. Hence, and as has been pointed out,[74] both steric effects and hydrogen bonding with amino acid residues near the ligand binding site are important factors in modulating the affinity of an iron porphyrin for an exogenous ligand.

4.4 Coordination of anions by Fe(III)

There is a dramatic change in ΔH values when SCN^- and N_3^- are coordinated by metMb compared to when they are coordinated by NAcMP11; log K_{eq} increases by 4.24 and 3.86, respectively (Table 3), but is offset by a decrease Δ(log K_{eq}) of 2.01 and 1.10 units, respectively, due to a significantly less favourable entropic factor. By contrast, the anions F^- and CN^- also bind preferentially to the haemoprotein, but the difference is now predominantly driven by the entropic factor. The steric factors that were important in discriminating against imidazole coordination by metMb are insufficient to fully account for the modulation of ligand binding affinities. We interpret the significantly more negative ΔH values for coordination of anions by metMb compared with NAcMP11 to be a consequence of the decreased polarity of the haem environment; in such an environment, the importance of the electrostatic interaction between the anionic ligand and residual positive charge at the metal centre would be enhanced, and the ionic contribution to the enthalpy change for metal–ligand binding would become more significant.

The importance of electrostatic effects in the ligand binding pocket of the haemoproteins has been documented. For example, Mauk and co-workers[75] studied the binding of cyanide to horse heart Mb and to some mutants. The introduction of a positive charge on the distal side of the haem (in V67R Mb) increased the affinity for cyanide (log K_{eq} = 6.96; wild type log K_{eq} = 5.63). This represents a stabilisation of 7.5 kJ mol^{-1}, presumably because of electrostatic compensation by the positively charged amino acid side chain for the binding of an anionic ligand. In simple haem compounds, the ratio of binding affinity of CO to O_2 may be several tens of thousands,[76] while in Mb compounds this ratio is much more favourable towards O_2 binding. Thus, the $K_{eq}(CO)/K_{eq}(O_2)$ ratio for sperm whale Mb is 14.5,[77] but 13400 in a "basket-handle" porphyrin with imidazole as the proximal ligand.[78] His64 plays a key role; it is hydrogen-bonded to coordinated O_2,[66] favouring its binding over CO, and stabilising oxyMb against autooxidation.[79] Elephant myoglobin (EMb) has Gln instead of His at position 64,[80] but the substitution does not significantly affect its affinity for CO when compared to other vertebrate myoglobins,[81] whilst in sperm whale Mb in which His64 has been mutated to a Gln(H64Q) there is a five-fold decrease in O_2 affinity and a three-fold increase in the rate of autooxidation.[82] The difference is that in EMb a Phe residue at position 29[83] (the residue is usually Leu) penetrates into the haem pocket and approaches bound O_2 within van der Waals contact, compensating for the H64Q change by an electrostatic interaction between the ligand and the positive edge of its multipole.

The ΔH differential for coordination to the exposed haem environment in NAcMP11 and the buried haem environment in metMb is much less significant for F^- and CN^-, and it is not immediately obvious why the thermodynamic parameters for coordination of these two ligands is significantly different to those for coordination of SCN^- and N_3^-. The polarity of the haem pocket is expected to have a significant effect on the apparent stability of the complex of the anion A^- of a weak acid, HA, with Fe(III) by perturbing the pK_a of HA.[84–87] As neutral HA is stabilised with a decrease in the polarity of the environment, ΔH for coordination of A^- by Fe(III) will become less and less negative, and, through the compensation effect noted above, ΔS will become more positive. This could explain

the results for CN^- (the pK_a of HCN is 9.04[89]), although if this factor is important, one might have expected the trend in ΔH and ΔS values for N_3^- (pK_a = 4.38[90]) to be similar to that for F^- (pK_a = 3.45[88]).

This work has demonstrated that there is a complex interplay of factors that control the affinity of an iron porphyrin for an exogenous ligand. Neutral N-donor ligands are readily coordinated by the relatively open porphyrin site of NAcMP11, but not by the buried site of metMb. Steric clashes with the distal protein environment, and the inability of some of these ligands to engage in appropriate hydrogen bonding with the amino acid residues in the distal environment results in their poor coordination. By contrast, several anionic ligands bind much more strongly to metMb than to NAcMP11. In some cases, the increased affinity is a consequence of a much more favourable enthalpic term which we attribute to an enhanced electrostatic interaction between the exogenous ligand and the residual positive charge at the metal centre in an environment of lower permitivity. This cannot be the only factor, however, as some ionic ligands are bound more strongly because of a more favourable entropic factor.

