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Identification of the Altered Pyrrole in the Isomeric Sulfmyoglobins: Hyperfine Shift Patterns as Indicators of Ring Saturation in Ferric Chlorins†

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ABSTRACT: Analysis of the ^{1}H NMR hyperfine shift patterns of isomeric sulfmyoglobins is carried out in the met-aquo and met-cyano states to determine the site of saturation in each protein. The utility of the patterns for structure elucidation is established by specific deuterium labeling of the heme methyls of the terminal base product. On the basis of the known saturation of ring B in this isomer [Chatfield, M. J., La Mar, G. N., Lecomte, J. T. J., Balch, A. L., Smith, K. M., & Langry, K. C. (1986) *J. Am. Chem. Soc. 108*, 7108-7110], the methyl resonance of the saturated ring is found to have strongly attenuated contact shift. Thus, the heme methyl contact shift pattern is diagnostic for the saturated pyrrole in the high-spin state. This rationale is then applied to analyze the assigned NMR spectra of the initial and terminal acid sulfmyoglobin products, revealing that the same ring B is saturated in each isomer. In contrast, the heme methyl contact shift pattern in low-spin ferric complexes reveals that the methyls both on the affected pyrrole and on the trans pyrrole are influenced similarly on sulfmyoglobin formation, precluding the use of this methyl shift pattern as a unique indicator of the site of saturation. Identification of exchangeable proximal histidine resonances for met-aquo sulfmyoglobin complexes with shifts similar to that in native myoglobin dictates inconsequential axial alterations in the sulfmyoglobins, while location of downfield meso proton resonances analogous to those of the native protein demonstrates the retention of the coordinate water in the active site of met-sulfmyoglobin.

Sulfmyoglobin (SMb)[1] is a green heme derivative of myoglobin (Mb) in which the native heme, **1**, has reacted with a sulfur atom in a manner that leads to saturation of the aromatic skeleton (Berzofsky et al., 1971). The chemical nature

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[1] Abbreviations: SMb, sulfmyoglobin; S_AMb, S_BMb, and S_CMb, isomeric forms of sulfmyoglobin; Mb, myoglobin; metMb, ferric myoglobin; NMR, nuclear magnetic resonance; ppm, parts per million; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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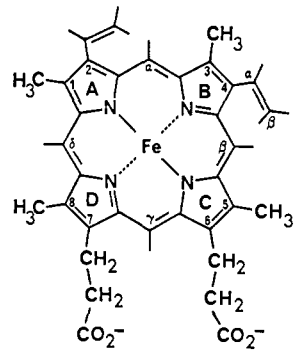

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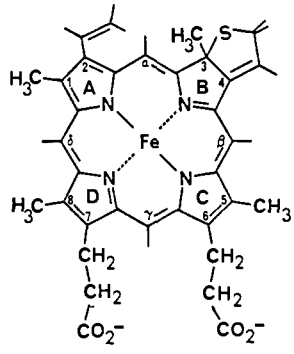
of the initially formed SMb has yet to be determined, but has been often envisaged as an episulfide across a pyrrole β–β bond (Berzofsky et al., 1972). Structure determination has been hampered by the very low stability of the modified prosthetic group with respect to reversion to the native state upon extraction from the protein (Berzofsky et al., 1972).

We have demonstrated recently that the SMb formed under standard preparative conditions is, in fact, heterogeneous (Chatfield et al., 1986a) and that at least three dominant species, labeled S_AMb, S_BMb, and S_CMb in order of formation, can be readily and selectively formed under specific solution conditions and oxidation/ligation state of the protein (Chatfield et al., 1987). The latter isomeric derivative, S_CMb, is formed at alkaline pH and only for Mb with a heme possessing a 4-vinyl substituent and has been shown to be reversibly extractable (Chatfield et al., 1986b,c). Selective isotopic labeling of the vinyl protons with deuterium, together with analysis of the spin multiplet structure, revealed that the 4-vinyl had reacted to yield structure **2** (Chatfield et al.,



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1986b). Derivatization of a similar extract has led to similar conclusions (Bondoc et al., 1986). Thus, the saturated pyrrole in S_CMb is ring B. The chemical natures of S_AMb, the precursor to S_CMb, and S_BMb, a terminal side product formed from S_AMb at acidic conditions, are unknown at this time. Two logical alternative hypotheses are that they all have ring B saturated, but with different functionality, or that they represent reaction of alternate pyrroles, with the conversion of S_AMb $\rightarrow$ S_CMb involving migration to a more stable site.

In this paper we address the question as to the identity of the saturated pyrrole in each of the three isomeric SMb using an indirect but nevertheless effective NMR method that relies on the analysis of the hyperfine shifts of the perturbed prosthetic group as influenced by the saturation of a pyrrole.

The elucidation of the structure of the prosthetic group of a hemoprotein by NMR is generally most effectively pursued by comparison of its detailed hyperfine shift pattern with that of well-characterized model complexes [La Mar & Walker (Jensen), 1978; La Mar, 1979; Budd et al., 1979]. To date, however, there have appeared few reports on the NMR spectra of paramagnetic iron chlorins (Stolzenberg et al., 1981; Strauss et al., 1985, 1987), and the reports have dealt only with synthetic porphyrin derivatives. A few studies of the NMR spectra of naturally occurring chlorins in proteins have also been reported, but the interpretations have been limited (Timkovich & Cork, 1982; Ikedo-Saito & Inubushi, 1987). Moreover, in no case have detailed and unambiguous assignments been presented. Therefore, we first seek to assign the NMR spectrum of S_CMb and to develop a basis for the interpretation of its hyperfine shift pattern in terms of the known saturation of ring B (Chatfield et al., 1986b). We then apply the model to interpreting the shift pattern of S_AMb and S_BMb. The oxidation state of choice is iron(III), for which detailed assignments and analyses are available for both model hemes and hemoproteins [La Mar & Walker (Jensen), 1978; La Mar, 1979; Satterlee, 1986].

