Assignment of the N.m.r. Spectrum of Iron(III) Protoporphyrin IX Dicyanide Using Paramagnetic Shift and Broadening Probes

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Summary Specific assignments of the paramagnetically shifted porphyrin ring proton resonances in the n.m.r. spectrum of iron(III)protoporphyrin IX dicyanide have been made by consideration of the perturbations induced in the spectrum by paramagnetic shift and relaxation probes.

ONE of the major problems in applying n.m.r. methods to the study of proteins is that of resolving and assigning resonances. One of the most successful approaches to this problem involves the use of paramagnetic ions to perturb the n.m.r. spectrum.[1] In hemoproteins the heme itself provides an excellent natural paramagnetic probe which can be used to monitor structural and electronic changes at and around the active site.[2,3] Detailed investigations of the electronic structure of the heme are required however if the full value of this probe is to be realised. In particular it is necessary to define the g-tensor directions in terms of the porphyrin substituents. A prior requirement is the development of assignment procedures for the heme resonances themselves. The n.m.r. spectra of low-spin hemin complexes have been investigated extensively and the

resonances have been correlated with the various types of porphyrin ring substituent protons.[3] Recent experiments in which the ring methyl substituents have been deuteriated have provided unambiguous assignments of the methyl group resonances for low-spin dicyano iron(III)protoporphyrin IX dimethyl ester complexes.[4] We now report a simple method for assigning further the n.m.r. spectrum of iron(III)protoporphyrin IX dicyanide (hemin-CN) using paramagnetic probes to selectively perturb the spectrum. This method has an advantage over that of selective deuteriation in that assignments are possible for protons of all types and not merely for those which are easily replaced by deuterium.

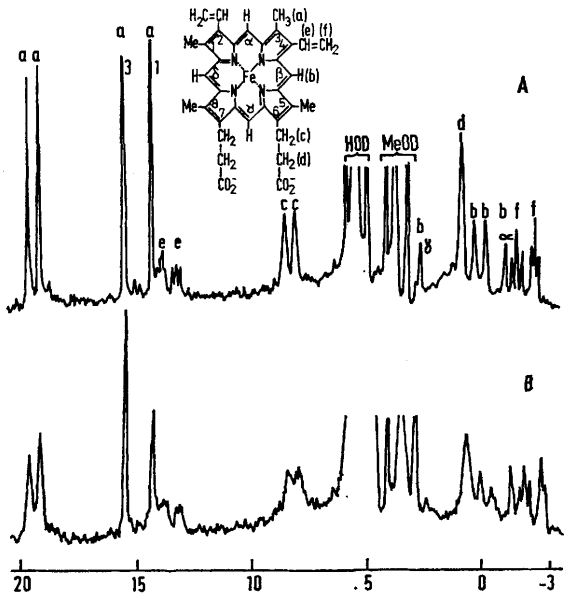

FIGURE. (a) 90 MHz spectrum of hemin-CN (6mM) in deuteriomethanol at 4°C showing assignments to porphyrin ring substituent groups.[3] (b) Spectrum of hemin-CN after addition of 0·2mM $Cr(big)_3^{3+}$.

Solutions of hemin-CN were prepared by dissolving hemin chloride in deuteriomethanol containing an excess of potassium cyanide, the pH of which had previously been adjusted by addition of DCl to *ca.* 5 using methyl red as an indicator. Spectra were recorded using a Bruker 90 MHz spectrometer equipped for pulsed Fourier transform operation. Me_4Si was used as an internal reference.

The spectrum of hemin-CN in deuteriomethanol at 4°C is shown in the Figure (A), together with the group assignments made previously by Wuthrich.[3] Titration of hemin-CN with aliquots of tris(biguanide)chromium(III) chloride, $[Cr(big)_3^{3+}]$ in deuteriomethanol results in broadening of the porphyrin proton resonances [Figure (B)]. A quantitative estimate of the relative degrees of broadening of the resonances was obtained by measuring the fractional decrease in peak height for various concentrations of $Cr(big)_3^{3+}$. Manganese(II) chloride caused even greater broadening. Resonances of the propionic acid side chains broadened most rapidly, with the methyl group resonances at 19·1 and 18·6 p.p.m. broadening more slowly and the methyl group at 14·2 p.p.m. even more slowly. Unfortunately measurement of the degrees of broadening of the other hemin-CN resonances was not possible due to precipitation at quite low Mn^{2+} concentrations. Similar problems of precipitation were encountered in using higher concentrations of europium(III) nitrate as a paramagnetic shift probe, but some hemin-CN resonances were observed to shift. The two methyl group resonances at lowest field were shifted upfield to the same degree whilst that at 15·2 p.p.m. remained unshifted and the other methyl group resonance at 14·2 p.p.m. was shifted slightly downfield (Table). The propionic acid β-CH_2 proton resonances at 8·2 and 7·8 p.p.m. were shifted equally upfield.

