Cytochrome c Oxidase Models: Synthesis and Reactivity of Iron(III)–Copper(II) Complexes of Deuterohaemin–Polybenzimidazole Dinucleating Ligands

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Deuterohaemin complexes modified through covalent linkage of bis- (**1**) or tris-(benzimidazole) (**2**) residues to one of the propionic acid side chains have been obtained and spectrally characterised. While in **1** the benzimidazole groups cannot bind intramolecularly to the iron centre, in **2** such an arrangement is possible because the carbon chain connecting the aromatic donor group to the propionic carbonyl group is slightly longer. As a consequence, while complex **1** binds two molecules of exogenous donor bases in two steps for **2** simultaneous binding affinity for two donor bases is very low. The intramolecular binding of benzimidazole markedly increases the reactivity of **2** in catalytic peroxidative reactions. Both complexes bond one copper(II) ion at the polybenzimidazole site. For **2** some competition between iron(III) and copper(II) for one of the ligand donor bases is evident from EPR measurements. Reduction experiments show that upon treatment with sodium ascorbate the partially reduced Fe^{III}–Cu^{I} complex is obtained, while the stronger reductant sodium dithionite produces the fully reduced Fe^{II}–Cu^{I} complex. Oxidation of the latter with dioxygen at low temperature (about −40 °C) occurs without any destruction of the porphyrin and gives the fully oxidised Fe^{III}–Cu^{II} complex. The EPR spectra (−150 °C) of the iron–copper complexes show a decrease in intensity of the Cu^{II} signal upon addition of small amounts of OH^-, but the marked tendency towards intermolecular association prevents a simple interpretation in terms of formation of an intramolecular Fe^{III}–OH–Cu^{II} bridge.

Cytochrome c oxidase is the terminal enzyme of the mitochondrial respiratory chain catalysing the four-electron–four-proton reduction of dioxygen to water.[1] The functional unit contains four redox-active metal centres ($haem_a$, Cu_A, $haem_{a3}$ and Cu_B), two of which ($haem_{a3}$, Cu_B) are in close proximity and constitute a unique dinuclear complex involved in dioxygen binding and reduction, and proton pumping. Strong coupling between $haem_{a3}$ and Cu_B in the oxidised, resting state of the enzyme, likely through an as yet unknown bridging ligand, is responsible for the absence of an EPR signal. The nature of the integral membrane protein and the structural complexity, due to the presence of several subunits, makes the clarification of the structure of this enzyme through X-ray crystallography unlikely in the near future. Therefore, modelling of the dinuclear $haem_{a3}$–Cu_B active site complex can be of considerable importance to elucidate its structure and the mechanism of dioxygen binding and reduction.

Most of the model systems reported so far aimed at mimicking the resting form of the enzyme dinuclear centre and were generally obtained by linking two separate iron(III)-porphyrin and copper(II) complexes by an imidazolate, oxygen, halogen or pseudohalogen, or sulfur ligands.[2–5] Only in a few cases have the donors for both the iron(III) and copper(II) centres been derived from a single, appropriately modified porphyrin ligand.[6] This approach is certainly more difficult from a synthetic point of view but prevents difficulties resulting from the establishment of a dissociation equilibrium between the two metal units in solution.

In this paper we report our first attempts to develop a new family of $haem_{a3}$–Cu_B models based on covalently modified, dinucleating porphyrin ligands. Our synthetic strategy involves the attachment of a polybenzimidazole residue to one of the propionic acid side chains of deuterohaemin [3,7,12,17-tetramethylporphyrin-2,18-dipropionato)iron(III)], resulting in complexes **1** and **2**. The polybenzimidazole moieties were chosen as easily accessible models for the polyimidazole environment of Cu_B in cytochrome c oxidase.[7]

Results and Discussion

Characterisation of the Haemin Complexes **1** *and* **2**.—The amino–polybenzimidazole residues to be linked to the carboxyl group of deuterohaemin were obtained by Phillips condensation of *N*-methyl-*o*-phenylenediamine and polycarboxylic acid derivatives (Scheme 1). *N*-Methylation at the benzimidazole rings was necessary to prevent side reactions in the following condensation with deuterohaemin. The coupling reaction between the amino–polybenzimidazole compounds and deuterohaemin to produce **1** and **2** follows a standard procedure in peptide synthesis, employing 1-hydroxybenzotriazole and dicyclohexylcarbodiimide, which allows very mild conditions to be used. We recently followed the same procedure to link peptide residues to deuterohaemin.[8] Compounds **1** and **2** are obtained as equimolar mixtures of the isomers with substitution at positions 2 and 18 of the porphyrin ring. Small amounts of derivatives containing substituents at both carboxyl groups of deuterohaemin are also produced during condensation, but these products are difficult to elute from the chromatographic columns and were not considered further.

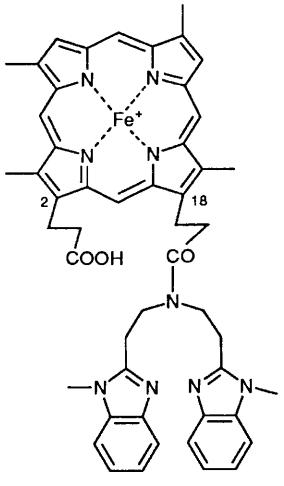
The deuterohaemin–polybenzimidazole derivatives have been characterised by FAB mass, NMR and optical spectroscopies. The FAB mass spectra of **1** and **2**, obtained from a glycerol matrix, gave peaks corresponding to the molecular ion clusters centred at the expected *m/z* values of 879 and 1051, respectively, in very good agreement with the simulated spectra, together with smaller peaks corresponding

to the adducts with a sodium ion, which was probably present as an impurity in the matrix. The electronic spectra of **1** and **2** are similar to that of deuterohaemin, except for the presence of the characteristic absorptions of the benzimidazole residues between 250 and 300 nm. The spectral data, including those associated with the Fe^{II} species, are summarised in Table 1. Aggregation[9] of the porphyrin complexes in solution is indicated by deviations of the spectra from Beer's law. These deviations are significant at concentrations as low as 5×10^{-6} mol dm^{-3} in solvents like methanol or dichloromethane, while in dimethyl sulfoxide (dmso) linearity in the absorbance *vs.* concentration plots is maintained up to about 3×10^{-5} mol dm^{-3}.

