Studies on Copper–Protoporphyrin–Iron(III) Complexes.† A Possible Model for Cytochrome *c* Oxidase

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Methods for preparing copper–protoporphyrin–iron(III) complexes are presented. Comparison of the properties of these complexes with haematin and μ-oxo-dimeric haematin clearly shows that the complexes are distinct new species. Infrared spectroscopy provides evidence for the copper binding to protoporphyrin propionate groups. Mössbauer and e.s.r. spectroscopy provide evidence for high-spin iron(III) and copper(II). There is some evidence for interactions between the two metal nuclei. A possible structure is presented and its properties are discussed in terms of the known behaviour of cytochrome *c* oxidase.

COMPLEXES formed between iron and the porphyrin group, or its derivatives, and with other macrocyclic ligands have provided useful models for haem proteins. The 'picket fence' iron porphyrin complexes synthesised by Collman *et al.*[1-4] as models for the oxygen transport proteins are one such example.

In this paper we present a method for the production of complexes formed between protohaem and metal ions, in particular copper(II) ions. Chemical and spectroscopic analyses suggest that added metal ions bind to the propionic carboxylate groups of the haem moiety. In 1923 Hamsik [5] reported the preparation of potassium, barium, calcium, silver, and lead salts of protoporphyrin IX–iron(III) but the stoicheiometries were not fully established. Such salts are of interest since they offer the possibility of bringing copper and iron atoms into close proximity and may thus provide insight into the interactions of such metals in the enzyme cytochrome *c* oxidase (E.C. 1.9.3.1). This latter is one of a small class of metalloproteins capable of catalysing the reduction of molecular oxygen to water.[6,7] Consideration of the thermodynamics of oxygen reduction suggests that donation of a single electron to oxygen would not be favoured.[8] It appears that cytochrome *c* oxidase circumvents this barrier by providing an oxygen binding site in which interaction between a copper atom (termed Cu_B) and a haem iron atom (of cytochrome a_3) play an important role in the process of donating electrons to dioxygen in multi-electron steps.[9-12]

Evidence (see Brunori *et al.*[7]) that Cu_B and the iron atom of cytochrome a_3 are antiferromagnetically coupled in the 'resting' fully oxidised form of the enzyme has come from the application of e.s.r. spectroscopy [13,14] and magnetic circular dichroism.[15] Coupling may be *via* a shared common ligand (*cf.* a histidine residue between the Zn and Cu atoms of superoxide dismutase [16]), possibly an oxygen atom donated by the protein (*i.e.* a carboxylate or phenolate),[17] or may even be derived from an oxygen molecule taking part in the catalytic cycle of the enzyme.[18] Recent extended *X*-ray absorption fine structure (EXAFS) studies provide some evidence for a sulphur atom acting as a bridging ligand.[19]

Investigations of binuclear iron–copper complexes as models for the oxygen binding site of cytochrome *c* oxidase have been reported by a number of workers recently.[20] The studies we describe here are in general agreement with these earlier reports. However, the complexes we discuss have the advantage of being both relatively easily prepared and, in so far as the binding site for metal ions involves the haem carboxylate groups, may actually occur naturally as these groups are present in haem proteins.

† Protoporphyrin IX = 3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropionic acid; haem = protohaem = [protoporphyrinate(2−)]iron(II); haemin = chloro[protoporphyrinate(2−)]iron(III); haematin = hydroxo[protoporphyrinate(2−)]iron(III).

EXPERIMENTAL

Copper–protoporphyrin–iron(III) complexes were prepared which had stoicheiometric ratios of 1 : 1 and 2 : 1 Cu : Fe. Haematin solutions were prepared from haematin (200 mg) (from bovine blood, Sigma) which was first dissolved in NaOH (5 cm^3, 1 mol dm^{-3}). The solution was then diluted with water to 100 cm^3. Copper nitrate solutions (25 cm^3) containing either 75 mg of $Cu(NO_3)_2 \cdot 4H_2O$ (for the 1 : 1 complex) or 150 mg (for the 2 : 1 complex) were then mixed with the haematin solutions to give the desired stoicheiometries. The pH was adjusted by addition of dilute nitric acid until precipitation occurred (pH 6.5 for 1 : 1 complex, 6.9—7.0 for 2 : 1 complex). The precipitates were filtered off and washed several times with water. The yields were about 90%. The compounds were used without further purification since it proved impossible to dissolve them to any extent without decomposition occurring.

