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^{13}C NMR Spectroscopy of Core Heme Carbons as a Simple Tool to Elucidate the Coordination State of Ferric High-Spin Heme Proteins

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Evidence is presented demonstrating that the magnitudes of the ^{13}C chemical shifts originating from heme meso carbons provide a straightforward diagnostic tool to elucidate the coordination state of high-spin heme proteins and enzymes. Pentacoordinate high-spin heme centers exhibit ^{13}C meso shifts centered at approximately 250 ppm, whereas their hexacoordinate counterparts exhibit ^{13}C shifts centered at approximately –80 ppm. The relatively small spectral window (400 to –100 ppm) covering the meso-^{13}C shifts, the relatively narrow lines of these resonances, and the availability of biosynthetic methods to prepare ^{13}C-labeled heme (Rivera, M.; Walker, F. A. *Anal. Biochem.* **1995**, 230, 295–302) make this approach practical. The theoretical basis for the distinct chemical shifts observed for meso carbons from hexacoordinate high-spin hemes relative to their pentacoordinate counterparts are now well understood (Cheng, R.-J.; Chen, P. Y.; Lovell, T.; Liu, T.; Noodleman, L.; Case, D. A. *J. Am. Chem. Soc.* **2003**, *125*, 6774–6783), which indicates that the magnitude of the meso-carbon chemical shifts can be used as a simple and reliable diagnostic tool for determining the coordination state of the heme active sites, independent of the nature of the proximal ligand. Proof of the principle for the ^{13}C NMR spectroscopic approach is demonstrated using hexa- and pentacoordinate myoglobin. Subsequently, ^{13}C NMR spectroscopy has been used to unambiguously determine that a recently discovered heme protein from *Shigella dysenteriae* (ShuT) is pentacoordinate.

Introduction

Rapid elucidation of the coordination and spin state is desirable because the advent of genomics and proteomics has brought about the discovery of new and interesting heme proteins. Many of these new proteins are thought to act as heme transporter, heme degrading, or heme storage proteins and enzymes.[1−4] Detailed understanding of how these proteins acquire heme, bind it tightly, or pass it on to another protein, often starts with an understanding of the heme coordination state and heme electronic structure. Novel heme proteins exhibiting a high-spin state can be either five-coordinate, with only one protein-provided axial ligand, or six-coordinate, with one strong-field axial ligand and a weak-field sixth ligand that can be protein provided or exogenous.

Spectroscopic elucidation of the coordination state of high-spin heme proteins as five- or six-coordinate is not straightforward. In this context, it is noteworthy that recent studies have shown that the study of core porphyrin carbon chemical shifts is a powerful approach to determine the electronic structure of model ferrihemes[5−11] and heme active sites.[11−14]

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Observation and assignment of core porphyrin carbons can be more challenging than the observation and assignment of their protonated counterparts because the proximity of core carbons to the heme iron makes their resonances more strongly affected by the unpaired electron. Nevertheless, efforts to detect, assign, and interpret core porphyrin carbon chemical shifts can be highly rewarding because relatively straightforward correlations permit one to gain valuable insights into the coordination state and electronic structure of ferrihemes.[12,15] In this report, we demonstrate that detection of core porphyrin carbon resonances from high-spin ferriheme active sites can be made in a relatively straightforward manner and that the magnitudes of these shifts lead to unambiguous determination of the coordination state.

