$^1H-^{13}C$ HETCOR Investigations on Heme-Containing Systems

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$^1H-^{13}C$ HETCOR studies were performed on some low-spin heme-containing systems. From the available data or our extended proton assignment, complete carbon assignments were performed. The carbon (and proton, when not yet already performed) chemical shifts were factored out into metal-centered pseudocontact shifts and contact (plus ligand-centered pseudocontact) shifts. The ratios between proton and carbon contact shifts were found to be constant for the symmetric bis(imidazole) protoporphyrin and to vary for the asymmetric protein systems. This feature is discussed also in the light of the data already available on other heme proteins.

Introduction

The improvement of NMR technologies permits the obtainment of further information on systems already largely investigated. ^1H reverse detection in heteronuclear correlation (HETCOR) spectroscopy of paramagnetic compounds may provide scalar hetero correlation even for broad signals and under natural-abundance conditions for the heteronucleus.[1-3]

Here we present investigations of bis(imidazole)-hemin ($PPIm_2$), metmyoglobin cyanide (MbCN), and cytochrome b_5 ($Cytb_5$). All of these systems contain low-spin iron(III). $PPIm_2$ represents a good model for the detection of all the expected cross peaks because concentrated solutions can be obtained. Indeed, $^1H-^{13}C$ NMR cross peaks are observed even for the fully bound imidazole nuclei which have the broadest proton lines (230 Hz). Reverse detection HETCOR studies are already available, with ^{13}C in natural abundance, for cytochromes c[2] and c_{551}[1] and oxidized high-potential iron sulfur proteins (HiPIPs).[3] A $^1H-^{13}C$ HETCOR direct study is available for MbCN,[4] which allows us to establish the superiority of reverse detection even in the case of fast-relaxing systems.

The assignment of the hyperfine-shifted proton signals is available for the three systems,[5-10] whereas the ^{13}C resonance assignment is known for some signals of MbCN[4] and for the bis(imidazole) adduct of a synthetic porphyrin.[11] No ^{13}C spectrum is available for $Cytb_5$. Actually, rat $Cytb_5$ is present in two isomers differing in the heme orientation inside the protein frame;[12] both isomers are investigated here. The magnetic anisotropy tensor, which provides the metal-centered pseudocontact shifts, is available for MbCN[13] and for the two isomers of $Cytb_5$.[14]

Once the carbon signals of the heme substituents are assigned, we examine the problem of a possible relationship between ^1H and ^{13}C hyperfine shifts. Such a problem has been investigated for pyridine,[15] triphenylphosphine,[16] and *N*-oxide[17] ligands bound to nickel(II) and cobalt(II); evidence for a complex spin density transfer mechanism had been found. Recently, Turner[18] addressed the problem for cytochrome c, separating the various contributions to the shifts for both ^1H and ^{13}C resonances and calculating the spin density on the resonating nuclei. He found that the hyperfine coupling constants of the proton nuclei of the methyls attached to sp^2 carbons of the heme pyrrole rings are dependent on the heme position.[18]

We now show that such variability is a general feature and that the extent of variability is quite large. Evidently, chemical shifts in paramagnetic molecules are sufficiently sensitive to be able to monitor very subtle structural inequivalences.

Materials and Methods

Hemin-iron(III) chloride was obtained from Sigma Chemical Co. and used without further purification. The bis(imidazole) complex was prepared by adding ≈4 equiv of imidazole to a solution of hemin chloride (20 mM) in deuterated dimethyl sulfoxide ($(C^2H_3)_2SO$).

Cytochrome b_5 was isolated from *Escherichia coli* strain TB-1, generously provided by Dr. S. G. Sligar. The protein was isolated and purified using the previously reported procedure.[19] The NMR sample (8 mM) was in 100 mM phosphate buffer (pH 7.1) in D_2O.

Sperm whale myoglobin was purchased from Sigma Chemical Co. and used without further purification. The metcyano complex was prepared by addition of small quantities of solid KCN to a 0.1 M NaCl-D_2O solution of the enzyme (10 mM) at pH 7.4.

