Sulfmyoglobin Derived from Deuterohemin Reconstituted Protein. 2. Molecular and Electronic Structure Determination of Two Extracted Prosthetic Groups

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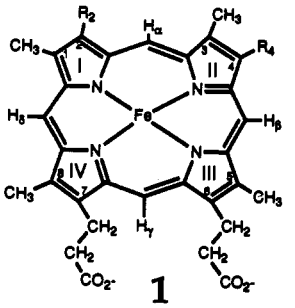
Abstract: The prosthetic groups of the intermediate reaction product of sulfmyoglobin, SMb, prepared from myoglobin reconstituted with deuterohemin, S_DMb, and the aerobic terminal reaction product in the presence of cyanide, S_EMb, reported elsewhere (Scharberg, M. A.; La Mar, G. N., preceding paper), have been extracted reversibly from the protein and their functional groups at all peripheral positions identified by a combination of NMR spectroscopy and mass spectrometry. For the intermediate reaction product, S_DMb, a stable chromophore could be extracted only upon first reacting S_DMb with iodoacetamide, a standard reagent for protecting thiol groups. 2D ^{1}H NMR of the directly extracted paramagnetic bis-cyano derivative of the two prosthetic groups reveals that each of the sulfhemins possesses altered functionalization solely at the 4-position when compared to the precursor deuterohemin. Fast atom bombardment mass spectral data show that the mass of the substituents in iodoacetamide-reacted S_DMb and S_EMb is 90 and 58 mass units, respectively. The former value is quantitatively consistent with the thioacetamide group, indicating that the intermediate reaction product contains 4-thiol-deuterohemin. The latter mass value, which increases by one unit when the aerobic reaction in the presence of cyanide is carried out in the presence of $^{13}CN^-$ and yields a ^{13}C NMR signal in the diamagnetic S_EMbCO complex that is diagnostic of a thiocyanate group, dictates that the chromophore of the terminal aerobic reaction product is 4-thiocyanato-deuterohemin. 2D ^{1}H NMR determination of the molecular structures of the directly extracted, paramagnetic chromophores is shown to be practical if the NOESY spectra are collected in viscous solvent. Moreover, the pattern of the heme methyl contact shifts in the bis-cyano complexes of a series of variably 4-substituted deuterohemins is shown to reflect the electronic nature of the 4-substituent, and the positions of the two extracted chromophores in this series are completely consistent with 4-thiol and 4-thiocyanato groups of the two sulfhemins. It is also shown that sulfhemin-E with the 4-thiocyanato group lead to significant equilibrium heme orientational disorder in Mb.

Introduction

The NMR and optical spectroscopic studies of sulfmyoglobin, SMb, and its subsequent reaction products derived from deuterohemin (**1**: $R_2 = R_4 = H$) and reconstituted myoglobin (Mb*, designated Mb* to differentiate it from native Mb that contains protohemin (**1**: $R_2 = R_4 =$ vinyl)), described in our companion report,[1] provide crucial information on the mechanism of formation of each species and whether the prosthetic group in a given species is a porphyrin or a chlorin. Two key products were identified[1] as resulting from the relatively unstable initially formed sulf-Mb* complex, S_AMb*: a porphyrin containing intermediate designated S_DMb*, and a porphyrin containing terminal aerobic reaction product in the presence of cyanide ion, designated S_EMb*. However, the poor ^{1}H NMR spectral resolution in the various intact SMb* complexes in even the optimal oxidation/ligation state renders a detailed molecular and electronic structure determination of the individual prosthetic groups impractical. The optimal route to the detailed characterization of the position and chemical nature of the functionalization of the various prosthetic groups is to reversibly extract the prosthetic groups for ^{1}H study free of the protein matrix. For native protohemin (**1**: $R_2 =$ vinyl, $R_4 =$ vinyl), the prosthetic group of the terminal sulfmyoglobin reaction product, S_CMb, designated sulfhemin-C, was found stable to acidic extraction, and detailed ^{1}H NMR isotope labeling, spin decoupling, and limited steady-state nuclear Overhauser effect (NOE) studies on the paramagnetic ferric bis-cyano complex showed its structure to involve a cyclized thiolene ring at pyrrole II.[2-4] The same structure was deduced based on ^{1}H NMR of the diamagnetic, esterified, and iron-free sulfporphyrin-C; however, partial modification of the reactive prosthetic group by the esterification procedure was noted.[3]



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Our interest in this report is to determine the complete molecular structure of the reversibly extractable prosthetic groups for sulfmyoglobin products of equine Mb reconstituted with deuterohemin (**1**: $R_2 = R_4 = H$). The goal is to carry out these structure determinations directly on the paramagnetic extracted prosthetic group rather than in a chemically modified form designed to yield diamagnetic species. The reason for this is twofold: the structure is determined directly on a prosthetic group whose reconstitution into apo-Mb establishes that the group is not modified by the extraction, and the unique contact shift pattern for a paramagnetic iron tetrapyrrole complex provides valuable information on the electronic nature of the peripheral functionalization.[3,5,6] However, with the more limited functionality of the 4-position in deuterohemin as compared to protohemin, spin-

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coupling analysis is considerably less useful, and the needed isotope labeled forms of deuterohemin are not readily available. Hence a more general method based on 2D NMR spectroscopy is desirable. While the line broadening in a paramagnetic complex yields much weaker scalar COSY-type cross peaks than in a diamagnetic system,[7] it has been shown that all spin-coupling patterns in low-spin ferric hemes and chlorins can be detected using appropriate experimental procedures.[8,9] Similarly, enhanced spin–lattice relaxation due to the unpaired electron strongly suppresses dipolar or NOESY cross peak intensities as compared to a diamagnetic analog, often below the practical limits of detection.[7,10] However, we have shown that the loss in intensity of cross peaks due to paramagnetism can largely be recovered by placing the molecule into a sufficiently viscous solvent.[6,10,11]

The preparation of the various cyano-met sulfmyoglobin complexes of deuterohemin-reconstituted equine Mb are reported in detail in our companion report.[1] Herein we describe solely the conditions for the extraction of stable derivatives and the ^1H NMR spectroscopy and mass spectrometry required to unambiguously establish the location and detailed chemical nature of all substituents. The structures determined here have been used in our comparison report[1] to develop a reaction mechanism consistent with the solution conditions facilitating their formation and to indirectly assign the structure of the initially formed complex designated S_AMb^* whose prosthetic group, like that of the S_AMb complex of protohemin,[12] is not stable to extraction.