The paramagnetic ^1H NMR spectra of complexes **1** and **2** show some solvent dependence, but the behaviour of the two complexes is similar. The spectra of the high-spin Fe^{III} species in $CDCl_3$ solution display a cluster of peaks near δ 40, associated with the porphyrin methyls, and less intense peaks near δ 80 and −40, attributable to the porphyrin pyrrole and *meso* protons, respectively (Fig. 1).[10] The broad signals above δ 30 partially overlapping the intense methyl signals are probably due to the α-CH_2 groups of the propionic side chains. The pattern of paramagnetic signals in these spectra is indicative of five-co-ordinated haemin species, according to the established trends.[10] By contrast, as for simple, unsubstituted natural haemins, the

Table 1 Optical spectral data for the complexes in methanol

	λ_{max}/nm					
Complex	Benzimidazole	δ	Soret	β		α
1 (Fe^{III})	249, 275, 282	345	390	478		580
2 (Fe^{III})	249, 275, 282	345	390	484		592
1 (Fe^{II})	249, 275, 282		416		540	
2 (Fe^{II})	249, 279, 282		414		540	



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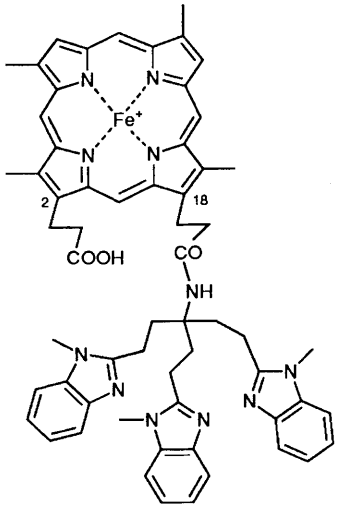

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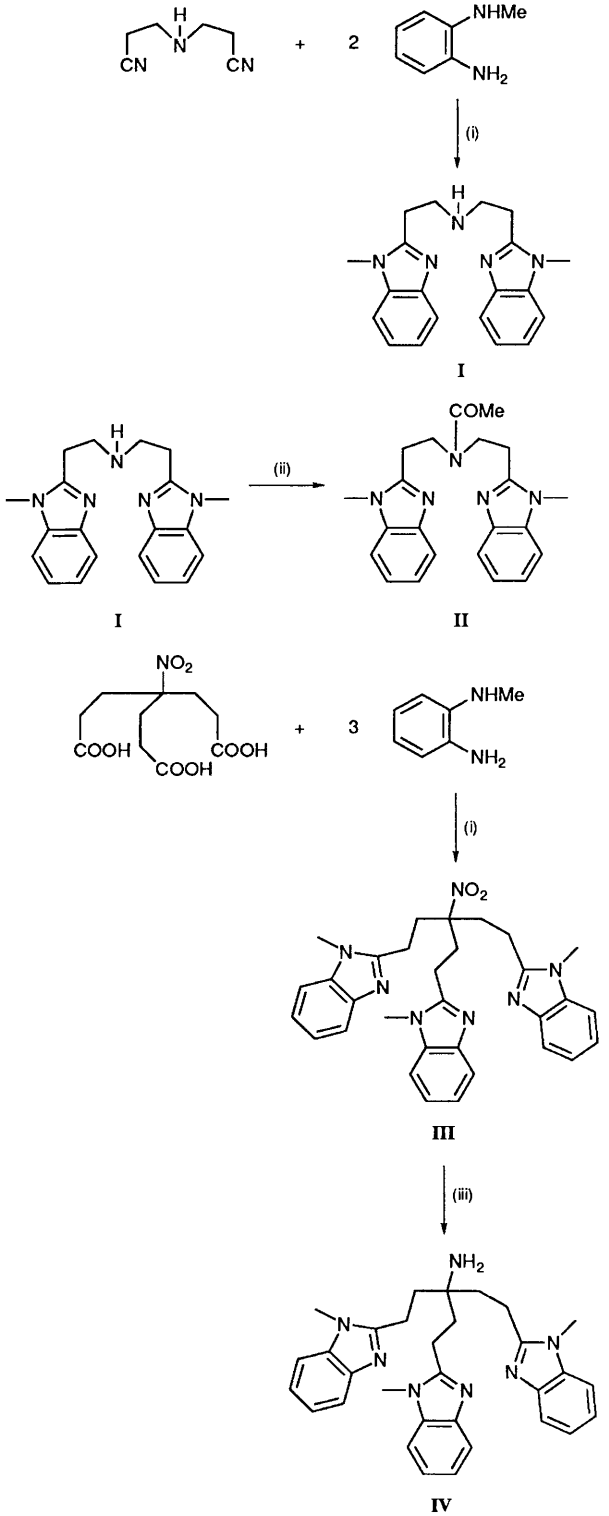


Scheme 1 (*i*) HCl; (*ii*) $(MeCO)_2O$–pyridine; (*iii*) Pd–C, H_2, 3 atm (≈3.04 × 10^5 Pa)

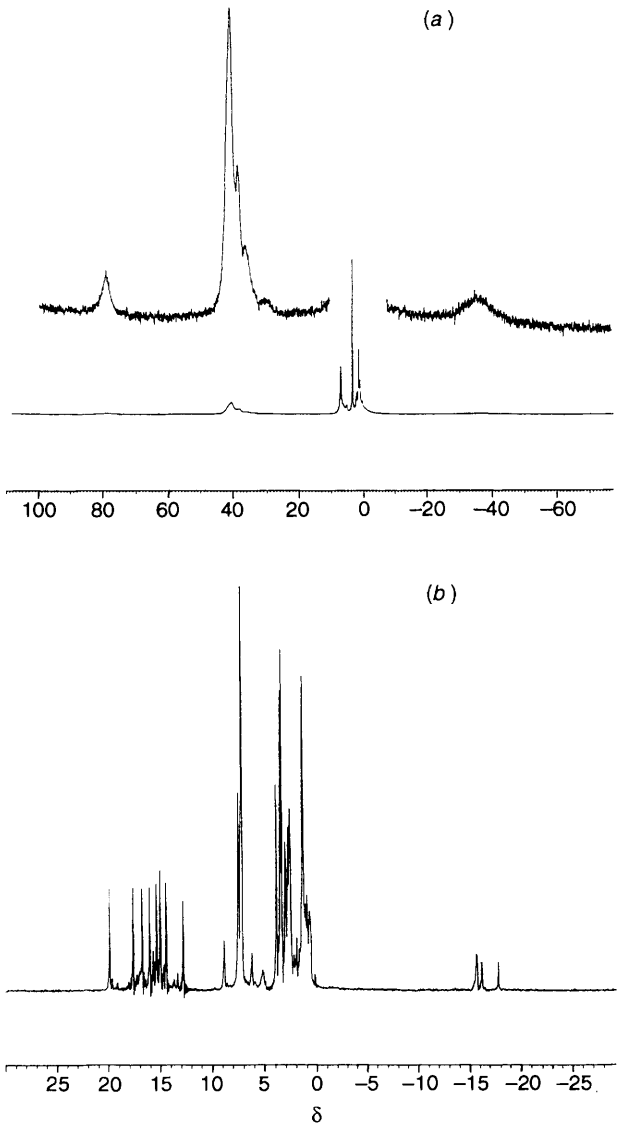


Fig. 1 Proton NMR spectra of complex **2** in $CDCl_3$ solution: (*a*) high-spin Fe^{III} species, (*b*) low-spin Fe^{III}–CN^- derivative