Analyses were carried out by the Microanalytical Laboratory, Department of Chemistry, University of Manchester, and are given in Table 1. The number of water molecules is uncertain on the basis of these data but one molecule has been included for calculating as this gives the best overall fit to the data in each case. Infrared spectra were recorded from mulls using a Perkin-Elmer 257 spectrometer. Electronic reflectance spectra of powders were recorded on a Unicam SP 700 spectrophotometer using magnesium oxide as reference.

Magnetic susceptibilities of powders were measured by the Gouy method at room temperature. Tubes were packed several times to check for orientation errors. E.s.r. spectra were obtained on the powdered solid at liquid-

helium temperatures and above using a Varian E9 instrument.

The Mössbauer spectra of the powders were recorded on a conventional constant-acceleration spectrometer previously described.[21] The source was ≈ 11 mCi * of ^{57}Co in rhodium (obtained from the Radiochemical Centre, Amersham) at 20 °C. The absorber was cooled in a RICOR MCH5 cryostat the temperature being controlled by a RICOR TC4B controller. The source was moved with a triangular-drive waveform giving mirror-image spectra, which were folded together and computer fitted. The velocity was calibrated and linearity of the waveform monitored by reference spectra of 10 mg cm^{-2} iron foil, to which isomer shifts are referred.

TABLE 1

Elemental analysis (%) *

	C	H	N	Fe	Cu
Cu–haematin–H_2O	58.0	4.40	7.30	7.30	8.70
($C_{34}H_{35}CuFeN_4O_7$)	(55.85)	(4.85)	(7.65)	(7.65)	(8.70)
Cu_2–haematin–H_2O	48.65	4.20	6.65	6.30	15.4
($C_{34}H_{37}Cu_2FeN_4O_9$)	(49.30)	(4.50)	(6.75)	(6.75)	(15.35)

* The calculated values (given in parentheses) for the 1 : 1 complex are based on structure A with the addition of one water molecule. The calculated values for the 2 : 1 complex are based on a structure containing one haematin moiety (minus two H^+ ions), two Cu^{2+} ions, two OH^- ions, and an additional water molecule.

RESULTS AND DISCUSSION

The analytical results for the two copper–protoporphyrin–iron(III) complexes are presented in Table 1. These clearly demonstrate the stoicheiometry of the complexes but do not rule out the possibility of co-precipitation of haematin and ' copper(II) hydroxide '. Haematin, however, usually precipitates at pH 4.6 and copper(II) hydroxide (depending on the concentration) at about pH 5.0. The complexes described here are obtained at pH 6.5—7.0.

Reflectance spectra of haematin as both monomers and dimers and the 1 : 1 copper(II)–haematin complex are shown in Figure 1. Clearly the band at 11 000 cm^{-1} (900 nm) in haematin is not present in the μ-oxo-dimeric haematin and is much attenuated in the copper complexes, being most reduced in the 2 : 1 Cu : Fe complex. This suggests that the complexes are either mixtures of μ-oxo-dimeric haematin and haematin co-precipitated with copper(II) hydroxide or that they are new materials. However, the first possibility may be considered unlikely as μ-oxo-dimeric haematin can generally be prepared only from aqueous solutions of a pH value of *ca.* 14. Thus the electronic spectra combined with the pH data at which the complexes were formed suggest true complex formation.

Infrared spectra of both copper complexes (Table 2) show changes in the carbonyl-stretching frequencies compared to haematin. Such changes rule out the presence of unreacted haematin and are consistent with structure A in which haem propionic carboxylate groups are bound to copper. Similar changes of the carbonyl-stretching frequencies are found for disodium protoporphyrinate compared to protoporphyrin-free acid (Table 2).

