Sulfmyoglobin Derived from Deuterohemin Reconstituted Protein. 1. Structure of the Initial Complex Based on the Mechanism of Formation of Subsequent Reaction Products

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Received December 3, 1992

Abstract: The preparation, reactivity patterns, and ^{1}H NMR and optical spectral properties of the sulfmyoglobin, SMb, complex formed from equine myoglobin reconstituted with deuteroheme are investigated to shed light on the structure of the prosthetic group. The characteristic ^{1}H NMR spectral properties in both paramagnetic and diamagnetic derivatives of the initially formed green SMb show it to contain an iron chlorin with molecular/electronic structure very similar to that obtained with native protohemin (Chatfield, M. J.; La Mar, G. N.; Kauten, R. J. *Biochemistry* **1987**, *27*, 6939–6950), except that the deuterohemin SMb is significantly less stable with respect to reversion to starting material. In low-spin ferric or ferrous derivatives, the initially formed green SMb undergoes a first-order rearrangement that regenerates a red hemin-containing SMb complex distinct from the starting material. In the absence of oxygen, this is a terminal product that slowly precipitates out of solution. In the presence of both oxygen and excess nucleophile such as azide or cyanide, this product reacts further to yield yet another red hemin-containing SMb, which ^{13}C NMR in the presence of $^{13}CN^-$ reveals to have incorporated the anion into the prosthetic group of this terminal reaction product. Using the identity of the perturbed 4-substituents as 4-thiol and 4-thiocyanato in the terminal anaerobic and aerobic SMb reaction products, respectively, as determined by 2D NMR and mass spectrometry of the extracted prosthetic groups (Scharberg, M. A.; La Mar, G. N., following paper), a detailed mechanism for the formation of these two products is presented where this initial episulfide rearranges by hydrogen migration to yield the 4-thiol group. This relatively unstable and reactive group, in turn, reacts with molecular oxygen to give a sulfenic acid for which the hydroxide ion is rapidly replaced by the stronger nucleophile, azide or cyanide. The retention of the incorporated sulfur atom (Berzofsky, J. A.; Peisach, J.; Horrecker, B. L. *J. Biol. Chem.* **1972**, *247*, 3783–3791) and its rearrangement to the 3-position of the terminal reaction product of native protohemin-containing Mb (Chatfield, M. J.; La Mar, G. N.; Lecomte, J. T. J.; Balch, A. L.; Smith, K. M.; Langry, K. C. *J. Am. Chem. Soc.* **1986**, *108*, 7108–7110), and to the 4-position of the terminal aerobic and anaerobic reaction products of deuteroheme-containing Mb, provide compelling evidence that the initially formed green SMb complex for both native protoheme and synthetic deuteroheme reconstituted protein is an episulfide across the β–β bond of pyrrole II.

Introduction

Sulfhemoglobin, SHb, is a green pigmented, inactive form of hemoglobin *in vivo*, Hb, formed under physiological conditions which has sulfur incorporated into one or two hemins in the protein tetramer.[1] The identical modification of the prosthetic groups in the Hb tetramer can be made *in vitro* by the sequential addition of peroxide, catalase, and sulfide.[2] Most of the early characterization of the mechanism of formation and the nature of the prosthetic group modification has been conducted on the analogous derivative of monomeric myoglobin, Mb, termed sulfmyoglobin, SMb.[3-7] The modification of red hemin, **1**, of native Mb or Hb, to yield the characteristic green color of SMb or SHb, has been attributed to the addition of a sulfur atom across a pyrrole β–β double bond to form a chlorin, **2**.[2,8] However, the instability of the initially formed derivative in either SMb or SHb has precluded any detailed structural characterization of the chromophore.[5] Progress in the characterization of SMb was made upon the recognition that the initially formed unstable complex, labeled S_AMb, can spontaneously rearrange with the protein matrix to yield a product, labeled S_CMb,[9,10] with a similarly green chromophore that is stable to extraction.[11-13] A majority of our quantitative understanding of S_AMb and its chemistry has resulted from the detailed ^{1}H NMR structural characterization of this extracted chromophore, which contains a cyclized thiolene ring.[11-13] This structure was shown to be consistent with a structure for the initially formed sulfhemin-A where the sulfur atom is added across the β–β bond of pyrrole II. It is thus clear that the characterization of the reaction products of S_AMb can provide valuable information not only on the structure of the initially formed complex but also on its chemistry that might aid in the design of a suitable drug to treat sulfhemoglobinemia, which is the pathological condition expressed by sulfhemoglobin.[1]

