The Effect of Axial Ligand Plane Orientation on the Isotropic Shifts of the Dichelated Protohemin Cyanide Complexes of Traylor and Berzinis

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Received July 1, 1999

In 1980 Traylor and Berzinis published the NMR spectrum of the mixture of two geometrical isomers of the dichelated protohemin cyanide prepared from the 6,7-bis-(3-*N*-imidazolylpropyl)propionamide of protohemin (*J. Am. Chem. Soc*. **1980**, *102*, 2844). We have prepared these same isomers and investigated their ^{1}H NMR spectra by modern 1- and 2D NMR techniques. The results show that the eight pyrrole−methyl resonances having a spread of 26.5 ppm at −57 °C divide into two sets of four widely spaced pyrrole−methyl resonances that have shifts 3,8 ≫ 5,1 for the isomer having the 6-substituted imidazole bound (isomer **I**), and 5,1 ≫ 3,8 for the isomer having the 7-substituted imidazole bound (isomer **II**). NMR techniques not available in 1980 (NOESY/EXSY, COSY, ROESY, DQF-COSY) have allowed nearly complete assignment of the NMR spectra, except for several *meso*-H resonances and the resonances of some of the protons of the alkyl side chains of each isomer, which all fall in a narrow chemical shift range in the diamagnetic region. The imidazole planes are found not to be lying directly over the porphyrin nitrogens, but are shifted by about 10° counterclockwise with respect to that line for isomer **I**, which yields the order 3 > 8 ≫ 5 > 1, and 10° clockwise of that line for isomer **II**, which yields the order 5 > 1 ≫ 3 > 8.

Introduction

For quite some time it has been known that the orientation of planar axial ligands determines the pattern of heme methyl and other substituent shifts in heme proteins. One of the first proofs of this dependence of heme methyl shifts on the orientation of planar axial ligands was provided by Traylor and Berzinis.[1] These authors studied the NMR spectra of the mixture of two geometrical isomers of the dichelated protohemin cyanide complexes prepared from the 6,7-bis-(3-*N*-imidazolylpropyl)-propionamide, **1**, and the 6,7-bis-(4-*N*-imidazolylbutyl)propionamide, **2**, of protohemin.[1] These complexes showed eight methyl resonances, four each from the 6- and 7-coordinated imidazole isomers of these mono-cyano, mono-imidazole-coordinated hemins. For **1** in particular, the spread of these eight resonances was quite large, namely, at least 17 ppm at room temperature,[1] a spread almost as large as observed for several heme proteins, including metmyoglobins cyanide,[2−4] hemoglobin cyanide,[5] insect hemoglobin cyanides,[6] and horseradish peroxidase cyanide.[7,8] Thus, as concluded by Traylor and Berzinis,[1] the orientation of the axial ligand plane is very important in determining the spread of the resonances. This work has been quoted by numerous authors ever since 1980, as evidence of the importance of axial ligand plane orientation on the spread of the methyl shifts of ferriheme proteins. However, at the time when Traylor and Berzinis published this work,[1] NMR methods were not routinely available that would allow assignment of the resonances, and the eight methyl peaks shown in Figure 2 of ref 1 have not previously been assigned. We have thus prepared the isomers of **1** once again and investigated their ^{1}H NMR spectra by modern 1- and 2D NMR techniques that have allowed us to assign the majority of the resonances, except for those of the CH_2 protons beyond the β-CH_2 of the 6- and 7-propionamides of each isomer and some of the *meso*-H resonances. We find that each isomer has a set of four widely spaced methyl resonances whose order and spread of chemical shifts is totally consistent with predictions based upon the orientation of the axial imidazole plane of each isomer being displaced by about 10° from the N−Fe−N axis of the pyrrole ring containing the propionate that provides the imidazole ligand.

Experimental Section

Reagents. Protohemin chloride was purchased from Mid-Century Chemicals; 1-(3-aminopropyl)imidazole was obtained from Aldrich. Solvents, triethylamine, KCN, NaCN, and other reagents were purchased from Fisher Chemical Company. NMR solvents (DMSO-d_6, DMF-d_7, D_2O) were purchased from Cambridge Isotopes Laboratories.