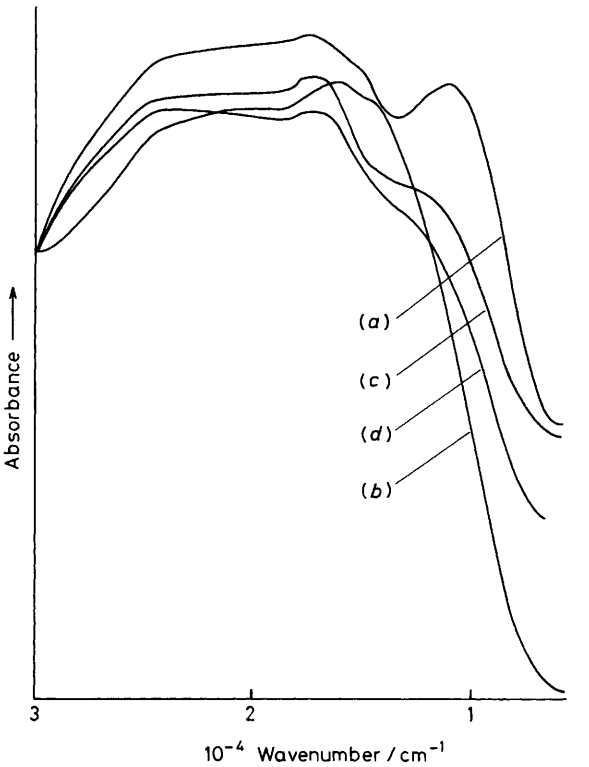


FIGURE 1 Reflectance electronic absorption spectra of (*a*) haematin; (*b*) μ-oxo-dimeric haematin; (*c*) 1 : 1 copper–protoporphyrin–iron(III) complex; (*d*) 2 : 1 copper–protoporphyrin–iron(III) complex

The Mössbauer data for haematin, μ-oxo-dimeric haematin, and the copper–haematin complexes are given in Table 3, and the spectrum of the 1 : 1 Cu : Fe complex is also shown in Figure 2. The data for the copper–haematin complexes are in keeping with the iron being high-spin iron(III) but with some indications of unusual spin coupling. The spectra obtained for the 1 : 1 Cu : Fe complex show some asymmetry but this does not show any variation between 80 K and room temperature, unlike other synthetic and biological ferrohaems.[22-28]

TABLE 2

Infrared absorption peaks in the region 1 400—1 800 cm^{-1} [a]

Haematin	PPIX	Na_2–PPIX	Cu–haematin	Cu_2–haematin
*1 720*s [b]	*1 700*s [b]		1 720vw	1 720vw
1 660 (sh)			1 660 (sh)	1 660 (sh)
1 630m				
		*1 550*s [c]	*1 560*vs [c]	*1 560*vs [c]
1 445m	1 435 (sh)	1 435 (sh)	1 445 (sh)	1 445 (sh)
1 410 (sh)	1 405 (sh)	*1 405*s [d]	*1 410*s [d]	*1 410*s [d]

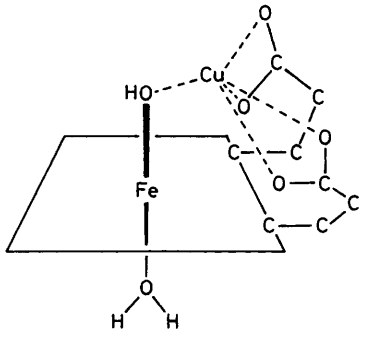
[a] All spectra were obtained from mulls. Strong peaks are italicised. PPIX = Protoporphyrin IX. [b] Assigned as C=O stretching of protonated carboxylic groups. [c,d] Asymmetric and symmetric C–O stretching vibrations of deprotonated carboxylic groups.

* Throughout this paper: 1 Ci = 3.7 × 10^{10} s^{-1}; 1 B.M. = 9.274 × 10^{-24} A m^2; 1 G = 10^{-4} T.

TABLE 3

^{57}Fe Mössbauer parameters for the haem complexes

Complex	T/K	δ/mm s^{-1}	Δ/mm s^{-1}	Γ/mm s^{-1}	
Haematin dimer	298	0.32(1)	0.58(2)	0.16(1)	
	80	0.40(1)	0.57(1)	0.17(1)	
Copper–protoporphyrin–iron(III) (1 : 1)	298	0.291(7)	0.677(8)	0.193(10)	0.235(14)
	80	0.405(4)	0.686(4)	0.194(6)	0.205(7)
Copper–protoporphyrin–iron(III) (2 : 1)	298	0.304(7)	0.638(7)	0.199(10)	0.210(12)
	80	0.399(3)	0.654(3)	0.190(5)	0.185(5)
Haematin	298	0.20(2)	0.78(2)	0.24(4)	0.51(13)
	80	0.384(13)	0.88(13)	0.263(12)	0.305(24)



A

Schematic representation of 1 : 1 copper–protoporphyrin–iron(III) complex showing the position of the copper atom above the haem plane and co-ordinated to the two propionic carboxylate groups and to a hydroxy group which acts as a bridge to the Fe atom. A scale model of this structure indicates that the Cu–O bond lengths are *ca.* 1.97 Å

The spectra cannot be explained as fitting a mixture of the μ-oxo-bridged dimeric haematin and haematin since such a mixture would be expected to show temperature-dependent Mössbauer spectra.

