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Linear Correlation between ^{1}H and ^{13}C Chemical Shifts of Ferriheme Proteins and Model Ferrihemes

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The $^{1}H\{^{13}C\}$ HMQC experiment at natural-abundance ^{13}C provides a very useful way of determining not only ^{1}H but also ^{13}C chemical shifts of most heme substituents, without isotopic labeling of the hemin. This is true both in model low-spin ferriheme complexes and in low-spin ferriheme proteins, even when the proton resonances are buried in the protein diamagnetic region, because the carbon shifts are much larger than the proton shifts. In addition, in many cases, the protohemin methyl cross peaks are fairly linearly related to each other, with the slope of the correlation, δ^{C}/δ^{H}, being approximately −2.0 for most low-spin ferriheme proteins. The reasons why this should be the case, and when it is not, are discussed.

Introduction

In 1973, Wüthrich and Baumann published the first paper on ^{13}C NMR spectra of several metalloporphyrins (low-spin Fe^{III} and Zn^{II}) at natural abundance.[1] They used a Varian XL-100 in the pulsed mode for the ^{13}C NMR spectra and acquired 20 000 transients for Fourier transformation. The signal-to-noise ratio was excellent, on the order of 50:1, for 20 mM samples in 12-mm-o.d. NMR tubes. $CDCl_3$ was used as the solvent for the Zn^{II}TPP (TPP = tetraphenylporphyrin) and -Proto(IX) complexes and 4:1 pyridine-d_5/D_2O for the Fe^{III}TPP and -Proto(IX) complexes, with an excess of KCN present to form the dicyanoiron(III) porphyrin complexes. In 1978 and 1983,[2,3] Goff and co-workers reported direct-detect spectra of high-spin iron(III) porphyrins, and in 1981, he reported a detailed study of bis(imidazole) complexes of several tetraphenylporphyrinatoiron(III) complexes.[4] Natural-abundance 1D ^{13}C direct-detect experiments have not changed that much since those days, although the availability of the instruments with which to detect them and the magnetic fields at which they can be carried out have changed a lot. What has also changed dramatically is the ease of ^{13}C enrichment, so that many more researchers are using ^{13}C-enriched starting materials to synthesize complexes that they wish to study, including all possible isotopic enrichments of the last committed precursor to heme synthesis, δ-aminolevulinic acid (ALA).[5,6] Some of the specifically enriched isomers of ALA have been available from Cambridge Isotopes at various times, but methods for the synthesis of the ^{13}C-labeled isomers of ALA that are not available commercially have also been developed.[7] Thus, specifically ^{13}C-labeled hemes can be synthesized and used for NMR investigations. Specific $^{1}H/^{13}C/^{13}C$ experiments have been developed[8,9] and used to completely assign the ^{13}C heme resonances of a rat outer mitochondrial membrane (OM) cytochrome b_5[10] and to learn detailed information about the heme electronic structure by studying specifically labeled heme isomers.[11−15]

In spite of these developments, many scientists still prefer to use nonenriched samples for investigating the ^{13}C resonances of various compounds, including hemes. The $^{1}H\{^{13}C\}$

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Ann Walker was born and raised in the small town of Adena, OH, in the tristate area of eastern Ohio, the panhandle of West Virginia, and western Pennsylvania. She earned her B.A. in chemistry at The College of Wooster in 1962 and her Ph.D. in physical inorganic chemistry at Brown University in 1966. She was an NIH Postdoctoral Fellow with the late Daniel Kivelson at UCLA. Ann began her career as a chemistry professor at Ithaca College in 1967 and moved to San Francisco State University in 1970. She was the recipient of an NIH Research Career Development Award for the period of 1976−1981. In 1990, she moved to the University of Arizona, where she is currently Regents Professor of Chemistry and Biochemistry and a member of the Center for Insect Sciences. In 2000, she was recipient of the Garvan-Olin Medal from the American Chemical Society. She was recipient of the Luigi Sacconi medal of the Italian Chemical Society in 2001. She spent a sabbatical leave in the Physics Institute at the University of Lübeck, Lübeck, Germany, with Professor Alfred X. Trautwein in 2003−2004, with support of an Alexander von Humboldt Senior Research Fellowship in Science. In 2006, she was recipient of the Alfred Bader Award in Bioinorganic Chemistry from the American Chemical Society. She spent the last 4 months of 2010 on sabbatical leave at the Institute of Molecular and Cell Biology, University of Rosario (IBR), Rosario, Argentina, in the laboratory of Professor Alejandro J. Vila. At the end of 2010, Ann retired as an Associate Editor of the *Journal of the American Chemical Society* after nearly 13 years.

HMQC experiment at natural abundance of ^{13}C (1.1%) is an available method that can be used by any researcher who has access to an inverse heteronuclear broad band or a ^{13}C (^{1}H-detect) probe. For paramagnetic model hemes and other macrocycles, or heme proteins, this experiment allows correlation of the ^{13}C and ^{1}H chemical shifts of heme substituents. We and others have found these spectra to be very helpful in identifying and confirming the ^{1}H chemical shifts of heme and nearby protein substituents