Experimental Section

Sample Preparation. The cyano-met complexes of S_AMb^*, S_DMb^*, and S_EMb^* were prepared as described in detail in the preceding paper.[1] The excess cyanide from the intermediate reaction product in the cyano-met form, 3 mM metS_DMb^*CN in ^2H_2O, was removed by ultrafiltration and the complex treated with 4 molar equiv of 0.1 M iodoacetamide (ICH_2CONH_2) in phosphate buffer in ^2H_2O at pH 7.0. The reaction was monitored by ^1H NMR, which revealed the gradual loss of the ^1H resonances, D_i, of metS_DMb^*CN, and the emergence of a new set of resonances, labeled D'_i, for the iodoacetamide reaction product, which we designate met$S_D'Mb^*CN$. The reaction was complete after 1 week at 22 °C. Extraction of prosthetic groups was carried out by the acid/2-butanone method of Teale.[13] The resulting hemins were dried under a stream of N_2 before dissolution in the appropriate solvent. For ^1H NMR studies, the dried hemin was dissolved in a mixture of 2:1 $(C^2H_3)SO/^2H_2O$ in the presence of 3 equiv of KCN or $Na^{13}CN$; the concentration of these bis-cyano complexes was ~3 mM. This particular solvent system was chosen because its high viscosity enhances negative nuclear Overhauser effects, NOEs, at −20 °C for paramagnetic hemins.[6,10,11] The iron was removed and the propionates esterified using $FeSO_4$ in concentrated hydrochloric and glacial acetic acid.[10] The resulting iron-free, sulfporphyrin-E was dissolved in diethyl ether for optical studies. Mass spectra were obtained on the extracted and dried sulfhemin dissolved in 2-butanone and on the di-esterified sulfporphyrin dissolved in diethyl ether.

NMR Spectra. ^1H and ^{13}C NMR spectral parameters for the protein complexes were described in the previous paper.[1] ^1H NMR reference spectra of the bis-cyano hemin complexes were obtained on GE Omega or NIcolet NT spectrometers operating at 500 MHz. The spectra consisted of 4096 scans of 8192 data points over a 30-kHz bandwidth using a 8.0-μs 90° pulse. Nonselective ^1H spin–lattice relaxation times, T_1, were obtained by the standard inversion–recovery experiment. ^{13}C NMR spectra of bis-cyano sulfhemin-E were recorded on a QE 300-MHz spectrometer in which the 5-mm broad-band probe had been tuned to 75.60 MHz for ^{13}C detection. The spectra consisted of 10^5 scans of 16 384 data points over a 50-kHz bandwidth using a 4.0-μs 24° pulse. ^{13}C NMR chemical shifts were referenced to tetramethylsilane via the natural abundance $(^{13}C^2H_3)_2SO$ signal which resonates at 39.0 ppm.[14]

Two-dimensional magnitude COSY[15] maps of the bis-cyano complexes of stable extracted prosthetic groups were collected at 25 °C on a GE Omega 500-MHz spectrometer with a 7.2-μs 90° pulse using 2048 complex points over a bandwidth of 34.4 kHz; 512 blocks of 64 scans per block were recorded with a total recycle time of 120 ms. The COSY pulse sequence contained a solvent presaturation of 60 ms with the decoupler frequency set at 4.76 ppm on the $^1HO^2H$ resonance. The total acquisition time was 1.2 h. The two-dimensional phase-sensitive NOESY maps of the bis-cyano complexes of the same prosthetic groups were collected at −20 °C on a GE Omega 500-MHz spectrometer with a mixing time of 50 ms, using the method of States *et al.*[9] The data were collected with a 10.5-μs 90° pulse over a bandwidth of 37 kHz using 1024 complex data points. The residual solvent signal was suppressed with a decoupler pulse during the relaxation delay. 512 blocks of 128 scans per block, with $t_1(\max) = 13.8$ ms, were collected with a recycle time of 500 ms. The total acquisition time was 8 h. The 2D magnitude COSY data were processed on a SUN SPARCS 1+ Workstation using GE Omega data processing software, Version 5.0. Both t_1 and t_2 dimensions were apodized by unshifted sine-bell-squared windows. The t_2 dimension was zero-filled to 1024 points so the resulting 2D data matrix was 1024 × 1024 complex points. The 2D NOESY data were processed on a Silicon Graphics Personal IRIS using FELIX software by Dennis Hare. Both t_1 and t_2 dimensions were apodized by a 30° shifted sine-bell-squared window. The t_2 dimension was zero-filled to 1024 points so the resulting 2D data matrix was 1024 × 1024 complex points. All ^1H chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) through the residual solvent resonance.

Optical Spectra. Optical spectra were recorded at ambient temperature on a Hewlett-Packard 8540A UV–visible spectrophotometer using 1-cm light path quartz cells. The spectrum of the bis-cyano sulfhemin complex was recorded on a solution of 2-butanone prepared by diluting 10 μL of the 3 mM stock solution to 2.0 μL in the presence of excess cyanide. The spectrum of the iron-free sulfporphyrin dimethyl ester was recorded on a ~1.5 μM solution in diethyl ether.

Mass Spectra. High-resolution fast atom bombardment (FAB^+) mass spectra were recorded on a VG Analytical High Sector mass spectrometer. A matrix of 7:2 dithiotheritol:dithioerythitol (DTT:DTE) and 3-nitrobenzyl alcohol in glycerol was used for the sulfhemins and iron-free sulfporphyrins, respectively. After smearing 2 μL of the matrix onto the probe, 2 μL of a 3mM sulfhemin in 2-butanone or sulfporphyrin in diethyl ether was mixed into the matrix and placed in a vacuum to remove solvent. The ionization was achieved by targeting the probe with a stream of xenon atoms operating at 8 keV at ambient temperatures.

Results

Sulfhemin-A. Attempts to extract the prosthetic group of a 70% metS_AMb^*CN sample yielded only deuterohemin with no evidence for ^1H resonances for any other species. Hence the prosthetic group of S_AMb^* is not stable under the extraction conditions with respect to reversion to deuterohemin. This is not surprising inasmuch as we found the initial S_AMb^* complex of deuterohemin to be even less stable with respect to reversion than the initially formed S_AMb complex of protohemin at neutral pH. The prosthetic group of this S_AMb complex shown has been also to be unstable to acid extraction.[12]

Sulfhemin-E. (a) Extraction/Reconstitution. The ^1H NMR spectrum of a ~60% metS_EMb^*CN sample prepared as described previously,[1] with peaks E_i (~35% metMb*CN with peak M_i, 5% minor species labeled e_i), is illustrated in Figure 1A. This spectrum reflects a sample that was allowed to react aerobically in the