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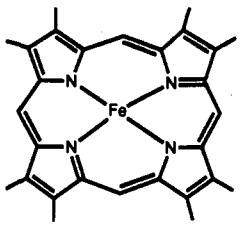

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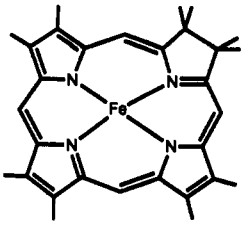

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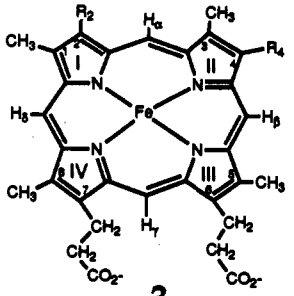

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While essentially all of the detailed structural work on SMb has been carried out on the native protein containing protohemin (3: R_2 = R_4 = vinyl),[8-18] we had reported earlier that Mb reconstituted with isopemptohemin (3: R_2 = H, R_4 = vinyl), or deuterohemin (3: R_2 = R_4 = H), yielded initial SMb complexes similar to the S_AMb complex.[14] Optical studies of SMb formed from protein reconstituted with deuteroheme and mesoheme (3: R_2 = R_4 = ethyl) have similarly shown[19] that essentially the same initial complex is formed whether the 2,4-substituents are vinyl groups, hydrogens, or ethyl groups. However, while the hemins with a 4-vinyl group converted to a terminal green complex defined as S_CMb (the 2-vinyl could be replaced by H without affecting the formation of the thiolene ring on pyrrole B), the hemin with a hydrogen at the 4-position appeared to convert to a red-pigmented derivative whose optical spectra was essentially the same as the unreacted protein, but for which the ^{1}H NMR spectra were distinct from those of the initial Mb complex.[14] As with the SMb complexes with protohemin,[10] ^{1}H NMR spectroscopy of the paramagnetic form of the SMb complexes of deuterohemin was shown[14] to be extremely well-suited for detecting the presence of optically very similar products.

In this report, and the comparison report that follows,[20] we conclude our studies of SMb with an investigation of the reactivity patterns of the SMb complexes formed from deuterohemin (3: R_2 = R_4 = H) reconstituted into equine Mb. These studies are pursued to obtain additional information on the molecular structure of the initially formed unstable S_AMb complex. We emphasize ^{1}H NMR spectroscopy as an analytical tool to both detect the various species formed, using the spectral characteristics of the various oxidation/ligation states to distinguish between hemin and chlorin prosthetic groups,[10,13,15,21,22] and delineate the solution conditions responsible for their formation. Using the detailed molecular/electronic structure of the extracted hemins determined by 2D NMR spectroscopy, isotope labeling, optical spectroscopy, and mass spectroscopy, which will be described in detail in the comparison paper,[20] we develop a reaction mechanism for formation of the intermediate and terminal products that provides additional strong support for an initially formed S_AMb complex that indeed has a sulfur atom added across the β–β bond of pyrrole II.

Although all of our initial ^{1}H NMR investigations of SMb complexes were carried out on sperm whale Mb,[9-11,13-16] the restricted availability of this protein, together with our observation that the initially formed SMb complex of deuterohemin reconstituted Mb is more stable to reversion to the native form for equine than sperm whale Mb, dictates the use of reconstituted equine Mb for this study. Previous ^{1}H NMR data have shown that SMb species with essentially the same ^{1}H NMR spectral parameters are obtained with native sperm whale[10] and equine[17,18] SMb. Moreover, the presently characterized SMb complex of equine Mb reconstituted with deuterohemin yields a ^{1}H NMR spectrum indistinguishable from the preliminary spectrum for the same heme reconstituted into sperm whale Mb.[14] It has been shown previously that native sperm whale and equine Mb yield essentially indistinguishable ^{1}H NMR spectra in the three paramagnetic forms vital to the characterization and the molecular/electronic studies of the chromophore, the high-spin ferric aquo or metMbH_2O,[23] the low-spin ferric cyano or metMbCN,[24] and the high-spin ferrous or deoxy Mb[25] derivatives.