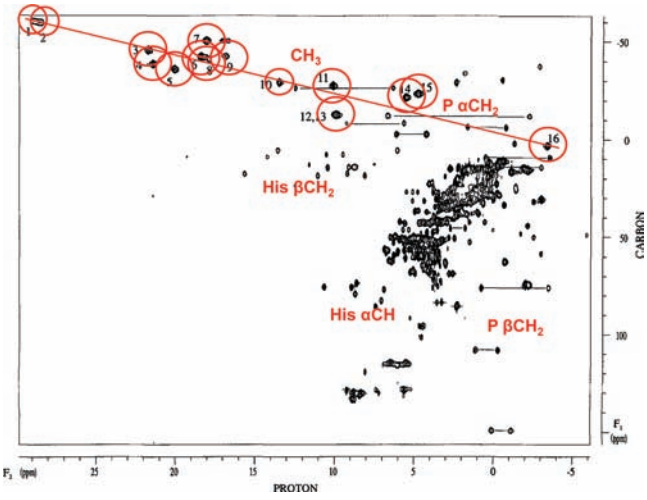

Figure 1. HMQC spectrum of the bis(histidine)-coordinated tetraheme protein from *Desulfovibrio vulgaris*, cytochrome c_3 (Hildenborough) at 38.3 °C and pH 9.0. The heme methyls of the four hemes in the protein are labeled **1**−**16**. A number of other resonances have been assigned, including most of the propionate α-CH_2, some of the β-CH_2, and the His α-CH and β-CH_2 resonances.[21] $\delta_C/\delta_H = -2$. Modified from ref 21 with permission from the Federation of European Biochemical Societies.

whose resonances may or may not be resolved outside the protein resonance envelope.[16−35] The first people to report a heteronuclear 2D spectrum of a low-spin ferriheme protein, in 1986, were Santos and Turner,[16] in that case a HETCOR. Timkovich was the first to do the inverse experiment, HMQC, on *Pseudomonas aeruginosa* cytochrome *c*-551, in 1991.[24] Banci et al. also used the HMQC experiment early to determine the ^{1}H and ^{13}C shifts of protohemin bis(imidazole) and met-Mb-CN in 1994.[25] Turner has used this method for aiding the assignment of heme protein resonances since 1995,[16−23] and it is particularly useful for the bis(histidine)-bound tetraheme

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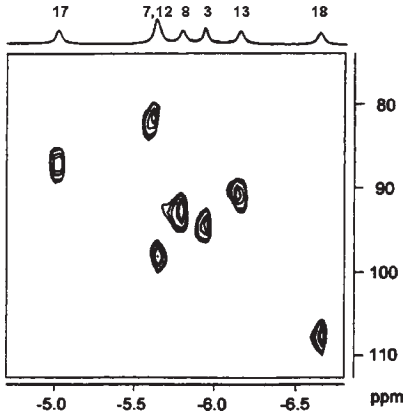

Figure 2. Pyrrole region of the 300 MHz $^1H-^{13}C$ HMQC spectrum of $[2\text{-ClTPPFe}^{III}(CN)_2]^-$ in methanol-d_4 at 294 K. No δ_C/δ_H correlation is observed probably because of the extremely small range of δ_C and δ_H observed, which magnifies the importance of the differences in the diamagnetic shifts. Modified from ref 18 with permission from the American Chemical Society.

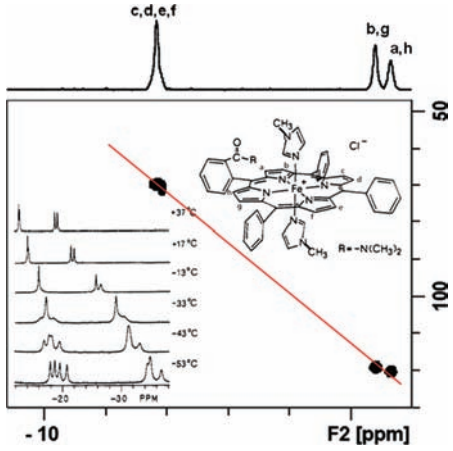

Figure 3. HMQC spectrum of the pyrrole hydrogen region of $[(o\text{-CONMe}_2)_1\text{TPPFe(NMIm)}_2]^+$, recorded at 500 MHz in $CDCl_3$ at 25 °C. The upper inset shows the structure of the complex and the eight types of pyrrole protons present (a−h). The lower inset shows the published temperature dependence of the spectra, recorded earlier, at 300 MHz.[51] Reprinted from Yang, F., unpublished, with her permission.

cytochromes c_3,[16,21,23] as shown in Figure 1. It has also been used to investigate model hemes such as the unsymmetrically pyrrole-substituted complex $[2\text{-ClTPPFe(CN)}_2]^-$[36] shown in Figure 2 and the unsymmetrically phenyl-substituted complex $[(o\text{-CONMe}_2)_1\text{TPPFe(NMeIm)}_2]^+$ shown in Figure 3 and other paramagnetic metal macrocyclic complexes such as the chloroiron(III) corrolate π-radicals, for example, $[ClFe^{III}(Corr^{\bullet 2-})]$, which are neutrally charged species with $S = 1$ and large chemical shifts but short electron-spin relaxation times,[37] as shown in Figure 4. However, it is worth noting that this is the only complex with $S > {}^1/_2$ of which we know, for which the ^1H and ^{13}C spin−lattice relaxation times (T_1's) are long enough to allow the ^1H and ^{13}C cross peaks to be observed.

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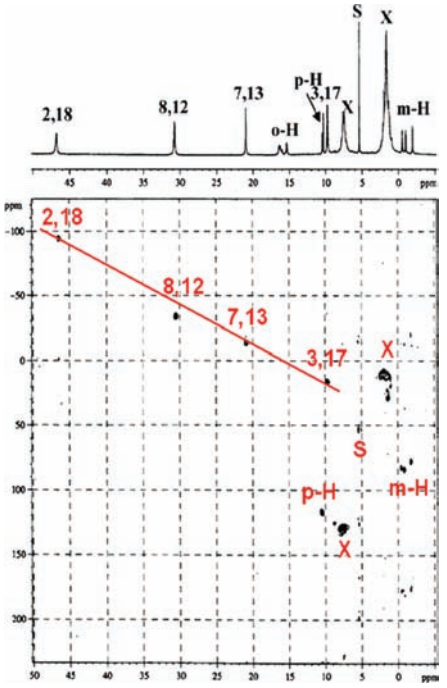

Figure 4. 1D ^1H and 2D $^1H\{^{13}C\}$ HMQC spectra of chloroiron(III) octamethyltriphenylcorrolate, (OMTPCorr)FeCl. The $^1H-^{13}C$ correlations for the four methyl, two *p*-phenyl, and four *m*-phenyl groups are shown; the relaxation times of the *o*-phenyl protons are too short to allow their correlations to be detected. Resonances marked X are those of corrole decomposition products. Recorded in CD_2Cl_2 at 25 °C and 500 MHz. Least-squares $\delta_C/\delta_H = -3.17$. Modified from ref 37 with permission of the American Chemical Society.