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References

1 P. A. Adams, D. A. Baldwin and H. M. Marques, in *Cytochrome c: A Multidisciplinary Approach*, eds. R. A. Scott and A. G. Mauk, University Science, Mill Valley, CA, 1996, pp. 635–692.
2 H. M. Marques, M. S. Shongwe, O. Q. Munro and T. J. Egan, *S. Afr. J. Chem.*, 1997, **50**, 166.
3 D. A. Baldwin, H. M. Marques and J. M. Pratt, *FEBS Lett.*, 1985, **183**, 309.
4 D. A. Baldwin, H. M. Marques and J. M. Pratt, *J. Inorg. Biochem.*, 1987, **30**, 203.
5 I. D. Cunningham, J. L. Bachelor and J. M. Pratt, *J. Chem. Soc., Perkin Trans. 2*, 1994, 1347.
6 P. A. Adams and C. Adams, *J. Inorg. Biochem.*, 1988, **34**, 177.
7 P. A. Adams and R. D. Goold, *J. Chem. Soc., Chem. Commun.*, 1990, 97.
8 P. A. Adams, *J. Chem. Soc., Perkin Trans. 2*, 1990, 1407.
9 A. M. Osman, J. Koerts, M. G. Boersma, S. Boeren, C. Veeger and I. M. C. M. Rietjens, *Eur. J. Biochem.*, 1996, **240**, 232.
10 D. A. Baldwin, H. M. Marques and J. M. Pratt, *J. Inorg. Biochem.*, 1986, **27**, 245.
11 D. W. Low, H. B. Gray and J. O. Duus, *J. Am. Chem. Soc.*, 1997, **119**, 1.
12 H. A. Harbury and P. A. Loach, *Proc. Natl. Acad. Sci. U.S.A.*, 1959, **45**, 1344.
13 D. W. Urry and J. W. Pettegrew, *J. Am. Chem. Soc.*, 1967, **89**, 5276.
14 J. Aron, D. A. Baldwin, H. M. Marques, J. M. Pratt and P. A. Adams, *J. Inorg. Biochem.*, 1986, **26**, 227.
15 G. Blauer, N. Sreerama and R. W. Woody, *Biochemistry*, 1993, **32**, 6674.
16 S. Mazumdar, O. K. Medhi and S. Mitra, *Inorg. Chem.*, 1991, **30**, 700.
17 S. Othman, A. Le Lirzin and A. Desbois, *Biochemistry*, 1994, **33**, 15437.
18 Y. P. Huang and R. J. Kassner, *J. Biol. Chem.*, 1981, **256**, 5327.
19 E. K. Yang and K. Sauer, in *Electron Transport and Oxygen Utilization*, ed. C. Ho, Elsevier-North Holland, New York, 1982, pp. 89–94.
20 J. S. Wang and H. E. Van Wart, *J. Phys. Chem.*, 1989, **93**, 7925.
21 O. Q. Munro and H. M. Marques, *Inorg. Chem.*, 1996, **35**, 3752.
22 A. D. Carraway, M. G. McCollum and J. Peterson, *J. Inorg. Biochem.*, 1988, **34**, 6885.
23 H. M. Marques and C. B. Perry, *J. Inorg. Biochem.*, 1999, **75**, 281.
24 O. Q. Munro, M. de Wet, H. Pollak, J. van Wyk and H. M. Marques, *J. Chem. Soc., Faraday Trans.*, 1998, **94**, 1743.
25 M. N. Hughes, in *Comprehensive Coordination Chemistry*, ed. G. Wilkinson, Pergamon Press, Oxford, 1987, vol. 4, pp. 614–717.
26 M. F. Perutz, *Nature*, 1972, **237**, 495.
27 R. Weiss, D. Mandon, T. Wolter, A. X. Trautwein, M. Muther, E. Bill, A. Gold, K. Jayaraj and J. Temer, *J. Biol. Inorg. Chem.*, 1996, **1**, 377.
28 T. L. Poulos and R. E. Fenna, *Metal Ions Biol. Syst.*, 1994, **30**, 25.
29 G. R. Moore and G. W. Pettigrew, *Cytochromes c: Evolutionary, Structural and Physicochemical Aspects*, Springer-Verlag, New York, 1990.
30 T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono and S. Yoshikawa, *Science*, 1995, **269**, 1069.
31 J. H. Dawson, *Science*, 1998, **240**, 433.
32 R. W. Estarbrook, *FASEB J.*, 1996, **10**, 202.
33 S. M. Musser, M. H. B. Stowell and S. I. Chan, *Adv. Enzym. Rel. Areas Mol. Biol.*, 1995, **71**, 79.
34 P. R. Rich, B. Meunier, R. Mitchell and A. J. Moody, *Biochim. Biophys. Acta*, 1996, **1275**, 91.