The method for characterizing the hyperfine shifts relies on forming the desired SMb derivative from the native protein reconstituted with hemin that is selectively deuteriated at individual methyl positions. Other hemin sites not previously addressed by isotope labeling that can provide useful structural probes, namely, the meso and propionate protons, will also be considered. The high-spin ferric form of hemes exhibits predominantly scalar or contact shifts that are sensitive to strong perturbation of the π system of the porphyrin skeleton (Budd et al., 1979; La Mar & Budd, 1979; Balch et al., 1985a,b). The low-spin ferric derivatives have been shown to yield detailed information on the nature of the heme orientation within the cavity (Shulman et al., 1971; La Mar et al., 1978; Davis et al., 1983), but a localized perturbation is readily propagated to the sites remote from the perturbation (Shulman et al., 1969; Mayer et al., 1974; La Mar et al., 1978).

The ^{1}H NMR spectra of the three met-aquo SMb complexes have been reported and differ from native met-aquo Mb primarily by exhibiting only three, rather than the usual four, heme methyl signals in the far downfield shifted region, indicating a strong perturbation of one pyrrole in each case (Chatfield et al., 1987). The assignment of these strongly perturbed methyl groups should lead to the identity of the altered pyrrole in each isomeric SMb. Speculative assignments on the ^{1}H NMR spectrum of one of the low-spin ferric met-cyano SMb complexes have been offered based on analysis of pH behavior of individual peaks, and inference has been drawn from these assignments as to the identity of the altered pyrrole (Timkovich & Vavra, 1985). The present unambiguous assignments of both the high-spin met-aquo and low-spin met-cyano complexes will therefore allow comparison of the two states as to their relative utility in identifying the saturated pyrrole of a chlorin.

Experimental Procedures

Sperm whale myoglobin was purchased from Sigma Chemical Co. and used as received. Apo-Mb was prepared by modification of the method of Teale (1959): Mb was dissolved in doubly distilled water to produce a solution of less than 0.5 mM protein, the pH adjusted on ice to 2.5[2] with 0.1 M HCl, the hemin extracted exhaustively into 2-butanone that had been chilled to −20 °C, and the straw-colored apoprotein dialyzed exhaustively against doubly distilled water. Following removal of ketone, the protein was dialyzed once against 50 mM phosphate buffer, pH 7.02, to adjust the pH without precipitation of the protein, which is observed at higher buffer concentrations. A final dialysis against water removed the

[2] This pH is optimal for complete hemin removal without the denaturation experienced at lower pHs.

buffer salts prior to lyophilization.

Deuterium-labeled hemins labeled at methyl 1 (~90% 1-C^2H_3), [1-C^2H_3]hemin (this compound also has 6,7-H_β approximately 50% deuteriated), methyl 5 (~65% 5-C^2H_3), [5-C^2H_3]hemin, methyl 8 (~65% 8-C^2H_3), [8-C^2H_3]hemin, methyls 1 and 3 (two samples were used, one with >80% deuteriation at both methyls and 50% deuteriation of the 6,7-H_βs and one with >60% deuteriation at the methyls and ~50% deuteriation of the 6,7-H_βs), [1,3-$(C^2H_3)_2$]hemin, and at meso $\alpha,\beta,\gamma,\delta$ (>70% ^2H), [meso-$^2H_4$]hemin, are the same materials reported in detail previously (Smith & Pandey, 1983; Smith et al., 1979, 1986a–c; La Mar et al., 1980). Reconstitutions of the proteins were accomplished by first dissolving the apoprotein in 0.2 M NaCl to give a solution <0.5 mM in apo-Mb and dissolving 1 mg of the desired hemin in 100 μL of 0.2 M NaO^2H. Then 0.9 molar equiv of hemin was slowly added to the apo-Mb solution at 0 °C, the slight excess of apo-Mb ensuring complete reconstitution of the hemin. The sample was allowed to equilibrate at 22 °C for 4 days as the met-aquo protein, pH 7.0–7.5, to remove the heme disorder (La Mar et al., 1983). The samples were then centrifuged to remove traces of precipitation and concentrated and exchanged into 0.1 M phosphate buffer, pH 8.0, in ^2H_2O or H_2O by ultrafiltration on an Amicon 8MC (YM5 membrane) to a final concentration of approximately 3 mM protein.

Solutions of Fe^{II}SMb (approximately 3 mM in either H_2O or ^2H_2O) were prepared by the successive addition of hydrogen peroxide, catalase, and ammonium sulfide to the myoglobin solution by the conditions defined previously (Berzofsky et al., 1971). Reference and methyl-labeled samples of $metS_AMbH_2O$ were prepared following chromatography of the met-aquo protein (Figure 2), those of $metS_BMbH_2O$ were prepared by acidic equilibration of $metS_AMbH_2O$ (Figure 3), and those of $metS_CMbH_2O$ were produced by equilibration of S_AMbCO followed by oxidation to give the met-aquo protein (Figure 1); these conditions have been described in detail previously (Chatfield et al., 1987). The cyanide complexes were then generated by the addition of 2 equiv of KCN. Labeling of the meso sites was performed under conditions that minimize formation of $metS_BMbH_2O$ and $metS_BMbCN$ (Chatfield et al., 1987), the resonances of which were found to obscure the meso resonances of all SMbs. This was performed by preparation of $metS_AMbH_2O$ and $metS_AMbCN$ in situ as previously described (Chatfield et al., 1987) using Mb reconstituted with [meso-$^2H_4$]hemin (Figures 2 and 5); chromatography and equilibration at 4 °C for 3 months as previously described (Chatfield et al., 1987) provided the [meso-$^2H_4$]hemin–$metS_CMbCN$ of Figure 4. The pH of the samples was determined by using a Beckman Model 3550 pH meter equipped with an Ingold microcombination electrode; the values are not corrected for isotope effects.

^1H NMR spectra were recorded on a Nicolet NTC-360 spectrometer operating at 360 MHz in the quadrature mode. Typical spectra consisted of (5–10) × 10^3 transients of 8192 points over a 110- or 50-KHz bandwidth using a 7-μs 90° pulse. All chemical shifts are given in ppm from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) referenced by the HO^2H resonance. NMR difference spectra were generated by using a subroutine of the NMC-1280 program as previously described (Chatfield et al., 1987). Resonances are labeled as previously (Chatfield et al., 1987), with A_i, B_i, and C_i designating resonances of $metS_AMbH_2O$, $metS_BMbH_2O$, and $metS_CMbH_2O$, respectively; A_i', B_i', and C_i' and M_i label resonances of $metS_AMbCN$, $metS_BMbCN$, $metS_CMbCN$, and metMbCN, respectively.