It has been known for quite some time that the most striking difference in the ^{1}H NMR spectra of five- and six-coordinate high-spin ferrihemes is the reversal in sign of the meso-H isotropic shift.[16−18] Thus, 6-coordinate ferrihemes exhibit meso-H resonances near 40 ppm, whereas the equivalent resonances in 5-coordinate ferrihemes occur near −25 ppm. The molecular orbital properties underlying this sign reversal brought about by heme coordination state are now fully understood[19] (discussed below). However, it is important to appreciate that perhaps the most important reason that this phenomenon has not been utilized to differentiate five- and six-coordinate high-spin ferriheme centers in proteins and enzymes is the extreme breadth of the meso-H resonances. This is illustrated in Figure 1 which allows comparison of the ^{1}H NMR spectrum of wild-type met sperm-whale myoglobin (sw-Mb) (Figure 1A) and that obtained from the corresponding H64V mutant (Figure 1B). In the ferric oxidation state, the heme iron of met-sw-Mb is six-coordinate high-spin, with a proximal histidine (His-93) and a distal H_2O serving as axial ligands.[17] The distal ligand (H_2O) in Mb is hydrogen bonded to the N_ϵ atom of the distal histidine (His 64); disruption of this hydrogen bond by the replacement of His-64 for Val results in a five-coordinate high-spin met-Mb (H64V-sw-Mb) devoid of the sixth H_2O ligand.[20,21] La Mar and co-workers demonstrated that the meso-H resonance in the ^{1}H NMR spectrum of the H64V mutant (Figure 1B) is buried under the methyl resonance originating from Val E11, near −29 ppm. In comparison, the meso-H signal in the six-coordinate wild-type sw-Mb resonates near 40 ppm (Figure 1-A). It is important to point out, however, that it is not trivial to assign meso-H resonances from high-spin ferrihemes because of the fast relaxation properties typical of $S = 5/2$ ferrihemes. Hence, although the magnitude of the meso-H shift is indicative of the coordination state in high-spin ferrihemes, the challenges associated with the assignment of very broad meso-H resonances render this approach impractical as an efficient method to establish coordination state. In this context, it is well-known that the relaxation rates of resonances affected by a paramagnetic center scale with the square of the magnitude of the gyromagnetic ratio.[22,23] Hence, the effect of paramagnetism on ^{13}C relaxation is reduced ∼16-fold with respect to ^{1}H, which results in narrower and therefore more readily observable ^{13}C resonances. We capitalized on this physical property to demonstrate that meso-carbon resonances from heme proteins are readily detected, thus providing a relatively straightforward spectroscopic diagnosis of the coordination state of high-spin ferric heme active sites.

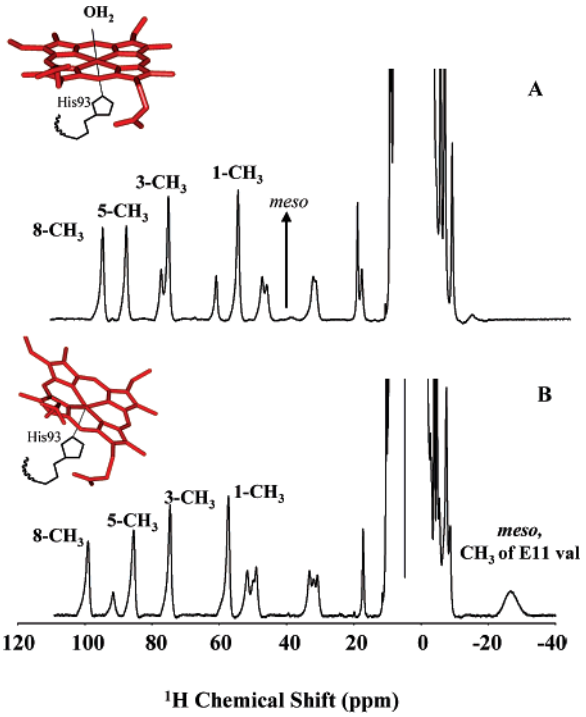

Figure 1. ^{1}H NMR spectra of hexacoordinate, wild-type sperm whale myoglobin (A) and pentacoordinate H64V sperm-whale myoglobin (B). The resonance assignments were obtained from ref 17. Note the broad line width and consequent low intensity of the meso-H resonance in A. The corresponding resonance in B is buried under the methyl group of Val E11.

Experimental Methods

General Methods. The recombinant pET 28b plasmid harboring a gene coding for wild-type sperm whale myoglobin was a generous gift from Dr. Mark Hargrove, Iowa State University. The corre-

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sponding H64V mutant gene was prepared using the Quickchange Mutagenesis Kit from Stratagene (La Jolla, CA). Oligonucleotides were synthesized at the Biotechnology Support Facility, University of Kansas Medical Center and used without further purification. Once the mutation had been confirmed by sequencing, the recombinant DNA plasmid was transformed into *Escherichia coli* BL21 (DE3) GOLD cells for subsequent protein expression.