35 H. A. Harbury, J. R. Cronin, M. W. Fanger, T. P. Hettinger, A. J. Murphy, Y. P. Myer and S. N. Vinogradov, *Proc. Natl. Acad. Sci. U.S.A.*, 1965, **54**, 1658.
36 H. M. Marques, D. A. Baldwin and J. M. Pratt, *J. Inorg. Biochem.*, 1987, **29**, 77.
37 F. Millar, J. M. Wrigglesworth and P. Nicholls, *Eur. J. Biochem.*, 1981, **117**, 13.
38 J. M. Pratt, in *Techniques and Topics in Bioinorganic Chemistry*, ed. C. A. McAuliffe, Macmillan, London, 1971, pp. 107–204.
39 M. P. Byfield and J. M. Pratt, *J. Chem. Soc., Chem. Commun.*, 1992, 214.
40 H. M. Marques, I. Cukrowski and P. R. Vashi, *J. Chem. Soc., Dalton Trans.*, 2000, 1335.
41 A. K. Churg and A. Warshel, *Biochemistry*, 1986, **25**, 1675.
42 A. G. Mauk and G. R. Moore, *J. Biol. Inorg. Chem.*, 1997, **2**, 119.
43 G. I. H. Hananaia, A. Yeghiayan and B. F. Cameron, *J. Biochem.*, 1966, **98**, 189.
44 M. W. Makinen and A. K. Churg, in *Iron Porphyrins, Part 1*, eds. A. B. P. Lever and H. B. Gray, Physical Bioinorganic Chemistry Series, Addison-Wesley, Reading, MA, 1983, p. 141.
45 W. A. Eaton and J. Hofrichter, *Methods Enzymol.*, 1981, **76**, 175.
46 H. M. Marques, O. Q. Munro, T. Munro, M. de Wet and P. R. Vashi, *Inorg. Chem.*, 1999, **38**, 2312.
47 P. George, in *Currents in Biochemical Research*, Interscience, New York, 1956, p. 338; Quoted, in E. Antonini and M. Brunori, *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North Holland Publishing, Amsterdam, 1971, p. 231.
48 R. C. Weast (ed.), *Handbook of Chemistry and Physics*, 53rd edn., CRC Press, Cleveland, OH, 1972, p. D122.
49 M. Brunori, G. Amiconi, E. Antonini, J. Wyman, R. Zito and A. Rossi Fanelli, *Biochim. Biophys. Acta*, 1968, **154**, 315.
50 D. H. Williams, M. S. Searle, J. P. Mackay, U. Gerhard and R. A. Mapelstone, *Proc. Natl. Acad. Sci. U.S.A.*, 1993, **90**, 1172.
51 M. S. Searle, M. S. Westwell and D. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1995, 141.
52 Y. Chen, G. Liu and W. Tang, *Inorg. Chim. Acta*, 1996, **249**, 239.
53 H. H. Perkampus and G. Prescher, *Ber. Bunsenges. Physik. Chim.*, 1968, **72**, 429.
54 P. George and G. I. H. Hananaia, *Biochem. J.*, 1952, **52**, 517.
55 T. Takano, *J. Mol. Biol.*, 1997, **110**, 569.
56 C. Lionetti, M. G. Guanziroli, F. Frigerio, P. Ascenzi and M. Bolognesi, *J. Mol. Biol.*, 1991, **217**, 409.
57 D. Ringe, G. A. Petsko, D. E. Kerr and P. R. Ortiz de Montellano, *Biochemistry*, 1984, **23**, 2.
58 K. A. Johnson, J. S. Olson and G. N. Phillips, *J. Mol. Biol.*, 1989, **207**, 459.
59 R. G. Little, K. R. Dymock and J. A. Ibers, *J. Am. Chem. Soc.*, 1975, **97**, 4532.
60 D. M. Collins, R. Countryman and J. L. Hoard, *J. Am. Chem. Soc.*, 1972, **94**, 2066.
61 W. R. Scheidt, S. R. Osvarth and Y. L. Lee, *J. Am. Chem. Soc.*, 1987, **109**, 1958.
62 T. Higgins, M. K. Safto and W. R. Scheidt, *Inorg. Chim. Acta*, 1991, **178**, 261.
63 M. K. Safo, G. P. Gupta, F. A. Walker and W. R. Scheidt, *J. Am. Chem. Soc.*, 1991, **113**, 5497.
64 E. Elkaim, K. Tanaka, P. Coppens and W. R. Scheidt, *Acta Crystallogr., Sect. B*, 1997, **43**, 457.
65 O. Q. Munro, H. M. Marques, P. G. Debrunner, K. Mohanrao and W. R. Scheidt, *J. Am. Chem. Soc.*, 1995, **117**, 935.
66 S. E. Phillips and B. Schoenborn, *Nature*, 1981, **292**, 81.
67 M. Bolognesi, S. Onesti, G. Gatti, A. Coda, P. Ascenzi and M. Brunori, *J. Mol. Biol.*, 1989, **205**, 529.