The asymmetric spectra observed for haematin are explained in the same way as for haemin by Blume [29] in terms of a temperature-dependent spin–spin relaxation process. The μ-oxo-dimeric haematin (which gives symmetric Mössbauer spectra) contains two high-spin ($S = \frac{5}{2}$) iron(III) ions antiferromagnetically coupled through the oxygen bridge. Since no fluctuations of the electron spins are possible under these conditions the Mössbauer spectrum is a symmetric doublet.

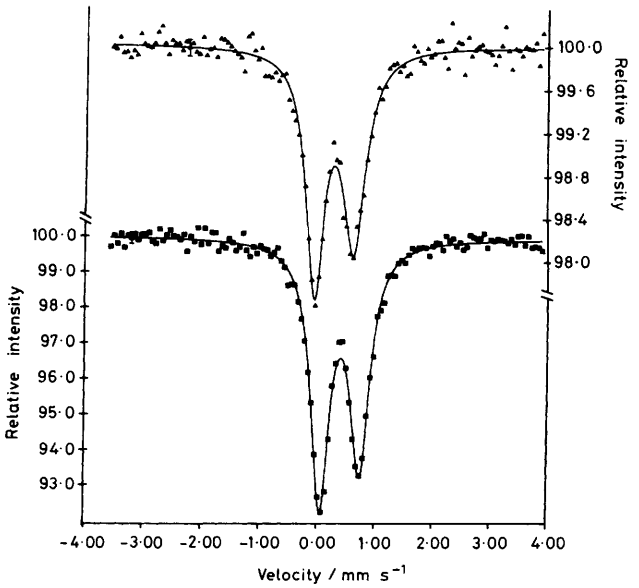


FIGURE 2 Mössbauer spectrum of 1 : 1 copper–protoporphyrin–iron(III) complex at 80 (■) and 298 K (▲)

The copper–protoporphyrin–iron(III) complexes, depending on structure, may also be antiferromagnetically coupled and the extent to which the coupling takes place will cause spin–spin relaxation to be manifest in the Mössbauer spectra, *i.e.* the spectra become symmetric. However, as such a coupling could not involve all the electrons some fluctuation of the electron spins may still be possible and cause the residual asymmetry observed in the spectra.

The quadrupole splittings found for the copper–protoporphyrin–iron(III) complexes are larger than those of the μ-oxo-dimeric haematin, but the isomer shifts are very close. It is significant that for the 2 : 1 and 1 : 1 copper–protoporphyrin–iron(III) complexes different quadrupole splittings are observed. This means that the copper ions are close enough to the iron centre to affect the Mössbauer quadrupole splittings, and they are bound differently in the two complexes. Indeed, in μ-oxo-dimeric haematin the iron atoms are antiferromagnetically coupled through an oxygen atom and it would seem likely that any antiferromagnetic coupling of the Cu to Fe in these complexes would also be through one atom giving support to structure A.

Preliminary e.s.r. spectra for the 1 : 1 copper–protoporphyrin–iron(III) complex as a solid show the material to be different from haematin and μ-oxo-dimeric haematin (Figure 3). The e.s.r. spectrum of copper–protoporphyrin–iron(III) complex in the region of $g = 6$ is narrower than the corresponding signal in haematin, and does not show the large broad signal around $g = 4$ shown by the dimer. This indicates that the material is not a co-precipitation of copper hydroxide and haematin.

The e.s.r. spectrum of the complex showed little or no effect of temperature on the linewidths of the signals but their intensities decreased with increasing temperature showing approximately Curie law behaviour. Our spectra are very similar in appearance over the temperature range 10—90 K to those reported by Gunter *et al.*[20a] The temperature dependence of the e.s.r. spectrum suggests to us that the two magnetic centres are largely but not completely independent of each other. When combined with the Mössbauer data this would suggest that the two centres are weakly coupled.

The electronic spectrum (Figure 4) of haematin dissolved in oxygen-free pyridine shows the presence of Fe^{II}, indicating that pyridine spontaneously and rapidly (within 15 s) reduces haematin. This is supported by the observation that the pyridine–haematin spectrum is unchanged by the addition of dithionite (aqueous). The 1 : 1 Cu : Fe complex dissolves slowly in pyridine under nitrogen. This solution, however, does not immediately show the presence of iron(II) but reduction occurs over several hours. The fact that the 1 : 1 copper protoporphyrin–iron(III) complex does not become reduced immediately on dissolving in pyridine (Figure 5) emphasises that this material is not a mixture of haematin and copper(II) hydroxide. The iron in this copper complex must be protected from the reduction in pyridine. A structure such as A would be consistent with this behaviour.