We have reported the natural-abundance $^1H\{^{13}C\}$ HMQC spectra of low-spin complexes of the nitrophorin proteins since 2003.[29−35] By using the information obtained from these HMQC spectra, we have been able to assign most of the heme substituent resonances of these protein complexes. For example, in Figure 5, the $^1H\{^{13}C\}$ HMQC spectra of NP2-(V24E)-ImH at pH* 7.0, the $^1H-^{13}C$ cross peaks of all heme substituents except for those of *meso*-CH are clearly observed for the **A** heme isomer and are fairly easy to assign using these $^1H\{^{13}C\}$ HMQC spectra in combination with $^1H-^1H$ NOESY spectra. For the **B** heme isomer, the heme methyl $^1H-^{13}C$ cross peaks, except for those of 5M and 8M, and the 2- and 4-Vα (which are broad because of chemical exchange processes) are also observed. [Note: For those readers unfamiliar with the two possible heme orientations, **A** and **B**, protohemin has no 2-fold rotation axes through the atoms of the heme plane, and thus when it binds to a protein, which is always asymmetric in its structure, two isomers are created. One of these has the heme substituents numbered 1−8 in a clockwise direction if viewed from above the heme plane (assuming the protein-provided ligand is bound below the plane), and the other has the heme substituents numbered 1−8 in a counterclockwise direction if viewed in the same way, as shown in the insets of Figures 5 and 6. The heme substituents 1−8 will thus be in different environments (have different protein residues near them) for each of these two isomers, and there will thus be two proton (and carbon) resonances for each substituent. Seldom are *all* of these resonances observed, but usually *most* of them are observed.] This particular NP2 mutant is one of the few that has a major **A** orientation of the heme; more commonly, it is the **B** heme

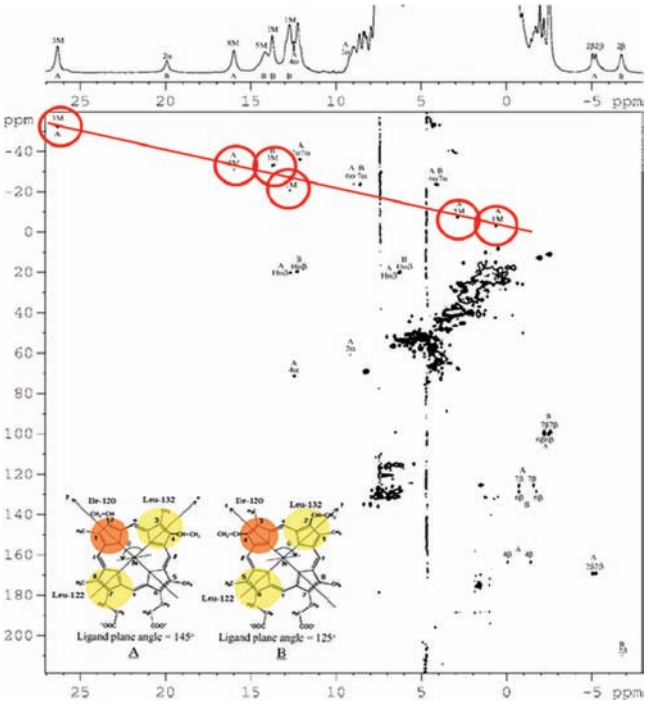

Figure 5. 1D and $^{1}H-^{13}C$ HMQC NMR spectra of NP2(V24E)-ImH in D_2O, buffered with 30 mM Na_2DPO_4/acetic acid-d_4 (pH* 7.0), recorded at 35 °C, 500 MHz. There are two sets of cross peaks: one from isomer **A** and the other from isomer **B**. The 5M isomer **B** ^{1}H resonance is broadened because of some sort of chemical exchange, and it does not give a $^{1}H-^{13}C$ cross peak. The **A** and **B** heme orientations are shown in the inset. Modified from ref 35 with permission from Elsevier Publishing Company.

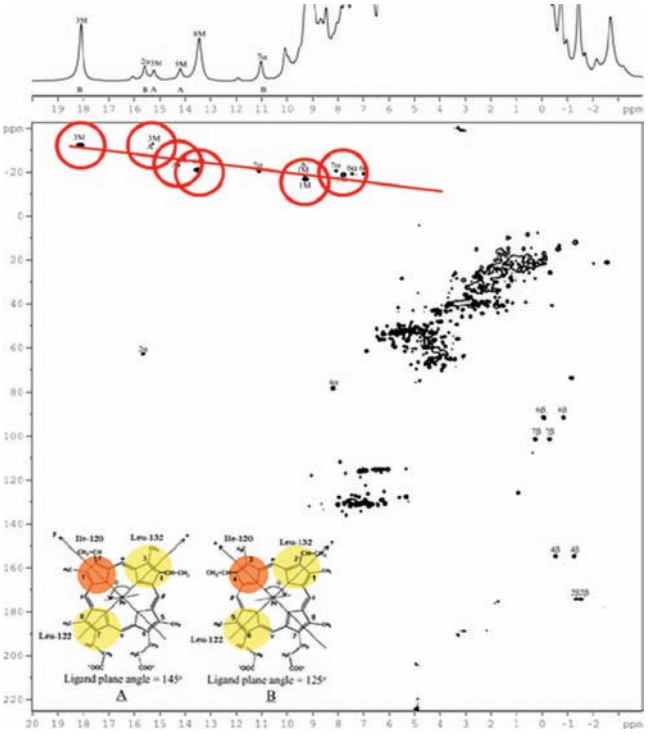

Figure 6. 1D and $^{1}H-^{13}C$ HMQC spectra of NP2-CN in D_2O, buffered with 30 mM Na_2DPO_4/acetic acid-d_4 (pH* 6.5), recorded at 35 °C and 500 MHz. The **A** and **B** heme orientations are shown in the inset. Modified from ref [35] with permission from Elsevier Publishing Company.