Synthesis. The dichelated protohemin chloride complex, precursor of the cyanide complex **1**, was prepared by a modification of the procedures reported by Traylor et al.[9,10] To a three-neck round-bottom flask equipped with a condenser, a rubber septum, a magnetic stirring bar, and an argon atmosphere were added 20 mL of freshly distilled $CHCl_3$ and 0.6 g of protohemin chloride. The flask was cooled in an ice bath, and 2 equiv of triethylamine was added via syringe over 10

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10.1021/ic990785d CCC: $18.00
Published on Web 11/20/1999

min. Next, 2 equiv of pivaloyl chloride was added slowly over a period of 30 min, followed by 2 equiv of 1-(3-aminopropyl)imidazole, which was added over a period of 20 min. After removal of the volatiles using a rotary evaporator, the product was chromatographed on TLC plates using 9:1 $CHCl_3$/MeOH. TLC revealed three spots, the diamide, monoamides, and unreacted protohemin. To separate the product from the side products and reactant, column chromatography was performed using silica gel as a solid support. The column was packed using 9:1 $CHCl_3$/MeOH; elution with this solvent provided the side products first, followed by the diamide, which eluted with a solvent mixture of 9:4 $CHCl_3$/MeOH. The solution was evaporated to dryness, redissolved in CH_2Cl_2, and treated with an aqueous solution of saturated NaCl to which a drop of concentrated aqueous HCl had been added, to convert the dichelated protohemin to the chloroiron form. The aqueous layer was removed and the sample evaporated to dryness. The monocyanide complexes, **1**, were produced by careful titration of 1 equiv of a saturated solution of KCN/D_2O into a DMSO-d_6 solution, or a NaCN/DMF-d_7 saturated solution into a DMF-d_7 solution of the dichelated protohemin, by following the change in the NMR spectrum as cyanide was added.

NMR Spectroscopy. 1- and 2D ^{1}H NMR spectra of **1** in DMSO-d_6 and DMF-d_7 were obtained at 300 MHz on a Unity-300 NMR spectrometer operating at 299.995 MHz. All 2D spectra were collected with 512−1024 t_2 data points and 128−160 t_1 increments. Usually, 80−256 transients were collected per t_1 increment. Phase sensitive NOESY/EXSY spectra of **1** in DMSO-d_6 were recorded at 35 °C over a bandwidth of 10 kHz using the standard pulse sequence. The recycle delay was 200 ms, and the mixing time was 80 ms. These data were processed using an unshifted Gaussian apodization function in both directions, and zero-filled to 1024 × 1024 data points before Fourier transformation. Low-temperature experiments: NOESY/EXSY, COSY, and ROESY spectra of **1** in DMF-d_7 were obtained at −57 °C. The spectra were acquired over a bandwidth of 17 kHz using standard pulse sequences. For the ROESY experiment a spin-lock field of 12 kHz was used during the mixing time of 60 ms. NOESY/EXSY and ROESY data were processed as above. COSY data were processed with an unshifted sine bell squared apodization function in both dimensions and zero-filled to give final matrices of 1024 t_1 × 1024 t_2 data points prior to Fourier transformation. Spectra were referenced to residual solvent protons.

Results and Discussion

Room Temperature NMR Data. The 1D and NOESY/EXSY spectra of **1** obtained at 35 °C are shown in Figure 1. The chemical exchange cross peaks arise from the exchange between methyl, vinyl, and propionate groups located at the same position of the two different geometrical isomers. There are four pairs of methyl resonances, labeled 1M to 8M in order of decreasing chemical shift: 1M (20.4 ppm) ↔ 5M (10.7 ppm), 2M (19.2 ppm) ↔ 6M (8.1 ppm), 3M (18.4 ppm) ↔ 7M (7.6 ppm), and 4M (14.2 ppm) ↔ 8M (4.8 ppm). There are also two pairs of vinyl β-H resonances labeled a−d in the 0 to −6 ppm region, with a ↔ c and b ↔ d. These are correlated to their respective vinyl α-H V_a−V_d by a DQF-COSY spectrum (not shown) to yield the same connectivities as reported by Traylor and Berzinis,[1] based upon 1D decoupling experiments. Consistent with this, the vinyl α-H show chemical exchange cross peaks V_a ↔ V_c and V_b ↔ V_d in Figure 1. Additional chemical exchange cross peaks between propionamide protons are also seen in Figure 1: 14.2 ↔ 3.8, 9.1 ↔ 7.1, 11.2 ↔ 2.1-(2), 6.4 ↔ 1.7(2), 1.0 ↔ 0.0(2). Some of these propionate protons are also correlated by the DQF-COSY spectrum (not shown).