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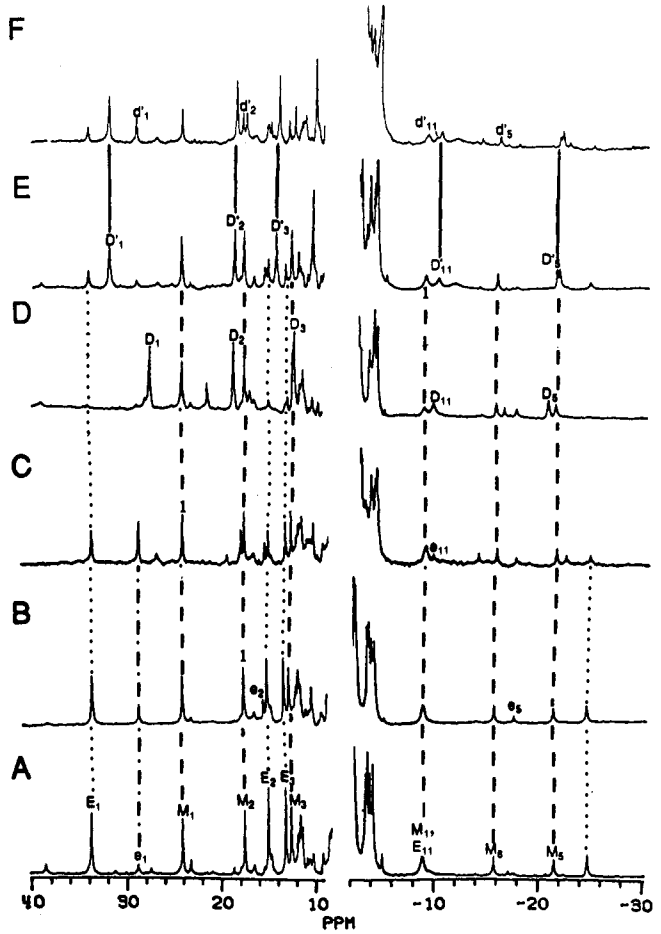

Figure 1. 500-MHz ^{1}H NMR spectra of various sulfmyoglobin complexes in ^2H_2O and pH 7.0 and 20 °C. (A) 60% metS$_E$Mb*CN with peaks E_i (35% unreacted metMb*CN with peak M_i, 5% species with peaks e_i) 4 weeks after the initial preparation; note the intensity ratio $E_i/e_i \sim 12$. (B) Same sample as in (A) after 4 months at 22 °C; note the increase in e_i peaks relative to E_i so that now $E_i/e_i \sim 3$. (C) Product of reconstitution of fresh apo-Mb with the red hemins extracted from the metS$_E$Mb*CN whose trace is shown in (A); note the same sets of peaks, E_i, M_i, and e_i but with the peak ratio E_i:$e_i \sim 1$; within 6 months, the ratio E_i/e_i reverts to 3, the same as shown in (B). (D) 70% metS$_D$Mb*CN with peaks D_i, 25% metMb*CN (with peaks M_i), and 5% metS$_E$Mb*CN (with peak E_i). (E) 60% metS$_D$'Mb*CN with peak D'_i, resulting from reacting metS$_D$Mb*CN with iodoacetamide. (F) metS$_D$'Mb*CN resulting from the reconstitution of fresh apo-Mb with the prosthetic groups extracted from the samples in (E): note the reappearance of the peaks D'_i and M_i, as well as the peaks d'_i, which essentially disappear within a week. Resonances for the cyano-met SMb* complexes are labeled the same as in our companion report:[1] χ_1–χ_3 for unassigned heme methyls, the χ_5, χ_6 for the pyrrole 2-H, 4-H respectively, and χ_{11} for the diagnostic upfield Ile 99(FG5) C$_\gamma$H peak.

presence of excess CN$^-$ for 4 weeks during which essentially all metS$_D$Mb*CN disappeared. However, allowing this sample to remain at 22 °C for an extended time after disappearance of metS$_D$Mb*CN led to a steady increase in the intensity of the minor component peaks e_i at the expense of the major component peaks E_i, until the ratio of the apparent methyl intensity E_i:e_i reached 3:1 after ~6 months, as shown in Figure 1B. The minor species that gives rise to peaks e_i hence gives the appearance of yet another reaction product even more stable than metS$_E$Mb*CN. However, this will be shown not to be the case (see below).

Standard acid extraction with 2-butanone[13] of the prosthetic group from the metS$_E$Mb*CN sample with the ^{1}H NMR trace as shown in Figure 1A yields red products which, when dissolved in 2:1 $(C^2H_3)_2SO/^2H_2O$ in the presence of excess cyanide, yield the ^{1}H NMR spectrum given in Figure 2B. Reconstitution of this mixture of extracted hemins into fresh apo-Mb yields

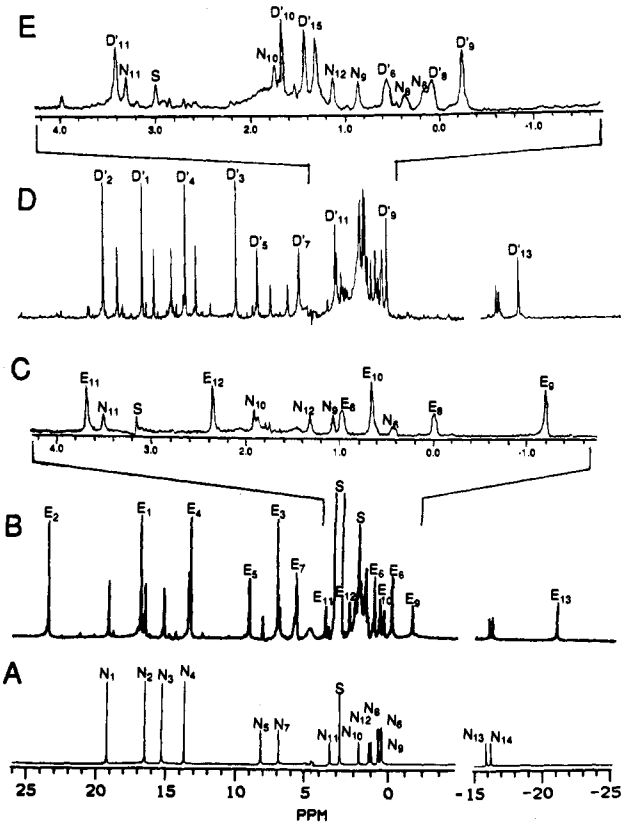

Figure 2. 500-MHz ^{1}H NMR spectra in 2:1 $(C^2H_3)_2SO$:^2H_2O at 25 °C of the bis-cyano complex of (A) deuterohemin, with peaks N_i, (B) sulfhemin-E with peaks E_i, and (D) the iodoacetamide complex of sulfhemin-D, designated sulfhemin-D', with peaks D'_i. Expansion of the crowded –1 to 4 ppm region collected in an inversion–recovery experiment, where the pulse repetition rate and delay time are selected to allow complete relaxation primarily for the most effective relaxed meso-H and to suppress solvent and impurity peaks for (C) sulfhemin-E and (E) sulfhemin-D'. The resonance labeling corresponds to i = 1–4 for methyls, 5–8 for propionate CH_2's 9–12 for meso-H's, and 13,14 for pyrrole-H's; individual assignments are given in Table I.

holoproteins with ^{1}H NMR spectra shown in Figure 1C; clearly observed are the peaks of unreacted metMb*CN (M_i), metS$_E$Mb*CN (E_i), and the species with peaks e_i, but with a significant increase in the intensity of the peaks labeled e_i at the expense of peak set E_i over that in Fgure 1, spectrum A or B. However, the nearly equally intense E_i and e_i peaks in Figure 1C change intensity with time with E_i increasing at the expense of e_i, until after approximately 6 months the ratio approaches 3:1, with the spectrum (not shown) essentially identical to that in Figure 1B. The half-life for this interconversion $e_i \rightarrow E_i$ is about 2 months at pH 7.0 and 20 °C in ^2H_2O. Note that the extracted chromophore from metS$_E$Mb*CN yields only one set of peaks for sulfhemin-E, even though it gives rise to two sets of peaks for a S$_E$Mb* product. Nevertheless, the above experiment clearly demonstrates that the sulfhemin-E chromophore from S$_E$Mb* is stable to extraction.