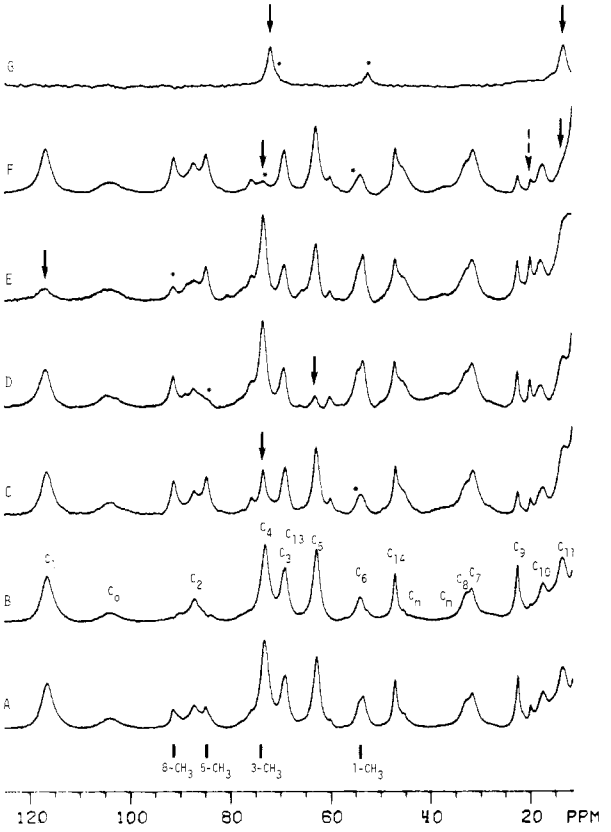

FIGURE 1: (A) 360-MHz ^1H NMR spectra of $metS_CMbH_2O$ at 20 °C in H_2O at pH 7.08. A stick diagram below gives the assigned methyl resonance positions in native $metMbH_2O$ (La Mar et al., 1980). (B) Computer-generated difference spectra of $metS_CMbH_2O$, with peaks C_i', formed by subtraction of the spectrum of $metMbH_2O$ (not shown) from the spectrum of trace A. (C, D, E, F) Analogous ^1H NMR traces of [1-C^2H_3]hemin–$metS_CMbH_2O$, [5-C^2H_3]hemin–$metS_CMbH_2O$, [8-C^2H_3]hemin–$metS_CMbH_2O$, and [1,3-$(C^2H_3)_2$]-hemin–$metS_CMbH_2O$ (also contains deuteriation at the β-propionates), respectively. (G) ^2H NMR trace of the sample of trace F. Asterisks designate loss of peak intensity due to the expected deuteriation of unreacted $metMbH_2O$; solid and dotted arrows designate loss of intensity due to deuteriation of methyl and single protons, respectively, in $metS_CMbH_2O$.

^2H NMR spectra were recorded on a Nicolet NT-500 spectrometer with a deuterium probe operating at a frequency of 76.76 MHz. These spectra were collected on 4096 points over a 34-kHz bandwidth with a 90° pulse of 25 μs and consisted of 5000 transients.

RESULTS

Figure 1A shows the low-field portion of the 360-MHz ^1H NMR spectrum of $metS_CMbH_2O$, with peaks of interest labeled C_i. This spectrum differs from that reported previously (Chatfield et al., 1987) only in that the solvent is 100% H_2O and an exchangeable proton signal is observed at 104 ppm. The individual resonances of this protein overlap those of the residual $metMbH_2O$, as indicated by the stick diagram of $metMbH_2O$ methyls shown below trace A, but may be separated by means of a computer-generated difference spectrum as shown in Figure 1B, which emphasizes the three apparent methyl peaks labeled C_1, C_4, and C_5. The peak at 70 ppm has been shown to be a composite of two one-proton peaks (Chatfield et al., 1987). Another candidate for a methyl peak is C_{11} on the low-field edge of the diamagnetic envelope, but its intensity is difficult to quantitate.

The influence of deuteriation of solely the 1-methyl group, the 5-methyl group, and the 8-methyl group is illustrated in parts C, D, and E, respectively, of Figure 1, which clearly identify the $metS_CMbH_2O$ peaks C_1, C_4, and C_5 as arising