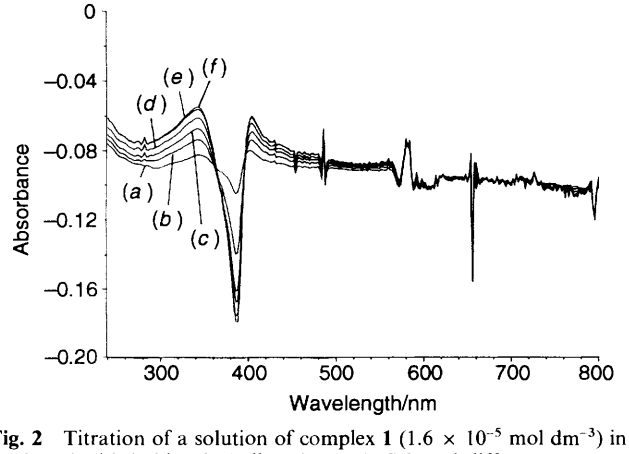


Fig. 2 Titration of a solution of complex **1** (1.6×10^{-5} mol dm^{-3}) in methanol with imidazole (cell path 1 cm). Selected difference spectra show the changes on addition of titrant corresponding to [Him]:[Fe] ratios of (*a*) 5:1, (*b*) 20:1, (*c*) 35:1, (*d*) 45:1, (*e*) 55:1 and (*f*) 60:1

spectra of complexes **1** and **2** in $(CD_3)_2SO$ show a pattern of paramagnetic signals typical for six-co-ordinated high-spin species.[10] Broad signals for porphyrin methyls, α-CH_2 groups and *meso* protons occur between δ 60 and 70, near δ 50 and at δ 40, respectively. In the diamagnetic region, relatively sharp signals for the benzimidazole protons, *N*-methyl groups and aliphatic methylene groups are clearly discernible in all cases. As usual, the NMR spectra of the low-spin Fe^{III}–CN^- species show much sharper signals. The eight peaks of equal intensity between δ 12 and 20 in Fig. 1 correspond to the four porphyrin methyl groups of the two structural isomers of **2**. Characteristic upfield signals for the pyrrole protons occur between δ −16 and −18.[10] Also well defined are the benzimidazole ring signals at δ 7–8. In general, the benzimidazole arms of the substituent seem little affected by the paramagnetic haemin groups in these spectra. We would expect some tendency of the benzimidazole nuclei to give stacking interactions with the porphyrin ring, but it is clearly difficult to establish whether some of the aromatic protons undergo upfield shifts, given the crowding of signals in the diamagnetic region and the problem of molecular association, which certainly occurs in the more concentrated solutions needed to record the NMR spectra.

The folding of the benzimidazole arms of complexes **1** and **2** above the haem plane and the eventual co-ordination of a donor group to the iron(III) porphyrin centre [FeP] can be assessed by competitive binding experiments with exogenous bases like imidazole (Him), as we carried out on haem–peptide complexes.[8] In the absence of steric restrictions, formation of a low-spin bis (adduct) of the haem with a nitrogen base (B) would be expected in one step, since generally $K_2 \gg K_1$ [equation (1)].[11]

$$[FeP] + B \overset{K_1}{\rightleftharpoons} [FeP(B)] + B \overset{K_2}{\rightleftharpoons} [FeP(B)_2] \quad (1)$$

Titration of **1** and **2** with imidazole elicits a different behaviour of their benzimidazole arms. For **1** binding of imidazole occurs in two steps. The first occurs with high affinity and is associated with little variation in the optical spectrum yielding $K_1 = 5000$ dm^3 mol^{-1} (Fig. 2). The second step involves more pronounced variation in the optical spectrum, resulting in the formation of the low-spin adducts, and requires a relatively high concentration of imidazole, yielding $K_2 = 350$ dm^3 mol^{-1} (Fig. 3). This suggests that binding of the first imidazole forces the benzimidazole-containing arm onto the opposite side of the haem plane, such that co-ordination of the second imidazole is sterically hindered. When complex **2** is titrated with imidazole, the low-spin adduct is obtained in one step, but surprisingly the binding affinity for imidazole is low, with $K = 110$ dm^3 mol^{-1} (Fig. 4). This behaviour can be explained by assuming that in **2** one of the benzimidazole (bzim) groups of the side arm is intramolecularly co-ordinated to the iron atom, but further co-ordination of imidazole to this species is hindered by the difficulty of the substituted benzimidazole ring to approach the porphyrin plane, following movement of the iron. Therefore, the low-spin species is the bis(imidazole) adduct obtained upon replacement of the co-ordinated benzimidazole by the exogenous ligand (Scheme 2).

To confirm the possibility of intramolecular axial binding to the iron by one of the benzimidazole groups in the side arm of complex **2**, but not in that of **1**, two additional experiments were performed. These were based on the assay of the catalytic activity of the complexes and on the estimate of the relative stability of their five-co-ordinate forms by molecular mechanics calculations. Iron(III) porphyrin complexes are known to be efficient catalysts for the oxidation of phenolic compounds by peroxide agents.[8,12] It is also known that the presence of an axial nitrogen base increases the rate of the catalytic oxidations.[8,13] When the rate of oxidation of *p*-cresol by *tert*-butyl hydroperoxide in the presence of catalytic amounts of **1** or **2** was measured under the same conditions, a remarkable six-fold difference in favour of the latter complex was found.

Molecular mechanics (MM) and molecular dynamics (MD) calculations were carried out to obtain the lowest energy minimum values for the four- and five-co-ordinated species of

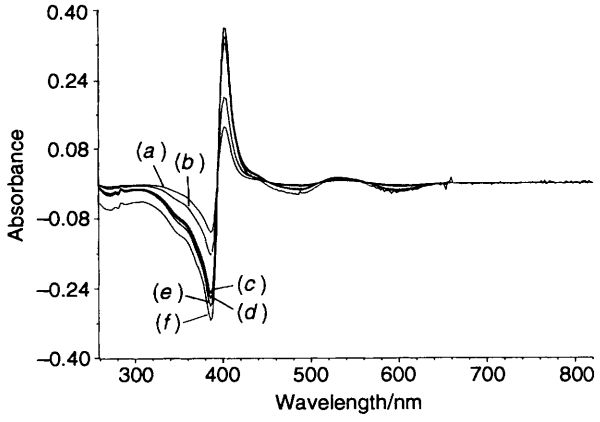


Fig. 3 Titration of a solution of complex **1** (1.6×10^{-5} mol dm^{-3}) in methanol with imidazole (cell path 1 cm). Selected difference spectra correspond to additions of the titrant in [Him]:[Fe] ratios of (*a*) 200:1, (*b*) 300:1, (*c*) 800:1, (*d*) 1000:1, (*e*) 1800:1 and (*f*) 11 000:1