The NMR spectra were recorded on a Bruker AMX 600 spectrometer equipped with a 5-mm inverse detection probe. The HMQC $^1H-^{13}C$ spectra consisted of 128-360 experiments acquired with 1K data points each, in the magnitude mode, by using the standard pulse sequence[20-22]

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Table 1. Proton and Carbon Chemical Shifts of Hyperfine-Shifted Signals and Assignments for $PPIm_2$ at 295 K[a]

assignt	δ_H (ppm)	δ^{diam} (ppm)[b]	δ^{pc} (ppm)	δ_H^{cont} (ppm)	δ_C (ppm)	δ^{diam} (ppm)[b]	δ^{pc} (ppm)	δ_C^{cont} (ppm)	$(\delta_C/\delta_H)^{cont}$
1-CH_3	14.1	3.8	−4.6	14.9	−32.1	13.0	−5.4	−39.7	−2.7
3-CH_3	11.8	3.8	−4.5	12.5	−26.3	13.0	−5.4	−33.9	−2.7
5-CH_3	18.0	3.7	−4.6	18.9	−41.4	11.7	−5.5	−47.6	−2.5
8-CH_3	18.4	3.7	−4.6	19.3	−42.0	11.7	−5.5	−48.2	−2.5
2α-CH	11.7	8.6	−4.8	7.9	58.5	131.5	−5.4	−67.6	
2β-CH_2	−3.5, −4.0	6.2, 6.5	−2.1, −2.7	*c*	173.2	119.6	−3.1	56.7	
4α-CH	10.9	8.6	−4.5	6.8	67.8	131.5	−5.5	−58.2	
4β-CH_2	−2.0, −2.5	6.2, 6.5	−2.2, −3.0	*c*	163.9	119.6	−3.2	47.5	
6α-CH_2	5.2, 5.2	4.7	−3.6, −5.0	*c*	−16.9	22.8	−5.5	−34.2	
6β-CH_2	0.4	3.6	−2.8, −3.4	*c*	83.0	38.8	−3.4	47.6	
7α-CH_2	4.9, 4.9	4.7	−3.7, −4.8	*c*	−16.5	22.8	−5.4	−33.9	
7β-CH_2	0.4	3.6	−2.0, −3.2	*c*	83.0	38.8	−3.0	47.2	
2-CH′	−7.0	8.0[d]	18.0	−33.0	124.1	134.8[d]	53.8	−64.5	
4-CH′	10.4	7.2[d]	22.4	−19.2	105.8	120.6[d]	56.7	−71.5	
5-CH′	7.7	7.2[d]	13.1	−12.6	99.6	120.6[d]	25.5	−46.5	

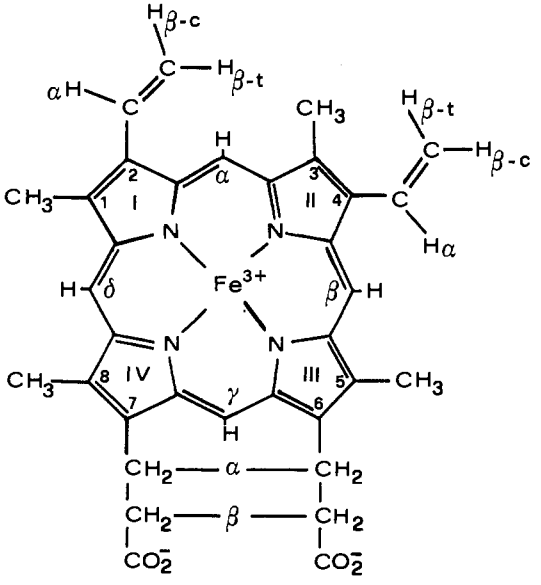
[a] Experimental values have errors of ±0.1 ppm. The estimated error on δ^{pc} is ±15%. [b] Taken from ref 29. [c] The contact contribution cannot be determined because the stereospecific assignment is not available. [d] Values measured for the free imidazole.

with or without decoupling during the acquisition time. A relaxation delay of 50−250 ms was utilized, and the $\Delta = 1/2J$ refocusing delay was set to 1.0−3.6 ms. The numbers of scans were 128 for hemin, 2048 for MbCN, and 1024 for $Cytb_5$.

The pseudocontact shifts were calculated, through the Kurland and McGarvey equation,[23] using the reported magnetic anisotropies and magnetic tensor axes.[13,14] The X-ray coordinates of the sperm whale myoglobin−CO adduct[24] and of the bovine $Cytb_5$ (Brookhaven Data Bank entry no. 3b5c)[25] proteins were used. The $PPIm_2$ system was taken as axial with a magnetic anisotropy derived from the EPR *g* values already published.[26]

Results

Assignment of the ^{13}C Signals. (a) Bis(imidazole)−Hemin. The HMQC $^1H-^{13}C$ map for this model system at a concentration of 20 mM is reported in Figure 1. The HETCOR spectrum correlates the shift of carbons in the F_1 dimension with the shifts of the attached protons in the F_2 dimension. All the carbons bearing one or more protons present in the system are observed in the map as cross peaks with the coupled protons. Also, the cross peaks for the imidazole ring CH's, which are close to the iron and are characterized by relatively broad lines, are detected with different acquisition parameters (inset of Figure 1). The shift values for ^1H and ^{13}C signals are reported in Table 1, together with their assignment, while structure **I** shows the atom labeling of the heme substituents.