Expression and Purification of the Wild-Type and Mutant Sperm Whale Mb Proteins. *E. coli* BL21 (DE3) GOLD cells (Stratagene, La Jolla, CA) were used as the protein expression host. A single colony of freshly transformed cells was cultured overnight in 5 mL of Luria Bertani (LB) medium containing 50 μg/mL kanamycin. The cells were subsequently subcultured into fresh 1 L LB−kanamycin medium and grown at 37 °C with continuous shaking at 225 rpm. Once the cell culture reached an optical density of 1.0, expression was induced by addition of isopropyl-β-D-thiogalactopyranose (IPTG) to a final concentration of 1 mM. Approximately 10 min after the induction of protein synthesis, 17 mg of δ-aminolevulinic acid (ALA) and 100 mg of $FeSO_4 \cdot 7H_2O$ were added to each liter of cell culture. The cells were grown for an additional 4−5 h at 30 °C and harvested by centrifugation. The harvested cells were resuspended and lysed, and the soluble protein fraction was recovered using previously described protocols.[24] Ammonium sulfate was added to the supernatant (60% saturation, 2.4 M), and the resultant mixture was centrifuged at 23 500 rpm for 1 h. The supernatant was recovered and treated with 20 mM Tris buffer (pH 8.0) to bring the concentration of ammonium sulfate to 1.9 M. The resultant solution was loaded onto a phenyl Sepharose column (2.6 cm i.d. × 30 cm length) pre-equilibrated with 1.9 M ammonium sulfate in 20 mM Tris buffer (pH 8.0) and eluted with a solution of 0.9 M ammonium sulfate in 20 mM Tris buffer (pH 8.0). Fractions with an absorbance ratio (A_{280}/A_{410}) of <1.0 were pooled, dialyzed extensively against phosphate buffer ($\mu = 0.10$, pH 6.0) at 4 °C, concentrated by ultrafiltration, and loaded onto a Sephadex G-50 column (2.6 cm i.d. × 30 cm length). Fractions with an absorbance ratio (A_{280}/A_{410}) of <0.5 were pooled, concentrated by ultrafiltration, and stored at −20 °C.

Expression and Purification of (ShuT). The recently discovered heme protein from *Shigella dysenteriae* (ShuT) was expressed and purified as previously described.[2] Briefly, a 100 mL subculture of *E. coli* BL21 (DE3) plysS harboring pETShuT in LB−ampicillin ($OD_{600} = 0.6$) was used to inoculate 1 L cultures to an OD_{600} of 0.06. The cells were grown to an OD_{600} of 0.6−0.8 and induced with 1 mM isopropyl-β-D-thiogalactopyranose (IPTG); then, they were grown for an additional 4 h at 30 °C. The cells were harvested by centrifugation for 20 min at 6000 g in a Beckman JA-20 rotor. The cells were lysed at 4 °C by stirring in 50 mM sodium phosphate (pH 8.0) containing 100 mg of lysozyme/100 mL of cell lysate, 0.2 mM PMSF, and a protease inhibitor cocktail (Roche Diagnostics GmBH).

The periplasmic fraction was isolated by centrifugation at 3000 g for 30 min, and the resulting supernatant was again centrifuged at 6800 g for 30 min. The clarified solution was applied to an Ni−NTA agarose column (1 × 10 cm), and the protein was eluted in 20 mM Tris-HCl (pH 7.8) containing 250 mM imidazole and 300 mM NaCl, following previous wash steps (20 column volumes) in the same buffer containing 20 mM imidazole. The peak fractions, as judged by SDS−PAGE, were pooled and dialyzed in 20 mM Tris-HCl (pH 7.5), concentrated to 10 mg/mL, and stored at −80 °C.