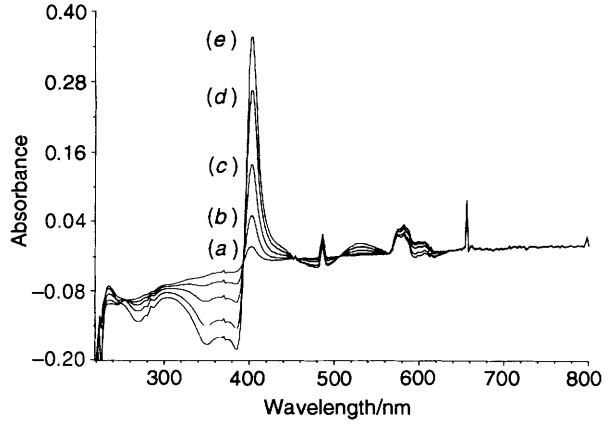


Fig. 4 Titration of a solution of complex **2** (8.2×10^{-6} mol dm^{-3}) in methanol with imidazole (cell path 1 cm). Selected difference spectra correspond to additions of the titrant in [Him]:[Fe] ratios of (*a*) 200:1, (*b*) 400:1, (*c*) 800:1, (*d*) 2000:1 and (*e*) 11 000:1

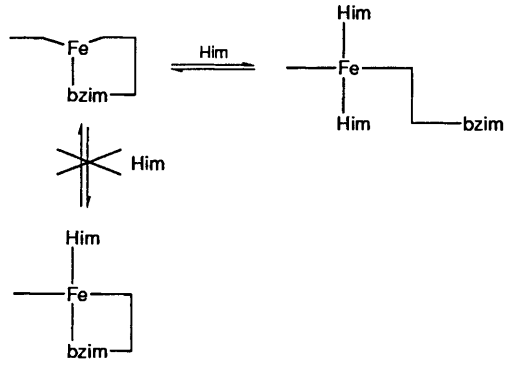
Table 2 Energy values for the four- to five-co-ordination transformation of complexes **1** and **2***

Complex	E_4	E_5	ΔE
1	39.0	27.4	−11.6
2	47.4	31.9	−15.5

* All energies in kcal mol^{-1} ($\approx$4.184 kJ mol^{-1}). E_4 and E_5 are the energies of the four- and five-co-ordinated forms, respectively. $\Delta E = E_5 - E_4$ is the energy required for five-co-ordination.

complexes **1** and **2**, where the fifth potential ligand is one of the benzimidazole residues on the side arm (Table 2). While it is well known that absolute energy values have little significance in MM calculations, the energy difference (ΔE) between the four- and five-co-ordinated species for a particular complex gives a meaningful indication of the relative tendency to adopt a particular co-ordination geometry. This kind of approach has already been successfully used to design iron porphyrin complexes with catalytic activity.[14] The energy values reported in Table 2 suggest that complex **2** has a greater tendency to adopt a five-co-ordinate structure than **1**. For the latter complex some strain in the folding of the side arm chain to allow one of the benzimidazole rings to co-ordinate to the iron axially is evident (Fig. 5).

Dinuclear Poly(benzimidazole)–Deuterohaemin–Copper Complexes.—Both complexes **1** and **2** bind a Cu^{II} very strongly, giving mixed dinuclear complexes. Binding of Cu^{II} occurs with



Scheme 2

only minor perturbations in the optical spectra of the deuterohaemin complexes, *i.e.* a small reduction in the intensity of the Soret band and small changes in the region of benzimidazole absorptions. However, the presence of Cu^{II} markedly affects the proton NMR spectra; in particular, in $CDCl_3$ the pattern of haem methyl signals becomes more complicated and broadened, and undergoes a downfield shift to about δ 50, while the porphyrin *meso* proton signal at high field is replaced by broad resonances at about δ −50 and −60. In $(CD_3)_2SO$ the haem methyl signals seem less affected, but considerable broadening and loss of resolution occurs for the resonances between δ 20 and 50. In all cases, severe broadening of the signals in the diamagnetic region is observed, particularly for those of the benzimidazole groups, which are the site of Cu^{II} binding.

The most informative technique to characterise the copper(II) sites of these dinuclear complexes is obviously EPR. The Cu^{II} signals can be conveniently monitored in frozen solutions at −150 °C, where those of the high-spin Fe^{III} centres are extremely weak or not detectable. Some of the EPR spectral data obtained under various conditions are collected in Table 3. In general, the spectra of the copper(II)–haemin complexes, as well as those of their simple Cu^{II} analogues with the ligands **II** (**5**) and **III** (**6**), show some solvent dependence, due to the availability of co-ordination positions at Cu^{II}. For the tris(benzimidazole) derivative of deuterohaemin the major Cu^{II} species observed in frozen solution (**4**) has a similar signal to complex **6**, indicating that the three benzimidazole donors of the porphyrin side arm prefer to bind the Cu^{II} rather than the Fe^{III} centre in the dinuclear complex. Therefore, although there is clearly some competition the major dinuclear complexes may be concluded to be **3** and **4** (S = solvent). The Cu^{II} spectra ($g_{\|} > g_{\perp}$, rather large $|A_{\|}|$ values) are indicative of tetragonal stereochemistry and for the Cu^{II}–tris(benzimidazole) units, the $|A_{\|}|$ values below 150×10^{-4} cm^{-1} suggest the presence of five-co-ordinated species, with two solvent molecules bound to the metal ion. From comparative experiments in different solvents, it is also apparent that the intensity of the signals observed for the dinuclear complexes is lower than that of the corresponding mononuclear analogues of copper(II) at the same concentration.

One of the most important characteristics of the binuclear harm–Cu site of cytochrome c oxidase is its redox behaviour in electron-transfer and dioxygen-reduction reactions.[1] Examining the redox behaviour of **3** and **4** we found that it is possible to reduce the Cu^{II} ion to Cu^{I} selectively in both cases, without affecting Fe^{III}, by anaerobic reaction with stoichiometric to moderate excess amounts of sodium ascorbate (Fig. 6). Copper(II) reduction is monitored by the disappearance of the EPR signal in the frozen solution. Reduction of both metal centres in the complexes occurs by treatment with sodium dithionite, which is a stronger reducing agent (Fig. 6). This result is of some interest because it reproduces the behaviour of the haem–Cu site of cytochrome c oxidase, which in the stepwise reduction process of its oxidised form undergoes one-