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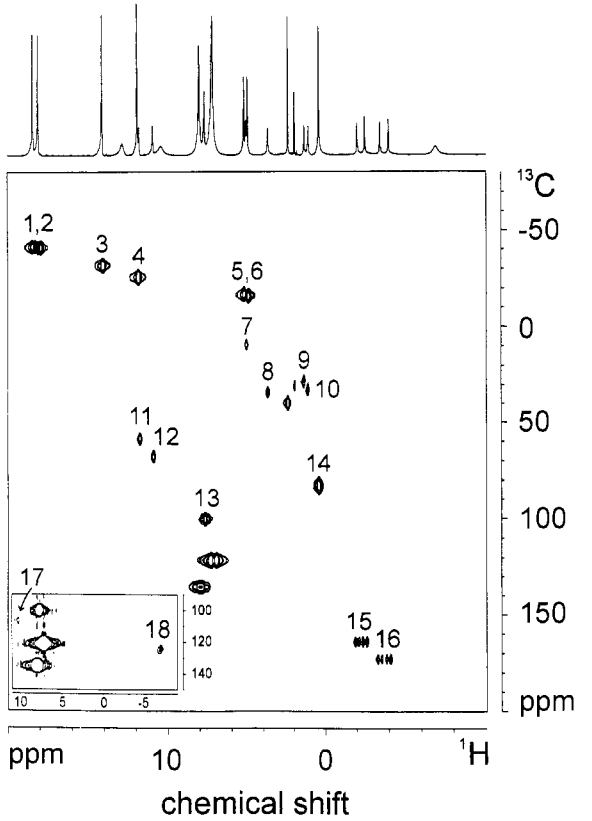

Figure 1. 600-MHz $^1H-^{13}C$ HMQC spectrum recorded on $PPIm_2$ at 295 K. The labeled cross peaks indicate the one-bond connectivities for the following protons: (1) 8-CH_3, (2) 5-CH_3, (3) 1-CH_3, (4) 3-CH_3, (5, 6) 6,7α-CH_2, (7) δ-meso, (8) α-meso, (9) β-meso, (10) γ-meso, (11) 2α-CH, (12) 4α-CH, (13) 5-CH′ Im, (14) 6,7β-CH_2, (15) 4β-CH_2, (16) 2β-CH_2. A recycle delay of 250 ms and $\Delta = 2.5$ ms were used. The cross peaks of (17) 4-CH′ and (18) 2-CH′ for bound imidazole are shown in the inset. The latter spectrum was obtained using a recycle delay of 50 ms and $\Delta = 1.0$ ms.

(b) Metcyanomyoglobin. Figure 2 shows the HMQC $^1H-^{13}C$ spectrum of MbCN. The methyl carbon signals, already assigned by Yamamoto,[4] are clearly identified in the upfield region of the carbon spectrum. The inverse detection probe allows a good resolution of the ^1H shifts because they are detected in the F_2 dimension. The acquisition of the ^1H

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Table 2. Proton and Carbon Chemical Shifts and Assignments for MbCN at 310 K[a]

