An Electron Spin Resonance Study of the Molecular Oxygen Adducts of the Apomyoglobin Complexes of Cobalt(II) Protoporphyrin IX and Cobalt-(II) 3,10,17,24-Tetrasulphonated Phthalocyanine

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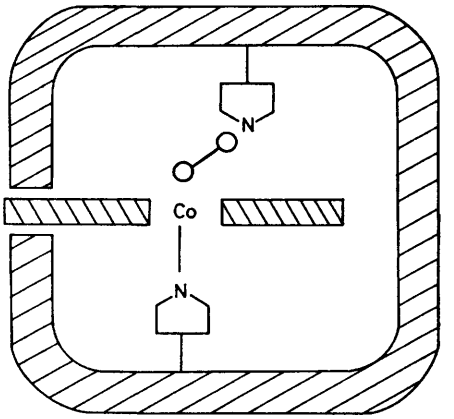
The apomyoglobin complexes of cobalt(II) protoporphyrin IX and cobalt(II) 3,10,17,24-tetrasulphonated phthalocyanine have been prepared and the e.s.r. spectra of their adducts with molecular oxygen recorded and quantitatively evaluated. The results obtained are compared with those from other work. The protein provides appropriate conditions for the oxygenation of cobalt(II) tetrasulphonated phthalocyanine at room temperature, and its influence on the e.s.r. parameters due to the molecular oxygen complex of the prosthetic group is discussed.

THE oxygenation of the iron(II) form of haemoglobin and myoglobin plays a vital role in the physiological transport and storage of molecular oxygen. The acquisition of molecular oxygen by these iron proteins is a complex process involving structural and electronic aspects of the prosthetic group as well as structural properties of the protein. In attempts to unravel the contributing factors to the oxygenation of the haem proteins one approach involves the replacement of the iron porphyrin by the corresponding cobalt(II) porphyrin to form a coboglobin, a strategy which makes possible the application of e.s.r. techniques to monitor the oxygenation process (see Scheme).



SCHEME
Schematic representation of a cobalt(II) porphyrin seen in elevation in the protein pocket, showing an axial interaction with the nitrogen atom of the proximal histidine and the molecular oxygen in an inclined position, lying close to the ring of the distal histidine group

In addition to changes in the metal ion, the nature of the prosthetic group may be further modified by the introduction of other macrocyclic structures into the protein such as water-soluble phthalocyanine derivatives. An earlier study has shown that cobalt(II) tetrasulphonated phthalocyanine, Co^{II}(tspc), undergoes the process of oxygenation once embedded in the globin structure,[1a] while the iron(II) state of Fe^{II}(tspc) introduced into apohaemoglobin is stabilized by the protein.[1b]

X-Ray crystallographic studies have shown that cobalt(II) protoporphyrin IX lies in the hydrophobic crevice of the apoprotein in a similar fashion to the iron porphyrin,[2] although it has been noted from resonance Raman and circular dichroism measurements that the packing of amino-acid side chains around copper(II) protoporphyrin, used as a reporter group (*i.e.* one sensitive to the environment), is different to that of metmyoglobin.[3] Kinetic measurements have shown that the rate of association (k_{on}) of molecular oxygen with the coboglobins is of the same order as those for myoglobin, whereas the dissociation rates, k_{off}, are about 10^2 times larger than for the corresponding myoglobin.[4] A further important observation is that the e.s.r. spectra of the molecular adducts of coboglobin are influenced by replacement of water, as solvent, by deuterium oxide. This effect has been interpreted as evidence of hydrogen bonding with the distal histidine group.[5] An opportunity to confirm the influence of the distal histidine group on the bonding of molecular oxygen is presented by those proteins where either the distal histidine group is replaced by some other amino-acid residue or where it is absent. The former possibility occurs in monomeric haemoglobin (Glycera) where the distal histidyl group is replaced by a leucyl residue.[6] E.s.r. measurements on the molecular oxygen complex of the cobalt(II) derivative of this particular protein have been interpreted as confirming the importance of the distal histidine group on molecular oxygen binding.[7] Again, in myoglobin (Aplysia), which possesses only one histidine group,[8] e.s.r., photodissociation equilibrium, and kinetic measurements of the molecular oxygen complex of cobalt(II) myoglobin (Aplysia) have shown that the bound oxygen interacts with some amino-acid residue adjacent to it since the e.s.r. spectrum due to the molecular oxygen complex is somewhat sharpened in the presence of deuterium oxide.