(b) Spectral Studies. The ^{1}H NMR spectrum of the extracted chromophores from a 70% metS$_E$Mb*CN sample (as in Figure 2B) is indicative of two species, the ~30% unreacted bis-cyano deuterohemin, with peaks labeled N_i, and the bis-cyano complex of the chromophore of S$_E$Mb*, designated sulfhemin-E, with peaks labeled E_i. The resonances for the former species are readily recognized upon comparison with the ^{1}H NMR spectrum of pure bis-cyano deuterohemin shown in Figure 2A. The resonances of the paramagnetic complexes in the crowded window –1 to 4 ppm in Figure 2B are more readily characterized in an inversion–recovery trace where the pulse repetition rate and delay time are selected to optimize the intensities of the fastest relaxing protons,

Table I. ^{1}H NMR Spectral Data for the Bis-Cyano Complexes of Sulfhemin-D′, Sulfhemin-E, and Deuterohemin in 2:1 DMSO:^2H_2O at 25 °C

		shift[b] (T_1[c])		
χ_i[a]	assign	deuterohemin (N_i)	sulfhemin-E (E_i)	sulfhemin-D′ (D'_i)
1	8-CH_3	19.11 (173)	16.80 (202)	17.16 (197)
2	5-CH_3	16.49 (216)	23.53 (130)	19.96 (163)
3	3-CH_3	15.16 (231)	6.98 (375)	10.48 (251)
4	1-CH_3	13.41 (260)	13.26 (245)	14.09 (255)
5	6-α-H_2	8.08 (216)	9.04 (216)	8.91 (214)
6	6-β-H_2	0.18 (289)	0.73 (231)	0.49 (220)
7	7-α-H_2	6.84 (231)	5.63 (*d*)	5.93 (207)
8	7-β-H_2	0.39 (216)	−0.28 (245)	0.01 (220)
9	α-meso-H	0.86 (72)	−1.46 (65)	−0.33 (68)
10	β-meso-H	1.69 (72)	0.43 (79)	1.57 (75)
11	γ-meso-H	3.37 (68)	3.59 (*d*)	3.35 (72)
12	δ-meso-H	1.09 (72)	2.17 (65)	1.24 (67)
13	2-H	−16.38 (58)	−21.31 (65)	−18.20 (60)
14	4-H	−16.59 (58)		
15	SCH_2ONH_2			1.33 (275)

[a] χ_1 corresponds to peaks labeled N_i, E_i, and D'_i in Figure 2. [b] Shifts in ppm from DSS. [c] T_1 in ms, with uncertainty ±15%. [d] Overlaps solvent resonance.

the meso-H's, and to suppress the slowly relaxing solvent and impurity signals. Such a trace is illustrated in Figure 2C and identifies the meso-H signals from bis-cyano sulfhemin-E, labeled E_9–E_{12}, as well as those from the residual bis-cyano deuterohemin, labeled N_9–N_{12}. Analysis of the spectra in Figures 2B,C indicates that bis-cyano sulfhemin-E exhibits a total of only 13 signals (4 three-proton, E_1–E_4; 4 two-proton, E_5–E_8; 5 single-proton intensity, E_9–E_{13}). A magnitude COSY spectrum of bis-cyano sulfhemin-E (Figure 3A) yields only two cross peaks which connect the two-proton intensity signals E_5 with E_7 and E_6 with E_8 and identify the spin-coupled $C_\alpha H_2$–$C_\beta H_2$ fragments of the two propionate groups; also detected are the same two cross peaks for the residual deuterohemin complex, N_5 to N_7 and N_6 to N_8.

A 2:1 DMSO/^2H_2O solution at −20 °C provides a solvent with sufficient viscosity to yield the NOESY cross peaks[6,10,11] that allow unique identification of all substituents in both the sulfhemin-E and unreacted deuterohemin bis-cyano complexes, as illustrated in Figure 4A. Correlation of the chemical shifts from −20 to 25 °C, carried out in 5 °C intervals (not shown), provides the relationship between the COSY and NOESY detected resonances. For bis-cyano deuterohemin whose methyls had been assigned by isotope labeling,[17] the cross peaks between the upfield pyrrole-H signals (N_{13}, N_{14}) to the two assigned methyls uniquely identify the 2-H and 4-H; methyl–meso-H cross peaks (not traced in Figure 4A because of crowding) complete the assignment for this complex. For bis-cyano sulfhemin-E, cross peaks between the propionates and two methyls locate the pair 5-CH_3, 8-CH_3; a cross peak between one of these methyls (8-CH_3) to a methyl not adjacent to a propionate (1-CH_3) leads to the unique identification of all four heme methyls and the two propionates. The cross peaks from each methyl to the rapidly relaxing meso-H signals, collectively located above, lead to the unambiguous assignment of the four meso-H's. Hence there remains only one resonance that is unassigned, the upfield single proton peak E_{13}, and its cross peak to 1-CH_3 identifies it as 2-H. Clearly the 4-H proton of the deuterohemin precursor is lost in sulfhemin-E, and only the 4-position is functionalized. The chemical shifts for the bis-cyano deuterohemin and sulfhemin-E complexes at 25 °C are listed in Table I; also included are the T_1 values.