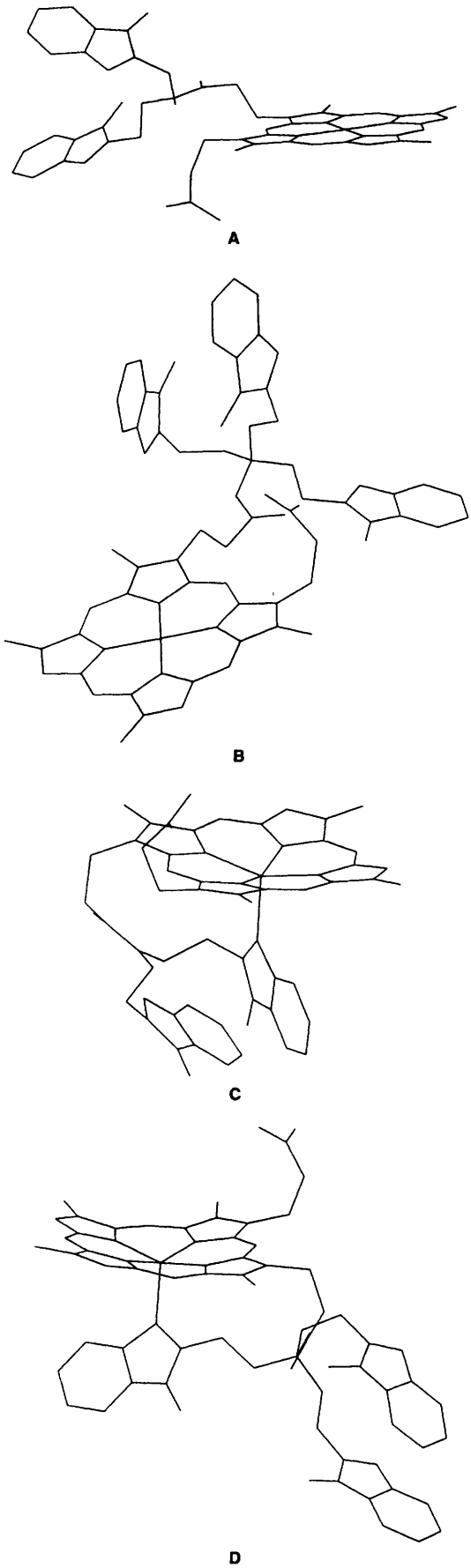


Fig. 5 Energy minimized structures for the four-co-ordinate (**A** and **B**) and five-co-ordinate (**C** and **D**) forms of complexes **1** and **2**, respectively containing an axially bound benzimidazole group

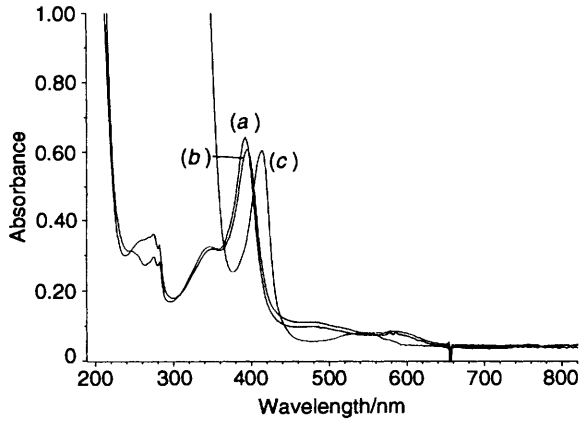
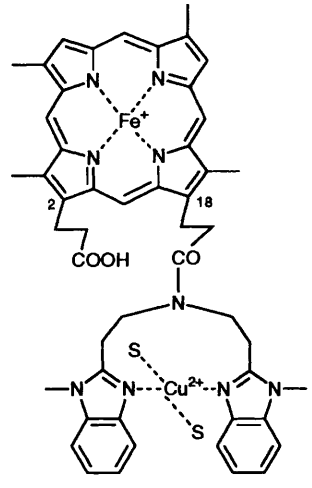


Fig. 6 Electronic spectra of complex **4** in aqueous methanol solution (*a*) before reduction, under an inert atmosphere (7.0×10^{-6} mol dm^{-3}), (*b*) after the addition of 2 mole equivalents of sodium ascorbate and (*c*) after the addition of excess of sodium dithionite. Complex **3** behaves similarly



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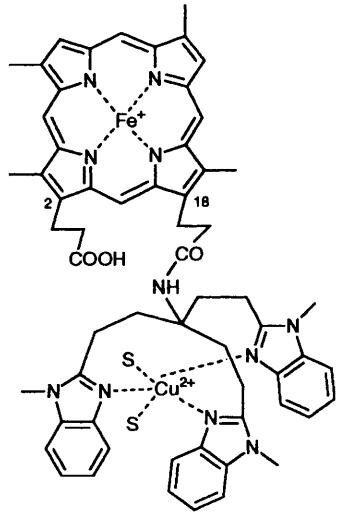

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electron reduction at the Cu^{II} centre first, followed by Fe^{III} reduction.[1] Apparently, both in cytochrome c oxidase and in these model systems, the reduction potential of Cu^{II} is higher than that of the haem Fe^{III}.

When fully reduced complexes **3** or **4** are treated with

Table 3 EPR spectral parameters for the copper(II) centres in frozen solutions (123 K)

Compound	Solvent	$g_{\|}$	$g_{\perp}$	$10^{-4}\|A_{\|}\|$/cm^{-1}
4	dmso	2.332[a]		148
	MeOH–MeCN (1:1)	2.332	2.075	148
6	dmso	2.337	2.078	149
	MeOH	2.334	2.075	148
3	dmso	2.310	2.058[b]	173
	MeOH[c]	2.321		152
5	dmso	2.311	2.065[d]	173
	MeOH	2.336	2.075	157

[a] Major complex formed. A $g_{\|}$ signal at 2.267 ($|A_{\|}| = 186 \times 10^{-4}$ cm^{-1}) is also observed in the mixture resulting from addition of Cu^{II} to complex **2**, corresponding to another minor species. [b] Multicomponent structure resolved with approximately 13.7 G (13.7 × 10^{-4} T) line separation. [c] Major species. [d] $|A_{\perp}| = 20 \times 10^{-4}$ cm^{-1}.

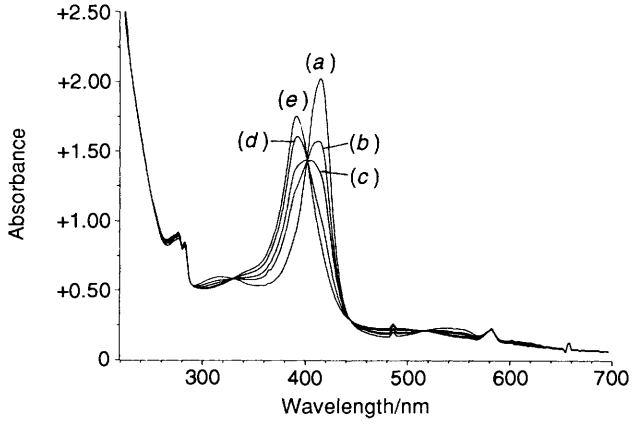

Fig. 7 UV/VIS Spectra recorded during the oxidation of fully reduced complex **4** in aqueous methanol (2.0×10^{-5} mol dm^{-3}) with dioxygen at about −45 °C. The spectra shown were taken after: (*a*) 0, (*b*) 90, (*c*) 105, (*d*) 130 and (*e*) 300 s. Complex **3** behaves similarly

dioxygen at room temperature immediate oxidation producing complete degradation of the porphyrin occurs. However, at sufficiently low temperature this destructive reaction is completely inhibited for both systems. As shown in Fig. 7 for complex **4**, operating at about −45 °C (the lowest temperature attainable with the cryostat available) complete conversion of the Fe^{II}–Cu^{I} species to the Fe^{III}–Cu^{II} species occurs. The presence of isosbestic points suggests that no intermediates or degradation products accumulate in these conditions. For both complexes **3** and **4** it is actually possible to perform several repetitive reduction–oxidation cycles at −45 °C, using alternate sodium dithionite/dioxygen reactions, without any detectable porphyrin degradation. This result is of some importance because it opens up the possibility of detecting intermediates in the reaction with dioxygen at lower temperatures.