Preparation of Mb and ShuT Samples Reconstituted with ^{13}C-Labeled Heme. ^{13}C-labeled δ-aminolevulinic acids (ALA) were prepared using a previously reported methodology[25] and were used as precursors for the biosynthetic preparation of ^{13}C-labeled protoheme IX (heme).[12,24,26] [5-^{13}C]-ALA was used to label heme at the meso (C_m) and α-pyrrole (C_α) positions, and [4-^{13}C]-ALA was used to label heme at the C_α and β pyrrole (C_β) carbons (see Figure 2). ^{13}C-labeled heme is prepared by capitalizing on the fact that the first committed precursor in heme biosynthesis is δ-aminolevulinic acid (ALA).[27,28] Hence, ^{13}C-labeled heme is biosynthesized in *E. coli* upon the addition of suitably ^{13}C-labeled ALA and trapped in the outer mitochondrial membrane cytochrome b_5 (OM cyt b_5), which is overexpressed in the bacterial host. The details of the biosynthetic protocol, which entails the expression and purification of OM cyt b_5 harboring ^{13}C-labeled heme, have been presented previously.[24] Reconstitution of the target protein with ^{13}C-labeled heme entails the removal of the isotopically labeled macrocycle from OM cyt b_5, followed by the formation of the heme-target protein complex. ^{13}C-labeled heme is extracted from OM cyt b_5 with the methods reported previously.[12,29,30] In short, the addition of 15 mL of pyridine to 2.5 mL of 1 mM OM cyt b_5 in phosphate buffer ($\mu = 0.10$, pH 7.0), followed by the slow addition of chloroform, typically 10−15 mL, results in the precipitation of the polypeptide, leaving the pyridine hemochrome in solution. The latter is separated from the denatured polypeptide by centrifugation, allowed to equilibrate at room temperature, and then dried over anhydrous $MgSO_4$. The desiccant is separated by filtration, and the filtered pyridine/chloroform solution transferred to a round-bottomed flask, where it is concentrated to dryness on a rotary evaporator. The solid is redissolved in 1.5 mL of DMSO, and the resultant solution is immediately used to reconstitute the target protein. In this study, ~2 μmol of Mb, H64V Mb, or ShuT was

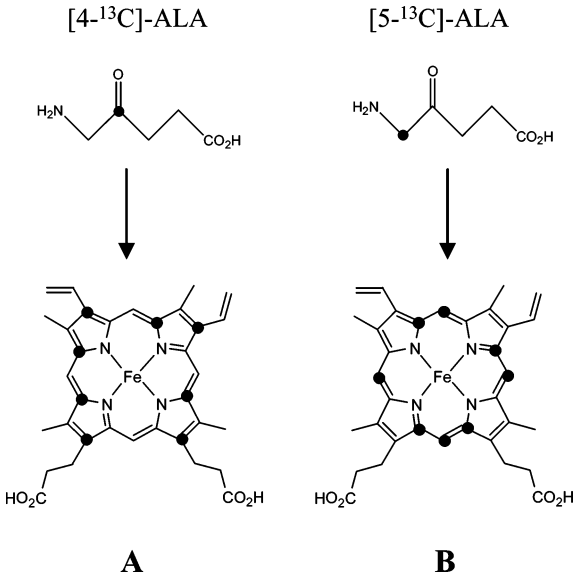

Figure 2. Heme labeled at the C_α and C_β positions was obtained using [4-^{13}C]-ALA as a precursor and heme labeled at the C_α and C_m positions was obtained from [5-^{13}C]-ALA.

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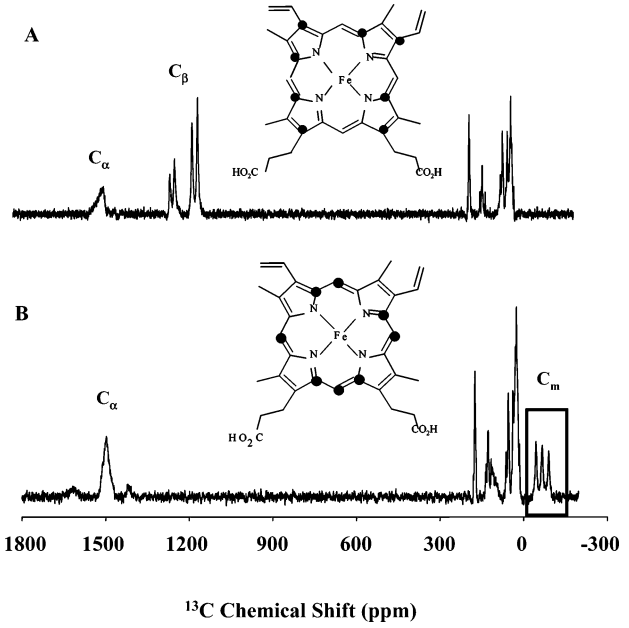

Figure 3. ^{13}C NMR spectra of wild-type sperm whale myoglobin, pH 6.0, reconstituted with (A) heme labeled at the C_α and C_β positions and (B) heme labeled at the C_α and C_m positions. The spectra were obtained at 30 °C over 213 kHz, with 100 ms acquisition time, 20 ms relaxation delay, and 450 000 scans.