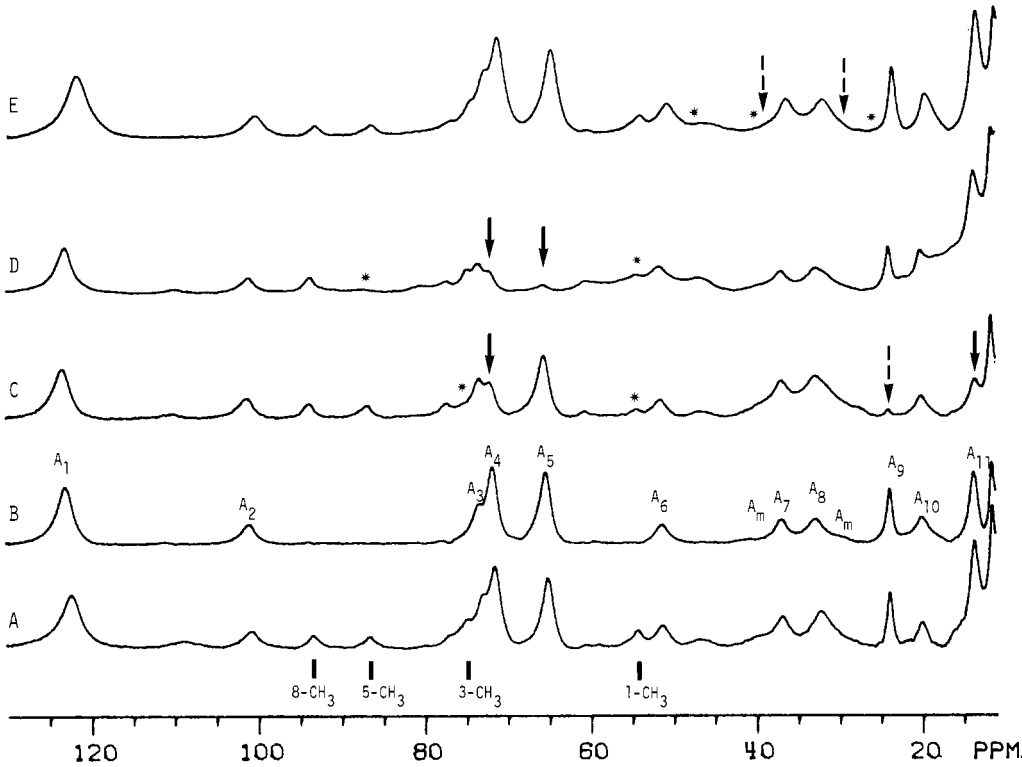

FIGURE 2: (A) 360-MHz ^1H NMR spectra of $metS_AMbH_2O$ at 20 °C in ^2H_2O at pH 7.1. A stick diagram below gives the assigned methyl resonance positions in native $metMbH_2O$ (La Mar et al., 1980). (B) Computer-generated difference spectrum of $metS_AMbH_2O$, with peaks A_i, pH 7.1, formed by subtraction of the spectrum of $metMbH_2O$ from trace A. (C, D, E) Analogous ^1H NMR traces of [1,3-$(C^2H_3)_2$]hemin–$metS_AMbH_2O$, pH 6.0 (also deuteriated at the β-propionates), [1,5-$(C^2H_3)_2$]hemin–$metS_AMbH_2O$, pH 6.0, and [meso-$^2H_4$]hemin–$metS_AMbH_2O$, pH 7.1, respectively; A_m labels meso resonances of $metS_AMbH_2O$. Asterisks designate loss of intensity due to expected deuteriation of the $metMbH_2O$ substituents. Solid and dotted arrows designate loss of intensity due to deuteriation of methyl and single proton resonances, respectively, of $metS_AMbH_2O$.

from the 8-CH_3, 1-CH_3, and 5-CH_3, respectively. The expected deuteriation of methyls for the residual $metMbH_2O$ is shown by asterisks. The location of the 3-methyl group is effected by the use of a hemin deuteriated at both the 1- and 3-methyl positions, as well as at the propionate β-position. The NMR trace of $metS_CMbH_2O$ reconstituted with the latter hemin is shown in part F of Figure 1. The loss of intensity of C_4 confirms the 1-CH_3 assignment, and the decrease in intensity at 13 ppm near the diamagnetic envelope indicates that C_{11} is 3-CH_3. This is confirmed by the ^2H NMR trace of the same complex, as shown in part G of Figure 1, which exhibits comparable intensity at the known 1-methyl position and at ~13 ppm. Thus, the "missing" heme methyl in the downfield-shifted region of $metS_CMbH_2O$ is 3-CH_3. The decrease in intensity of the narrow single proton peak C_9 in Figure 1F establishes that it arises from a 6- or 7-propionate H_β. The established assignments are listed in Table I.

The methyl assignments of $metS_AMbH_2O$, the precursor to $metS_CMbH_2O$, are pursued by using a pair of doubly labeled hemins. The trace for this initially formed complex in ^2H_2O is illustrated in part A of Figure 2; in H_2O, an additional exchangeable one proton peak (A_0) is observed at ~104 ppm (not shown). The residual $metMbH_2O$ peaks are recognized by comparison to the native spectrum with the stick diagram given below trace A. The computer difference trace of solely $metS_AMbH_2O$ is reproduced in part B of Figure 2, which reveals three apparent methyl peaks, A_1, A_4, and A_5, in the far downfield region, and one potential methyl peak at ~13 ppm. The effects of deuteriation of the 1,3-methyls (as well as 6,7-propionate H_βs) and the 1,5-methyls are shown in parts C and D, respectively, of Figure 2, which clearly provide the assignment of A_4, A_5, and A_{11} to 1-CH_3, 5-CH_3, and 3-CH_3, respectively. The remaining methyl peak, A_1, can thus be assigned to 8-CH_3. The relatively narrow single proton peak, A_9, also loses intensity in trace D, identifying it as a propionate H_β. Trace E displays the spectrum of $metS_AMbH_2O$ formed with [meso-$^2H_4$]hemin; a decrease in intensity of two very broad resonances, labeled A_m, near 30 and 40 ppm is observed.

Table I: ^1H NMR Chemical Shifts (ppm) of Assigned Resonances of the Isomeric Met-aquo Sulfmyoglobin Complexes, pH 7.1, 20 °C, in ^2H_2O

peak χ_i[a]	assignment	S_AMb	S_BMb	S_CMb	Mb[b]
1	8-CH_3	121.4	107.5	120.6	92.8
4	1-CH_3	70.9	72.3	74.7	54.0
5	5-CH_3	64.5	76.8	64.4	86.3
11	3-CH_3	13.5	16.5	13.2	74.4
9	β-propionate	23.6	24.0	22.8	20.0
m	meso H	30, 40	*c*	38, 45	27, 38, 48
0	N–H	104.2	104.2	104.2	103.6

[a] Where χ = A, B, or C (see text). [b] Assignments from La Mar et al. (1980). [c] Not determined.