The fast ion bombardment (FAB$^+$) mass spectrum in the molecular range 500–650 of a mixture of 70% sulfhemin-E and

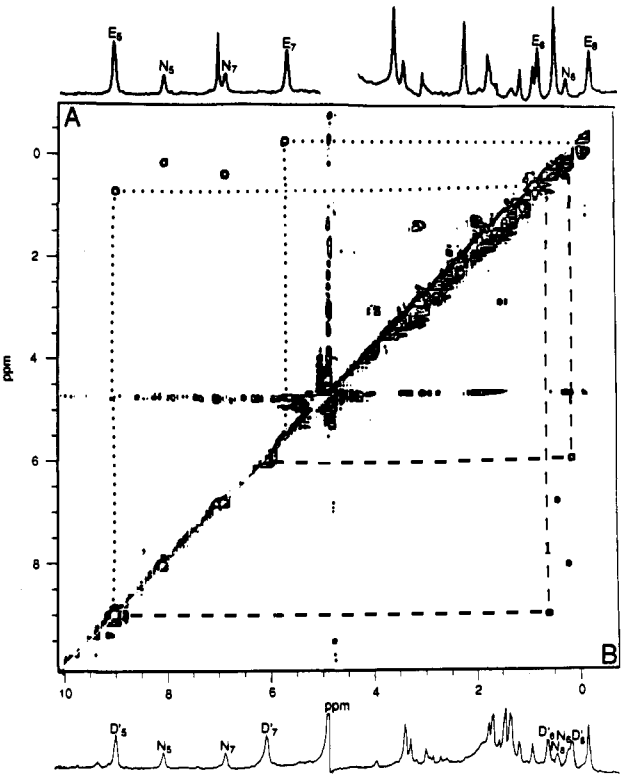

Figure 3. Split diagonal portions of the 500-MHz ^{1}H NMR magnitude COSY spectra in 2:1 $(C^2H_3)_2SO$:^2H_2O at 25 °C of bis-cyano complexes: (A) ~70% sulfhemin-E and 30% deuterohemin (the reference trace, with peak E_i, is shown above the COSY map), and (B) ~70% sulfhemin-D′ and ~30% deuterohemin (the reference trace, with peaks D'_i, is shown below the COSY map). The scalar connectivities for χ_5–χ_6 and χ_7–χ_8 for sulfhemin-E and sulfhemin-D′ are indicated by dotted and dashed lines, respectively. The cross peaks for unreacted deuterohemin peaks N_5 to N_6 and N_7 to N_8 are also observed, but not labeled to avoid crowding.

30% deuterohemin is illustrated in Figure 5A. The peak labeled N_{564} is the same as that observed for pure deuterohemin (not shown) and corresponds to the parent deuterohemin molecule ($C_{30}H_{26}N_4O_4Fe$, $M^+ = 564$). Previous mass spectrometric studies of metalloporphyrins[18] have observed pseudomolecule ions M^+ to M^{3+}, which have been attributed to hydrogen uptake; such ions are also observed for deuterohemin. The second major ion must arise from sulfhemin-E, is indicative of a species heavier than N_{564} by 57 mass units, and is labeled E_{621}; it also shows weaker pseudomolecular ions to M^{2+}. The demonstrated loss of 4-H dictates that the mass of the substituent is 58. Esterification of sulfhemin-E (and the $^{13}CN^-$ generated sulfhemin-E) with methanol and removal of the iron yielded the sulfporphyrin-E dimethyl ester and its ^{13}C labeled derivative. Poor quality FAB$^+$ mass spectra yield the deuteroporphyrin dimethyl ester parent ion at 529 and new parent ions 596 and 597 for sulfporphyrin-E DME and its ^{13}C labeled derivative, respectively (not shown: see supplementary material), again dictating a 4-substituent in sulfhemin-E with mass 58 (59 in the ^{13}CN derivative).

The optical spectra of 70% bis-cyano sulfhemin-E and 30% deuterohemin and pure bis-cyano deuterohemin in 2-butanone are essentially the same, with sulfhemin-E exhibiting a small (5 nm) red shift of the Soret band in comparison to deuterohemin. Spectra of low-spin hemins are known to be relatively insensitive to peripheral substitution.[19] Esterification of the propionates and removal of the iron, however, yielded an optical spectrum of the 70% sulfporphyrin-E dimethyl ester with a strong blue shift of the lowest energy band (464 nm) as compared to deuteroporphyrin dimethyl ester (625 nm) (not shown: see supplementary material).

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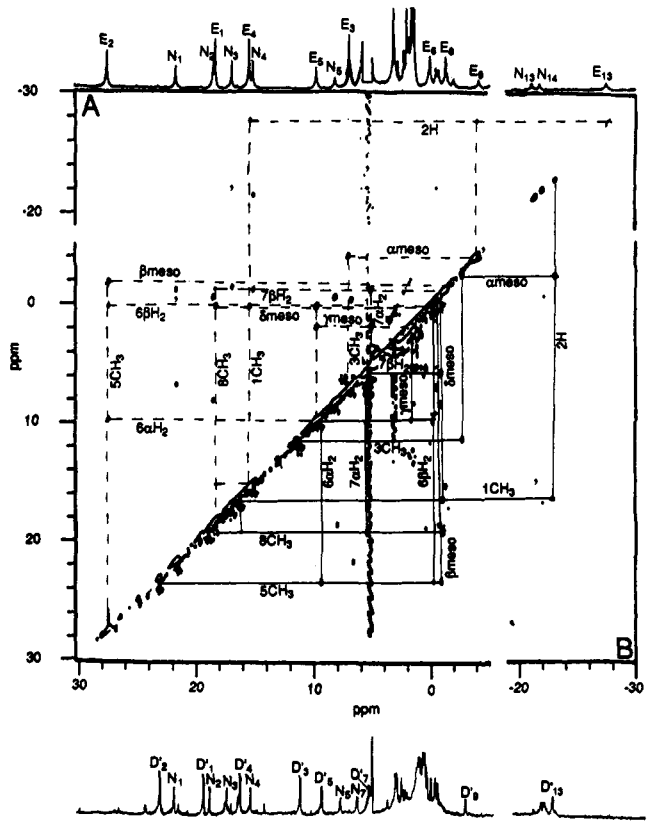

Figure 4. Split diagonal portions of the 500-MHz ^1H NMR NOESY spectra in 2:1 $(C^2H_3)_2SO$:^2H_2O at −20 °C of bis-cyano complexes: (A) ~70% sulfhemin-E and ~30% deuterohemin (the reference trace with prominent peaks E_f and N_f labeled is shown above the NOESY map); and (B) ~70% sulfhemin-D′ and ~30% deuterohemin (the reference trace is shown below the map with prominent peaks D'_f and N_f labeled). The dipolar connectivities needed to assign all resonances of the sulfhemin-E and sulfhemin-D′ complex are traced in dashed and solid lines, respectively. Because of the crowding, the cross peaks to completely assign the deuterohemin complex are not labeled.