Another aspect of the reactivity of the mixed haem–Cu complexes which is relevant for modelling cytochrome oxidase is the attempt to couple the metal centres intramolecularly through a small bridging ligand. In the oxidised state, the magnetic properties of the enzyme,[15] and the absence of EPR signals for an odd-spin species,[16] are consistent with a strong antiferromagnetic interaction ($-J > 200$ cm^{-1}) between the Fe^{III} and Cu^{II} sites, yielding an $S = 2$ ground-spin state. Recently, two synthetic oxo-bridged Fe–O–Cu compounds, obtained by 'assembling' an iron(III) porphyrin complex and a copper(II) complex have been shown to exhibit such magnetic properties.[5] In the systems reported here, we investigated the possible formation of an intramolecular Fe^{III}–X–Cu^{II} bridge in solution by addition of OH^-. The reaction of **3** or **4** with OH^- can be followed by optical spectroscopy, to monitor the changes occurring at the haem site, and EPR spectroscopy, to monitor the effects at the Cu^{II} site. However, the two spectroscopic methods require different concentration ranges (about 10^{-6} for optical spectra and about 10^{-4} mol dm^{-3} for EPR) so that the influence of intermolecular association, *e.g.* porphyrin–porphyrin aggregation, is not the same. In general, in fact, the addition of OH^- causes marked reduction or even complete depletion of the Cu^{II} EPR signal at little more than the stoichiometric amount, while the optical spectra are essentially unaffected. But the same effect is noted on adding equimolar amounts of OH^- to solutions of the simple copper(II) complexes **5** or **6**, where it is likely that strongly coupled bis(μ-hydroxo)-bridged dinuclear species are formed. Although signals attributable to a low-spin Fe^{III}–O–Fe^{III} complex are not observed in the presence of OH^-, from the EPR data currently available (at −150 °C) it is not possible to confirm the presence of an intramolecular Fe^{III}–OH–Cu^{II} bridge. Detailed EPR measurements in liquid helium, where the Fe^{III} signals should be observable, may be useful to distinguish between the effects of molecular association and possible bridge formation. However, our current efforts are directed toward the synthesis of further covalently modified haemins in order to reduce and possibly eliminate their spontaneous aggregation.

Experimental

Materials and Instrumentation.—Reagents and solvents from commercial sources were of the highest purity available and used as received. Dimethylformamide (dmf) was refluxed under vacuum over barium oxide to remove dimethylamine, stored over calcium hydride, and distilled under reduced pressure before use. Elemental analyses were performed at the microanalytical laboratory of the Chemistry Department in Milano. NMR spectra were recorded on Bruker WP-80 or AC-200 spectrometers, operating at 80 MHz and 200 MHz, respectively, and optical spectra on Perkin-Elmer Lambda 5 or HP 8452A diode-array spectrophotometers. Infrared spectra were recorded on a JASCO FTIR-5000 instrument, mass spectra with a VG 7070 EQ spectrometer, and EPR spectra were measured in frozen solutions using a Varian E-109 spectrometer operating at X-band frequencies and a V-4000 variable-temperature-control apparatus. The spectra of air sensitive solutions were obtained in optical or EPR cells fitted with Schlenk connections. Deuterohaemin was prepared from haemin according to a literature method.[17]

Preparations.—N,N-*Bis*[2-(1-*methylbenzimidazol*-2-*yl*)-*ethyl*]*amine* **I**. 3,3′-Iminodipropionitrile (11.4 mmol) and *N*-methyl-*ortho*-phenylenediamine dihydrochloride (23.9 mmol) were dissolved in 6 mol dm^{-3} hydrochloric acid (30 cm^3) and the mixture was allowed to react under gentle reflux in the dark for 100 h. The solution was cooled to room temperature and neutralised under stirring in an ice bath with concentrated ammonia. The light white precipitate so formed was filtered off and washed several times with diluted ammonia. The product was crystallised from ethanol–water (1:1) (yield 67%) (Found: C, 71.95; H, 6.90; N, 21.10. Calc. for $C_{20}H_{23}N_5$: C, 72.05; H, 6.95; N, 21.00%); $\tilde{\nu}_{max}$/cm^{-1} (Nujol) 3300s, 1618m, 1514s, 1468s, 1444s, 1131m, 866w and 754s; $\delta_H(CDCl_3)$ 7.8–7.6 and 7.3–7.1 (m, 8 H, phenyl H + benzimidazolyl H), 3.70 (s, 6 H, CH_3N), 3.25 (t, 2 H, benzimidazole CH_2), 3.05 (t, 2 H, CH_2N), 1.70 (s, 1 H, NH).

N-*Acetyl*-N,N-*bis*[2-(1-*methylbenzimidazol*-2-*yl*)*ethyl*]-*amine* **II**. *N*,*N*-Bis[2-(1-methylbenzimidazol-2-yl)ethyl]amine (2.4 mmol), triethylamine (4.8 mmol) and acetic anhydride (2.5 mmol) were mixed in dichloromethane (40 cm^3) and kept under stirring at room temperature for 2 h. The solution was

evaporated to dryness under vacuum, the solid dissolved in the minimum amount of dichloromethane and chromatographed on silica gel, using dichloromethane–methanol (9:1, v/v) as eluent. The main fraction was collected and evaporated to dryness under vacuum giving a white solid (yield 90%) (Found: C, 70.15; H, 6.90; N, 18.55. Calc. for $C_{22}H_{25}N_5O$: C, 70.35; H, 6.70; N, 18.65%); $\tilde{\nu}_{max}/cm^{-1}$ (Nujol) 1638s, 1618w, 1522m, 1458s and 726s; $\delta_H(CDCl_3)$ 7.8–7.6 and 7.3–7.1 (m, 8 H, phenyl H + benzimidazolyl H), 4.1–3.8 (m, 4 H, benzimidazole CH_2), 3.76 (s, 3 H, CH_3N), 3.58 (s, 3 H, CH_3N), 3.3–2.9 (m, 4 H, CH_2N), 2.11 (s, 3 H, CH_3CO).

Tris[2-(1-*methylbenzimidazol*-2-*yl*)*ethyl*]*nitromethane* **III**. Nitromethylidynetripropionic acid (6 mmol) and *N*-methyl-*o*-phenylenediamine dihydrochloride (20 mmol) were dissolved in 6 mol dm^{-3} hydrochloric acid (80 cm^3) and the mixture was allowed to react under gentle reflux in the dark for 20 h. The solution was cooled to room temperature, diluted with 100 cm^3 of cold water to precipitate the compound, and filtered off. The light brown crude product was dissolved in hot water (100 cm^3) and precipitated again under stirring with concentrated ammonia at 0 °C. The white precipitate was collected by filtration, washed several times with cold water, and dried under vacuum (yield 50%) (Found: C, 65.70; H, 6.65; N, 16.95. Calc. for $C_{31}H_{33}N_7O_2\cdot 2H_2O$: C, 65.15; H, 6.50: N, 17.15%); $\tilde{\nu}_{max}/cm^{-1}$ (Nujol) 3454m, 1618w, 1537s, 1512m, 1458s, 1334m, 746m, 733m and 679w; $\delta_H(CDCl_3)$ 7.8–7.6 and 7.3–7.1 (m, 12 H, phenyl H + benzimidazolyl H), 3.70 (s, 9 H, CH_3N), 2.96 (s, 12 H, CH_2N, benzimidazole CH_2), 1.90 (s, 2 H, H_2O).