orientation that is most abundant for NP2 and for which most of the heme resonances are resolved. It is also frequently the rule that the $^{1}H-^{13}C$ cross peak for the most hyperfine-shifted methyl resonance is not observed because of chemical exchange processes;[38] in fact, the only pH at which the 3M resonance of the **A** heme orientation for any NP2-ImH complex is observed is pH 7.0 for this V24E mutant shown in Figure 5.

β-Vinyl and α- and β-propionate C−H cross peaks can be spotted because pairs of protons have cross peaks to the same carbon. Examples are the 6α- and 7α-CH_2 propionate cross peaks for the **A** heme isomer of Figure 5, with a ^{13}C chemical shift at −23.9 ppm, ^{1}H chemical shifts at +9.0 and +4.2 ppm and a ^{13}C chemical shift −36.1 ppm, ^{1}H chemical shifts at +12.2 and +12.1 ppm, respectively, for the **A** heme orientation of Figure 5. The corresponding β-CH_2 resonances are at +100.0, −2.2, and −2.5 ppm and +125.6, −0.8, and −1.6 ppm, respectively, for the **A** heme orientation. (^{1}H resonance assignments of the 6α-, 7α-, and β-propionate resonances and the 2α, 4α, and 4β vinyls are easily confirmed by their NOESY cross peaks.) Other CH_2 groups that are easily recognized are those of His57 β-CH_2, ^{13}C chemical shift at +20.2 ppm and ^{1}H chemical shifts at +12.7 and +6.3 ppm for the **A** heme orientation and ^{13}C chemical shift at +18.6 ppm and ^{1}H chemical shifts at +12.3 and +6.2 ppm for the **B** heme orientation. The His α-CH resonances are very closely spaced at $^{1}H-^{13}C$ shifts of 8.3, 69.1 ppm and 8.2, 68.8 ppm. Additional $^{1}H-^{13}C$ cross peaks from protein methyls of I120 and L132, which are very close to the heme, are also seen, with ^{13}C chemical shifts of 14−16 ppm and ^{1}H chemical shifts of −1.7 to −2.3 ppm, as will be discussed in further detail elsewhere (Shokhireva, T. K.; Yang, F.; Walker, F. A., in preparation). For wild-type NP2-CN, the $^{1}H-^{13}C$ cross peaks for all heme substituents except *meso*-CH are observed, as shown in Figure 6. His57 β-CH_2 cross peaks are also not seen.

To observe these $^{1}H-^{13}C$ cross peaks in the HMQC spectrum of a low-spin iron(III) heme protein requires at least a 1 mM concentration (2 mM is preferable) of ferriheme protein at natural-abundance ^{13}C and requires an approximate 24-h experiment time. Any modern NMR spectrometer equipped with an inverse (^{1}H observe) ^{13}C or broad-band probe can be used, and 500 MHz as the ^{1}H Larmor frequency is certainly sufficient for all systems that have been reported thus far; all of the $^{1}H\{^{13}C\}$ HMQC spectra presented in the author's group's papers have been recorded at 500 MHz.[29−35] Because inverse ^{13}C or inverse broad-band probes are common on modern spectrometers, the use of this experiment is strongly recommended to find the heme ^{1}H resonances that are obscured by the protein resonances, and to then look for these resonances in the WEFT-NOESY spectra of the proteins, to permit complete assignment of the heme resonances. Protein side chains that are close to the heme iron, especially the histidine ligand β-CH_2 and α-CH cross peaks, as well as methyl-, methylene- and aromatic-containing side chains of amino acids that are within ~4 Å of Fe also have unusual $^{1}H-^{13}C$ chemical shifts because of the pseudocontact interaction (see below) and can then be followed in the NOESY spectra to assign the side chains of those amino acids.

As shown in Figures 1 and 3−6, and even more so in Figures 7 and 8, which have been created from ^{1}H and ^{13}C

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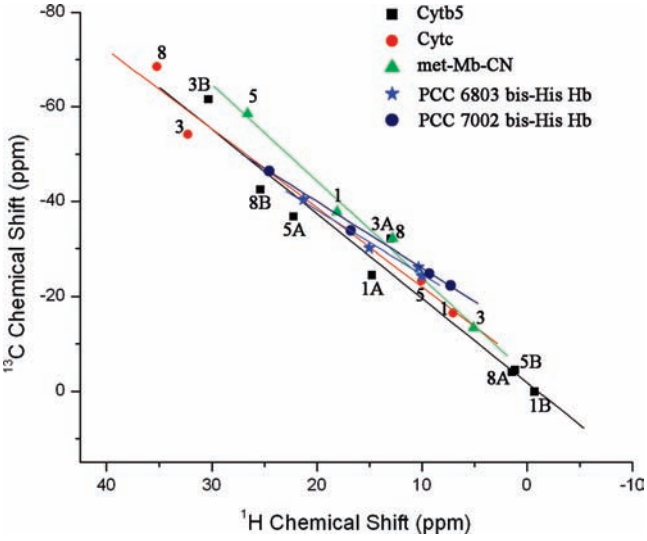

Figure 7. Plot of ^{13}C vs ^{1}H chemical shifts for horse cytochrome *c*,[24] rat OM cytochrome b_5,[10] met-Mb-CN,[27] and two cyanobacterial 6-coordinate (bis-His) met-Hbs.[28] Note that in the first three cases the 3-methyl points deviate from the best-fit line. Including 3M, for cyt *c*, $\delta_C/\delta_H = -1.67$; without 3M, it is -1.82; for met-Mb-CN, $\delta_C/\delta_H = -2.04$ with 3M, $\delta_C/\delta_H = -1.9$ without 3M. Including 3MA and 3MB, for OM b_5, $\delta_C/\delta_H = -1.78$; without 3MA and 3MB, it is -1.59. For the cyanobacterial 6-coordinate met-Hbs, 3M does not deviate from the line.