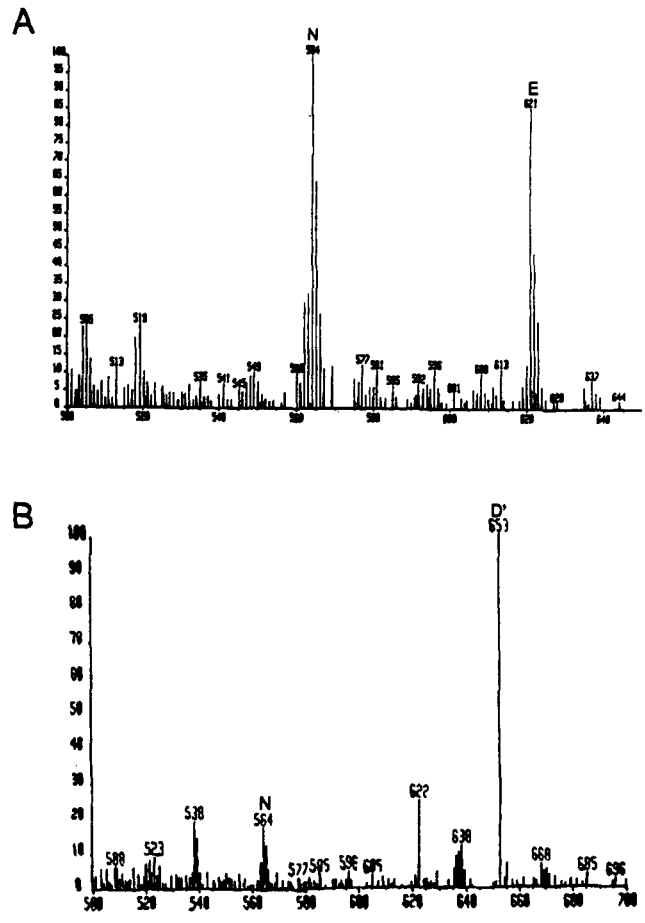

Figure 5. High-resolution FAB$^+$ mass spectra of two samples: (A) ~60% sulfhemin-E and 40% deuterohemin displaying the molecular ion (M^+) range from 500 to 650 (the M^+ peak at 621 (labeled E_{621}) arises from sulfhemin-E, while the M^+ peak at 564 (labeled N_{564}) is due to deuterohemin); and (B) ~70% sulfhemin-D′ and 30% deuterohemin displaying the molecular ion (M^+) range from 500 to 700 (the M^+ peak at 653 (labeled D'_{653}) results from sulfhemin-D′, while the M^+ peak at 564 (labeled N_{564}) is due to deuterohemin).

The ^{13}C chemical shifts of the paramagnetic bis-cyano complex of sulfhemin-E, or the intact met[^{13}C]S_EMb*CN prepared in the presence of ^{13}CN, do not have an interpretive basis in terms of functionality because of large contributions from the contact shift in these paramagnetic derivatives.[5] However, reduction of sulfhemin-E within the protein matrix readily affords the diamagnetic S_EMb*CO complex described in our companion report.[1] Figure 6A illustrates the ^{13}C NMR spectrum of met[^{13}C]S_EMb*^{13}CN prepared from $^{13}CN^-$, which exhibits two signals not present when $^{12}CN^-$ is used, a peak labeled $^{13}CN^-$ for free cyanide and peak E. Reduction of this sample with dithionite in the presence of CO, followed by chromatography to remove the displaced $^{13}CN^-$ ion, yields the [^{13}C]S_EMb*CO complex with the ^{13}C NMR trace shown in Figure 6B; only one signal, also labeled E, is retained at 110 ppm. Reoxidation of the [^{13}C]S_E-Mb*CO sample in Figure 6B with $K_3Fe(CN)_6$ in the presence of $^{12}CN^-$ yields the met[^{13}C]S_EMb*^{12}CN sample with the ^{13}C NMR trace shown in Figure 6C. The resonance E at 126 ppm is clearly associated with the sulfhemin-E prosthetic group in metS_EMb*CN, and its chemical shift in the diamagnetic S_E-Mb*CO complex is 110 ppm.

Sulfhemin-D. (a) Derivatization/Extraction/Reconstitution. Attempts to extract the red chromophore from metS_DMb*CN via the standard acid/2-butanone method[13] of a sample of 70% metS_DMb*CN, with the ^1H NMR spectrum as in Figure 1D, led to extensive precipitation with only the bis-cyano deuterohemin identified by ^1H NMR in the extract. Moreover, the amount of deuterohemin detected is consistent with that arising solely from the 30% unreacted metMb*CN in the original sample. Hence the prosthetic group is unstable to extraction, but it appears to yield insoluble coprecipitate with the protein rather than reverting back to deuterohemin. On the basis of the likely structure of sulfhemin-E as 4-thiocyanato-deuterohemin (see below), however, the possibility exists that the prosthetic group of S_DMb* may possess a free thiol group. Hence a solution of 70% metS_DMb*CN in ^2H_2O at pH 7.0 with excess cyanide removed via ultrafiltration was reacted for 1 week at 22 °C with 4 equiv of iodoacetamide, ICH_2CONH_2, a standard reagent for protecting free thiol groups.[20] This addition of iodoacetamide led to the complete loss of the resonances D_f of metS_DMb*CN, as shown in Figure 1D (without affecting the intensity of the unreacted metMb*CN resonances), and the appearance of a new set of peaks labeled D'_f for an apparent iodoacetamide adduct we will designate as metS_D′Mb*CN. The ^1H NMR spectrum of metS_D′Mb*CN is illustrated in Figure 1E, with three low-field heme methyl peaks D'_1–D'_3, upfield 2-H peak D'_5, and Ile 99 (FG5) $C_\gamma H$ peak D_{11}, all characteristic[1] of a new heme rather than a chlorin prosthetic group, which we designate sulfhemin-D′. The metS_D′Mb*CN complex was found stable to decomposition, precipitation, and further reaction in the presence of free CN^- over a period of several months.

Most importantly, the sulfhemin-D′ prosthetic group for the sample in Figure 1E was stable to the standard acid/2-butanone extraction,[13] which yielded a red product whose dissolution in 2:1

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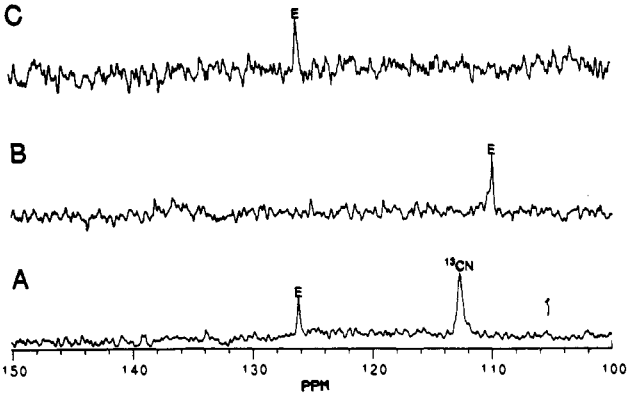

Figure 6. 100–150-ppm region of the 75-MHz ^{13}C NMR spectrum in ^2H_2O at 20 °C and pH 7.0 of (A) the paramagnetic metS_E[^{13}C]-Mb*^{13}CN complex prepared[1] by aerobically reacting the initially formed sulfmyoglobin complex in the presence of 90% labeled $^{13}CN^-$. The peak at 126.9 ppm labeled E arises from the ^{13}C label incorporated into the paramagnetic prosthetic group, while that labeled $^{13}CN^-$ is due to free cyanide ion in solution; the ^{13}C NMR resonance from the coordinated $^{13}CN^-$ is too broad to detect. (B) Spectrum of the diamagnetic S_E[^{13}C]-Mb*CO complex prepared from the sample used in (A) by dithionite reduction in the presence of unlabeled carbon monoxide, followed by removal of free $^{13}CN^-$ by ultrafiltration; the peak at 110.0 ppm labeled E arises from the ^{13}C label incorporated into the diamagnetic prosthetic group. (C) Spectrum of the paramagnetic metS_E[^{13}C]Mb*CN complex resulting from oxidizing with ferricyanide, in the presence of unlabeled CN^-, the complex in (B); comparison with (A) shows that only the peak from the label in the metS_E[^{13}C]Mb*CN remains.