Experimental Section

Sulfmyoglobin Preparation. Myoglobin from equine skeletal muscle was purchased from Sigma and used without further purification. Apomyoglobin was prepared by a modified method of Teale[26] and was reconstituted with deuterohemin as described previously.[27] Sulfmyoglobin was prepared by the sequential addition of peroxide, catalase, and sulfide using the modified method of Chatfield *et al.*[10] The resulting deoxy-sulf-Mb product was chromatographed at 4 °C on a G25 fine sephadex column equilibrated with 0.1 M potassium phosphate buffer at pH 8.0. After eluting from the column, the deoxy-sulf-Mb product was converted to the desired oxidation/ligation state. In contrast to an earlier report,[19] our preparations off the column yielded ^{1}H NMR spectra that exhibited resonance only for the deoxy-SMb complex, and no detectable resonance for metSMb (see below).

Interconversion of Oxidation/Ligation States for a Fixed Chromophore. Interconversion of the sulfMb species denotes a change in the iron oxidation and/or ligation state that does not affect the chemical nature of the chromophore. This interconversion occurs rapidly with respect to any chemical change of the chromophore. Aquo-met sulfMb was prepared by oxidizing a deoxy sulfMb sample with 25 μL of 0.2 M $K_3Fe(CN)_6$. Excess $K_3Fe(CN)_6$ was removed by ultrafiltration using an Amicon 8MC with a YM5 membrane while exchanging into 0.1 M potassium phosphate buffer at pH 6.8 in ^2H_2O. Deoxy sulfMb was converted to the carbon monoxide product by bubbling CO into the protein sample at 0 °C. A azide met sulfMb product was prepared by adding 4 equiv of NaN_3 dissolved in ^2H_2O to an aquo-met sulfMb sample at pH 7.0. Cyano-met sulfMb complexes were prepared by adding 4 equiv of KCN dissolved in 0.1 M potassium phosphate buffer to aquo-met sulfMb at pH 7.0.

Reactions at the Chromophore. These reactions alter the chemical nature of the chromophore of a sulfMb in a given oxidation/ligation state of the protein and are carried out under strict control of the conditions of pH (usually 7.0), temperature (22 °C), and a fixed quantity of endogenous ligand. Aerobic conditions were provided by simply working in the laboratory atmosphere; anaerobic conditions were provided by flushing the sample repeatedly with gaseous nitrogen. The progress of such a reaction is slow and was monitored periodically by ^{1}H NMR.

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Comparative studies investigating the influence of ligand concentration, pH, or absence/presence of O_2 were carried out on split samples of a common preparation of the initial sulf-Mb species to guarantee the identical sample composition.

Optical Spectra. Optical spectra were recorded at ambient temperatures on a Hewlett-Packard 8540A UV–visible spectrophotometer using 1 cm light path quartz cells referenced against water. Optical spectra were obtained by diluting 20 μL of 3 mM protein into 2 mL of H_2O in the optical cell. The positions of optical maxima for the various protein species are listed in the supplementary material.

NMR Spectra. GE Omega 300 MHz, Nicolet NTC-360 MHz, and Nicolet NT-500 MHz spectrometers operating at 300, 360, and 500 MHz, respectively, were used to obtain the ^1H NMR spectra. Typical spectra consisted of 10^3–10^4 transients of 4096 complex points over an 8–80 kHz bandwidth using a 7.0-μs 90° pulse. The residual water pulse was suppressed by a decoupler pulse. Limited 1D nuclear Overhauser effect (NOE) experiments were carried out on the met-cyano complexes of ferric sulf-Mb species containing hemin prosthetic groups to identify the upfield hyperfine shifted Ile 99(FG5) CγH peak by its characteristic intra-residue NOE pattern and NOE to the lowest field heme methyl.[28] The 1D NOE difference spectra were obtained by subtracting a set of scans with the decoupler on-resonance for 150 ms from an identical set with the decoupler well off-resonance. All chemical shifts are referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). NMR difference spectra were generated by using a subroutine of the GE Omega NMR software, Version 5.0, or a subroutine of the Nicolet NMC-1280 program. ^{13}C NMR spectra were recorded on a GE QE-300 MHz spectrometer in which the 5-mm broad-band probe had been tuned to 75.60 MHz for ^{13}C nucleus detection. The spectra consisted of 10^4 scans of 16 384 data points over a 50-kHz bandwidth using a 4.0-μs 24° pulse at 25 °C. ^{13}C NMR chemical shifts were referenced to an external standard, natural abundant $[^{13}C^2H_3]O^2H$, which resonates at 49.0 ppm[29] at 25 °C.