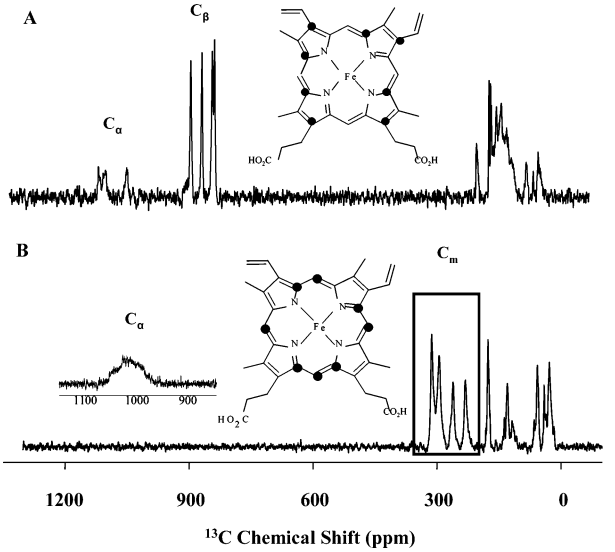

Figure 4. ^{13}C NMR spectra of the H64V mutant of sperm whale myoglobin at pH 6.0 reconstituted with (A) heme labeled at the C_α and C_β positions and (B) heme labeled at the C_α and C_m positions. The spectra were obtained at 30 °C over 213 kHz, with 100 ms acquisition time, 20 ms relaxation delay, and 450 000 scans.

reconstituted with a freshly prepared solution of ^{13}C-labeled heme until the A_{280}/A_{Soret} ratio no longer changed. The resultant solution was incubated at 4 °C overnight and then purified by size exclusion chromatography at the same temperature. The Mb−^{13}C-labeled heme complexes were purified in a Sephadex G-50 column (90 cm × 2.6 cm i.d.), equilibrated and eluted with 10 mM phosphate buffer (pH 6.0). The ShuT−^{13}C-labeled heme complex was purified in a Sephacryl S-100 column (2.6 cm i.d. × 90 cm length) also equilibrated and eluted with 10 mM phosphate buffer (pH 8.0). Fractions containing pure protein were concentrated in Amicon centrifugal concentrators to ∼1 mL and then transferred to smaller Centricon concentrators to exchange the protein into deuterated phosphate buffer.

NMR Spectroscopy. ^{1}H and ^{13}C spectra were acquired on a Varian Unity Inova spectrometer operating at a 599.74 MHz ^{1}H frequency and a 150.92 MHz ^{13}C frequency. The ^{1}H spectra were referenced to the residual water peak at 4.8 ppm, and the ^{13}C spectra were referenced to an external solution of dioxane (60% v/v in D_2O) at 66.66 ppm. Proton spectra were acquired with presaturation of the residual water peak over 49 k data points, a spectral width of 125 kHz, a 200 ms acquisition time, no relaxation delay, and 1024 scans. The acquisition parameters used to obtain ^{13}C NMR spectra are given in the appropriate figure captions.

Results and Discussion

Wild-type sperm whale Mb was reconstituted with hemin labeled with ^{13}C at the C_α and C_β positions (insert of Figure 3A) and with hemin labeled at the C_α and C_m positions (insert in Figure 3B). The ^{13}C NMR spectrum in Figure 3A displays the paramagnetically affected resonances between 1100 and 1500 ppm, whereas the spectrum in Figure 3B shows a set of peaks centered near 1500 ppm and a second set of resonances (enclosed in a box) near −80 ppm. The ^{13}C labeling patterns in Figure 3A and B have in common the isotopic enrichment of C_α. It is therefore straightforward to conclude that resonances centered near 1500 ppm originate from C_α, resonances centered at ∼1200 ppm originate from C_β, and resonances near −80 ppm correspond to meso carbons. A similar set of experiments was conducted with the H64V mutant, which is known to be pentacoordinated and high-spin.[20,21] The spectrum shown in Figure 4A, obtained from a sample of H64V Mb reconstituted with heme labeled at the C_α and C_β positions, was acquired with a spectral window covering from −200 to 1200 ppm. This spectrum shows two sets of peaks originating from the heme: one near 800 ppm and the second at ∼1000 ppm. The spectrum of H64V sw-Mb reconstituted with heme labeled at the C_α and C_m positions is shown in Figure 4B. In this spectrum, which was acquired with a spectral window covering from −200 to 1200 ppm, a set of peaks (enclosed in a box) centered near 280 ppm is apparent. However, when the spectrum was acquired using the same sample with a smaller spectral window (700−1400 ppm), a set of broad peaks centered near 1000 ppm became evident. These observations permit the straightforward assignment of the resonances at ∼800 ppm to C_β, those near 1000 ppm to C_α, and the ones near 280 ppm to C_m.