$(C_2H_3)_2SO/^2H_2O$ with excess cyanide yielded the ^1H NMR spectrum illustrated in Figure 2D, with peaks labeled D'_i; the unreacted deuterohemin complex again yields the set of peaks labeled N_i. Reconstitution of the extracted mixture of prosthetic groups into fresh apo-Mb yielded holoproteins with the ^1H NMR trace illustrated in Figure 1F. All of the peaks of metS_D'Mb*CN (as well as residual metMb*CN) reappeared, indicating reversible extraction and reconstitution. However, a new set of resonances, labeled d'_i, also appears after reconstitution that were not detected prior to extraction. These resonances, d'_i, lose intensity with time and essentially disappear (<5% intensity) after a week (not shown).

(b) Spectral Studies. The ^1H NMR trace of 65% bis-cyano sulfhemin-D' and 35% bis-cyano deuterohemin under slow pulsing conditions is shown in Figure 2D; the resonances for the latter complex have been identified above. The expanded –1 to 4 ppm region of an inversion–recovery trace, designed to emphasize the rapidly relaxing meso-H's and suppress the slowly relaxing solvent lines, is shown in Figure 2E. The sulfhemin-D' complex exhibits 14 separate signals, 4 three-proton peaks, D'_1–D'_4, five two-proton signals, D'_5–D'_8, D'_{15}, and five single-proton peaks, D'_9–D'_{13}. The –1 to 4 ppm portion of a fast repetition rate spectrum shown in Figure 2E identifies the four fastest relaxing single-proton signals, D'_9–D'_{12}, that must be attributed to meso-H's. A COSY spectrum in Figure 4B identifies the pairs D'_5/D'_7 and D'_6/D'_8 as the C_2H_2–$C_\beta H_2$ fragments of the two intact propionate groups whose NOESY spectrum at –20 °C in Figure 5B exhibits cross peaks to adjacent methyls and meso-H's, uniquely identifying the four methyls, four meso-H's, the two propionates, and the 2-H signal. The 4-H is clearly lost, indicating that, as in bis-cyano sulfhemin-E, the 4-position alone of sulfhemin-D' is modified from that of deuterohemin. A new relatively weakly relaxed two-proton signal, D'_{15}, fails to exhibit either COSY or NOESY cross peaks and must originate from the iodoacetamide CH_2 group associated with position 4. The chemical shifts and T_1 values are listed in Table I.

The fast atom bombardment, FAB^+, mass spectrum of a 65% sulfhemin-D' and 35% deuterohemin mixture is shown in Figure 6B. The parent molecular ion, M^{+1}, for deuterohemin at 564 (labeled N_{564}) is observed, as for the pure complex. In addition, we observe a new ion at 653 (labeled D'_{653}) that must be associated with sulfhemin-D'. The loss of 4-H from deuterohemin dictates that the functional group at the 4-position must have a mass of 653 – (564 – 1) = 90.

Discussion

Molecular Structure of Sulfhemins. The 2D NMR spectra demonstrate that all functional groups of the precursor deuterohemin are retained in the extractable prosthetic groups of each of the two SMb* reaction products, except for the hydrogens at the 4-positions. The spectral characteristics of the chromophore in the intact SMb* complexes in a variety of oxidation/ligation states have previously shown both of the chromophores to be hemins rather than iron chlorins.[1] For sulfhemin-D', the mass of 90 for the 4-substituent, together with the fact that sulfhemin-D' is the stable product of sulfhemin-D and iodoacetamide, dictates that the 4-substituent is the thioacetamide group (SCH_2CONH_2, mass = 90) or that sulfhemin-D' is 4-thioacetamide-deuterohemin, *i.e.*, **1** with R_2 = H and R_4 = SCH_2CONH_2). The only direct NMR support for the 4-position functionality is the observation of a weakly relaxed two-proton signal D'_{15}. Moreover, since the 4-substituent of sulfhemin-D' is that expected for a thiol reacting with iodoacetamide, we assign the structure of sulfhemin-D as 4-thiol-deuterohemin, *i.e.*, **1** with R_2 = H and R_4 = SH.

The mass spectral data for sulfhemin-E prepared with unlabeled cyanide dictates a 4-substituent of mass 58, and the likely incorporation of a cyanide, reflected in the introduction of a ^{13}C label via $^{13}CN^-$, indicates the remainder of the 4-substituent has the mass of a retained sulfur atom. Here the NMR data on the 4-substituent are more direct in identifying its electronic nature. The ^{13}C chemical shift in diamagnetic S_EMb*CO for the label introduced via $^{13}CN^-$ is 110 ppm, which is in the narrow range exhibited by thiocyanate groups.[14] The optical spectrum of the iron-free sulfhemin-E exhibits a strong blue shift for its lowest energy UV–visible band, as compared to deuteroporphyrin. This shift is characteristic of a rhodo-type spectrum that reflect the presence of a strongly electron withdrawing group on the porphyrin periphery.[19] Hence we conclude that sulfhemin-E is 4-(thiocyanato)deuterohemin, *i.e.*, **1** with R_2 = H and R_4 = SCN.

Extraction and Reconstitution of Chromophores. The prosthetic groups of S_EMb* and S_D'Mb* are clearly reversibly extractable and provide stable chromophores suitable for detailed structural studies. For each of sulfhemin-E and -D', however, the reconstitution into apoMb* regenerated not only all of the resonances for metS_EMb*CN and metS_D'Mb*CN observed prior to extraction but also peaks for new species with peaks labeled e_i (in Figure 1C) and d_i (Figure 1F), which immediately after reconstitution exhibited intensity comparable to those of E_i and D'_i, respectively. For metS_D'Mb*CN, the peaks labeled d'_i lost intensity quickly with time (1 week), indicating that they are from a metastable form which is not significantly populated at equilibrium. This metastable form can be readily attributed to the sulfhemin-D' rotated by 180° about the α,γ-meso axis, as previously characterized for initially reconstituted Mb with a variety of hemins.[21,22]

The two sets of resonances, E_i, e_i, obtained upon reconstituting sulfhemin-E into Mb must have the same origin in heme orientational disorder, with the species with peaks e_i representing the orientation 180° rotated about the α,γ-meso axis from that of the product formed in the reaction. However, the species with peaks e_i loses intensity only very slowly and does not disappear, but reaches an apparent equilibrium ratio of intensity $E_i/e_i \sim$

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3/1 (Figure 1C). This unusually strongly perturbed orientational preference for sulfhemin-E is also apparent without extracting and reconstituting the chromophore. Thus after metS_EMb*CN is formed essentially quantitatively within a few days from aerobic metS_DMb*CN in the presence of cyanide,[1] the sample appears to very slowly (over 6 months) equilibrate further to yield a new species with peaks e_i, as shown in Figure 1C. These peaks, however, are the same as those generated with much higher intensity upon reconstitution and dictate that the equilibration of metS_EMb*CN to a new apparent sulfmyoglobin complex is nothing more than the *in situ* chemically modified prosthetic group reorienting about the α,γ-meso axis to accommodate altered contacts to the protein matrix.