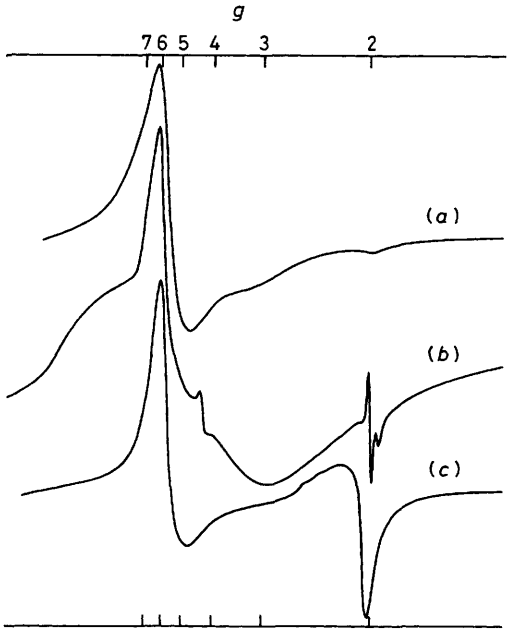


FIGURE 3 E.s.r. spectra of (*a*) haematin, (*b*) μ-oxo-dimeric haematin, and (*c*) 1 : 1 copper–protoporphyrin–iron(III) complex. Spectra are obtained at 20 K, 2 mW power, and modulation 20 G

It has proved impossible to prepare a haematin copper complex starting from the methyl ester of protoporphyrin–iron(III). No copper haem salt or even a co-precipitation product is formed. We take this as supporting evidence that it is the anionic propionate groups that bind to the copper.

Room-temperature magnetic susceptibility studies gave $\mu_{eff.}$ values of 4.1 and 4.4 B.M. for the 1 : 1 Cu : Fe and 2 : 1 Cu : Fe complexes respectively. Both complexes appeared to obey the Curie law over the temperature range 80—300 K. A simple spin-only treatment is entirely inadequate to interpret these results, especially when it is considered that the 1 : 1 complex has an even number of electrons and the 2 : 1 complex an odd number.

The results might suggest the presence of iron of spin lower than $\frac{5}{2}$ but this is not compatible with the e.s.r. spectrum which is typical of high-spin Fe^{III} or with the Mössbauer spectrum which shows only high-spin Fe^{III}.

Conclusions.—All the studies reported here show that both the copper haem complexes are discrete compounds and not co-precipitated mixtures. The i.r. data provide evidence for the presence of COO^- groups. Taken together the above results lead us to postulate structure A for the 1 : 1 copper–protoporphyrin–iron(III) complex. Using molecular models it is possible to demonstrate that this structure can be formed incorporating typical Cu–O bond lengths (*ca.* 1.97 Å) and without undue strain. It is suggested that the fifth and sixth ligands to the central iron atom are OH^- and H_2O respectively on the basis of charge balance and the analytical results of Table 1.

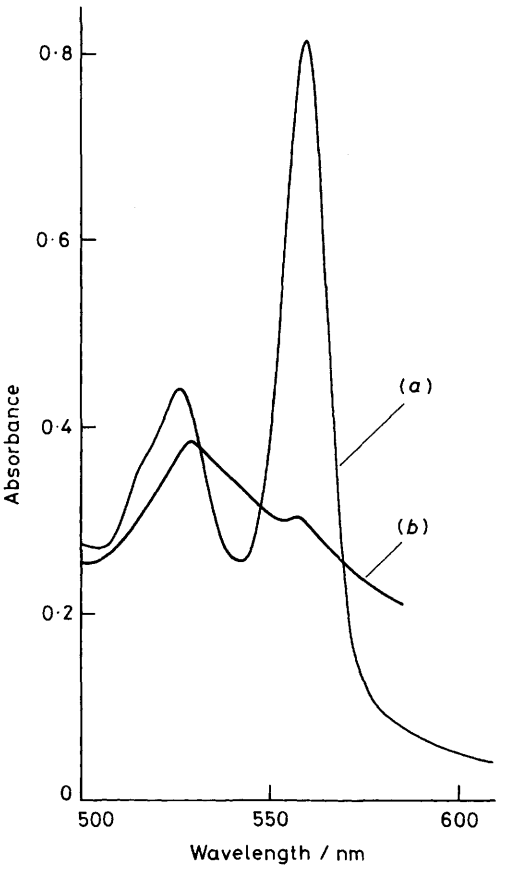


FIGURE 4 Absorption spectra of haematin complexes: (*a*) haematin in pyridine in the absence of air; (*b*) haematin in the presence of air