It is obvious from the large differences in isotropic shifts between methyl groups located at the same ring position that the spin density distributions of the two isomers of **1** are drastically different. The only difference between the two geometrical isomers is the angle of orientation of the axial imidazole ligand. Therefore, it is clear that the degeneracy-lifting effect of the axial ligand plane is responsible for the differing isotropic shifts of exchanging methyl groups, as discussed in ref 1. As illustrated by the electron density distributions of the eight methyl resonances seen in the 1D NMR spectrum of the imidazole chelated protohemin **1**[1] shown in Figure 2, the four resonances which are the most isotropically shifted are expected to belong to methyl groups at pyrrole positions 3 and 8 of isomer **I** and 1 and 5 of isomer **II**. This is because molecular models (CPK) indicate that the dihedral angle of the imidazole plane of isomer **I** should be 90 ± 20° with respect to the *x*-axis of Figure 2, while that of isomer **II** should be 0 ± 20° with respect to that axis. The molecular models also indicate that there could be some fluxionality in the axial imidazole for each isomer, and that although the ligand planes of the two isomers are expected to have the average orientations just mentioned, there is likely to be some variation about that average orientation.

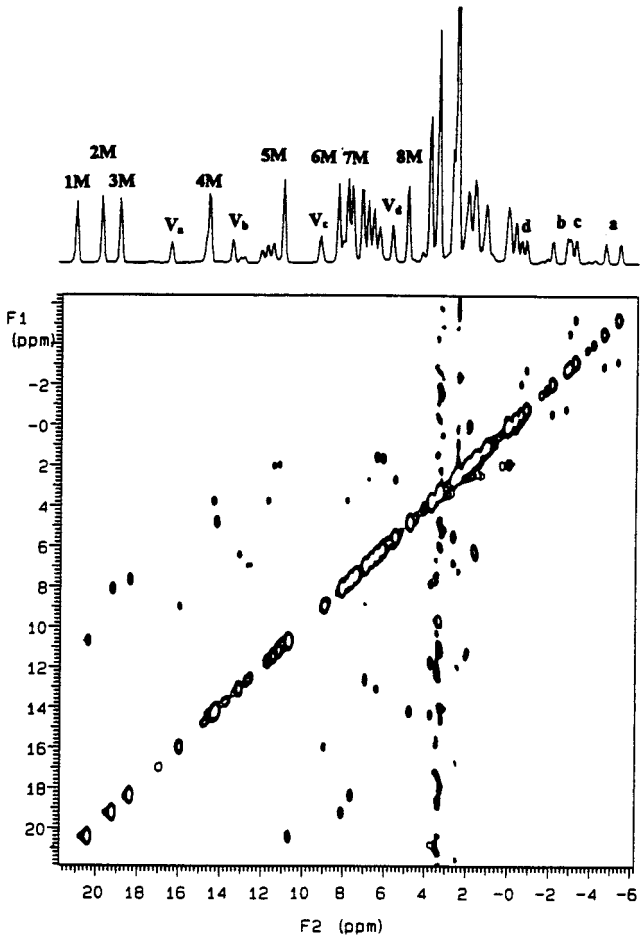

Figure 1. 1D and NOESY/EXSY spectrum of the dichelated protohemin cyanide isomers recorded at 300 MHz in DMSO-d_6 at 35 °C. Note the strong chemical exchange cross peaks that result from exchange of the 6- and 7-imidazole groups and thus link the resonances of one isomer to their partners in the other isomer. As is evident, heme methyl resonances are linked 1M ↔ 5M, 2 ↔ 6, 3 ↔ 7, and 4 ↔ 8 by chemical exchange.

It has been shown that, for many naturally occurring heme proteins, the order of methyls in the 3,8 pair can be switched as a function of ligand plane orientation, but for methyls in the 1,5 pair the order is always the same, with the isotropic shift of the methyl at position 5 always greater (3−5 ppm or more) than that at position 1.[11] On the basis of this finding and the

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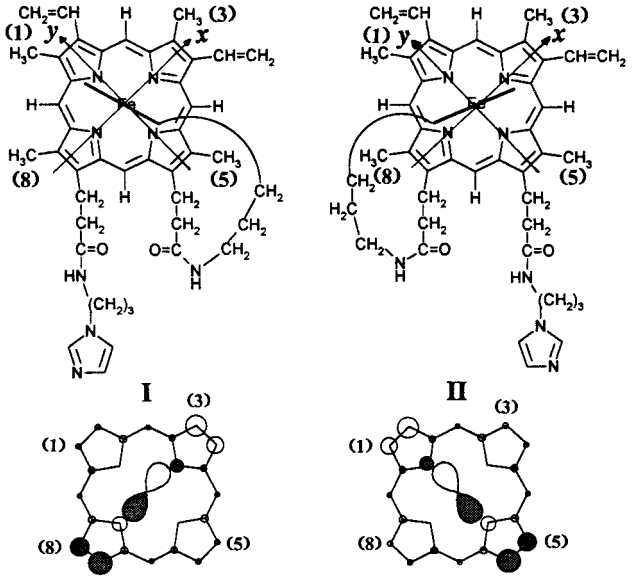