68 M. Bolognesi, A. Coda, F. Frigerio, G. Gatti, P. Ascenzi and M. Brunori, *J. Mol. Biol.*, 1990, **213**, 621.
69 M. Bolognesi, F. Frigerio, C. Lionetti, M. Rizzi, P. Ascenzi and M. Brunori, in *Structure and Function of Invertebrate Oxygen Carriers*, eds. S. N. Vinogradov and O. H. Kapp, Springer-Verlag, New York, 1991, pp. 161–170.
70 A. Mattevi, G. Gatti, A. Coda, M. Rizzi, P. Ascenzi, M. Brunori and M. Bolognesi, *J. Mol. Recogn.*, 1991, **4**, 1.
71 J. Qin, G. N. La Mar, F. Ascoli, M. Bolgnesi and M. Brunori, *J. Mol. Biol.*, 1992, **224**, 891.
72 M. Rizzi, P. Ascenzi, A. Coda, M. Brunori and M. Bolgnesi, *Rend. Fis. Acc. Lincei IX*, 1993, **4**, 65.
73 E. Conti, C. Moser, M. Ricci, A. Mattevi, C. Lionetti, A. Coda, P. Ascenzi, M. Brunori and M. Bolognesi, *J. Mol. Biol.*, 1993, **233**, 498.
74 B. A. Springer, S. G. Sligar, J. S. Olson and G. N. Phillips, *Chem. Rev.*, 1994, **94**, 69.
75 E. Lloyd, B. C. King, F. M. Hawkridge and A. G. Mauk, *Inorg. Chem.*, 1998, **37**, 2888.
76 E. C. Niederhoffer, J. H. Timmons and A. E. Martell, *Chem. Rev.*, 1984, **84**, 137.
77 K. Moffat, J. F. Deatherage and D. W. Seybert, *Science*, 1979, **206**, 1035.
78 M. Momenteau, B. Loock, D. Lavalette, C. Tetreau and J. Mispelter, *J. Chem. Soc., Chem. Commun.*, 1983, 962.
79 R. E. Brantley, S. J. Smerdon, A. J. Wilkinson, E. W. Singleton and J. S. Olson, *J. Biol. Chem.*, 1993, **268**, 6995.
80 H. Dene, M. Goodman and A. E. Romero-Herrera, *Proc. R. Soc. London, Ser. B*, 1980, **207**, 111.
81 A. E. Romero-Herrera, M. Goodman, H. Dene, D. E. Bartnicki and H. Mizukami, *J. Mol. Evol.*, 1981, **17**, 140.
82 B. A. Springer, K. D. Egeberg, S. G. Sligar, R. J. Rohlfs, A. J. Mathews and J. S. Olson, *J. Biol. Chem.*, 1989, **264**, 3057.
83 D. A. Bisig, E. E. Di Iorio, K. Diederichs, K. H. Winterhalter and K. Piontek, *J. Biol. Chem.*, 1995, **270**, 20754.
84 A. Brancaccio, F. Cutruzzola, C. T. Allocatelli, M. Brunori, S. R. Smerdon, A. J. Wilkinson, Y. Dou, D. Keenan, M. Ikeda-Saito, R. E. Brantley and J. S. Olson, *J. Biol. Chem.*, 1994, **269**, 14443.
85 J. Mintorovitch and J. D. Satterlee, *Biochemistry*, 1988, **27**, 8045.
86 M. Ikea-Saito, H. Hori, L. Andersson, R. C. Prince, I. J. Pickering, G. N. George, C. R. Sanders, R. S. Lutz, E. J. McKelvey and R. Mattera, *J. Biol. Chem.*, 1992, **267**, 22843.
87 Y. Dou, J. S. Olson, A. J. Wilkinson and M. Ikeda-Saito, *Biochemistry*, 1996, **35**, 7107.
88 Ref. 48, p. D129.
89 H. M. Marques, K. L. Brown and D. W. Jacobsen, *J. Biol. Chem.*, 1988, **263**, 12378.
90 H. M. Marques, E. L. J. Breet and F. F. Prinsloo, *J. Chem. Soc., Dalton Trans.*, 1991, 2941.