assignt	δ_H (ppm)	δ^{diam} (ppm)[b]	δ^{pc} (ppm)	δ_H^{cont} (ppm)	δ_C (ppm)	δ^{diam} (ppm)	δ^{pc} (ppm)	δ_C^{cont} (ppm)	$(\delta_C/\delta_H)^{cont}$
1-CH_3	18.3	3.6	−3.4	18.1	−38.4	13.0	−4.3	−47.1	−2.6
3-CH_3	4.8	3.8	−5.7	6.7	−14.2	13.0	−7.4	−19.8	−2.9
5-CH_3	26.4	2.5	−3.3	27.2	−58.2	11.7	−4.1	−65.8	−2.4
8-CH_3	12.5	3.6	−6.5	15.4	−32.0	11.7	−8.2	−35.5	−2.3
2α-CH	17.6	8.4	−3.6	12.8	51.3	131.5	−4.2	−76.0	
2β-CH_2	−1.5, −2.3	5.7, 5.7	−1.8, −2.8	−5.4, −5.2	185.7	119.6	−2.7	68.8	
4α-CH	5.7	8.6	−6.2	3.4	80.8	131.5	−7.1	−43.6	
4β-CH_2	−1.5, −0.4	6.3, 6.6	−2.5, −3.6	−5.3, −3.4	141.0	122.8	−3.9	22.1	
6α-CH_2	9.2, 7.5	4.2	−2.6, −3.9	7.6, 7.2	−27.3	22.8	−3.7	−46.4	
6β-CH_2	1.7, −0.4	3.6	−0.1, −3.1	−1.8, −0.9	108.1	38.8	−1.9	71.2	
7α-CH_2	1.2, 0.2	4.2	−6.0, −6.8	3.0, 2.8	−8.3	22.8	−7.5	−23.6	
7β-CH_2	1.6, 0.8	3.6	−2.4, −2.9	0.4, 0.1	67.2	38.8	−3.4	31.8	

[a] Experimental values have errors of ±0.1 ppm. The estimated error on δ^{pc} is ±10%. [b] Taken from ref 37. [c] Taken from ref 29.

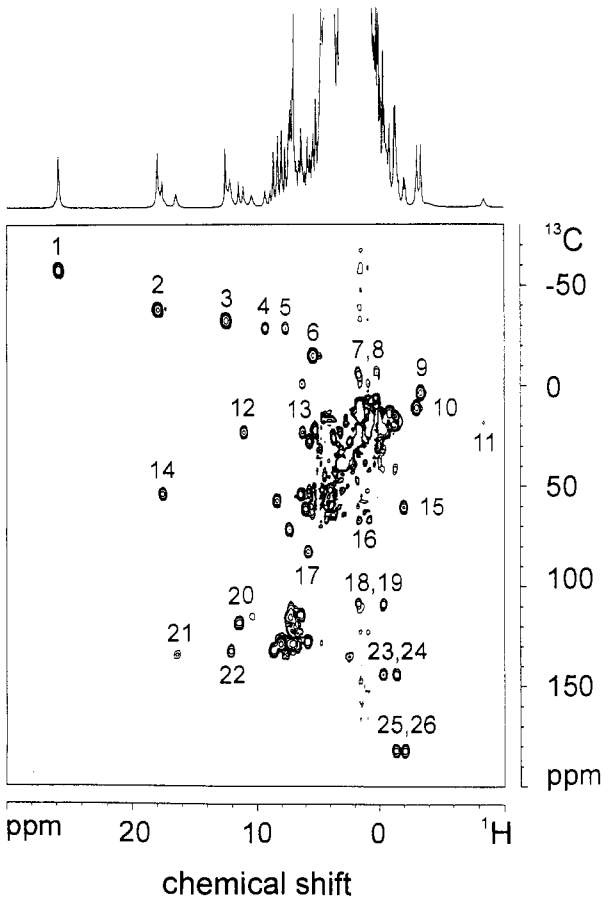

Figure 2. 600-MHz ^{1}H−^{13}C HMQC spectrum recorded on MbCN at 310 K. The labeled cross peaks indicate the one-bond connectivities for the following protons: (1) 5-CH_3, (2) 1-CH_3, (3) 8-CH_3, (4, 5) 6α-CH_2, (6) 3-CH_3, (7, 8) 7α-CH_2, (9) δ-CH_3 Ile FG5, (10) γ-CH_3 Ile FG5, (11) γ-CH Ile FG5, (12, 13) β-CH_2 His F8, (14) 2α-CH, (15) α-CH Val E11, (16) 7β-CH_2, (17) 4α-CH, (18, 19) 6β-CH_2, (20) δ-CH His E7, (21) ζ-CH Phe CD1, (22) ε-CH Phe CD1, (23, 24) 4β-CH_2, (25, 26) 2β-CH_2.

resonances, characterized by faster relaxation rates and higher sensitivity with respect to ^{13}C resonances, also allows us to perform a reasonable number of experiments in relatively short experiment times.

The straightforward assignment, based of the available assignments of the ^{1}H spectra,[7] is reported in Table 2. Direct detection ^{13}C−^{1}H 2D spectra of this protein had been previously recorded using considerably longer acquisition times, except when few experiments were performed.[4,27] In the latter case, the low resolution present in the F_1 dimension (^{1}H shifts) led in an early paper to an erroneous assignment of one of the heme methyl groups.