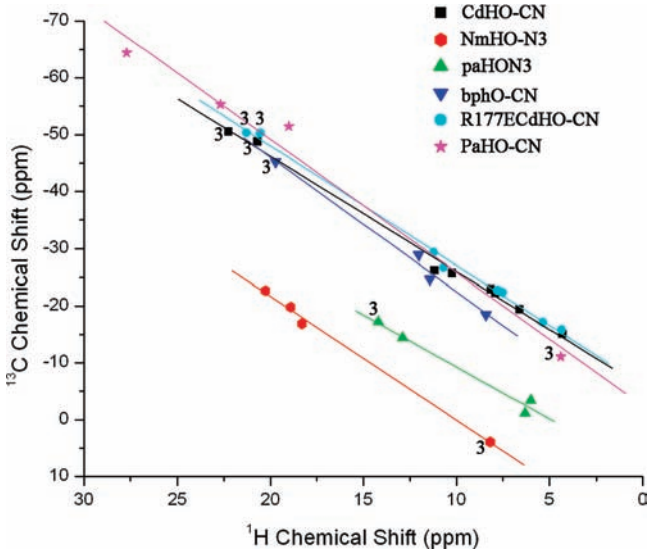

Figure 8. Plot of ^{13}C vs ^{1}H chemical shifts for several bacterial heme oxygenases as the cyanide and azide complexes. Note that the 3-methyl points do not appear to deviate from the best fit lines.

data of ^{13}C-enriched heme proteins published by Rivera and co-workers[10−15] and others who have used the ^{1}H{^{13}C} HMQC experiment on nonenriched heme proteins,[24−28] the β-pyrrole CH_3 ^{1}H−^{13}C cross peaks of ferriheme proteins usually present a fairly linear correlation of the ^{13}C and ^{1}H chemical shifts, with the exception being the case of the pyrrole hydrogen and carbon resonances of the Fe^{III}TPP low-spin complex shown in Figure 2.

The unique shifts and linear correlations of methyl H,C shifts are a result of the combination of the diamagnetic, contact, and pseudocontact contributions to the chemical shifts of both nuclei[24]

$$\delta^{H} = \delta^{H}_{dia} + \delta^{H}_{con} + \delta^{H}_{pcM} \quad (1)$$

$$\delta^{C} = \delta^{C}_{dia} + \delta^{C}_{con\pi} + \delta^{C}_{pcM} + \delta^{C}_{pcL\pi} \quad (2)$$

where δ^{H}_{dia} and δ^{C}_{dia} are the shifts of the same nuclei in a diamagnetic complex [usually zinc(II)-substituted complexes are used to evaluate these[27,39−41]], δ^{H}_{con} is the proton contact shift contribution, and $\delta^{C}_{con\pi}$ is the corresponding carbon contact shift for delocalization of the unpaired electron from the metal to the ligand through π bonds. The pseudocontact (formerly called dipolar[39,40]) shifts, δ^{H}_{pcM} and δ^{C}_{pcM}, are the result of the through-space dipolar coupling between the nucleus of interest (H or C) and the electron, which is centered on the metal, because of the magnetic anisotropy of d orbitals, which produce an angular and distance dependence on the size of the pseudocontact shift and can be calculated if one knows the distance and angular relationship between each nucleus and the metal center from the X-ray crystal structure.[24,39−41] In addition, for nuclei larger than hydrogen, there is an additional contribution, $\delta^{C}_{pcL\pi}$, which arises because of spin delocalization from the metal to the macrocycle via π bonds and then through space to aliphatic carbons that are connected to the carbon of the π system. Wüthrich and Baumann considered all of these terms in 1973, in the first report of the natural-abundance direct-detect ^{13}C NMR spectra of synthetic and natural hemin complexes,[1] but they also included potential σ contributions to the contact, metal-centered pseudocontact, and ligand-centered pseudocontact shifts of the carbons, which have since been shown to be unimportant for low-spin iron(III) complexes, where the unpaired electron is in a π-symmetry d orbital.[24,39−41]

The situation for low-spin iron(III) is that the diamagnetic shifts of all protons and carbons of substituents at the heme β-pyrrole positions are similar, the metal-centered pseudocontact shifts are fairly small, and the ligand-centered pseudocontact shifts vary linearly with the contact shifts,[42] which are large. Thus, the combination of these contributions, with the dominance of the contact shifts, moves these cross peaks well outside the normal ranges observed for the two nuclei in diamagnetic compounds and thus makes them easy to recognize and assign.