The methyl assignments for the acidic terminal SMb product, $metS_BMbH_2O$, are pursued by using the same two doubly methyl labeled hemins. The difficulty with the assignments of this complex is that it cannot be prepared without having present appreciable amounts of the precursor, $metS_AMbH_2O$, as well as unreacted $metMbH_2O$ (Chatfield et al., 1987). The trace of a preparation containing ~25% $metS_BMbH_2O$ is illustrated in part A of Figure 3. The computer difference trace of $metS_AMbH_2O$ is reproduced in trace B. By computer difference correction for both native $metMbH_2O$ and $metS_AMbH_2O$, we generate the trace for pure $metS_BMbH_2O$, as shown in part C of Figure 3. Again, we find three apparent methyl peaks, B_1, B_4, B_5, in the far downfield region and one near the diamagnetic envelope, peak B_{11} at ~15 ppm. Preparation of the desired proteins using

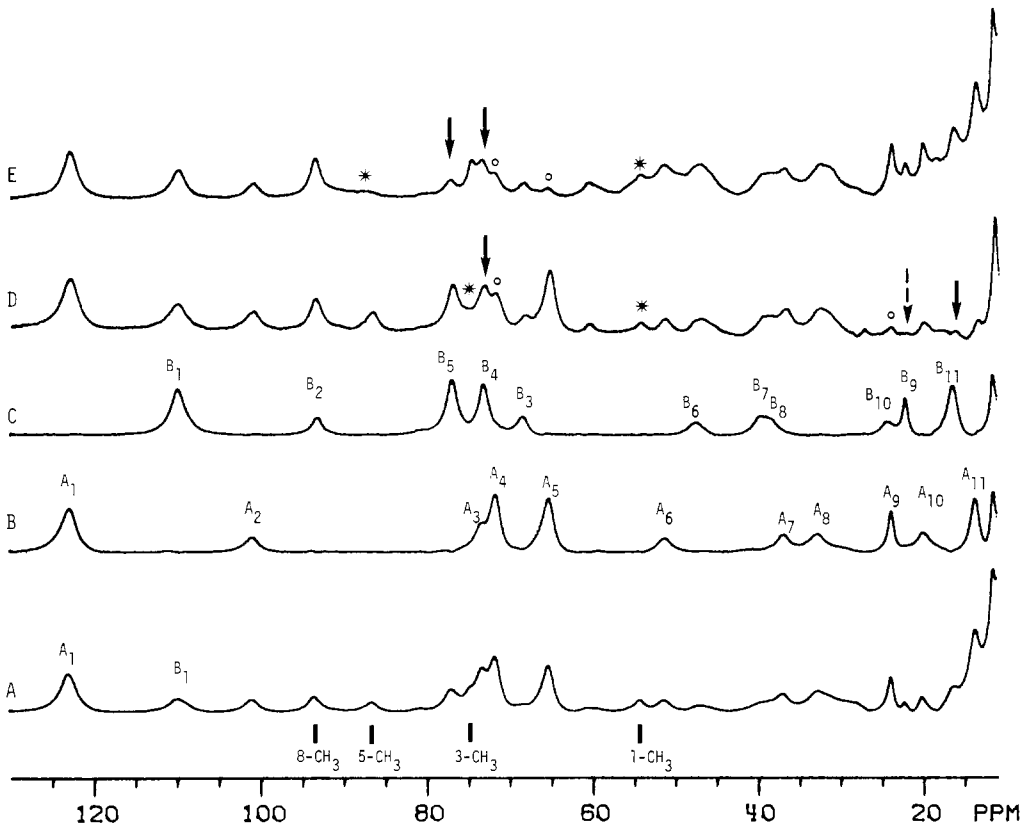

FIGURE 3: (A) 360-MHz ^1H NMR spectra of $metS_BMbH_2O$ (25%, peaks designated B_i) in the presence of $metS_AMbH_2O$ (65%, peaks labeled A_i) and 10% $metMbH_2O$ at 20 °C, in ^2H_2O at pH 6.06. A stick diagram below provides the assigned methyl resonance positions in $metMbH_2O$ (La Mar et al., 1980). (B) Computer-generated difference spectrum of $metS_AMbH_2O$ from Figure 2B. (C) Computer-generated difference spectrum of $metS_BMbH_2O$, with peaks labeled B_i, formed via sequential subtraction of the resonances of $metMbH_2O$ and of $metS_AMbH_2O$ (trace B) from trace A. (D, E) Analogous ^1H NMR traces of [1,3-$(C^2H_3)_2$]hemin–$metS_AMbH_2O$ and –$metS_BMbH_2O$ (55:25) (also deuteriated at the β-propionates) and [1,5-$(C^2H_3)_2$]hemin–$metS_AMbH_2O$ and –$metS_BMbH_2O$ (50:25), respectively. Asterisks and open circles designate expected deuteriation of $metMbH_2O$ and residual $metS_AMbH_2O$ (Figure 2), respectively. Solid and dotted arrows indicate loss of intensity of methyl and single proton resonances, respectively, due to deuteriation of the $metS_BMbH_2O$ substituents.

the hemins labeled at the 1,3- and 1,5-methyls yields traces D and E of Figure 3, respectively. Here the expected deuteriations of residual native protein are again marked by asterisks, and the now anticipated deuteriation of $metS_AMbH_2O$ peaks (from Figure 2) are marked by open circles. The loss of intensity of peaks B_4 and B_{11} in trace D and of peaks B_4 and B_5 in trace E establishes the assignments of B_4, B_5, and B_{11} to 1-CH_3, 5-CH_3, and 3-CH_3. The remaining methyl peak, B_1, hence must arise from the 8-CH_3, and the narrow single proton peak B_9 is due to a propionate H_β. The presently known assignments of the three met-aquo SMb complexes are listed in Table I.

Met-cyano Sulfmyoglobin Complexes. The resolved portions of the 360-MHz ^1H NMR spectrum of $metS_CMbCN$, with peaks C_i, relevant to assignment are illustrated in part B of Figure 4; the trace of pure native metMbCN is shown in trace A, which allows identification of the residual native protein peaks, M_5, M_1, and M_8, known to arise from the 5-CH_3, 1-CH_3, and 8-CH_3 (Mayer et al., 1974; La Mar et al., 1983). The computer difference corrected trace of pure $metS_CMbCN$ is given in part C of Figure 4, with peaks C_1', C_4', and C_{12}' having intensity consistent with methyl peaks. Peaks C_{19}', C_{17}', and C_{18}' have been shown previously to originate in the 4-ethylidene H_α and H_βs, while C_5' arises from the 2-vinyl H_α (Chatfield et al., 1986b). Spectra of $metS_CMbCN$ prepared from hemin deuteriated at 1-methyl, 5-methyl, both 1,3-methyls, and 8-methyl are shown in traces D, E, F, and G, respectively, of Figure 4, and clearly identify C_1' as 1-CH_3, C_4' as 5-CH_3, and the upfield peak C_{12}' as 3-CH_3. Trace G indicates the C_{20}' at –1.7 ppm is due to 8-CH_3 (confirmed by ^2H detection, not shown). Thus, all four heme methyls are unambiguously assigned in $metS_CMbCN$. Formation of $metS_CMbCN$ with [meso-$^2H_4$]hemin provides a sample with the spectrum in trace H, showing reduced intensity of resonance C_m', with an additional meso assigned at 10.0 ppm (not shown).