Turning to the results obtained by substitution of the iron in haemoglobin by cobalt(II), some prospects of unravelling the complicated effects due to interventions between the subunits arise from a study of the dioxygen affinity of the cobalt in the macrocyclic allied with the individual α and β chains. Such studies[9] have shown

that the oxygen affinity of the individual chains is higher than in cobalt(II) haemoglobin and independent of pH. The e.s.r. spectra of the oxy-cobalt α chain show distinctly narrowed hyperfine structure in comparison with the oxy-cobalt β chain, indicating different environments around the paramagnetic centres, the source of the inequivalence between the α and β chain being thought to be near the distal histidine group. Other studies on the subunit interaction in iron–cobalt hybrid haemoglobins in oxy- and deoxy-forms show that the electronic structure of the prosthetic groups in deoxy-α subunits is more closely related to the state of the quaternary structure of the haemoglobin molecule than is the deoxy-β subunit.[10] An opportunity to measure the effect of the distal histidine on oxygen binding by the cobalt derivative of haemoglobin is made possible by the molecular oxygen complex of cobalt(II)-substituted haemoglobin (Zürich) where the distal histidine group in the β subunits is replaced by arginine which has an effect on the entrance of ligands into the haem product.[11] A study of the oxygenation of cobalt(II) haemoglobin (Zürich) showed that the oxygen affinity was higher, although the co-operativity smaller, than in cobalt(II) haemoglobin (Aplysia).[12] The e.s.r. spectral variation in going from water to deuterium oxide as solvent suggests the absence of hydrogen bonding between the distal amino-acid residue and the bound dioxygen in the abnormal β subunits.

Recent studies [13-15] have shown that it is possible to extract structural information, namely the angular disposition of the molecular oxygen moiety with respect to the cobalt atom in monomeric molecular oxygen adducts of cobalt(II) compounds, from e.s.r. spectral data. In addition, the magnetic parameters associated with the e.s.r. spectra of these complexes have been shown to vary with the substituent groups associated with the ligand. In the light of these developments, the e.s.r. spectrum of the molecular oxygen complex of coboglobin has been studied, and a comparison made of the e.s.r. parameters associated with the molecular oxygen complexes of similar compounds, with the aim of establishing the influence of the protein on the structural disposition of the molecular oxygen. To focus attention on the ability of the protein to provide a suitable environment for the formation of the molecular oxygen adduct by the prosthetic group, the oxygenation of the Co^{II}(tspc)–apomyoglobin complex has been investigated, and the magnetic parameters for this system determined.

RESULTS

Preparation and Oxygenation of the Cobalt(II) Protoporphyrin IX–Apomyoglobin Complex (Cobomyoglobin).— The substitution of cobalt(II) protoporphyrin IX and related materials into the protein pockets of a number of the apoproteins of the respiratory iron proteins involved in oxygen transport and storage has been described on a number of occasions. More recently, the preparative procedures have been extended to include the synthesis of chlorophyll derivatives, apomyoglobin complexes.[16,17] Some aspects of the preparative conditions used in the synthesis of cobomyoglobin are summarized in Table 1 which indicates that reliable conditions for the preparation of the metalloproteins are those described by Yonetani *et al.*[5] In the present study it was confirmed that the synthesis of the coboglobins was successful using these conditions although the yields were often disappointingly low. Some difficulties are encountered by the use of high-pH conditions which are necessary for the stabilization of the metalloporphyrin but which may lead to some denaturation of the apoprotein. Alternatively, the metalloporphyrin may be dissolved in neutral aqueous solutions containing the amine polyethylene oxide non-ionic detergent, Teric 18M20. The resulting aqueous solution provides mild conditions for the formation of the cobomyoglobin by insertion of cobalt(II) protoporphyrin IX into the protein product of apomyoglobin. Indeed it was found that an aqueous solution containing the non-ionic detergent and cobalt(II) protoporphyrin IX, along with a suitably small amount of ascorbic acid to prevent oxidation of the product, favoured the formation of the cobalt(II) protoporphyrin–apomyoglobin complex when apomyoglobin was introduced into the solution. The cobomyoglobin formed was separated by elution from a Sepha-

TABLE 1

Preparative conditions for coboglobin

Insertion conditions	Reducing agent	Comment	Visible spectral data (nm) Soret	β	α	Ref.
Cobalt(II) protoporphyrin IX in 10% pyridine–water to apomyoglobin; in air	Hydrosulphite in excess	Reproducibility problems; large amount of cobalt(III) products	423	538	568	*a*
Excess of cobalt(II) protoporphyrin IX in minimal amount of pyridine added to apomyoglobin in 0.1 mol dm^{-3} phosphate buffer, pH 7.0; in air	Dithionite in excess	Large amount of cobalt(III) products	426	539	574	*b*
Cobalt(II) protoporphyrin IX in 50% pyridine to apomyoglobin in phosphate buffer, pH 7.0 exclusion of oxygen	Dithionite in excess	Reproducibility problems solved, little cobalt(III) products formed	426	539	577	*c*
Cobalt(II) protoporphyrin IX in 0.5% non-ionic detergent to apomyoglobin; exclusion of air	Ascorbate or dithionite	Good yields; reproducible	426	539	577	*d*

[a] B. M. Hoffman and D. H. Petering, *Proc. Natl. Acad. Sci.*, 1970, **67**, 637. [b] J. W. Chien and L. C. Dickinson, *Proc. Natl. Acad. Sci.*, 1972, **69**, 2783. [c] Ref. 31. [d] This work.

dex G-50 column followed by further chromatographic separation on a Whatman C-25 cation-exchange column. The e.s.r. spectrum, recorded at 140 K, of an aqueous solution of the isolated cobalt(II) protoporphyrin IX–apomyoglobin complex is quite similar to that reported by Yonetani *et al.*[5]

Computer simulation of the e.s.r. spectrum due to the molecular oxygen adduct of cobalt(II) protoporphyrin IX–apomyoglobin was achieved using the procedure outlined previously [13] which takes into account the non-coincidence of the g and A axes in the XY plane (Figure 1). Errors were obtained from goodness of fit by eye.