Thus, the approximately linear correlation is basically a result of the fact that the contact contribution dominates the chemical shifts of both ^{1}H and ^{13}C in low-spin iron(III) compounds, at least for those present at the β-pyrrole positions. The McConnell equation relates the hyperfine coupling constants a_H and a_C, which are directly proportional to the contact shifts of each nucleus, for the β-pyrrole positions to the electron density at the β-pyrrole carbons,[43] and this is true for both the ^{1}H and ^{13}C contact shifts:

$$a_H = Q_H\rho_C/2S \text{ for } ^{1}H \quad (3)$$

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Table 1. ^{1}H and ^{13}C Chemical Shifts of the Heme Methyls and Best Slopes δ_C/δ_H for a Number of Ferriheme Proteins

				^{1}H chemical shifts, ppm				^{13}C chemical shifts, ppm					
protein	temp (°C), pH	heme	L_1/L_2	1M	3M	5M	8M	1M	3M	5M	8M	δ_C/δ_H w, w/o 3M	ref
D. vulgaris cyt *c*	38.3, 9.0	I	His/His	18.35	9.72	18.01	28.44	−42.32	−12.14	−50.28	−59.97	−1.98	21
		II		4.69	21.23	5.58	21.81	−23.10	−38.27	−21.50	−45.85		
		III		13.47	9.91	20.04	−3.44	−29.09	−26.99	−35.80	3.39		
		IV		17.64	9.72	16.65	28.92	−40.52	−12.14	−42.15	−60.63		
S. frigidimarina cyt *c*	35.7, 6.1	I	His/His	9.25	18.54	11.70	36.29	−22.4	−21.6	−29.7	−68.0	−1.68, −1.53	
		II		14.66	12.21	23.57	21.6	−35.4	−22.3	−51.4	−45.7		
		III		17.19	9.88	18.72	37.09	−34.6	−11.4	−47.1	−66.5		
		IV		7.37	23.11	10.96	32.00	−23.1	−44.8	−32.7	−61.9		
Horse cyt *c*	50, 6.7		His/Met	7.65	30.10	10.84	32.43	−16.6	−49.4	−22.8	−63.0	−1.66, −1.83	16
Horse cyt *c*	30, 5.3		His/Met	7.09	31.88	10.16	34.46	−17.62	−54.44	−24.06	−67.92	−1.67, −1.83	18
Horse cyt *c*	25, 7.0		His/Met	7.04	32.29	10.03	35.23	−16.5	−54.2	−23.3	−68.5	−1.67, −1.82	24
Anabena sp. cyt c_6	34, 4.3		His/Met	15.40	35.99	21.10	38.02	−22.3	−48.3	−28.8	−59.7	−1.52, −1.69	22
M. braunii cyt c_6	34, 5.5		His/Met	13.83	35.00	17.42	37.10	−20.9	−49.5	−26.0	−61.2	−1.58, −1.75	22
Ps. aeruginosa cyt *c*-551	32, 7		His/Met	24.80	13.47	31.25	17.17	−46.9	−21.8	−54.3	−35.9	−1.76, −1.31	24
horse cyt *c*-CN	44, 6.9		His/CN^-	15.46	11.46	21.29	20.79	−37.55	−20.37	−44.47	−49.77	−2.60, −1.64	20
met-Mb-CN	RT, 7.4		His/CN^-	18.3	4.8	26.4	12.5	−38.4	−14.2	−58.2	−32.0	−2.04	25
met-Mb-CN	RT, ~7.5		His/CN^-	18.1	5.1	26.6	12.8	−37.8	−13.4	−58.5	−32.2	−2.04	27
Synechocystis sp. PCC 6803 bis-His Hb	25, 7		His/His	15.03	9.99	21.28	10.33	−30.2	−24.3	−40.4	−26.2	−1.6	28
Synechococcus sp. PCC 7002 bis-His Hb	25, 7		His/His	16.77	7.28	24.55	9.30	−33.8	−22.3	−46.4	−24.8	−1.6	28
Rat OM cyt b_5	RT, 7.0	A	His/His	14.75	12.99	22.23	1.43	−24.5	−32.1	−36.8	−4.0	−1.78, −1.59	10
		B		−0.71	30.33	1.16	25.38	0.1	−61.6	−4.5	−42.5		
Ps. aerug. HO-CN	10, 7.4		His/CN^-	22.69	4.41	27.71	19.01	−55.32	−11.10	−64.44	−51.45	−2.33	11
C. diphth. HO-CN	10, 7.4	A	His/CN^-	4.32	20.69	7.91	10.23	−15.12	−48.82	−22.28	−25.67	−2.02, −1.66	12
		B		6.60	22.26	8.15	11.17	−19.41	−50.46	−22.94	−26.18		
C. diphth. HO R177E-CN	10, 7.4	A	His/CN^-	4.36	20.55	7.52	11.21	−15.82	−50.26	−22.41	−29.30	−2.10	12
		B		5.34	21.29	7.78	10.68	−17.15	−50.32	−22.73	−26.69		
		B′		19.94	6.05	27.47	22.81	−45.41	−15.77	−60.14	−53.87		
bphO-CN	30, 7.0		His/CN^-	8.41	19.73	11.42	12.03	−18.43	−45.17	−24.64	−28.91	−2.36	14
N. meningitides HO-N_3	25, 7.0		His/N_3^-	18.90	8.17	18.31	20.26	−19.77	3.90	−16.91	−22.70	−2.17	13
Ps. aeruginosa HO-N_3	25, 7.0		His/N_3^-	6.29	14.15	5.96	12.91	−1.12	−17.21	−3.40	−14.38	−1.82	13
NP2-Hm	35, 5.5	A	His/Hm	−1.5	30.4	~10.4	16.0	−2.7	−63.6	----	−32.0	−1.87	35
		B		14.6	15.0	15.3	0.2	−24.1	−36.8	−30.5	−5.4		
NP2(V24E)-ImH	35, 7.0	A	His/ImH	0.6	26.4	2.9	16.0	−3.4	−52.5	−7.4	−31.1	−1.90	35
		B		12.8	13.8	14.2	2.5	−20.8	−33.2	-----	------		
NP1-Hm	20, 7.0	A	His/Hm	−1.3	29.3	13.7	13.8	−1.6	−64.1	−27.0	−39.1	−2.03	31
		B	12.9	13.9		13.4	39.1	−21.8	−39.1	−28.2	−5.4		
NP1-CN	20, 7.0	A	His/CN^-	10.8	16.5	13.4	7.3	−22.7	------	−27.9	−21.3	−1.15	31
		B		8.2	19.8	7.3	12.9	−18.8	------	−21.3	−26.8		
NP4-CN	20, 7.0	A	His/CN^-	10.2	16.9	12.9	7.7	−22.2	------	−27.6	−22.8	−1.07	31
		B		8.6	19.4	7.9	12.5	−19.5	------	−22.3	−26.2		
NP2-CN	30, 7.0	A	His/CN^-	9.3	15.8	14.2	7.9	−18.2	−34.9	−20.5	−20.2	−1.28, −0.62	31
		B		7.9	18.4	7.8	13.4	−19.6	−34.6	−20.2	------		
$Fe^{III}PPIX(ImH)_2$	RT, DMSO		ImH/ImH	14.1	11.8	18.0	18.4	−32.1	−26.3	−41.4	−42.0	−2.41	25
$[(o\text{-}CONMe_2)_1TPPFe(NMeIm)_2]^+$	25, $CDCl_3$			−13.6	−13.6	−20.7	−21.3	72	72	119	120	−6.85	[a]
[OMTPCorrFeCl]	25, CD_2Cl_2			46.4	9.7	20.9	30.6	−93.3	+17.4	−12.5	−32.7	−3.17	37