The observed differences in the degrees of broadening of individual resonances in the spectrum of hemin-CN upon titration with $Cr(big)_3^{3+}$ or manganese(II) chloride can be interpreted in terms of the relative distances (broadening $\propto r^{-6}$) of the various porphyrin side-chain protons from the probe binding site. The cationic probes used bind symmetrically at the carboxylate side chains of the hemin since the resolved resonances of the propionic acid β-CH_2 protons broaden to an identical extent and are shifted equally by Eu^{3+}. We assume that the metal ion binding is symmetrical with respect to a plane perpendicular to the plane of the heme and passing through the α- and γ-meso protons. (No distinction needs to be made between equal binding to the two separate carboxylate anions and simultaneous

TABLE

Constituent group		Chemical shift/p.p.m.[a]	Peak height with 0·2mM $Cr(big)_3^{3+}$ [b]	Peak height with 0·1mM Mn^{2+} [b]	Resonance shift with 1·2mM Eu^{3+}/p.p.m.	Assignment
-Me		19·1	0·38	0·55	+0·35	5- or 8-methyl
		18·6	0·43	0·55	+0·35	8- or 5-methyl
		15·2	0·94	0·97	0·0	3-methyl
		14·2	0·50	0·92	−0·13	1-methyl
meso-H		2·5	0·36	0·26		γ-meso
		0·0	0·58			β or δ-meso
		−0·4	0·50			δ or β-meso
		−1·1	0·95			α-meso
Propionic acid	α-CH_2	0·7	0·52	~0		
	β-CH_2	8·2	0·51	0·27	+0·26	
	β-CH_2	7·8	0·51	0·28	+0·26	
Vinyl-CH		13·7	*ca.* 0·80	1·0		2-vinyl CH
		13·1	*ca.* 0·71	*ca.* 0·9		4-vinyl CH
Vinyl-CH_2		−1·8	0·75			4-vinyl CH_2
		−2·5	0·85			2-vinyl CH_2

[a] Relative to Me_4Si. [b] Peak height expressed as fraction of the height in the absence of broadening probes.

binding to both.) We may now determine the relative distances of all of the protons from the probe position and hence assign the resonances to specific side chains (Table).

The two methyl group resonances at lowest fields, 19·1 and 18·6 p.p.m., which are shifted equally by Eu^{3+} and broaden together to a greater extent than the other two methyl group resonances at 15·2 and 14·2 p.p.m. can be assigned immediately to methyl group protons at position 5 and 8 of the porphyrin ring, adjacent to the propionic acid groups. Of the two remaining methyl group resonances, that at 15·2 p.p.m. is essentially unperturbed by $Cr(big)_3^{3+}$ and is therefore assigned to the methyl group most distant from the probe site, that is, the 3-methyl substituent. The remaining resonance which displays intermediate broadening effects is assigned to the methyl group at the 1-position.

Considering now the *meso*-proton resonances, that at 2·5 p.p.m. is broadened most rapidly by $Cr(big)_3^{3+}$ and is therefore assigned to the γ-hydrogen atom which is closest to the probe binding site. Two of the other three *meso*-proton resonances broaden at very similar rates while that at −1·1 p.p.m. broadens more slowly and is assigned to the α-*meso*-proton. This leaves the resonances at 0·0 and −0·4 p.p.m. to be assigned to the β- and δ-protons which are equidistant from the probe site and cannot be distinguished by the present method. Broadening of the vinyl CH proton resonances is most marked for that at 13·1 p.p.m. but all effects are relatively slight and thus only a tentative assignment to the 4-vinyl substituent is made. (Construction of models shows the 4-vinyl to be nearer to the probe site than the vinyl group at the 2-position). Finally the vinyl CH_2 proton resonance at −1·8 p.p.m. is most broadened by $Cr(big)_3^{3+}$ and can therefore also be assigned to the 4-vinyl group. The assignments are summarized in the Table.

Spectra of the dicyano complexes of 1,8-, 1,3- and 5,8-di(deuteriomethyl) protoporphyrin(IX)dimethyl esters provide assignments[4] which are in agreement with those of the present study and, in addition, enable assignment of the resonances at 19·1 and 18·6 p.p.m. to methyl substituents at positions 8 and 5 respectively on the porphyrin ring. The assignments can be used to describe the *g*-tensor of the heme.

These assignments are also useful in analyses of biosynthetic pathways; the methods used can give a complete assignment of a coenzyme, vitamin B_{12}, where assignment is of particular value in the understanding of synthetic routes.[5]

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