A comparison of the properties of the 1 : 1 copper–protoporphyrin–iron(III) complex with those of the binuclear site of cytochrome *c* oxidase is appropriate here. The optical data (Figure 1) show that the band at 900 nm, characteristic of haematin, disappears on complex formation. There is no comparable counterpart in the enzyme, where this region of the spectrum is dominated by the absorbance of the other, magnetically isolated, copper atom present.[7]

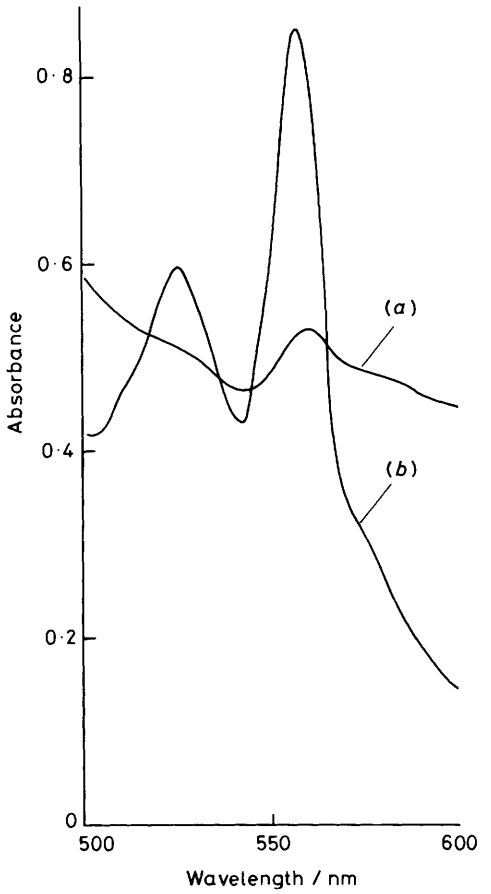


FIGURE 5 Absorption spectra of haematin complexes: (*a*) 1 : 1 copper–protoporphyrin–iron(II) complex in pyridine in the absence of air; (*b*) as (*a*) plus sodium dithionite

Magnetic susceptibility data for the enzyme are most conveniently explained in terms of a high-spin iron atom antiferromagnetically coupled to a copper(II) ion forming an $S = 2$ unit.[15,30] The corresponding data for the complex cannot be interpreted in a similar fashion. Indeed, the unusual $\mu_{eff.}$ values we report are at this time unexplained, as also seems to be the case for other such model complexes.[20a] The Mössbauer measurements on both the enzyme [31] and the complex show the presence of high-spin iron(III) possessing similar chemical shifts in both cases. However, the quadrupole splitting in the enzyme is 1.3 mm s^{-1} whereas in the complex a value of 0.68 mm s^{-1} was obtained, indicating a greater degree of electronic asymmetry is present in the ligand environment of the high-spin iron in the enzyme. We note, however, that the behaviour of the oxidised enzyme at low temperatures [31] shows features similar to those of the complex. In particular, the spectra have a more symmetrical character than would be expected by comparison with other haem proteins.[31]

In agreement with the magnetic measurement the e.s.r. data [13,14] for the enzyme are compatible with strong antiferromagnetic coupling between metal sites. The e.s.r. spectra of the complexes, however, taken together with the corresponding Mössbauer data show that any such coupling must be weak. It remains for a more quantitative e.s.r. study to determine the exact extent and nature of such coupling.

Although this model does not mimic accurately the physical properties of cytochrome *c* oxidase, it nevertheless has some appealing features primarily because no specialised architectured molecule has had to be prepared for the Cu–Fe complex formation. We note that if such a coupled binuclear centre existed in oxidase it would have interesting properties *vis-à-vis* its redox behaviour. In particular, reduction of Cu^{II} to Cu^{I} may result in ligand exchange on this atom in view of the well established preferences of Cu^{I} and Cu^{II} for different ligands. One might envisage one of the propionic carboxylates ligated to Cu^{II} in this model being replaced on reduction to Cu^{I} by another ligand, *e.g.* phenolate oxygen. The flexibility and mobility of the propionic acid side chains which bear the carboxylate groups might play an important role in any such ligand-exchange processes.

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