[a] Yang, F. Unpublished.

where ρ_C is the spin density on the carbon which is part of the π system, to which the methyl group is bound, $Q_H \sim +70-75$ MHz for methyl groups attached to sp^2-hybridized carbons that are part of the π system,[44,45] and

$$a_C = Q_C\rho_C/2S \text{ for } ^{13}C \quad (4)$$

where ρ_C is the spin density on the carbon which is part of the π system, to which the methyl group is bound, $Q_C \sim -39$ MHz for sp^3-hybridized carbons that are bound to sp^2-hybridized carbons that are part of the π system,[18,46,47] with a ligand-centered pseudocontact shift for ^{13}C that is linearly related to the ^{13}C contact shift.[24,42]

For systems in which there is a very small difference in the chemical shifts of the pyrrole hydrogen resonances, such as the case of the $[2\text{-ClTPPFe(CN)}_2]^-$ complex[36] shown in Figure 2 (overall ^1H range = 1.6 ppm), the diamagnetic chemical shifts of the pyrrole hydrogen and carbon resonances, which must differ because of the substituent effect of the 2-chloro group, probably contribute to masking of a clear linear correlation of the H−C cross peaks for this complex and for other $[2\text{-XTPPFe}^{III}(CN)_2]^-$ where the ^1H chemical shift range is significantly larger[36] because there still is not a linear correlation of the ^1H and ^{13}C chemical shifts. This is probably due to the additional contributions to the carbon diamagnetic shifts because of the substituent and to the ligand-centered pseudocontact contributions to the paramagnetic shifts (eq 2).[24] However, for phenyl-substituted Fe^{III}TPP bis(imidazole) complexes, which have sufficiently large spreads of the β-pyrrole hydrogen and carbon resonances, linear correlations are observed, but with much steeper slopes, as shown in Figure 3, as will be discussed further below. We have observed these linear correlations for methyl resonances of $S = {}^1/_2$ ferrihemin complexes, as well as for the $S = 1$ iron corrolate complex shown in Figure 4, where the slope is approximately double that for a $S = {}^1/_2$ system (Table 1).

For the His/Met-coordinated ferriheme centers of horse cytochrome *c*, cyanobacterial cytochromes c_6, and bacterial cytochrome *c*-551, the His/His-coordinated cytochromes c_3, bis-His met-Hbs, cytochrome b_5, and the cyanide complexes of myoglobin and bacterial heme oxygenases, as well as the azide complexes of the latter, the observed slope of this line is typically −1.8 to −2.2. This slope appears to be a hallmark of the $(d_{xy})^2(d_{xz},d_{yz})^3$ electron (or $d_\pi{}^1$ hole) configuration of the ferriheme center because His/Met- and His/His-coordinated ferriheme complexes invariably have this ground state. The same is true for the imidazole and histamine complexes of the nitrophorin proteins, as summarized in Table 1, although the range in the slope is somewhat larger (−1.7 to −2.3).[35] This larger range is undoubtedly because of the strongly nonplanar nature of the nitrophorin hemes,[48,49] which tends to mix in some d_{xy} character.[31] If only the contact shift of eqs 1 and 2 were to contribute, then according to eqs 3 and 4, the ratio $Q_C/Q_H = -0.52$ to −0.56. When corrected for the difference in the magnetogyric ratio of ^1H and ^{13}C ($Q_C\gamma_H/Q_H\gamma_C$), the ratio of $\delta^C{}_{con\pi}/\delta^H{}_{con}$ is predicted to be −2.08 to −2.24, which is very close to what is observed for the slope of the lines for the majority of $S = {}^1/_2$ ferriheme complexes (see Table 1).

Although the correlation and actual slopes of many of the lines are, in fact, between −1.8 and −2.2, the steeper slope of the $[(o\text{-CONMe}_2)_1\text{TPPFe(NMeIm)}_2]\text{Cl}$ complex (−6.85) suggests that this case of β-pyrrole carbons and their directly bound protons behaves with a different relationship than is present for methyl groups (eqs 3 and 4). This is, in part, due to the fact that the Q values are somewhat different in this case, but there are more differences to be considered; in fact, the much greater slope for this model complex is due to the combination of three factors: (1) the direct one-bond relationship between the carbon of the π system and the pyrrole protons of interest,[50] (2) the substituent effect of the single *o*-dimethylamide on one phenyl ring,[45] and (3) the dynamics of the complex.[51] The Q_C value for an sp^2-hybridized carbon that is part of an extended π system is +54.6 MHz,[50] and the Q_H of a proton directly attached to this sp^2-hybridized carbon is −65.8 MHz,[50] which together yield a predicted slope $\delta^C{}_{con\pi}/\delta^H{}_{con}$ of −3.32. However, instead of a slope of −3.32, one that is approximately double that value, −6.85, is observed. This is a result of the fact that the dimethylamide substituent on one ortho position of one phenyl group of the TPP provides a substituent effect that makes the chemical shifts of the pyrrole protons different from each other *and also* hinders, but does not completely prevent, the rotation of the *N*-methylimidazole ligand bound on that side of the heme plane.