The assignments of $metS_AMbCN$ and $metS_BMbCN$ are pursued simultaneously in preparations containing both species, as shown in Figure 5. The trace of a ~65% $metS_AMbCN$, ~25% $metS_BMbCN$ mixture (10% metMbCN) is illustrated in trace B; the very small residual metMbCN methyl peaks, M_5, M_1, M_8, are recognized by comparison with its trace in part A of the figure. The computer-difference traces of pure $metS_AMbCN$ and $metS_BMbCN$, generated from trace B and that of the initially produced nearly pure $metS_AMbCN$ (not shown), are reproduced in parts C and D of Figure 5, respectively; small impurity peaks in the latter trace are marked by x. Clearly recognizable are three apparent methyl peaks, A_1', A_4', and A_{12}' and B_1', B_4', and B_{12}', for each species. Any one of these three methyl peaks serves as an indicator of the amount of that species present when any other pair of heme methyls is deuteriated if the presence of a single proton resonance of $metS_AMbCN$ residing directly under A_4 and that of another under B_{12} are taken into account.

Preparations using hemin deuteriated at the 1,3-methyl and the 1,5-methyl yield NMR traces E and F, respectively, of Figure 5. In trace E, the A_4', B_4' peak intensity dictates that both pairs of peaks A_1', A_{12}' and B_1', B_{12}' lose intensity, establishing that they all arise from 1-CH_3 or 3-CH_3. Trace F shows the presence of comparable amounts of $metS_AMbCN$

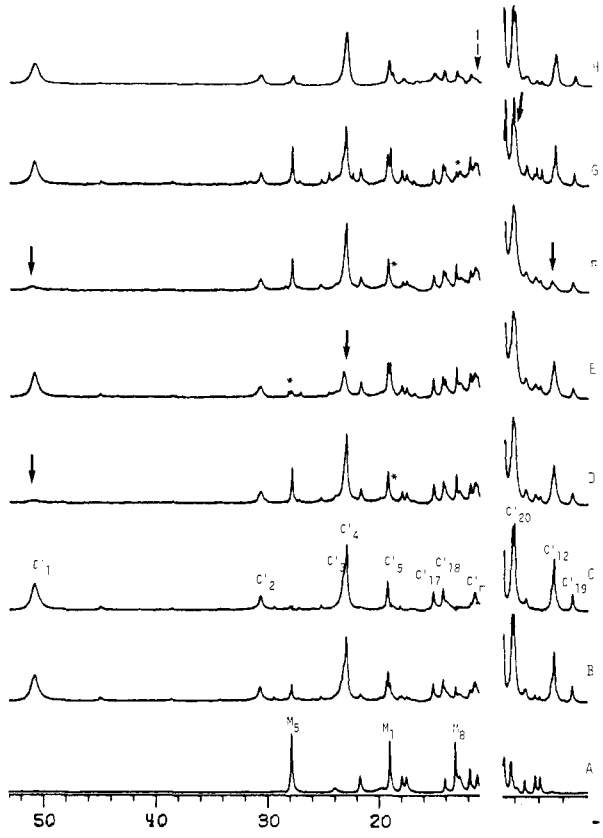

FIGURE 4: (A) 360-MHz ^1H NMR spectra of native metMbCN provided for reference with resonances M_i at 20 °C in H_2O at pH 7.1. (B) MetS_CMbCN at 20 °C in H_2O at pH 7.1. (C) Computer-generated difference spectrum of metS_CMbCN, with peaks C_i' formed following subtraction of the resonances of metMbCN (trace A) from those of metS_CMbCN (trace B). (D, E, F, G, H) Analogous ^1H NMR traces of [1-C^2H_3]hemin–metS_CMbCN, [5-C^2H_3]hemin–metS_CMbCN, [1,3-$(C^2H_3)_2$]hemin–metS_CMbCN (partially deuteriated at the β-propionates), [8-C^2H_3]hemin–metS_CMbCN in H_2O, and [meso-$^2H_4$]hemin–metS_CMbCN in ^2H_2O, respectively. The upfield region is vertically scaled to 50% of the downfield section. Asterisks designate expected deuteriation of unreacted metMbCN (Mayer at al., 1974; La Mar et al., 1983). Arrows designate loss of intensity due to deuteriation of the metS_CMbCN substituents.