Table 3. Further Proton and Carbon Assignments for MbCN at 310 K[a]

assignt	δ_H (ppm)	δ_C (ppm)	assignt	δ_H (ppm)	δ_C (ppm)
H_ϵ Phe CD1	12.1	131.7	γ-CH_3 Ile FG5	−3.2	12.1
$H_{\delta 2}$ His E7	11.6	118.2	δ-CH_3 Ile FG5	−3.5	5.2
β-CH_2 His F8	11.1, 6.2	21.0	$H_{\gamma 1}$ Ile FG5	−8.7	20.1
H_α Val E11	−2.1	59.3			

[a] Experimental values have errors of ±0.1 ppm.

The detection of new, additional cross peaks allows us now to assign the ^{13}C signals of almost all the rest of the heme substituents. The α carbons of the propionate moieties are now detected in the upfield region through the connectivities with 6α and 7α protons, which had been previously assigned.[7,9] The assignment of the vinyl α-CH groups is the same as that proposed by La Mar's group by using labeled hemins in reconstituted MbCN.[28] Their carbon chemical shifts are not in the upfield region, because their diamagnetic shifts are quite downfield (131.5 ppm in the zinc(II) protoporphyrin dicyanide complex).[29] The hyperfine shift is always negative due to the large amount of direct spin delocalization through π-MO onto the carbon rings. The carbon chemical or hyperfine shifts of the β-CH_2 vinyl moieties are downfield with respect to the olefinic region, and each one gives two cross peaks. Additional cross peaks that appear outside the diamagnetic envelope are those involving nuclei, either carbons or protons, which belong to residues not directly coordinated to the iron ion but which feel the unpaired electron through dipolar interaction.

(c) **Cytochrome b_5.** In Figure 3 the HMQC ^{1}H−^{13}C spectrum of Cytb$_5$ is reported together with the ^{1}H 1D NMR spectrum, for which most of the paramagnetically shifted signals have been already assigned. The protein is produced with the heme ring bound in two different orientations (named A and B), and the relative amounts of the two forms are almost equal.[12] The spectrum, therefore, shows two sets of signals of similar intensities for the heme protons, at variance with Cytb$_5$ from other sources,[5,30,31] which have one dominant form. Taking advantage of the already assigned ^{1}H NMR signals,[8,10] the assignment of the corresponding ^{13}C signals is straightforward

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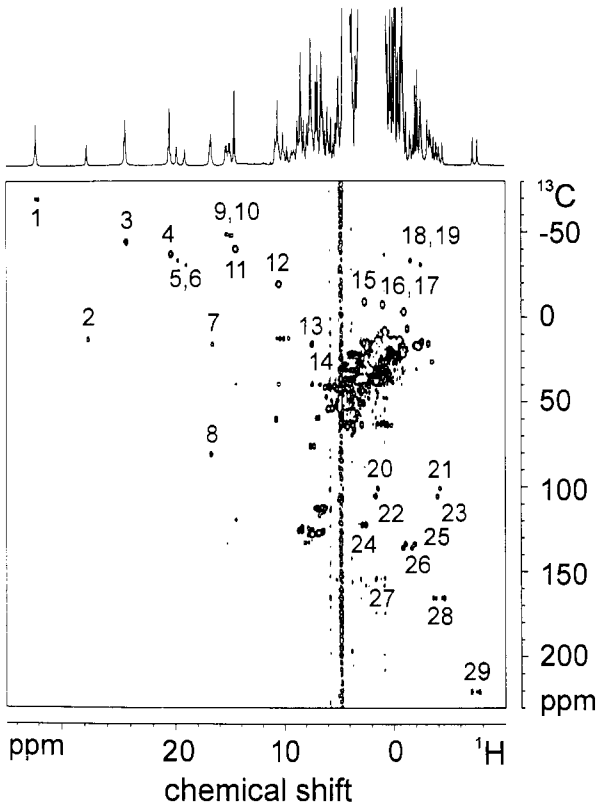

Figure 3. 600-MHz $^1H-^{13}C$ HMQC spectrum recorded on $Cytb_5$ at 295 K. The resonances of the two isomers are indicated as A and B, respectively. The labeled cross peaks indicate the one-bond connectivities for the following protons: (1) B3-CH_3, (2) A2α-CH, (3) B8-CH_3, (4) A5-CH_3, (5) A7α-CH, (6) B6α-CH, (7) Aβ-H 39, (8) B4α-CH, (9) B7α-CH_2 (10) A6α-CH_2, (11) A3-CH_3, (12) A1-CH_3, (13) Aβ-H 39, (14) A4α-CH, (15) A8-CH_3, (16) B5-CH_3, (17) B1-CH_3, (18) A7α-CH, (19) B6α-CH, (20, 21) B6β-CH_2, (22, 23) A7β-CH_2, (24) A4β-CH_2, (25) A6β-CH_2, (26) B7β-CH_2, (27) B4β-CH_2, (28) B2β-CH_2, (29) A2β-CH_2.