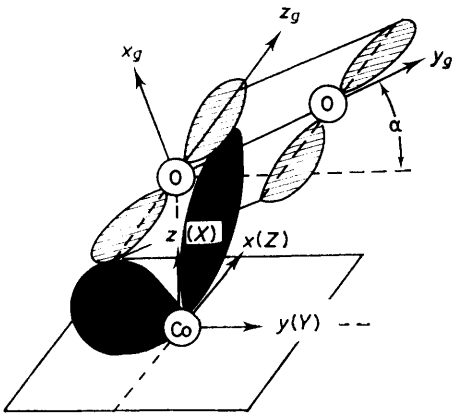


FIGURE 1 Simplified molecular structure of cobalt(II)–O_2 adducts. Principal g axes are x_g, y_g, and z_g. Hyperfine (A) principal axes are $X(z)$, $Y(y)$, and $Z(x)$. Symmetry is C_s. The planar part of porphyrin and tspc molecules is outlined but more evidence would be needed to establish the orientation unequivocally. The unpaired electron π^* orbital hatched and the relevant part of the cobalt d_{xz} orbital (filled in) are depicted schematically to illustrate the point that, as α increases, π^*–d_{xz} overlap should increase and possibly go through a maximum at α_m

We do not repeat the arguments relating to the effects of low symmetry [18] but merely emphasize that it is not necessarily correct to read hyperfine splitting directly from the experimental charts of frozen-solution spectra. The spin-Hamiltonian parameters obtained from the computer simulation of the experimental spectrum are given in Table 2, which also shows the results obtained by Yonetani *et al.*,[5] along with the molecular oxygen adducts of related materials for which a typical result is shown in Figure 2. All the other results are based upon digitization of the spectra published in the various references listed in Table 2.

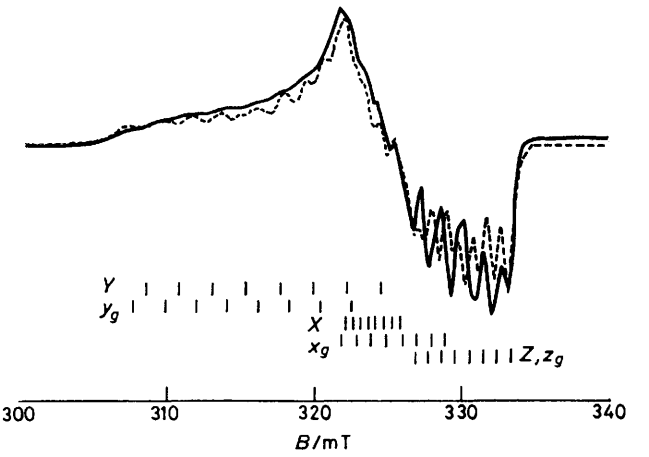


FIGURE 2 Digitized e.s.r. spectrum of the oxygen adduct of cobalt(II) mesoporphyrin-substituted sperm whale myoglobin based on Figure 4A of ref. *b* in Table 2. The dotted curve is the simulated spectrum using parameters given in Table 2

Preparation and Oxygenation of the Cobalt(II) *Tetrasulphonated Phthalocyanine–Apomyoglobin Complex.*—The cobalt(II) chelate of tetrasodium 3,10,17,24-tetrasulphonated phthalocyanine, Na_4[tspc], a water-soluble anionic compound, gives

TABLE 2

E.s.r. parameters associated with the molecular oxygen adducts of cobalt(II) porphyrins in protein pockets

Apoprotein	Cobalt(II) porphyrin	Solvent conditions	g_x	g_y	g_z	$10^4A_X(z)$ [a]	$10^4A_Y(y)$ [a]	$10^4A_Z(x)$ [a]	α/°	Ref.
						cm^{-1}				
Sperm whale myoglobin	Mesoporphyrin	H_2O	2.0123	2.0782	1.9850	5.5	21.3	8.5	18	*b*, Figure 4A
	Mesoporphyrin	D_2O	2.0136	2.0790	1.9850	5.0	22.0	8.5	22	*b*, Figure 4A
	Protoporphyrin	Phosphate buffer, pH 7.0	2.0130	2.0748	1.9878	4.0	20.0	8.0	23	*b*, Figure 2A
	Protoporphyrin IX	Phosphate buffer, pH 7.0	2.0127	2.0760	1.9905	5.5	19.0	7.7	23	*c*
	Protoporphyrin IX	H_2O pH 7.0	2.0027	2.0627	1.9870	5.0	20.5	8.0	23	*b*, Figure 2A
Haemoglobin (Glycera)	Mesoporphyrin	H_2O	2.0008	2.0680	1.9778	4.0	16.5	6.8	23	*b*, Figure 4B
Myoglobin (Aplysia)	Mesoporphyrin	H_2O pH 7.0	2.0068	2.0885	1.9836	5.8	21.5	9.6	22	*d*, Figure 2
Haemoglobin (α chain)	Protoporphyrin IX	H_2O	2.0077	2.0760	1.9870	5.0	17.8	7.9	25	*e*, Figure 4
(β chain)	Protoporphyrin IX	H_2O	2.0095	2.0680	1.9912	4.0	20.5	7.9	26	*e*, Figure 4
(Zürich)	Protoporphyrin IX	H_2O	2.0093	2.0680	1.9850	4.0	19.8	7.9	26	*f*, Figure 5