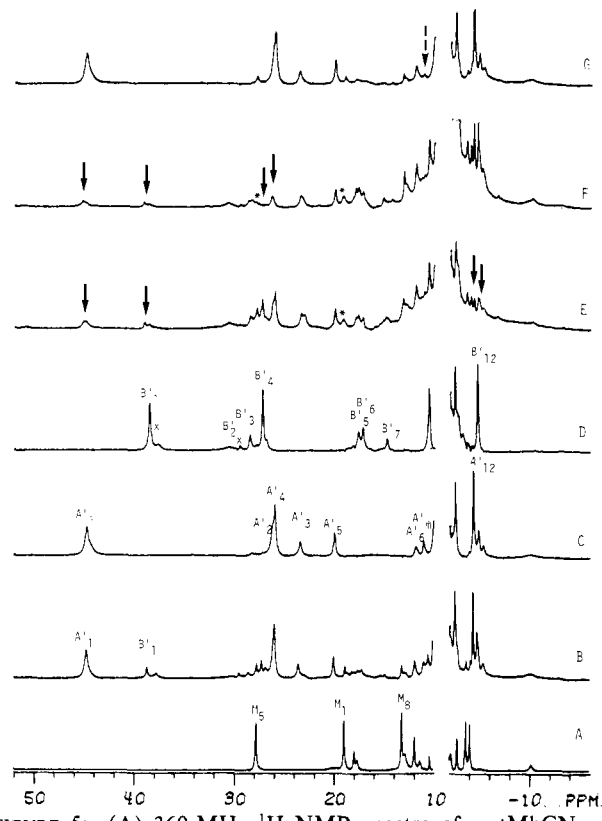

FIGURE 5: (A) 360-MHz ^1H NMR spectra of metMbCN with resonances labeled M_i at 20 °C, in ^2H_2O at pH 7.1. (B) Mixture of metS_AMbCN (peaks A_i') and metS_BMbCN (peaks B_i') (65:25). (C) Computer-generated difference spectrum of metS_AMbCN formed by subtraction of the spectrum of metMbCN (trace A) from that of a pure sample of metS_AMbCN (not shown). (D) Computer-generated difference spectrum of metS_BMbCN prepared by sequential subtraction of the resonances of traces A and C from that of trace B. x designates small impurity peaks (Chatfield et al., 1987). (E, F, G) Analogous ^1H NMR traces of [1,3-$(C^2H_3)_2$]hemin–metS_AMbCN and –metS_BMbCN (55:25) (partially deuteriated at the β-propionates), [1,5-$(C^2H_3)_2$]hemin–metS_AMbCN and –metS_BMbCN (50:25), and [meso-$^2H_4$]hemin–metS_AMbCN, respectively. Asterisks designate expected deuteriation of unreacted metMbCN. Solid and dotted arrows designate loss of intensity due to deuteriation of the methyls and single proton peaks, respectively, of the metS_AMbCN and metS_BMbCN complexes.

and metS_BMbCN by the intensity of peak A_{12}', B_{12}', and the sharply decreased intensity of the two pairs of signals A_1', B_1' and A_4', B_4' dictates that they arise from 1-CH_3 or 5-CH_3. The combination of data from traces E and F therefore uniquely dictates that A_1' and B_1' both originate in 1-CH_3, A_4' and B_4' both are due to 5-CH_3, and A_{12}' and B_{12}' both arise from 3-CH_3. The 8-CH_3 of metS_AMbCN was found by isotope-labeling to resonate in the crowded region at −0.84 ppm (not shown); the corresponding peak of metS_BMbCN was not located. No additional intensity loss was observed for resonances corresponding to deuteriation of the 6,7-propionate H_βs. Trace G displays the spectrum of metS_AMbCN deuteriated at the meso positions. A single resonance downfield of 10 ppm, A_m', exhibits a loss of intensity, assigning it to a meso proton; a second partially resolved meso resonance is found at 9.6 ppm (not shown). Because this region is obscured in mixtures of metS_AMbCN and metS_BMbCN, meso assignments in the latter protein were not attempted. The assigned resonances in all three met-cyano SMb complexes are listed in Table II.

Table II: ^1H NMR Chemical Shifts (ppm) of Assigned Resonances of the Isomeric Met-cyano Sulfmyoglobin Complexes, pH 7.1, 20 °C, in ^2H_2O

peak χ'[a]	assignment	S_AMb	S_BMb	S_CMb	Mb[b]
1	1-CH_3	44.7	38.5	50.7	18.9
4	5-CH_3	25.9	27.6	23.0	27.7
12	3-CH_3	−4.3	−4.7	−5.2	5.1
20	8-CH_3	−0.8	*c*	−1.7	13.1
m	meso H	11.0, 9.6	*c*	11.5, 10.0	*c*

[a] Where χ = A, B, or C (see text). [b] Assignments from Mayer et al. (1974) and La Mar et al. (1983). [c] Not determined.

DISCUSSION

Structural Indicators of Chlorins. The NMR spectral properties of both the high-spin and low-spin ferric complexes of the chlorin-like prosthetic group in S_CMb differ from those of analogous complexes of native Mb (Tables I and II). For the met-aquo complex, the methyl assignments clearly reveal that large contact shifts are observed for the three unperturbed pyrroles A (1-CH_3), C (5-CH_3), and D (8-CH_3) of metS_CMbH_2O, which are similar to those found in the native complex (La Mar et al., 1980) and that the saturated pyrrole (B) exhibits a dramatically reduced contact shift. Thus, the difference in contact shift pattern from those of the native heme are both qualitative and quantitative. The selective and localized perturbation on the saturated pyrrole is due to direct attenuation of transferred spin density to the affected pyrrole B. Similarly sharp and selective attenuations of contact shifts for substituents on the affected pyrrole have been noted in

model complexes where a carbene inserts into a Fe–N bond (Balch et al., 1985a) or where a single pyrrole nitrogen is alkylated (Balch et al., 1985b). Moreover, selective and localized perturbations have been observed in native metMbH_2O when HgI_3^- was intercalated over pyrrole A (La Mar & Budd, 1979). Thus, the presently observed selective decrease in contact shift of the 3-CH_3 in metS_CMbH_2O is completely consistent with, and apparently diagnostic for, its known site of saturation.

In a more detailed analysis, we note that metS_CMbH_2O exhibits similar methyl contact shifts for pyrroles A and C adjacent to the saturated pyrrole, while pyrrole D trans to the saturation site exhibits a somewhat larger contact shift. It then appears that ring saturation not only abolishes effective spin transfer to the saturated pyrrole but also appears to enhance spin transfer to the trans pyrrole. Thus, the high-spin ferric state of chlorins appears to serve as an ideal state for which the detailed characterization of the contact shift pattern could yield the identity of the saturated pyrrole(s).