Aminotris[2-(1-*methylbenzimidazol*-2-*yl*)*ethyl*]*methane* **IV**. Tris[2-(1-methylbenzimidazol-2-yl)ethyl]nitromethane (2.44 mmol) was dissolved in acetic acid–water (1:3, v/v) (50 cm^3). Palladium charcoal (10% palladium content) (0.5 g) was added and the mixture was hydrogenated at 3 atm ($\approx 3.04 \times 10^5$ Pa) under stirring for 24 h. The catalyst was removed by filtration and the filtrate was evaporated to dryness under vacuum. The crude oily product thus obtained was treated with 3 mol dm^{-3} hydrochloric acid to form a white precipitate. The product was filtered off washed several times with water and dried under vacuum (yield 90%) (Found: C, 64.30; H, 6.50; N, 16.90. Calc. for $C_{31}H_{35}N_7\cdot 2HCl$: C, 64.35; H, 6.45; N, 16.95%); $\tilde{\nu}_{max}/cm^{-1}$ (Nujol) 3392s (br), 1615s, 1566s, 1533s, 1464s, 1104m, and 760s; $\delta_H(CDCl_3)$ 7.8–7.6 and 7.3–7.1 (m, 12 H, phenyl H + benzimidazolyl H), 3.70 (s, 9 H, CH_3N), 3.2–2.9 (m, 6 H, benzimidazole CH_2), 2.3–2.1 (m, 6 H, CH_2N), 1.80 (s, 2 H, NH_2).

Complexes **1** *and* **2**. Deuterohaemin chloride (1.0 mmol) was dissolved in anhydrous dmf (15 cm^3). 1-Hydroxybenzotriazole (3.0 mmol) and, after 0.5 h, dicyclohexylcarbodiimide (1.0 mmol) were added to the solution under stirring. The mixture was allowed to react at 0 °C for 1 h. Then, the compounds **I** or **IV** (1.1 mmol) and triethylamine (2.0 mmol) were added and the mixture was allowed to react at 0 °C for 4 h, followed by 32 h at room temperature. The crude product was precipitated by addition of diethyl ether. The solid was dissolved in the minimum amount of butanol–acetic acid–water (4:2:1, v/v/v) and chromatographed on a silica-gel column (4 × 30 cm), using the same solvent mixture as eluent. The eluted fractions consisted of unreacted deuterohaemin and the condensation product. The products were recovered by evaporation to dryness under vacuum and showed a single spot on TLC analysis.

Concentrations of solutions of the modified haemins were determined spectrophotometrically using $\varepsilon = 93\,000$–$97\,000$ $dm^3\ mol^{-1}\ cm^{-1}$ for the Soret bands of **1** and **2**, respectively. These values were determined in methanol solution by the pyridine haemochromogen method.[18]

Complexes **3** *and* **4**. The mixed-metal complexes were obtained by treating methanolic solutions of **1** or **2** under stirring with methanolic solutions containing equimolar amounts of copper(II) perchlorate hexahydrate. The solutions were evaporated under vacuum for measurements in different solvents.

Binding Experiments.—The equilibrium constants for ligand–adduct formation were determined by spectrophotometric titrations at 25 °C. The data were analysed as described previously.[8]

Catalytic Oxidations by Complexes **1** *and* **2**.—The catalytic activity of **1** and **2** in the oxidation of *p*-cresol by *tert*-butyl hydroperoxide was studied. Since the kinetics of oxidation follows the trend typical for substrate saturation behaviour,[8] the comparison between the catalytic activity of the complexes was made through the evaluation of their maximum velocities, *i.e.* the initial rates under substrate saturation conditions. The experiments were performed as follows. The reaction mixtures contained deuterohaemin catalyst [$(2–4) \times 10^{-6}$ mol dm^{-3}] *p*-cresol (1×10^{-4}–5×10^{-3} mol dm^{-3}) and *tert*-butyl hydroperoxide (10^{-3} mol dm^{-3}) in methanol. Initial rates were calculated following the increase of absorbance at 316 nm, corresponding to the apparent maximum of absorption of the product mixture. The spectrum of the product mixture was identical in all experiments, indicating that the composition was the same. The values of the rates expressed as $(\Delta A_{316}/\Delta t)$/[catalyst] are the following (averages of 8 experiments): $100 \pm 30\ \Delta A$ $dm^3\ mol^{-1}\ s^{-1}$ for **1**, $620 \pm 50\ \Delta A$ $dm^3\ mol^{-1}\ s^{-1}$ for **2**. The addition of 1 equivalent of Cu^{II} to **1** or **2** did not appreciably affect the rates of oxidations.

Reduction Experiments.—The complexes **3** and **4** (approximately 10^{-5} mol dm^{-3}) were dissolved in water–methanol (1:3, v/v) under an argon atmosphere. A slight excess of the reductant dissolved in a small amount of the same degassed solvent was added to the solutions and UV/VIS spectra were recorded. Similar experiments were performed to follow reduction of the Cu^{II} signal by EPR, but higher concentrations of the complexes were used (approximately 1×10^{-4} mol dm^{-3}).

Reactivity with Dioxygen at Low Temperature.—These experiments were performed using a double-jacketed quartz cuvette fitted with Schlenk connections and connected to a Haake K cryostat to allow the system to reach −45 °C. The complexes were dissolved in a degassed solution of water–methanol (1:4, v/v) and the desired amount of solution was transferred with a syringe to the quartz cuvette kept in an inert atmosphere. An excess of sodium dithionite was added to the solution and spectra were recorded during reduction of iron. The solution was exposed to dioxygen contained in a syringe (5 cm^3) and the changes in the absorption spectra of the complexes were recorded during oxidation.

Molecular Mechanics and Molecular Dynamics.—The MM and MD calculations were performed using Insight and Discover software packages[19] installed on a Silicon Graphic Indigo workstation. Force-field parameters suitable to describe iron in a porphyrin environment were developed as described elsewhere.[14] The energy values reported in Table 2 were obtained as follows. The iron(III) porphyrin complexes **1** and **2** were constructed using the graphic facilities in Insight. Models for both the four-co-ordinated complexes, where the benzimidazole residues do not interact covalently with the iron atom and the five-co-ordinate complexes, where the nitrogen atom of one benzimidazole residue is axially bonded to the iron atom in the fifth co-ordination position were considered. The four-co-ordinated models obtained were submitted to energy minimisation in a vacuum using an algorithm of 1000 steps of steepest descent followed by one with 10 000 steps of conjugate gradient. To allow the complexes to scan the potential energy hypersurface better and consequently reach the absolute energy minimum every complex was submitted to 10 000 MD steps (1fs) at 300 K in a vacuum for a total of 10 ps simulation time. The conformations obtained every 1000 steps were energy minimised as described above and the lowest energy minimums are considered in the Results and Discussion section. This

procedure can be considered as a satisfactory method of obtaining absolute energy minima owing to the intrinsic rigidity of the molecules studied here.