[a] *A* values assumed negative for hyperfine analysis. [b] F. J. Kayne and T. Yonetani, *J. Biol. Chem.*, 1977, **252**, 4882. [c] This work. [d] M.Ikeda-Saito, M. Brunori, and T. Yonetani, *J. Biol. Chem.*, 1978, **253**, 173. [e] F. J. Kayne and T. Yonetani, *J. Biol. Chem.*, 1977, **252**, 620. [f] Ref. 12.

rise to solute–solute interactions in aqueous solution such that it is present in aggregated forms; the presence of a polar solvent such as dimethylformamide (dmf) is thus required to provide the conditions necessary for the occurrence of the monomeric form.[12] The formation of a molecular oxygen complex of Co^{II}(tspc) by its reaction with molecular oxygen in aqueous solution has been studied and shown to take place in the presence of 10% v/v dmf at pH *ca.* 12. The presence of 10% v/v of dmf in aqueous solutions containing Co^{II}(tspc) provides conditions for the occurrence of monomeric metallophthalocyanine species and the introduction of apomyoglobin into such a solution leads to the formation of the Co^{II}(tspc)–protein complex within 1 h of mixing at room temperature. The metallophthalocyanine–protein complex was isolated from aqueous solutions containing phosphate buffer, apomycoglobin, and Co^{II}(tspc) present in some 10% excess over that required for a 1 : 1 mole ratio of protein to metallophthalocyanine. After storage at room temperature, the reaction mixture was subjected to chromatography to separate the metallophthalocyanine–protein complex.

Aqueous solutions of Co^{II}(tspc)–apomyoglobin were prepared under nitrogen by dissolution of the isolated metalloprotein in water. Exposure of the aqueous solution to molecular oxygen gives rise to the u.v.–visible spectrum shown in Figure 3 which depicts changes similar to that observed previously [1a] for the Co^{II}(tspc)–apohaemoglobin complex and provides evidence for the reversible formation of

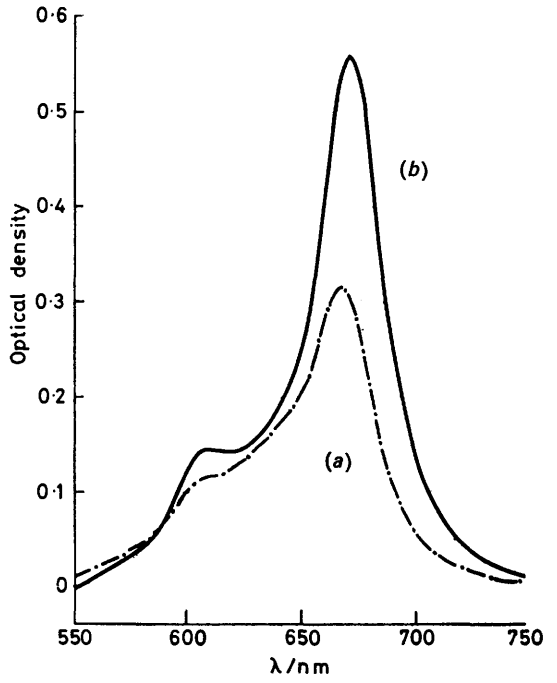


FIGURE 3 Visible spectrum of an aqueous solution, pH 7.0 (0.1 mol dm^{-3} phosphate buffer), containing (*a*) Co^{II}(tspc)–apomyoglobin (7.5×10^{-6} mol dm^{-3}) in N_2 atmosphere, (*b*) Co^{II}(tspc)–apomyoglobin and exposed to molecular oxygen at room temperature (293 K)

a molecular oxygen complex. The e.s.r. spectrum at 110 K of the Co^{II}(tspc)–apomyoglobin complex in aqueous solution is shown in Figure 4 where the extra hyperfine lines in the $g_{\parallel}$ region of the spectrum undoubtedly arise from the interaction of the unpaired electron in an out-of-plane orbital on cobalt(II) with the nucleus of the nitrogen atom of the proximal histidine group present in the hydrophobic pocket of the protein. The e.s.r. spectrum of Co^{II}(tspc) at 110 K in an aqueous solution containing 10% v/v dmf, shown in