Since the initially formed S_DMb*, S_D'Mb*, and S_EMb* must retain the orientation of the prosthetic group as in the native deuterohemin or protohemin, the reorientation of sulfhemin-E to yield a 3:1 rather than ~20:1 preference for the native orientation[22] indicates that there are destabilizing interactions between the 4-substituent of sulfhemin-E and the local protein matrix. The 4-SCN group is more rigid than the 4-vinyl group (or 4-thioacetamide group), and hence it appears not to be accommodated as readily by the protein matrix. It is of interest that the only other derivatized deuterohemin that has exhibited such a strong change in the orientational preference in the Mb pocket is 4-ethyldeuterohemin, as detected by single-crystal X-ray diffraction.[23] The much slower reorientation (by a factor of $\sim 10^2$) of sulfhemin-E as compared to protohemin[22] or deuterohemin[21] may reflect an electronic effect of the 4-substituent on the lability on the axial bond to the His F8 and CN^-, both of which must be broken to allow reorientation of the heme.

Structure Determination in Paramagnetic Hemins. The 2D ^1H NMR experiments on the directly extracted paramagnetic, low-spin ferric ($S = {}^1/_2$) sulfhemins provide all assignments necessary to identify the site of functionalization of the two derivatives of interest and further confirm that it is not necessary to make diamagnetic derivatives for structural determination. In order to make the NOESY experiment effective for a paramagnetic system, however, it was found necessary to use 2:1 $(C^2H_3)_2SO$–/^2H_2O, whose viscosity at −20 °C is 2–3 times that of ^2H_2O at 25 °C. Since the electronic T_1, and hence nuclear T_1's, of low-spin hemins are independent of the mobility of the complex,[11] but ^1H–^1H cross relaxation increases linearly with viscosity, the intensity of NOESY cross peaks, particularly those involving the more strongly relaxed meso-H's, are significantly increased and hence more readily detected.

The combination of mass spectra and ^{13}C NMR is sufficient to completely determine the chemical nature of the 4-substituents of sulfhemin-D' and sulfhemin-E to be the thioacetamide, $SCH_2CO(NH_2)$, and thiocyanate, SCN, groups, respectively. However, the NMR of the retained methyl substituents of the two chromophores can provide independent confirmation of the functionality of the 4-position. The dominant interaction contributing to the hyperfine shifts for a low-spin hemin is the contact shift that arises from the spin delocalized due to porphyrin-to-iron π charge transfer.[5] The placement of a methyl on each pyrrole allows the methyl contact shift to serve as an indicator of the asymmetry of the delocalized π spin density. The electron donating/withdrawing properties of methyls and propionates differ inconsequentially. Thus bis-cyano mesohemin (**1**: R_2 = R_1 = ethyl) exhibits essentially identical contact shifts for the four methyls.[17] It had previously been shown that, as the 2- and/or 4-substituents are made more electronic withdrawing, the π spin density, as reflected in the spread of the four methyl contact shifts, increases while the mean of the four methyl contact shifts remain minimally perturbed.[17]

The heme methyl shifts in bis-cyano complexes of deuterohemin

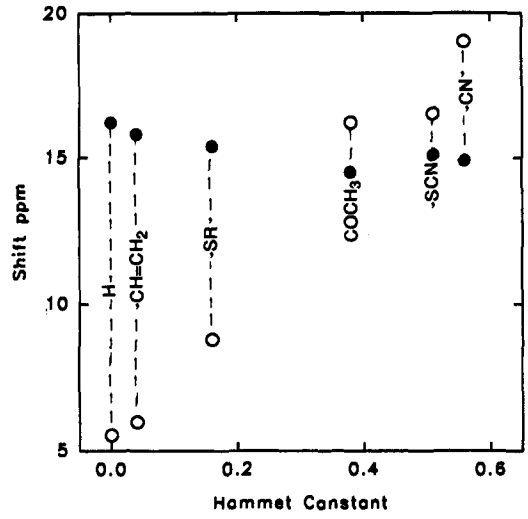

Figure 7. Plot of the mean of the four methyl shifts (open marker), in ppm, and the spread of the four methyls shifts (closed markers), in ppm, for bis-cyano complexes of various 4-R substituted deuterohemins with variable R_4, as a function of the Hammet σ constant; R_4 is given for each data point. Also included are the data for sulfhemin-E with R_4 = thiocyanate and sulfhemin D' with $R_4 = SCH_2CO(NH_2)$. Note the essential independence of R_4 for the mean chemical shifts, while the spread increases monotonically with the Hammet constant, σ.

substituted solely at position 4, *i.e.* structure **1** (R_2 = H with variable R_4), have been reported for R_4 = H,[21] vinyl, acetyl,[17] and cyanide.[24] The average and total spread of the four heme methyl shifts for these four characterized hemins are plotted versus the Hammet σ constant[25] in Figure 7. The plot shows that the mean of the four methyl shifts is largely invariant at 15.0 ± 0.5 ppm, and that the spread in the shifts increases monotonically with σ. The mean of the four methyl shifts for the bis-cyano sulfhemin-D' and -E is 15.4 and 15.1, respectively, and fall into the range for the others in Figure 7. The heme methyl shift spread for sulfhemin-E is 16.5 ppm, dictating that its 4-substituent is strongly electron withdrawing with the Hammet σ constant between that of acetyl (0.38) and cyanide (0.56) groups. This is clearly satisfied by the thiocyanate group, with σ = 0.51,[25] as shown in Figure 7. Similarly, the heme methyl shift spread of 8.8 ppm dictates the electronic influence of the 4-substituent in sulfhemin-D' is between that of a vinyl (0.04) and the acetyl (0.38) group, but much closer to the former. A σ value for SR of 0.16[25] again places the data point on the trend determined by the other 4-substituents in Figure 7. Hence the pattern in the heme methyl shifts in the bis-cyano sulfhemin-D' and -E provides direct confirmation of the chemical functionality of their unique 4-substituents.

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Supplementary Material Available: Two figures showing the optical spectrum and mass spectra of sulfporphyrin-E dimethyl ester (2 pages). Ordering information is given on any current masthead page.

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