The above-described observations indicate that hexa- and pentacoordinated Mb's exhibit distinct differences in the chemical shifts of their corresponding core carbons. Among these differences, those corresponding to C_m shifts stand out: C_m resonances from hexacoordinated Mb are centered at approximately −80 ppm (Figure 5A), whereas the C_m resonances from pentacoordinated H64V sw-Mb occur near 250 ppm (Figure 5B). We propose that these chemical shift differences can be used as a diagnostic tool for the relatively straightforward determination of the coordination state of high-spin heme proteins. Two important properties make this approach readily applicable to this task: (i) C_m resonances from penta- and hexacoordinated heme active sites are relatively sharp and resonate relatively close to the typical

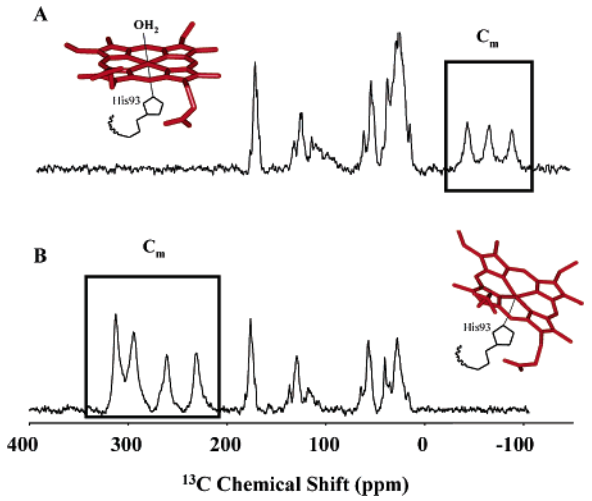

Figure 5. ^{13}C NMR spectra demonstrating the difference in the magnitude of meso-carbon chemical shifts obtained from (A) hexacoordinate high-spin sw-Mb and (B) pentacoordinate high-spin H64V-sw-Mb. The spectra were obtained at 30 °C over 84 kHz, with 100 ms acquisition time, 20 ms relaxation delay, and 110 000 scans.

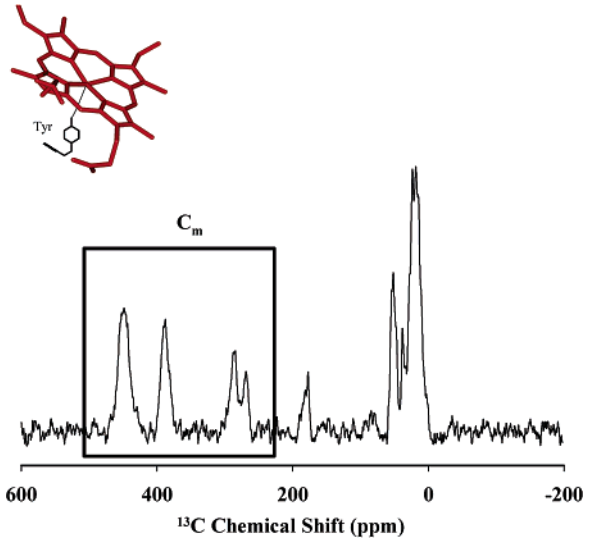

Figure 6. ^{13}C NMR spectrum of ShuT at pH 8.0 reconstituted with heme labeled at the C_α and C_m positions. The C_m chemical shifts centered at ~350 ppm unambiguously demonstrate that the ferriheme center in ShuT is pentacoordinate and high spin. The spectrum was obtained with the aid of a cryoprobe in a Bruker instrument operating at 125.76 MHz (^{13}C− frequency) and 30 °C over 88 kHz, with 100 ms acquisition time, 20 ms relaxation delay, and 50 000 scans.