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References

1 B. G. Malmström, *Chem. Rev.*, 1990, **90**, 1247; R. A. Capaldi, *Annu. Rev. Biochem.*, 1990, **59**, 569; S. I. Chan and P. M. Li, *Biochemistry*, 1990, **29**, 1; G. T. Babcock and M. Wikström, *Nature (London)*, 1992, **356**, 301; B. G. Malmström, *Acc. Chem. Res.*, 1993, **26**, 332.

2 S. E. Dessens, C. L. Merrill, R. J. Saxton, R. L. Ilaria, jun., J. W. Lindsey and L. J. Wilson, *J. Am. Chem. Soc.*, 1982, **104**, 4357; R. J. Saxton, L. W. Olson and J. L. Wilson, *J. Chem. Soc., Chem. Commun.*, 1982, 984; R. J. Saxton and L. J. Wilson, *J. Chem. Soc., Chem. Commun.*, 1984, 359; V. Chunplang and L. J. Wilson, *J. Chem. Soc., Chem. Commun.*, 1985, 1761.

3 A. C. Cutler, T. Brittain and P. D. W. Boyd, *J. Inorg. Biochem.*, 1985, **24**, 199; C. T. Brewer and G. Brewer, *Inorg. Chem.*, 1987, **26**, 3420; C. T. Brewer and G. Brewer, *J. Chem. Soc., Chem. Commun.*, 1988, 854; B. Lukas, J. R. Miller, J. Silver and M. T. Wilson, *J. Chem. Soc., Dalton Trans.*, 1982, 1035; C. A. Koch, C. A. Reed, G. A. Brewer, N. P. Rath, W. R. Scheidt, G. Gupta and G. Lang, *J. Am. Chem. Soc.*, 1989, **111**, 7645.

4 B. R. Serr, C. E. L. Headford, C. M. Elliott and O. P. Anderson, *J. Chem. Soc., Chem. Commun.*, 1988, 92; B. R. Serr, C. E. L. Headford, O. P. Anderson, C. M. Elliott, C. K. Schauer, K. Akabori, K. Spartalian, W. E. Hatfield and B. R. Rohrs, *Inorg. Chem.*, 1990, **29**, 2663; B. R. Serr, C. E. L. Headford, O. P. Anderson, C. M. Elliott, K. Spartalian, V. E. Fainzilberg, W. E. Hatfield, B. R. Rohrs, S. S. Eaton and G. R. Eaton, *Inorg. Chem.*, 1992, **31**, 5450.

5 A. Nanthakumar, M. S. Nasir, K. D. Karlin, N. Ravi and B. H. Huynh, *J. Am. Chem. Soc.*, 1992, **114**, 6564; S. C. Lee and R. H. Holm, *J. Am. Chem. Soc.*, 1993, **115**, 5833; A. Nanthakumar, S. Fox, N. N. Murthy, K. D. Karlin, N. Ravi, B. H. Huynh, R. D. Orosz, E. P. Day, K. S. Hagen and N. J. Blackburn, *J. Am. Chem. Soc.*, 1993, **115**, 8513.

6 M. J. Gunter, L. N. Mander, K. S. Murray and P. E. Clark, *J. Am. Chem. Soc.*, 1981, **103**, 6784; C. K. Chang, M. S. Koo and B. Ward, *J. Chem. Soc., Chem. Commun.*, 1982, 716; V. Bulach, D. Mandon and R. Weiss, *Angew. Chem., Int. Ed. Engl.*, 1991, **30**, 572.

7 S. I. Chan, *Ann. N.Y. Acad. Sci.*, 1988, **550**, 207; J. P. Hosler, M. M. J. Tecklenburg, Y. Kim, G. T. Babcock, R. B. Gennis and S. Ferguson-Miller, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 4786; J. Minagawa, T. Mogi, R. B. Gennis and Y. Anraku, *J. Biol. Chem.*, 1992, **267**, 2096.

8 L. Casella, M. Gullotti, L. De Gioia, E. Monzani and F. Chillemi, *J. Chem. Soc., Dalton Trans.*, 1991, 2945; L. Casella, M. Gullotti, L. De Gioia, R. Bartesaghi and F. Chillemi, *J. Chem. Soc., Dalton Trans.*, 1993, 2233.

9 W. I. White, in *The Porphyrins*, ed. D. Dolphin, Academic Press, New York, 1978, vol. 5, p. 303.

10 D. L. Budd, G. N. La Mar, K. C. Langry, K. M. Smith and R. Naygir-Mazhir, *J. Am. Chem. Soc.*, 1979, **101**, 6091; H. M. Goff, in *Iron Porphyrins, Part I*, eds. A. B. P. Lever and H. B. Gray, Addison-Wesley, Reading, MA, 1983, p. 237; I. Bertini and C. Luchinat, *NMR of Paramagnetic Molecules*, Benjamin/Cummings, Menlo Park, CA, 1986, ch. 7.

11 F. A. Walker, M. W. Lo and M. T. Ree, *J. Am. Chem. Soc.*, 1979, **98**, 5552; T. Yoshimura and T. Ozaki, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 2268.

12 P. Jones, D. Mantle and I. Wilson, *J. Inorg. Biochem.*, 1982, **17**, 293.

13 D. Mansuy, *Pure Appl. Chem.*, 1990, **62**, 741; P. W. White, *Bioorg. Chem.*, 1990, **18**, 440; B. Meunier, *Chem. Rev.*, 1992, **92**, 1411; B. Meunier, in *Metalloporphyrins Catalyzed Oxidations*, eds. F. Montanari and L. Casella, Kluwer, Dordrecht, 1994, p. 1.

14 L. Angelucci, L. De Gioia and P. Fantucci, *Gazz. Chim. Ital.*, 1993, **123**, 111; P. Dauber-Osguthorpe, V. A. Roberts, D. J. Osguthorpe, J. Wolff, M. Genest and A. T. Hagler, *Proteins: Struct. Func. Genet.*, 1988, **4**, 31.

15 M. F. Tweedle, L. J. Wilson, L. Garcia-Iniguez, G. T. Babcock and G. Palmer, *J. Biol. Chem.*, 1978, **253**, 8065; T. H. Moss, E. Shapiro, T. E. King, H. Beinert and C. Hartzell, *J. Biol. Chem.*, 1978, **253**, 8072.

16 G. W. Brudvig, R. H. Morse and S. I. Chan, *J. Magn. Reson.*, 1986, **67**, 189.

17 J. H. Fuhrhop and K. M. Smith, *Laboratory Methods in Porphyrin and Metalloporphyrin Research*, Elsevier, Amsterdam, 1975, p. 17.

18 Ref. 17, p. 48.

19 Insight II/Discover, 2.7 Molecular Modeling Software, Biosym Technologies Inc., San Diego, CA, 1991.

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