The NMR spectral characteristics of low-spin metS_CMbCN differ quantitatively from those of native metMbCN but retain the same characteristic asymmetry properties, and the affected pyrrole exhibits only a small and structurally nondiagnostic change in hyperfine shift. The dominant heme contact shift pattern in native metMbCN is that the methyls on pyrroles A (1-CH_3) and C (5-CH_3) are shifted strongly downfield and those on pyrrole B (3-CH_3) and D (8-CH_3) strongly upfield (Mayer et al., 1974; La Mar et al., 1983) compared to the mean methyl shift or that of a fourfold symmetric model compound [La Mar & Walker (Jensen), 1978]. This pattern is retained in metS_CMbCN, although the relative and absolute magnitudes of the 1-CH_3 and 5-CH_3 low-field shifts differ. It is noteworthy that the upfield bias of 8-CH_3 and 3-CH_3 is almost the same in spite of the fact that only pyrrole B (with appended 3-CH_3) is affected in forming metS_CMbCN. More importantly, the known saturated pyrrole B (3-CH_3) exhibits an upfield shift, which, while further upfield than in native metMbCN (La Mar et al., 1986), is completely within a window of 3-CH_3 shifts of the unperturbed hemin of legume metcyanohemoglobin that exhibits otherwise the same qualitative hyperfine shift pattern (Kong, 1982). It has been noted that intercalation of xenon and cyclopropane over pyrrole A of metMbCN causes the largest shift alteration at sites far removed from the perturbation (Mayer et al., 1974). Thus, we conclude that the hyperfine shift patterns of low-spin ferric chlorins are clearly not diagnostic for the site of saturation. Certainly the strongly low-field shifted 1-CH_3 does not lend itself to any obvious interpretation in terms of the saturation of ring B.

An attempt to infer the site of saturation in a met-cyano complex of SMb on the basis of speculative assignments of the low-field methyls has been reported (Timkovich & Vavra, 1985). The present conclusion is that even the correct assignments are not likely to yield the correct site of saturation in the low-spin met-cyano complex but that such a determination of structure is likely based on correct assignment of heme methyl peaks in the high-spin ferric met-aquo complex.

Structural Comparison of Isomeric Sulfmyoglobins. The question as to whether the same pyrrole or different pyrroles are saturated in S_AMb or S_BMb than in S_CMb is readily answered by the detailed methyl assignments of the respective met-aquo complexes, as summarized in Table I. In both S_AMb and S_BMb, not only are the patterns of the four methyl shifts very similar to those of S_CMb, but in each case 3-CH_3 exhibits a uniquely attenuated contact shift and 8-CH_3 exhibits a somewhat larger contact shift than in native Mb. These data serve as compelling evidence that each of the three isomeric SMbs have the same pyrrole B saturated and that the structural difference must reside in altered chemical functionality of the affected pyrrole. These results are consistent with, but do not in any way prove, that the precursor, S_AMb, possesses an episulfide across the β–β bond of pyrrole B (Berzofsky et al., 1972), which rearranges to yield S_CMb.

The detailed assignments of heme methyl peaks in the low-spin met-cyano complexes of S_AMb and S_BMg again reflect the same identity for similarly shifted signals. Thus, although the detailed shift patterns may not clearly reveal the affected pyrrole, the essentially identical pattern for the three isomeric SMbs argues for similar rhombic perturbations in the three complexes. Detailed interpretation of the hyperfine shift pattern in low-spin ferric chlorin-like complexes will be deferred until information becomes available on such model complexes outside the protein matrix. This is necessary because it is known that the heme–protein interaction, in the form of the axial histidyl imidazole bond, is the major cause of the in-plane asymmetry in such protein complexes (Shulman et al., 1971; Traylor & Berzinis, 1980). Such protein-based perturbations of high-spin ferric hemes are insignificant by comparison (Budd et al., 1979) and provide the dominant rationale as to why the selective perturbation in the met-aquo, but not in the met-cyano, complexes can be interpreted uniquely in terms of the site of saturation.

It may be noted that the attempted assignment (Timkovich & Vavra, 1985) of the low-field methyls of metS_AMbCN using indirect arguments based on titration behavior of the peak position resulted in one correct assignment (peak A_4 to 5-CH_3) and one incorrect assignment (peak A_1 to 8-CH_3). However, even the correct assignment of metS_AMbCN fails to provide direct evidence for the now known site of saturation of ring B (Chatfield et al., 1986b; Bondoc et al., 1986).

Other Structural Indicators in Sulfmyoglobin. The exchangeable proton resonance at 104 ppm in metS_CMbH_2O (Figure 1) and metS_AMbH_2O (not shown) must originate from the labile ring proton of the proximal histidyl imidazole (La Mar & de Ropp, 1979; La Mar, 1979). The very similar, necessarily contact shift of this proton to that in native metMbH_2O (103.8 ppm) (La Mar & de Ropp, 1979) further supports the contention that sulfglobin formation does not significantly alter the proximal axial protein interaction. We had previously shown that a similar conclusion for both the proximal and distal sides could be inferred from the NMR spectral characteristics of the ferrous deoxy and carbonyl ligated forms of the isomeric SMbs (Chatfield et al., 1987).

The single assigned propionate H_β peaks (A_9, B_9, and C_9) in the met-aquo sulf-Mbs exhibit similar shifts and line widths to those for a native metMbH_2O peak that was specifically assigned to the 7-propionate group and shown to be consistent with its unique orientation (Unger et al., 1985). We similarly assign peaks A_9, B_9, and C_9 to this proton. Thus, the orientation of at least the 7-propionate group is inconsequentially influenced by sulfglobin formation.

Each of the three met-aquo SMb isomers fails to exhibit any detectable broad peaks on the upfield side of the diamagnetic envelope (Chatfield et al., 1987) but does show evidence for very broad peaks in the low-field 20–40 ppm region where the meso-Hs of the native protein resonate (La Mar et al., 1980). Such peaks are labeled C_m and A_m in Figures 1 and 2. The loss of intensity of two of these broad components in metS_AMbH_2O (Figure 2E) upon meso deuteriation reveals meso-H hyperfine shifts very similar to those

in the native protein. By comparison of the spectra of $metS_AMbH_2O$ with those of $metS_CMbH_2O$, we similarly assign the broad resonances C_m in Figure 1 to meso Hs of $metS_CMbH_2O$. Inasmuch as clearly five-coordinate high-spin ferric model complexes of both porphyrins and chlorins (Budd et al., 1979; Stolzenberg et al., 1985) reveal strongly upfield shifted meso-H signals, the presently located downfield meso-H peaks in ferric high-spin SMbs indicate that they are indeed six-coordinate and hence properly titled as met-aquo complexes.

Current studies are addressed toward obtaining a more quantitative description of the NMR spectral properties of model chlorin complexes to be used in assessing the influence of protein folding on the spectral properties.

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