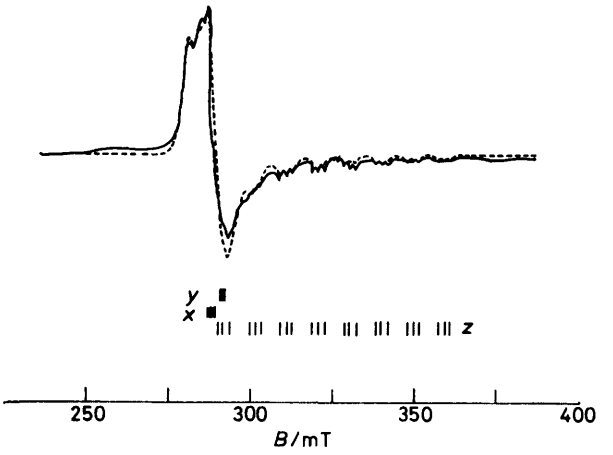


FIGURE 4 E.s.r. spectrum at 110 K of an aqueous solution containing Co^{II}(tspc)–apomyoglobin (1.0×10^{-3} mol dm^{-3}) in 0.1 mol dm^{-3} phosphate buffer, pH 7.0, under N_2. Microwave frequency 9.149 GHz. The dotted curve is the line simulation using the parameters outlined in the text

Figure 4, is typical of a low-spin monomeric state of cobalt(II), and may be described by three g values and three hyperfine constants. These were obtained by means of computer simulation [19] of the spectra; $g_x = 2.268 \pm 0.003$; $g_y = 2.234 \pm 0.003$; $g_z = 2.008 \pm 0.003$; $A_x = 0.0000 \pm 0.0002$; $A_y = 0.0000 \pm 0.0002$; $A_z = 0.0090 \pm 0.0002$; $^{N}A_z = 0.0017 \pm 0.0001$ cm^{-1}. The last parameter is the largest hyperfine constant for interaction with a single axial nitrogen. The e.s.r. spectrum at 110 K of an aqueous solution of Co^{II}(tspc)–myoglobin exposed to molecular oxygen is shown in Figure 5.

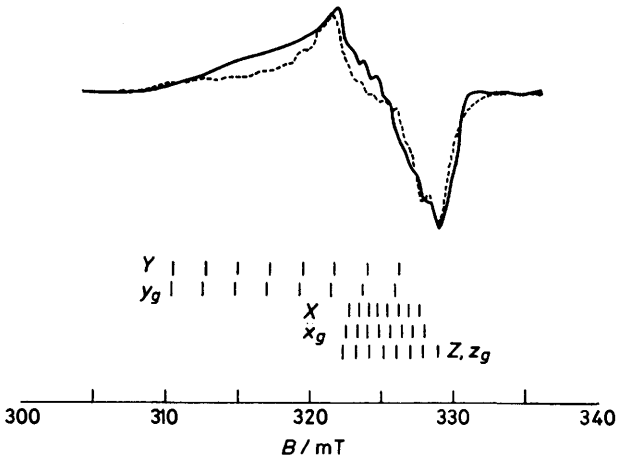


FIGURE 5 E.s.r. spectrum at 110 K of an aqueous solution, pH 7.0 (0.1 mol dm^{-3} phosphate buffer), containing Co^{II}(tspc)–apomyoglobin (1.0×10^{-3} mol dm^{-3}) after exposure to molecular oxygen at room temperature. Microwave frequency 9.149 GHz. The dotted curve is the line simulation using the parameters outlined in Table 3

TABLE 3

E.s.r. parameters associated with molecular oxygen adducts of cobalt(II) tetrasulphonated phthalocyanines in protein pockets [a]

Molecular oxygen adduct	g_x [b]	g_y [b]	g_z [b]	α [c]/°	$10^4A_X(z)$	$10^4A_Y(y)$	$10^4A_Z(x)$	10^4a_x	10^4a_y	10^4a_z	$f+h$	$g+h$
					cm^{-1}							
Co^{II}(tspc)–apomyoglobin	2.009	2.054	2.007	10	6.5	21.5	8.8	4.0	−8.7	4.8	−0.9	14.9
Co^{II}(tspc), water–dmf, pH 12	2.005	2.084	2.001	10	8.0	18.0	9.0	3.2	−5.8	2.7	0.6	10.5

[a] All hyperfine constants assumed negative. [b] ±0.002. [c] ±5°.

Computer simulations of the e.s.r. spectrum of the molecular oxygen adduct (Figure 4) of Co^{II}(tspc)–apomyoglobin carried out as for the protoporphyrin IX complex give the parameters summarized in Table 3 where they may be compared with those derived from the e.s.r. spectrum of the molecular oxygen adduct of Co^{II}(tspc) formed in an aqueous solution containing 10% v/v dmf at pH 12.0.[14]