Figure 2. Geometrical isomers **I** and **II**, showing the numbering system for the heme methyls and the relative expected amounts of spin density at the pyrrole carbon to which each methyl is attached. Dihedral angles of axial ligand planes are measured counterclockwise from the *x*-axis.

expected orientations (±20°), there are six plausible assignments for the two sets of four methyl peaks in terms of their β-pyrrole positions: 8 > 5 > 3 > 1, 8 > 3 > 5 > 1, 3 > 8 > 5 > 1, 5 > 1 > 8 > 3, 5 > 1 > 3 > 8, and 5 > 3 > 1 > 8.

Low-temperature NMR experiments were required to determine which of these possibilities is the correct assignment. At low temperature, chemical exchange between geometrical isomers is very slow on the NMR time scale, so that NOESY experiments should show only NOE connectivities. However, the resonances seen in the 1D spectrum at −57 °C are shifted so dramatically from their positions at 35 °C that definitive assignment required additional low-temperature experiments such as COSY, which would give information about vinyl protons and propionamide−imidazole chain proton positions. A ROESY experiment was also carried out at −57 °C to see if there was any detectable chemical exchange.

Figure 3 shows the COSY (below the diagonal) and NOESY (above the diagonal) spectra of **1** in DMF-d_7 obtained at −57 °C. One can see the connectivity between the vinyl α-H and β-*cis*-H for all four vinyl groups of the two isomers. The assignments are summarized in Table 1. The ROESY spectrum obtained at −57 °C (not shown) confirmed that chemical exchange could not be detected.

The NOESY map obtained at −57 °C, Figure 3, above the diagonal, shows that there are NOEs between 1M and β-vinyl b, between 4M and β-vinyl a, and between 5M and β-vinyl d that identify these methyls as either β-pyrrole methyl-3 or methyl-1 in one or the other of the geometrical isomers. Combining this with the chemical exchange information derived from Figure 1 and the expectation, mentioned above, that 5-CH_3 should always have a larger chemical shift than 1-CH_3, it is clear that 4M must be a 1-CH_3 resonance, and thus 1M and 5M must be 3-CH_3 resonances. Because of chemical exchange between 4M and 8M, the latter must be the other 1-CH_3 resonance. Thus, one isomer must have the methyl resonance order 3 > *x* > *x* > 1, and unless both isomers have the *same* order, with different average resonance values (a *highly* unlikely possibility[11]), 1M (3-CH_3) and 8M (1-CH_3) belong to the same isomer, **I**, while 4M (1-CH_3) and 5M (3-CH_3) belong to isomer **II**. Thus, there are only two possible assignments of the methyl