(see Table 4). The ^1H signals of 4α-CH and 4β-CH_2 are also assigned for both orientations, with the aid of a NOESY map (data not shown). The corresponding ^{13}C signals are also observed as cross peaks in the HETCOR map (Figure 3). It should be noted that, while the ^{13}C shifts of all the heme CH_3 groups for both heme orientations are in a relatively narrow range of values, the corresponding ^1H shifts experience a much larger distribution of values.

Factorization of the Observed Chemical Shifts. The experimental shifts for those nuclei which sense the unpaired electron are the result of several contributions,[32] which should be evaluated in order to be analyzed.[33-35] First of all, knowledge of the diamagnetic contribution to the shift, i.e. the shift that the considered nucleus would have if it were in the same, but diamagnetic, molecule, is needed in order to obtain the isotropic hyperfine shift.

In the case of Mb and $Cytb_5$, the proton shifts are available for the low-spin reduced forms, which are diamagnetic.[36-39] For $PPIm_2$ the shift values were taken from the bis(cyano) adduct of zinc protoporphyrin IX.[29] For the ^{13}C nuclei no diamagnetic values are available except for those of the bis(imidazole)zinc-(II) protoporphyrin IX complex.[29] These values have been used also for the two heme proteins (see footnotes to Tables 1-4). Since the hyperfine shift is large, the error made taking as diamagnetic shifts those of the model compound is expected to be lower than a few percent. Once the diamagnetic contribution is subtracted, the isotropic hyperfine shift remains, which in turn, is the sum of three terms:

$$\left(\frac{\Delta\nu}{\nu}\right)^{\text{hyperfine}} = \left(\frac{\Delta\nu}{\nu}\right)^{\text{MCpc}} + \left(\frac{\Delta\nu}{\nu}\right)^{\text{LCpc}} + \left(\frac{\Delta\nu}{\nu}\right)^{\text{cont}}$$

The first term is due to the coupling, through space, between the nucleus and the unpaired spin density on the metal itself. It can be calculated when the magnetic anisotropy and the orientation of the magnetic susceptibility tensor are known and the structure of the system is available. The orientation of the magnetic susceptibility tensors has already been determined for both MbCN[13] and $Cytb_5$ (both heme orientations)[14] by fitting the hyperfine shift values of nuclei belonging to residues not directly coordinated to the metal ion. By taking the reported magnetic tensor data and structure and by using the well-known equation for the metal-centered pseudocontact shift,[32,40] this contribution was calculated for the protons and the carbons assigned in the spectra. The calculated values are reported in Tables 1, 2, and 4.

We are left with the values which are the sum of two contributions: contact and ligand-centered pseudocontact shifts. The former contribution is due to the presence of unpaired spin density at the resonating nucleus. The latter is significant only for carbon nuclei which bear spin density in pπ orbitals. For nuclei one bond away, the ligand-centered pseudocontact shift is expected to be small. For the nuclei of the heme substituents (i.e. methyl, vinyl, and propionate groups), the contact contribution is determined by the unpaired spin density on the adjacent carbon in the heme ring, which is involved in the π molecular orbital.[41] As the source of the unpaired spin density on a given carbon atom and its attached proton(s) for each substituent is the same, the contact shift on carbons and protons should be related through proportionality constants (Q_{C-C} for carbons and Q_{C-C-H} for protons)

$$\left(\frac{\Delta\nu}{\nu}\right)^{\text{cont}} = S(S+1)Q\varrho$$

where ϱ is the spin density at the heme ring carbon. The constant Q_{C-C} has been proposed to be −39 MHz for carbons[42] and to be in a large range for protons (+5 to +120 MHz)[43-45] depending on the nature of the group bearing the protons. Indeed, the Q_{C-C-H} constant for protons depends on the dihedral angle between the C−C−H plane and the C−C plane perpendicular to the heme ring.[46-48] This dependence makes the analysis of the shifts of groups other than methyls more