DISCUSSION

The e.s.r. spectrum due to the molecular oxygen adduct of the cobomyoglobin-containing cobalt(II) protoporphyrin IX observed in the present investigation is very similar to that obtained previously and, as expected, the magnetic and structural parameters associated with the two e.s.r. spectra are in reasonable agreement. This provides confidence in the validity of the simulation of the remaining e.s.r. spectra reported by Yonetani *et al.*[5] and summarized in Table 2. An inspection of the results obtained from the e.s.r. spectra of sperm whale apomyoglobin containing cobalt(II) mesoporphyrin, where the value of α increases from 18 to 22° as a result of replacement of the solvent water by deuterium oxide, could be considered support for the concept of a structural change brought about by the release of hydrogen bonding from the distal histidine group in deuterium oxide. To appreciate the effects on the spectra resulting from small changes in α, Figure 6 of the preceding paper [20] may be consulted. This view is strengthened by the results from cobalt(II) myoglobin (Aplysia) where the value of α is 22° and again in cobalt(II) haemoglobin (Glycera) where α is 23°. However, it should be noted that there are important changes in g_y, which is a reflection of the energy of excitation of the electron in the $2p\ \pi^*$ orbitals of the superoxo-group, such that its value increases to 2.0885 in the molecular oxygen complex of cobalt(II) myoglobin (Aplysia) and falls to 2.068 in that of cobalt(II) haemoglobin (Glycera). This indicates that the superoxo-group, which is usually very sensitive to its surroundings, experiences environmental changes within these proteins.

A comparison of the results obtained from the apomyoglobin-containing cobalt(II) protoporphyrin IX with those of the protein-containing cobalt(II) mesoporphyrin show that the substituent effects, arising from certain regions of the periphery of the porphyrin, are transmitted to the superoxo-group so as to increase the value of α and make a discernible change in the value of g_y although they have little effect on the hyperfine tensors.

Turning to the results associated with the substituted haemoglobin, the expected differences in the environments of the superoxo-group in the α and β subunits are borne out by a significant difference in the value of g_y for these subunits. Although the values of the angle α are not very different for the α and β subunits, they are higher than those found in the myoglobins. Here, for the first time, the values of the hyperfine tensor, A_Y, are different. The results for the cobalt-substituted haemoglobin (Zürich) suggest that the environment of the superoxo-group within this protein is closely similar to one which would be experienced within the β chain of normal haemoglobin.

Biological evolutionary processes have perfected the protein structure to deal efficiently with the accommodation of the prosthetic group in the protein pocket that provides an internal environment which makes possible the acquisition and retention of molecular oxygen at room temperature and in aqueous media. In order to appreciate the capacity of the protein to fulfil these tasks, the prosthetic group of natural occurrence is here replaced by a cobalt(II) macrocyclic chelate which does not exist in living systems, but which bears some resemblance to the structure of the porphyrins. An earlier study has shown that cobalt(II) chelates of tetrasulphonated phthalocyanine undergo the process of oxygenation once embedded in the globin structure.[1] The many contacts which the peripheral groups of the natural prosthetic group, iron(II) protoporphyrin IX, make with the protein may be expected to be greatly different for the Co^{II}(tspc). However, an essential structural requirement for oxygenation, namely the axial interaction of the metal centre with the proximal histidine group, is satisfied. The u.v.–visible spectral data show that oxygenation of Co^{II}(tspc)–apomyoglobin occurs at room temperature in aqueous media, a clear indication of the ability of the protein to promote the addition of molecular oxygen to the cobalt(II) ion of Co^{II}(tspc) held within the hydrophobic pocket of the protein. The e.s.r. data suggest that the structural disposition of the molecular oxygen with respect to the cobalt centre is very similar in both metallophthalocyanine and its apomyoglobin complex. However, the magnetic parameters show differences which may be expected to arise as a result of changes in the axial base introduced as a result of incorporation into the protein.

E.S.R. Parameters for Cobalt(II)–Dioxygen Adducts.—Attempts to explain the e.s.r. properties of Co–O_2 adducts have focused attention either on the possible formation of an O_2^- radical ion resulting from almost

complete electron transfer from Co^{II} to molecular oxygen [21] or, particularly through the work of Drago *et al.*[22-24] and some recent results for cobalt myoglobin by Dickinson and Chien,[25] by means of spin pairing, resulting in the formation of a σ bond. Participation of a cobalt d_{xz} orbital through direct overlap with the unpaired electron in a π^* orbital is considered responsible for the occurrence of cobalt(II) hyperfine structure in the O_2^- model.[21] Electron transfer is seen to occur by different pathways in the two models. In the former, the unpaired electron is thought to be transferred from cobalt to oxygen, while in the spin-pairing picture, electron transfer occurs in the bonding region, the unpaired electron being one of the π^* electrons always on the oxygen molecule.