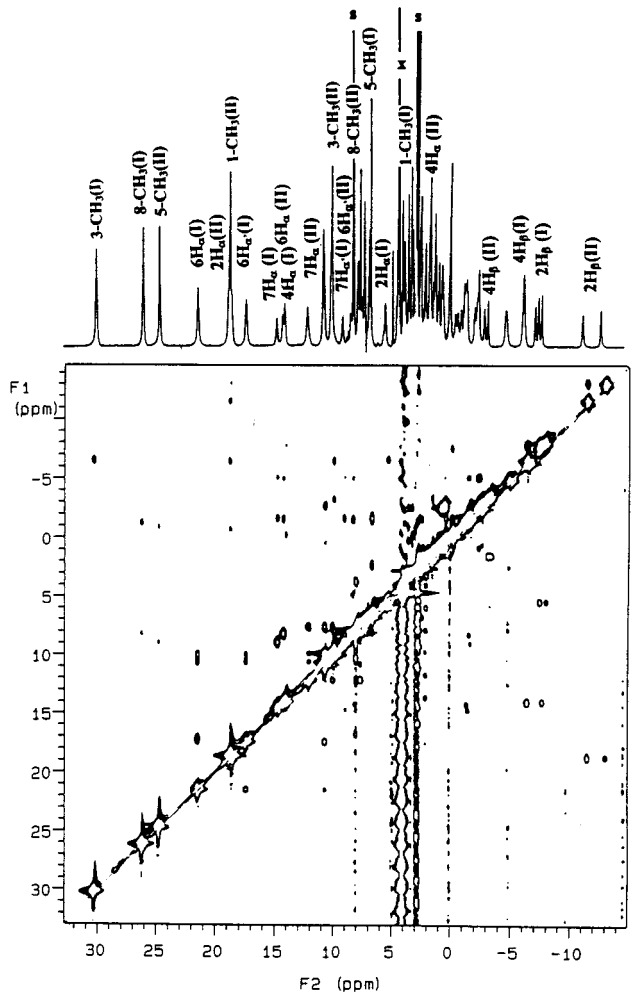

Figure 3. 1D and 2D NOESY and COSY spectra of **1** at −57 °C in DMF-d_7. The COSY spectrum (below the diagonal) shows the connectivities between α-H and β-H within each vinyl group. The NOESY spectrum (above the diagonal) shows NOEs between 3-CH_3-(**I**) (30.2 ppm) and 4H-β_t(**I**) (−6.4 ppm); 5-CH_3(**II**) (18.4 ppm) and 6H$_{\alpha'}$(**II**) (9.0 ppm); 1-CH_3(**II**) (18.6 ppm) and 2H-β_t(**II**) (−11.5 ppm); 3-CH_3(**II**) (9.9 ppm) and 4H-β_t(**II**) (−3.1 ppm); 8-CH_3(**I**) (26.1 ppm) and 7H$_{\alpha'}$(**I**) (8.1 ppm).

resonances:[12,13] 3(**I**) > 5(**II**) > 8(**I**) > 1(**II**) > 3(**II**) > 5(**I**) > 8(**II**) > 1(**I**) and 3(**I**) > 8(**I**) > 5(**II**) > 1(**II**) > 3(**II**) > 8(**II**) > 5(**I**) > 1(**I**). In both cases, the order 3 > 8 ≫ 5 > 1 is predicted for isomer **I** and 5 > 1 ≫ 3 > 8 for isomer **II**, and the average methyl shift is very similar (16.9 vs 16.7 ppm and 15.3 vs 15.5 ppm, respectively, for isomers **I** and **II**). Also in both cases, the approximate chemical shifts are predicted from the plot of Figure 5 of ref 11 if the dihedral angle of the 6-imidazole plane from the *x*-axis of Figure 2 is 100° for isomer **I** and that for the 7-imidazole plane is 170° for isomer **II**, i.e., 90° + 10° and 0° − 10°, respectively. However, this plot was constructed assuming equal substituent effects for the 5- and 8-methyl groups,[11] which, for the angles of 100° and 170°, predicts the same chemical shifts for the 5-CH_3 of one isomer and the 8-CH_3 of

(12) In a recent chapter on NMR of paramagnetic metalloporphyrins,[13] it was stated that only one unique assignment was possible (the first of the two listed). However, since that chapter was printed, it has become clear that there are two possible assignments, as listed, and that the second is most likely correct.

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Table 1. Dichelated Protohemin Cyanide Peak Assignments for Isomers **I** and **II** in DMSO-d_6 at 35 °C and in DMF-d_7 at −57 °C, with Their Ligand Plane Orientations as Listed

	chemical shift (ppm)					
	isomer **I** (100°)			isomer **II** (170°)		
proton	35 °C	−57 °C		35 °C	−57 °C	
1-Me	4.8	3.7		14.2	18.6	
3-Me	20.4	30.2		10.7	9.9	
5-Me	7.6	7.4		18.4	24.7	
8-Me	19.2	26.1		8.1	8.0	
meso-β		−0.8			−1.0	
meso-δ		−1.4				
2-H_α	9.0	5.1		15.9	18.7	
2-$H_{\beta c}$	−3.2	−8.0		−5.2	−13.0	
2-$H_{\beta t}$	−2.9	−7.4		−4.5	−11.5	
4-H_α	13.1	13.9		6.4	1.6	
4-$H_{\beta c}$	−2.8	−7.7		−0.8	−3.4	
4-$H_{\beta t}$	−2.0	−6.4		−0.6	−3.1	
6-H_α		12.0			14.6	
6-$H_{\alpha'}$		7.6	bound		9.0	free
6-H_β		10.7			−1.5	
6-$H_{\beta'}$		10.0			−5.0	
7-H_α		13.9			21.4	
7-$H_{\alpha'}$		8.1	free		17.3	bound
7-H_β		−1.4			10.8	
7-$H_{\beta'}$		−4.9			10.1	

the other, and the same in return for the 8- and 5-CH_3. And while the chemical shifts of 2M and 3M are similar to each other, as are those of 6M and 7M, the two resonances of each group are certainly not superimposed.