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Table 4

(a) Proton and Carbon Chemical Shifts and Assignments for $Cytb_5$-A at 295 K[a]

assignt	δ_H (ppm)	δ^{diam} (ppm)[b]	δ^{pc} (ppm)	δ_H^{cont} (ppm)	δ_C (ppm)	δ^{diam} (ppm)[c]	δ^{pc} (ppm)	δ_C^{cont} (ppm)	$(\delta_C/\delta_H)^{cont}$
1-CH_3	10.6	3.3	−3.3	10.6	−19.3	13.0	−3.8	−28.5	−2.7
3-CH_3	14.5	3.4	−4.3	15.4	−40.2	13.0	−5.3	−47.9	−3.1
5-CH_3	20.4	3.5	−3.7	20.6	−37.2	11.7	−4.2	−44.7	−2.2
8-CH_3	2.6	3.7	−7.3	6.2	−9.1	11.7	−8.6	−12.2	−2.0
2α-CH	27.7	7.4	−1.1	21.4	13.3	131.5	−1.2	−117.0	
2β-CH_2	−7.1, −7.6	5.1, 5.4	−0.5, −0.9	−11.7, −12.1	220.0	119.6	−0.8	101.2	
4α-CH	4.4	8.3	−6.2	2.3	28.6	131.5	−8.1	−94.8	
4β-CH_2	2.5, 2.9	6.0, 6.0	−3.5, −5.0	0.0, 1.9	122.1	122.8	−5.2	4.5	
6α-CH_2	15.1, 14.9	4.7[c]	−1.0, −1.1	*d*	−47.6	22.8	−1.2	−69.2	
6β-CH_2	−1.0, −1.8	3.6[c]	−0.2, −0.3	*d*	133.7	38.3	−0.5	95.9	
7α-CH_2	19.7, −1.5	4.7[c]	−4.3, −4.7	*d*	−33.6	22.8	−6.1	−50.3	
7β-CH_2	1.7, −3.9	3.6[c]	−2.3, −3.1	*d*	105.3	38.3	−3.8	70.8	

(b) Proton and Carbon Chemical Shifts and Assignments of $Cytb_5$-B at 295 K[a]

assignt	δ_H (ppm)	δ^{diam} (ppm)[b]	δ^{pc} (ppm)[e]	δ_H^{cont} (ppm)	δ_C (ppm)	δ^{diam} (ppm)[b]	δ^{pc} (ppm)[e]	δ_C^{cont} (ppm)	$(\delta_C/\delta_H)^{cont}$
1-CH_3	−0.9	3.5	−7.0	2.6	−3.2	13.0	−8.3	−7.9	−3.0
3-CH_3	32.2	2.9	−1.0	30.3	−69.9	13.0	−1.0	−81.9	−2.7
5-CH_3	1.0	3.7	−7.5	4.8	−7.4	11.7	−8.9	−10.2	−2.1
8-CH_3	24.3	3.6	−3.1	23.8	−44.3	11.7	−3.6	−52.4	−2.2
2α-CH	10.8	7.9	−4.6	7.5	60.0	131.5	−6.3	−65.2	
2β-CH_2	−3.7, −4.5	5.7, 6.2	−2.5, −4.0	−6.9, −6.7	165.7	119.6	−3.9	50.0	
4α-CH	16.8	8.4	−3.1	11.5	80.4	131.5	−3.2	−47.9	
4β-CH_2	1.6, 0.8	5.2, 4.6	−1.3, −1.2	−2.3, −2.6	154.0	122.8	−1.7	32.9	
6α-CH_2	19.0, −2.5	4.7[c]	−5.3, −5.3	*d*	−30.6	22.8	−6.5	−46.9	
6β-CH_2	1.5, −4.2	3.6[c]	−2.1, −2.9	*d*	100.5	38.3	−3.2	65.4	
7α-CH_2	15.4, 15.3	4.7[c]	−0.9, −1.2	*d*	−48.6	22.8	−1.0	−70.4	
7β-CH_2	−0.9, −1.6	3.6[c]	0.0, 0.7	*d*	135.9	38.3	0.2	97.4	

[a] Experimental values have errors of ±0.1 ppm. The estimated error on δ^{pc} is ±10%. [b] Taken from ref 39. [c] Taken from ref 29. [d] The contact contribution cannot be determined because the stereospecific assignment is not available. [e] Values calculated using the coordinates of the heme of $Cytb_5$ crystal structure but rotated 180° about the α,γ-meso axis with respect to its position.

complex. In the case of fast-rotating methyl groups, the angular dependence averages to $^1/_2$.[49]

The ligand-centered pseudocontact contribution to the hyperfine shift of nuclei of heme substituents in principle depends on the π-spin density on the adjacent heme carbon and should be proportional, through different constants, to tbe same quantity, i.e. the π spin density ϱ. As anticipated, it should be small for carbon and even smaller for protons.