diamagnetic range of ^{13}C resonances, which accounts for their relatively simple detection. This is in stark contrast with the difficult detection and assignment of the corresponding meso-^{1}H resonances, as has been illustrated in Figure 1. (ii) The electronic properties that give rise to the distinct C_m shifts are now well understood[19] and therefore provide a firm basis for consistent correlations between C_m shifts and heme coordination state. In short, five-coordinate ferrihemes place the metal out of the porphyrin plane, whereas in six-coordinate ferrihemes, the metal sits within the porphyrin plane. In high-spin ferrihemes (five- or six-coordinate), spin delocalization through σ-bonding interactions with the iron $d_{x^2-y^2}$ orbital is dominant[19] and results in very high spin densities at the C_α and C_β positions and therefore in large downfield C_α and C_β shifts[31,32] (Figures 3 and 4). In addition, the out-of-plane displacement of the metal in five-coordinate ferrihemes promotes interactions between the metal d_{z^2} orbital and the porphyrin a_{2u} orbital that are not possible in six-coordinate high-spin ferrihemes.[19] These orbital interactions in five-coordinate ferrihemes allow delocalization of spin density from the porphyrin a_{2u} orbital into the metal d_{z^2} orbital, thus placing positive unpaired electron density at C_m, which results in downfield C_m shifts. In this context, it is noteworthy that the C_m positions are located at the nodes of the σ-bonding system and therefore are insulated from direct σ-delocalization. Hence, interactions between the d_{z^2} and a_{2u} orbitals in five-coordinate ferrihemes result in positive spin density at the meso positions and therefore downfield shifts ranging from 250 to 600 ppm (Figure 5B). In contrast, the placement of the iron in the porphyrin plane in six coordinate ferrihemes prevents similar orbital interactions, thus leaving the C_m positions insulated from direct spin delocalization. The negative C_m shifts, −30 to −100 ppm (Figure 5A), are therefore a consequence of polarization from large spin density at the C_α position, which places negative spin density at the meso carbons.

Results from the DFT calculations also suggest that the porphyrin to iron-d_π (d_{xz} and d_{yz}) spin delocalization is more efficient in six- than in five-coordinate ferrihemes.[32] This phenomenon should be manifested in larger C_α and C_β shifts in six-coordinate complexes relative to the equivalent shifts in their five-coordinate counterparts. Indeed, spectra obtained from the six-coordinate Mb and from the five-coordinate H64V Mb (Figures 3 and 4) show that the C_α and C_β shifts corresponding to Mb are larger than those obtained from the H64V mutant. This would suggest that the relative magnitude of the C_α and C_β shifts can be used to determine the coordination state of a ferriheme in an active site. However, the large chemical shifts and broader lines of the C_α and C_β shifts makes this approach less practical than simply measuring the magnitude of the C_m shifts, which are significantly easier to observe. In the following discussion, we apply this concept to the determination of the coordination state of a recently discovered heme protein implicated in the heme uptake system of *S. dysenteriae* (ShuT).[2]

^{13}C NMR analysis of the Coordination State of the Heme in ShuT. ShuT is a 28.5 kDa monomeric periplasmic heme protein thought to be involved in transporting exogenously acquired heme across the periplasm of *S. dysenteriae*.[4] Exhaustive mutagenesis and spectroscopic (MCD and resonance Raman) studies have suggested that the ferriheme center in ShuT is five-coordinate, with Tyr-94 serving as the axial ligand.[2] We have used this recently discovered protein to illustrate the simplicity with which the coordination state of high-spin heme proteins can be elucidated using ^{13}C NMR spectroscopy.

A sample of ShuT was purified to homegeneity as described in the Experimental Methods. After purification, the sample of ShuT consisted of a mixture of molecules with

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and without heme in an approximate 1:1 ratio. A solution containing heme labeled at the meso carbons was added to a solution of ShuT to reconstitute labeled heme into those molecules devoid of heme. The resultant solution was purified from excess free heme and then concentrated for subsequent NMR analysis. A set of C_m resonances centered near 350 ppm in the ^{13}C NMR spectrum of this protein (Figure 6) demonstrates that ShuT harbors a five-coordinate ferric heme center. The spectrum was obtained in ~2 h from a 250 μL solution (2.0 mM) of ShuT using a cryoprobe. The relatively short analysis time required for the ShuT sample, which was labeled with ^{13}C heme to approximately a 50% enrichment level, underscores the simplicity and the potential of utilizing the C_m shift in the determination of coordination state of ferric heme centers in novel proteins. In comparison, spectroscopic methods commonly used to determine the coordination state of high-spin ferriproteins (MCD and resonance Raman) rely strongly on the availability of suitable model complexes for assignment and interpretation of the pertinent signals.[2]

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