Cobalt hyperfine structure is accounted for in the O_2^- picture in terms of covalent bonding of the unpaired electron and cobalt *d* orbitals and involves a direct mechanism. The extreme spin-pairing model involves indirect hyperfine coupling caused by spin polarization of the bonding electrons by the unpaired electron. A more detailed discussion may be found in the preceding paper.[20]

After correcting for direct dipolar contributions, one finally obtains parameters $f + h$ and $g + h$ which describe the anisotropic part of the hyperfine structure. It is postulated that g arises from direct coupling to d_{xz} orbitals and f and h from indirect coupling. If, as is found, $f + h < 0$, then one may conclude that indirect effects are not occurring. (For the definition of a_x, a_y, a_z, f, g, and h see the preceding paper.[20]) Tables 3 and 4 give results for a_x, a_y, a_z, $f + h$, and $g + h$ where we have used the relations (1) and (2). In general terms, the values of $g + h$ are remarkably constant and imply, in the limit where $h = 0$, that the d_{xz} mixing coefficient, α'', is such that $\alpha''^2 = 0.06$. The amount of direct mixing of d_{xz} is, therefore, quite small and not inconsistent with a model in which indirect effects are quite significant.

$$f + h = \tfrac{7}{6}(a_x - a_z) \quad (1)$$

$$g + h = \tfrac{7}{6}(a_x - a_y) \quad (2)$$

Figure 6, which shows the correlations $(f + h)$ *versus* $(g + h)$, $(f + h)$ *versus* α, and $(g + h)$ *versus* α, may be broadly interpreted as in the preceding paper.[20] Involvement of (d_{xy}, d_{z^2}) orbitals would appear to provide a direct rather than an indirect spin-polarization contribution to hyperfine structure as evidenced by negative

TABLE 4

Cobalt hyperfine analysis for oxygenated cobalt porphyrins in protein pockets. Based on data in Table 2

Apoprotein	Cobalt(II) porphyrin	Solvent conditions	$10^4 a_x$	$10^4 a_y$	$10^4 a_z$	$f + h$	$g + h$	Ref.
				cm^{-1}				
Sperm whale myoglobin	Mesoporphyrin	H_2O	3.8	−9.0	5.3	−1.7	15.6	*a*
	Mesoporphyrin	D_2O	3.8	−9.7	5.8	−2.3	15.8	*a*
	Protoporphyrin IX	Phosphate buffer, pH 7.0	3.2	−8.8	5.7	−2.9	14.0	*a*
			3.5	−7.8	4.2	−0.8	13.2	*b*
	Protoporphyrin IX	H_2O pH 7.0	3.7	−8.8	5.2	−1.7	14.6	*a*
Haemoglobin (Glycera)	Mesoporphyrin	H_2O	2.8	−6.9	4.1	−1.5	11.3	*a*
Myoglobin (Aplysia)	Mesoporphyrin	H_2O pH 7.0	3.2	−8.7	5.5	−2.7	14.6	*c*
Haemoglobin (α chain)	Protoporphyrin IX	H_2O	2.9	−7.0	4.3	−1.6	11.9	*d*
(β chain)	Protoporphyrin IX	H_2O	3.4	−9.2	5.8	−2.8	14.7	*d*
(Zürich)	Protoporphyrin IX	H_2O	3.2	−8.7	5.6	−2.8	14.6	*e*

[a] See footnote *b*, Table 2. [b] See footnote *d*, Table 2. [c] See footnote *e*, Table 2. [d] Ref. 12. [e] I. M. Ruzic, Ph.D. Thesis, Monash University, 1981.

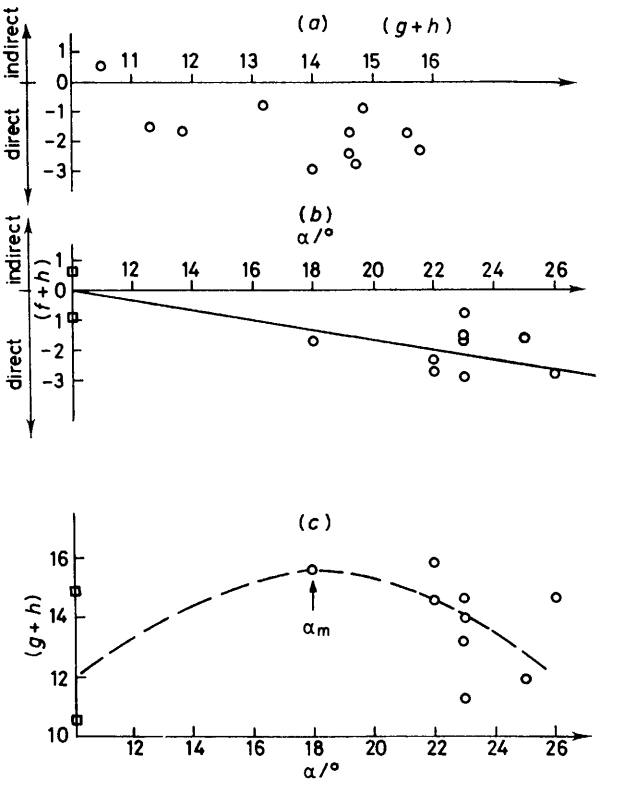


FIGURE 6 Correlation diagrams for cobalt–oxygen complexes: (*a*) $(f + h)$ *versus* $(g + h)$; (*b*) $(f + h)$ *versus* α; and (*c*) $(g + h)$ *versus* α. Cobalt porphyrin–O_2 complexes in myoglobin and haemoglobin (○) (Tables 2 and 4) and Co(tspc)–O_2 complexes (□). Error bars are not given but $(f + h)$ and $(g + h)$ values are probably in error by ± 1. (For a schematic molecular-orbital diagram see preceding paper,[20] Figure 4)

$f + h$ values. The variations of $(f + h)$ with α [Figure 6(*b*)] suggest that overlap between the oxygen unpaired electron orbital π^* and (d_{yz}, d_{z^2}) increases as α increases. On the other hand, the $(g + h)$ *versus* α plot has the vaguest suggestion that $(g + h)$ may have a maximum value. As α is increased, one would expect the π^*–d_{xz} overlap to increase and even possibly to go through a maximum.