Typically, in model heme complexes, the planar axial ligands rotate extremely rapidly, on the order of hundreds of thousands to a million times per second;[52,53] this makes model heme complexes very different from heme proteins, where axial ligands are held in fixed orientations by the design of the protein. This rapid rotation of model heme axial ligands averages the differences in pyrrole hydrogen chemical shifts to the differences observed for such complexes due to the substituent effect of a unique substituent (up to 3 ppm).[45] However, the bulky dimethylamide substituent was designed to hinder the rotation of one of the *N*-methylimidazole ligands in order to simulate the fixed position of axial ligands in proteins. However, although its rotation is slowed considerably, it is not stopped, which gives the pyrrole protons quite variable chemical shifts as the temperature is changed.[50] As shown in the lower inset of Figure 5, at low temperatures a total of seven resolved resonances of the eight protons are resolved, which collapse to three at the highest temperature studied in the original work at 300 MHz.[50] As the temperature is raised, the slope decreases but does not reach the predicted $\delta^C{}_{con\pi}/\delta^H{}_{con}$ of −3.32 by temperatures near the boiling point of the solvent. Hence, model heme complexes, even with bulky substituents, cannot fully simulate the major effect of the fixed orientation of one axial ligand (or both in the case of cytochromes b_5, *c*, and c_3 and the cyanobacterial methemoglobins) discussed above. The methyls of the

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bis(imidazole) complex of iron(III) protoporphyrin IX,[25] however, where the ligands can spin very rapidly, shows a slope δ^C/δ^H only slightly larger than the expected slope, of −2.41. This slightly larger slope is probably due to the substituent effect of the two vinyl groups, which lead to somewhat different diamagnetic shifts for the 1M and 3M and the 5M and 8M.

In contrast to the typical $\delta^C_{con\pi}/\delta^H_{con} \sim -2.0$ for the His/His, His/Met, and His/cyanide-bound complexes of most proteins, for the cyanide complexes of the nitrophorins, the slope is typically −0.8 to −1.4,[31,33,35] which appears to be a hallmark of the $(d_{xz},d_{yz})^4(d_{xy})^1$ electron configuration of the ferriheme center.[31] {There are no other ferriheme protein systems to which the nitrophorins can be compared, because most hemin cyanide complexes of α-helical heme proteins do not have the $(d_{xz},d_{yz})^4(d_{xy})^1$ electron configuration or at best exhibit a mixed electronic ground state.[31] This is because most ferriheme proteins do not have ruffled hemes; a ruffled distortion of the ferriheme has been shown to be required to stabilize the $(d_{xz},d_{yz})^4(d_{xy})^1$ electron configuration of the ferriheme center,[31,54,55] because only when there is a ruffling distortion of the heme does a d_{xy} unpaired electron on iron have the proper symmetry to accept π donation from a filled π orbital on the porphyrin [the $a_{2u}(\pi)$ orbital].} In this electronic ground state, the majority of the spin density is on the *meso*-carbons, and we have not as yet detected either the proton or the carbon signals of the *meso*-carbons of the cyanide complexes of the nitrophorins. Thus, the much smaller amount of spin density present at the methyl groups of the protohemin macrocycles of these cyanide complexes is more prone to variations in the diamagnetic and metal-centered pseudocontact shifts of eqs 1 and 2 than are the larger spin densities of the His/His and His/Met-coordinated heme proteins.

Comments about specific low-spin ferriheme protein systems that should be made include the following:

1 The 3M cross peak often deviates from the linear correlation, as seen in Figures 5−7 but not in Figure 1 or Figure 8. For Figure 1, the four hemes each have cross peaks that deviate from the best line, but while points 4, 10, 12, and 17 are the four 3M cross peaks, points 3, 7, 9, and 14 are the four 5M cross peaks and point 15 is a 1M cross peak.

2 Although we first noticed that there was a correlation between ^{1}H and ^{13}C shifts of methyl groups for the nitrophorins, they actually follow this correlation more poorly than do other heme proteins, probably in large part because they are significantly ruffled and also because dynamics prevent several important cross peaks from being observed. Ruffling not only creates the $(d_{xy})^1$ ground state, but it also changes the pseudocontact shifts by reversing the sign ($g_{\|} < g_{\perp}$) and decreasing the magnitude of the magnetic anisotropy to $\sim^1/_4$ or less of its magnitude for the $(d_{xz},d_{yz})^3$ ground-state systems and also causes a very slightly variable distance between iron and the methyl groups. In that respect, 3M is in a different geometrical position with respect to the propionate groups than are 1M, 5M, and 8M, and thus its chemical shifts are expected to be differently altered by the nonplanarity of the heme. In addition, the effects of macrocycle ruffling on the diamagnetic shifts in the absence of the half-filled d_{xy} orbital are unknown and may well be variable. The histamine and imidazole complexes tend to follow a linear correlation better than do the cyanide complexes, in line with the fact that the cyanide complexes are the most nonplanar and have a large degree of $(d_{xy})^1$ character to the orbital of the unpaired electron.[31]

3 Although the plots for the bacterial heme oxygenase−cyanide (HO−CN) complexes are more linear than those of the cytochromes b_5 and c and the nitrophorins, the corresponding azide complexes ($HO-N_3$) have smaller ^{13}C shifts compared to the others, which appears to make the azide complexes stand out as different from the cyanide complexes. This adds strength to the conclusion that the spin state for the azide complexes is not the same as that of the cyanide complexes; the authors had concluded that the azide complexes had a major contribution from an $S = {}^3/_2$ spin-state species.[13]

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