Although it should be possible to identify the 8-CH_3 resonance in each isomer by its NOE to the same *meso*-H as is observed for the 1-CH_3 resonance of the same isomer, the critical NOEs to the 1-CH_3 of **I** and the 8-CH_3 of **II** are obscured by t_1 noise. Thus, we can only assign the 5- and 8-CH_3 resonances of each isomer on the basis of the relative substituent effect observed for dicyanoprotohemin (no axial ligand planes) in methanol,[14] dimethyl sulfoxide[15] or ethylene glycol,[16] 8 > 5 in each case. On this basis, the second assignment of the eight methyl resonances, 3(**I**) > 8(**I**) > 5(**II**) > 1(**II**) > 3(**II**) > 8(**II**) > 5(**I**) > 1(**I**), is almost certainly the correct one. Consistent with this, of the *meso*-H resonances that can be assigned, β(**I**) > δ(**I**), as has been observed previously for dicyanoprotohemin.[15,16]

Intense NOE cross peaks between the methyl in the 5 position of isomer **II** and the 6-α-CH_2 group (9.0 ppm) of the propionamide part of the free imidazole chain and between the methyl in the 8 position of isomer **I** and the 7-α-CH_2 group (8.1 ppm) of the propionamide part of the free imidazole chain were found in the NOESY map, Figure 3, above the diagonal. These NOEs were the key to assignment of the CH_2 (both α and β) of the propionamide part of the imidazole chains. For the free imidazole chains (7(**I**) and 6(**II**)) the chemical shifts of the protons in both α- and β-CH_2 positions are very similar for the two isomers, while for the bound imidazole chains the chemical shifts of protons in the α-CH_2s are different for the two isomers, but the protons in the β-CH_2s have the same chemical shifts (Table 1). For the free imidazole chains, the similar α-CH_2 shifts suggest similar average Karplus angles for the individual protons of each isomer, while for the bound imidazole chains the different α-CH_2 shifts for the two isomers indicate different spin densities for the individual protons of each isomer, but likely similar Karplus angles. The other CH_2 protons (of the propylimidazole moieties) of both free and bound imidazole chains overlap strongly in the diamagnetic region of the NMR spectrum and could not be assigned.

The assignments of all methyl, vinyl, and propionate portions of the four imidazole chains of **1** accomplished by low-temperature NOESY and COSY spectra, as well as the NOESY/EXSY spectrum obtained at 35 °C, are listed in Table 1. These data, together with the predicted patterns of methyl shifts for isomers **I** and **II** (Figure 2, lower section), identify the isomer with methyl shift pattern 3 > 8 ≫ 5 > 1 as **I** and that with methyl shift pattern 5 > 1 ≫ 3 > 8 as isomer **II**. In each case, the total spread of the methyl resonances is large (26.5 and 16.7 ppm, respectively, at −57 °C and 15.6 and 10.3 ppm, respectively, at 35 °C) and is quite comparable to those of heme proteins, as originally suggested by Traylor and Berzinis.[1] The significant difference in spread of the methyl resonances of **I** and **II** is totally consistent with spin density predictions due to the unsymmetrical nature of protohemin[11] and is similar to those of the cyanide complexes of lignin peroxidase for isomer **I** (3 > 8 > 5 > 1, dihedral angle = 120°,[17] methyl resonance spread = 29.3 ppm[8]) and methemoglobin α and β chains for isomer **II** (5 > 1 > 8 > 3, dihedral angle = 178°,[18] methyl resonance spread = 12.9 ppm[5]). Thus, the cyanide complexes of the two geometrical isomers of the dichelated protohemin **1** are excellent models for the ferriheme proteins, where histidine imidazole ligands are held in fixed positions by a combination of covalent attachment to the protein backbone, hydrogen bonding of the N−H of the imidazole ring to backbone amide carbonyls, and, possibly, steric crowding by other protein side chains.

Acknowledgment. Financial support from the National Institutes of Health, Grant DK-31038, and the University of Arizona Materials Characterization Program is gratefully acknowledged.

IC990785D

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