Nuclear Magnetic Resonance Studies of the Binding of ^{15}N-Labelled Ligands to Haemins and Haemoproteins. Solvent Effects on the ^{15}N Paramagnetic Shifts of Iron-Bound $C^{15}N^-$ in Low-Spin Haemin Cyanide Complexes and Cyano Haemoproteins

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Summary ^{15}N n.m.r. isotropic shifts for the iron-bound ^{15}N labelled cyanide of the low spin ferric mono- and dicyano complexes of natural porphyrins are shown to be very sensitive to the solvent; protic solvents were most effective in causing upfield bias of the $C^{15}N$ shift and the sizable ^{15}N shift induced by pH variation for cyano myoglobin was interpreted in terms of possible involvement of hydrogen bonding between distal histidine and haem-bound cyanide.

In recent years ^1H n.m.r. spectroscopy has become a powerful tool for structural studies of haemoproteins, particularly in elucidating electronic states of the haem, characterizing the ligand bonding situation, and delineating the haem–apoprotein interaction.[1] Most of these studies have dealt with the isotropic shift of peripheral porphyrin substituents in the haemoproteins or in model compounds. These studies have recently been complemented by ^{13}C n.m.r. investigations of haem-bound ^{13}CO[2] and R–$N^{13}C$[3] in the diamagnetic low-spin iron(II) myoglobin and haemoglobin. We are currently investigating the n.m.r. isotropic shift of the ^{15}N-labelled axial ligand bound to the paramagnetic haem iron in haemoproteins and in model compounds.[4] Structural changes in the haem environment within the protein or on going from the model compound to the protein have been shown to be sensitively reflected in the isotropic shifts of the haem-bound ligand molecule.[4] We report here some n.m.r. studies of solvent effects in low-spin iron(III) haemin cyanide complexes and cyano haemoproteins using the ^{15}N isotropic shift of iron-bound $C^{15}N^-$. We have studied the ^{15}N n.m.r. of mono- and dicyano-complexes of natural porphyrins in various solvents and of cyano myoglobin and cytochrome c at different pH values.†

Spectral changes in the $C^{15}N$ resonances of dicyano haemin complex with varying amounts of D_2O in dimethyl sulphoxide (DMSO) solution were observed. The iron-bound $C^{15}N$ signal, which was shifted downfield far beyond the diamagnetic spectral region, exhibits substantial upfield shift with increasing D_2O content. Similar spectral shifts were also encountered for iron-bound $C^{15}N$ resonances in dicyano and pyridine–cyano complexes of haemin in pyridine–H_2O mixed solvents. The results are given in the

† The ^{15}N n.m.r. spectra were recorded in the pulse Fourier transform mode with a Jeol PFT-100 spectrometer operating at 10·15 MHz. All measurements on samples of horse myoglobin (Sigma, Type III), horse cytochrome c (Sigma, Type VI) and protohaemin (Sigma, Type III) were made in a 10 mm n.m.r. tube in the presence of ^{15}N enriched-KCN (Prochem, 96·7% atom ^{15}N) in appropriate solvents. 5—50 K transients were collected using a spectral width of 10 kHz and 8 K data points, and a pulse repetition time of 0·42 s. Chemical shifts are reported in p.p.m. from internal $^{15}NO_3^-$.

Table. Observed chemical shifts of $C^{15}N^-$ co-ordinated axially to haemin and haemoproteins (at 25 °C)[a]

Haemin	Solvent[c]	Chemical shift p.p.m.	Haemoprotein	pH	Chemical shift p.p.m.
Protohaemin$(C^{15}N^-)_2$	DMSO	+732	Myoglobin$(C^{15}N^-)$	5·7	+931
	DMSO+D_2O(18:1)	+716		6·0	+939
	DMSO+D_2O(18:2)	+699		6·9	+948
	DMSO+D_2O(18:4)	+671		8·0	+936
	Py+D_2O(20:4)	+657		10·0	+940
	Py+D_2O(20:5)	+652		11·7	+940
	Py+D_2O(20:7)	+637			
	MeOD	+506	Cytochrome c$(C^{15}N^-)$	5·2	+841
	MeOD+D_2O(1:1)	+480		6·6	+842
				7·8	+847
Protohaemin(Py)$(C^{15}N^-)$[b]	Py+H_2O(5:1)	+1000		9·0	+848
	Py+H_2O(2:1)	+941			
	Py+H_2O(3:2)	+931			

[a] Positive sign signifies downfield shift from internal $^{15}NO_3^-$. [b] For concentrations of haemin chloride, cyanide, and pyridine (solvent) in the preparation of pyridine–cyano complexes of protohaemin, see W. S. Caughey, C. H. Barlow, J. C. Maxwell, J. A. Volpe, and W. S. Wallace, *Ann. New York Acad. Sci.*, 1973, **206**, 296. [c] Abbreviations used are: DMSO, dimethyl sulphoxide; Py, pyridine.