The estimate of these two contributions (contact and ligand-centered pseudocontact contributions) is a difficult task. However, in the case of methyl groups attached to the heme ring, if the C−C and the C−H bonds have the same length for all the groups, the $[(\Delta\nu/\nu)^{cont} + (\Delta\nu/\nu)^{LCpc}]$ ratio between the carbon and the attached proton would be the same for all the groups. On this basis, a method for the factorization of the metal-centered pseudocontact shift was proposed.[50] The above values together with the absolute values of the hyperfine shifts are meaningful with respect to the spin delocalization on the heme ring. The ratios for the methyl groups of the systems here investigated are reported in the last columns of Tables 1, 2, and 4.

Discussion and Concluding Remarks

This is the most extensive assignment of the hyperfine-shifted ^{13}C signals through HETCOR studies on paramagnetic proteins. All the carbons of the heme ring substituents have been assigned by taking advantage of the signal assignment of the protons attached to the carbons. The assignments for the lacking protons have been obtained here through the detection of dipolar connectivities. The use of reverse detection techniques makes the NMR characterization of heteronuclei practical for systems in natural abundance. We wish to stress the relevance of these extensive ^{13}C signal assignments, as they open new possibilities for the understanding of the mechanisms for spin density delocalization. Furthermore, the knowledge of the magnetic susceptibility anisotropies and of the directions of the principal axes of the magnetic tensor allows us to calculate the pseudocontact contributions to the hyperfine paramagnetic shifts and to factor them out from the observed values. The contact plus the ligand-centered shift values for the assigned proton and carbon nuclei can thus be obtained. It is from the analysis of these values that further information becomes available about the spin delocalization processes.

The ratio between the contact (and ligand-centered) shifts of ^{13}C and its bound ^1H nucleus (i) is found to be variable. This happens when more than one spin transfer mechanism is operative.[15-17] When only a π-spin transfer mechanism is operative on the C nuclei, the ratios between the contact shifts of the carbon of the heme methyls and those of the attached proton are expected to be equal for all four groups. When σ and π mechanisms are operative and they contribute to a different extent to the various porphyrin positions, then the ratios are expected to be different.

We find that in $PPIm_2$ the $^{13}C/^1H$ contact shift ratio is almost constant (2.6 ± 0.1), which nicely fits the expectation. Therefore, although the heme is not 4-fold symmetric, the spin densities on the four pyrrole carbons bearing the methyl groups are the same. This is due to the fast rotation of the axial ligands.

In the proteins, the restricted motion of the imidazole ring removes the pseudocylindrical symmetry. In MbCN and in the major isomer of $Cytb_5$, the contact shifts for the methyl groups are spread over a large range of values and the ratios between the contact shifts of the carbon and of its attached protons for the methyl groups are quite scattered. The same behavior is found also in cytochrome *c*, where it was suggested that different Q_{C-C-H} values should be used.[2] In the minor isomer of $Cytb_5$,

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the 3- and 8-methyl groups on one side of the heme and the 1- and 5-methyl groups on the other side have pairwise similar shifts as a result of the projection of the axial imidazole plane being along pyrroles 2 and 4.[5,30] Even so, the $^{13}C/^1H$ shift ratios do not correlate with the shift pattern. In principle, the nonplanarity of the FeN_4 moiety may introduce some σ-spin density into the heme ring, which then would account for different ratios. We can conclude that, whereas the contact shifts are consistent with dominant π-spin density on the π orbital of the heme carbons, the different ratios presumably monitor two mechanisms of different extents. However, the overall marked lack of symmetry in the proteins by itself may be responsible for the different chemical bond lengths which are monitored through the contact shifts. When propionate and vinyl groups are considered, the problem is further complicated by the angular dependence of the proton contact shifts, and no efforts to understand the contribution to the hyperfine shifts have been attempted.

Note Added in Proof. A recent extension of the proton assignments for $Cytb_5$ is in complete agreement with our results (Lee, K.-B.; et al. *Biochim. Biophys. Acta* **1993**, *1202*, 189).

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