For the single-crystal spectrum of cobalt(II) myoglobin –dioxygen, data reported by Dickinson and Chien,[25] for one of the two centres at low temperatures, centre II, yield $g + h = 12.4$ and $f + h = 6.1$, consistent with their general conclusions. The actual values differ from their reported spin densities as a result of a correction needed to the orientation of the direct dipolar interaction. Centre II [25] does not have quite the geometry suggested by Figure 1.

In a very recent single-crystal study of cobalt myoglobin–dioxygen, Hori *et al.*[26] found evidence for a single centre near room temperature, consistent with *X*-ray results, and two centres at low temperatures in broad agreement with Dickinson and Chien.[25] In contrast to most previous models of Co–O_2 adducts, it is claimed that $g_{max.} = 2.056$ is *not* along the O–O direction in the high-temperature site. Their low-temperature g and A values, however, differ slightly from those reported by Dickinson and Chien. Hori *et al.*[26] have argued that the process of freezing crystals may be responsible for the existence of two low-temperature sites. It may be that of the rate of cooling is important and might, in principle, point the way to a reconciliation of the results.

In comparing the cobalt myoglobin–dioxygen crystalline data [27] with the other substituted porphyrins, it is clear that whereas $f + h$ is positive in the former, it is always negative in the cases given in Table 4. Either indirect spin polarization is not occurring or the model is incomplete and needs further modification. On the other hand, the single-crystal results of Jörin *et al.*[28] for B_{12r}·O_2 lead to $f + h = 4.1$ and $g + h = 16.3$, consistent with the model proposed.

EXPERIMENTAL

Apomyoglobin was prepared from sperm whale myoglobin, type III, obtained from Sigma Chemical Company, using the method outlined in the literature.[27] The complex Co^{II}(tspc) was prepared by the method described by Weber and Busch.[29]

Preparation of Co^{II}(tspc)–*Apomyoglobin.*—An aqueous solution of Co^{II}(tspc) (2.60×10^{-3} mol dm^{-3}) was prepared under nitrogen. An aliquot (3 cm^3) of this solution was diluted five-fold with an aqueous solution of apomyoglobin (5.0×10^{-4} mol dm^{-3} in haem site [30]), at pH 6.0, maintained by a phosphate buffer (0.1 mol dm^{-3}) at room temperature. The reaction mixture was stored under nitrogen for 1 h and then subjected to a chromatographic procedure similar to that described by Yonetani *et al.*[31] The metalloprotein solution (15 cm^3) was applied to a chromatographic column (length 45 cm, diameter 2.5 cm) of Sephadex G50, which had been equilibrated with phosphate buffer (0.1 mol dm^{-3}, pH 6.0). The column was eluted with an aqueous phosphate buffer (0.1 mol dm^{-3} pH 6.0), the eluate being collected in a rotary fraction collector. The fraction containing the metalloprotein, identified by u.v.–visible spectrophotometric measurements, was dialysed against distilled water and further chromatographed on a column (20 × 2.5 cm) of the cation-exchanger Whatman CM-52, equilibrated with an aqueous phosphate buffer solution at pH 6.0, which is just below the isoelectric point of pH 7.0 of horse myoglobin. The cation exchanger was chosen so as not to compete for the anionic Co^{II}(tspc) prosthetic group introduced into the protein. Independent experiments were carried out to show that Co^{II}(tspc) is strongly adsorbed from aqueous solution by anion exchangers of the type Whatman DE-52. After application of the dialysed aqueous solution of the Co^{II}(tspc)–apomyoglobin complex to the Whatman CM-52 column, the column was washed with phosphate buffer, pH 6.0 (5×10^3 mol dm^{-3}), and the material adsorbed on the column eluted by aqueous solutions of potassium phosphate, whose concentration was progressively increased from 5×10^{-3} to 2×10^{-1} mol dm^{-3}. The fractions were monitored using u.v.–visible spectrophotometry. The fraction containing the metalloprotein was dialysed against a number of changes of distilled water and finally isolated by removal of water at diminished pressure.

Preparation of the Cobalt(II) *Protoporphyrin* IX–*Apomyoglobin Complex.*—The cobalt(II) protoporphyrin IX–apomyoglobin complex was prepared as described by Yonetani *et al.*[31] or by the modified procedure using non-ionic detergents described earlier.

E.s.r. spectra were obtained using a Varian E12 EPR spectrometer operating at *ca.* 9.15 GHz. Low-temperatures were achieved using a Varian liquid-nitrogen accessory.

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