Table, which also contains the ^{15}N shifts for the dicyano species in methanol and methanol–water mixed solvents. There is a striking difference between the bound $C^{15}N$ isotropic shifts in aprotic and protic solvents, the latter having an upfield bias. This trend is also observed with the mono-cyano complex.

A similar solvent effect was found by Frye and La Mar[5] for proton isotropic shifts of porphyrin peripheral groups in the dicyano haemin complex, with a downfield bias of at most *ca.* 2—3 p.p.m. on going from aprotic (*e.g.* DMSO) to protic (*e.g.* CD_3OD) solvents. This proton shift markedly contrasts with the present result in which a large $C^{15}N$ shift (as much as 200 p.p.m.) is observed for the corresponding change in solvent. The solvent-induced proton shift has been interpreted primarily in terms of variation in magnetic anisotropy with the change of solvent polarity, responsible for dipolar (or pseudo-contact) shifts of the porphyrin peripheral groups. The upfield bias of the iron-bound $C^{15}N$ resonance with protic solvents is consistent with a substantial change in the g value anisotropy. From data available, $g_{\parallel}$ *ca.* 1·8 and $g_{\perp}$ *ca.* 3·6 in $CDCl_3$, and $g_{\parallel}$ *ca.* 2·0 and $g_{\perp}$ *ca.* 2·9 in D_2O a dipolar ^{15}N downfield shift of 130 p.p.m. in $CDCl_3$ and 57 p.p.m. in D_2O for the iron-bound $C^{15}N$ is expected, resulting in an upfield shift of 73 p.p.m. on going from weak to strong proton donating solvents. However, the present result for the ^{15}N solvent shift of 200 p.p.m. appears to be too large to be attributed only to this anisotropy effect. Although the intrinsic origin of the isotropic shift of the haem-bound axial ligand, especially of the nucleus located very close to haem iron, has remained unresolved from the theoretical point of view,[6] there could be a substantial contribution from the contact shift for the haem-bound $C^{15}N$ resonance. The hydrogen bonding of the solvent molecule to the co-ordinated cyanide, which weakens the axial ligand field, reduces the magnetic anisotropy[5] and σ-basicity towards the metal ion, decreasing the spin transfer from iron to cyanide. The upfield bias of the $C^{15}N$ shift in CH_3OD compared with that in DMSO can be interpreted along these lines. This solvent-induced change in the ligand binding situation is also consistent with the proton shift study of Frye and La Mar.[5]

The effect of solvent on the haem-bound $C^{15}N$ resonance in the haemin cyanide complexes prompted us to explore solvent effects in cyano haemoproteins. We have also studied the pH-dependent features of the ^{15}N resonance of haem-bound $C^{15}N$ in cytochrome c and myoglobin cyanide complexes, together with the ^1H n.m.r. spectra. These results are also shown in the Table. In the pH range examined, there was no substantial ^{15}N shift for cytochrome c (*ca.* 7 p.p.m.), while we obtained a sizeable pH-dependent shift for myoglobin cyanide. This shift (16·5 p.p.m.) for myoglobin with a pH variation from 5·7 to 6·9 could be reasonably interpreted in terms of a possible hydrogen bond effect between distal histidine and haem-bound axial cyanide.[7] With lowering pH, protonation at the distal histadine may enhance this hydrogen bond, resulting in an upfield $C^{15}N$ resonance shift. Such a large shift was not encountered for cytochrome c cyanide, probably because there is no such distal histidine in the proximity of the haem in cytochrome c.[8] The rather complex profiles of the ^{15}N shift above pH 7 for myoglobin cyanide appear to reflect a structural change in the haem environment caused by ionization of an amino acid residue in the vicinity of the haem.

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