Results

The symbol Mb is reserved for native protein possessing the natural protohemin prosthetic group, **3** (R_2 = R_4 = vinyl). Myoglobin reconstituted with the synthetic hemin, deuterohemin (**3**: $R_2 = R_4 = H$), is designated by Mb*. Consistent with labeling of the various derivatives of SMb of the protohemin containing native Mb, we label the initially formed complex as S_AMb^*. Having used the subscripts B and C to designate the acidic and alkaline sulfMb reaction products of native Mb,[10] we label new rearrangement or reaction products of S_AMb^* as S_DMb^*, S_EMb^*, S_FMb^*, etc. The preparation of sulfMb* and the majority of its subsequent reactions depends on the oxidation/ligation states of the iron. We present the reactions that are essentially quantitative in the flow chart illustrated as Scheme I. The vertical direction is reserved for the rapid interconversion of oxidation/ligation states for a given chromophore, using the agents indicated; oxidation and reduction are effected by ferricyanide and dithionite, respectively. Reactions involving chemical modification of the chromophore periphery are shown along horizontal lines.

Both NMR spectroscopy[9–18] and optical spectroscopy[3–5,10,19] provide well-established spectral characteristics that distinguish between a porphyrin group and a chlorin prosthetic group for a given sulfmyoglobin protein complex. The optical spectra for the deoxy and aquo-met derivatives of the green protein species provide unique absorption bands in the 600- and 700-nm regions which distinguish them from those of the red porphyrins containing species in the same oxidation/ligation state. For the low-spin cyano-met protein form, however, the optical spectra are much less useful in distinguishing between chlorin and porphyrin containing proteins.[11] ^1H NMR spectroscopy provides alternate, and in some cases superior, discrimination between heme and iron chlorin systems.[10,15,16,21,22,30–33] The resonances whose shifts are diagnostic for hemes versus chlorins and which are readily assigned because of their unique spectral properties are labeled similarly in all derivatives, using the general index χ_i, where χ indicates the particular chromophore in $S\chi Mb^*$ (χ = A, D, E, F, or G), and i identifies the particular peak or sets of peaks: χ_1–χ_4 for heme methyls, χ_5 and χ_6 for the pyrrole 2,4-H positions, χ_7–χ_{10} for the heme meso-H's, χ_{11} for the Ile 99 (FG5) CγH, χ_{12} for the axial His F8 ring labile proton, NδH, and χ_{13} for the Val 68(E11) $C\gamma H_3$. The symbol M_i is reserved for unreacted Mb*. The characteristic differences in signal position for one or more of those designated functional groups in one or more oxidation/ligation states between heme and iron chlorin complexes have been considered in detail previously for the SMb complexes of native sperm whale Mb.[10]

The criteria to be applied are as follows. For deoxy complex, the His F8 ring labile NδH signal (χ_{12}), assigned by comparison of ^1H_2O and ^2H_2O spectra, resonates at ~80 ppm at 20 °C for a heme,[34] but only at ~65 ppm for an iron(II) chlorin.[10,22,33] In the diamagnetic ferrous carbon monoxide complex, the meso-H signals (χ_7–χ_{10}) experience smaller low-field and the Val E7 $C\delta H_3$ (χ_{13}) displays smaller upfield ring current shifts in an iron chlorin[10,31,32] than heme[30] system. The high-spin ferric complex of a hemin displays all four heme methyl signals (M_1–M_4) in the low-field region 50–100 ppm,[35] while a ferric chlorin exhibits only three low-field shifted methyls[13,15,21] (the methyl of the saturated pyrrole appears near the diamagnetic envelope). The cyano-met Mb complexes containing a hemin exhibit three resolved low-field heme methyl signals[36] (χ_1–χ_3), strongly upfield shifted 2-H, 4-H (χ_5, χ_6), and a characteristic rapidly relaxed (T_1 ~ 60) upfield signal near –9 ppm that arises from Ile 99 (FG5); the latter signal is readily assigned by its characteristic NOE pattern to the other protons of the same residue and the low-field methyl signal.[28] The low-spin ferric complex of an iron chlorin, in contrast, reveals only two low-field methyl signals (χ_1, χ_2) with larger shifts than for a hemin,[15,21,22] upfield pyrrole-H signals (χ_5, χ_6), but no clearly resolved Ile 99 (FG5) CγH signal; the smaller upfield CγH shift is due to its exclusive dipolar origin and the significantly reduced magnetic anisotropy of iron chlorin relative to a hemin.[16] The spin equilibrium met-azide complexes of iron chlorin have not been reported previously but are expected to exhibit properties intermediate between those of met-cyano and met-aquo complexes.

Initially Formed Sulfmyoglobin Complexes (S_AMb^*). (a) Deoxy S_AMb^*. The 360 MHz reference ^1H NMR trace of unreacted deoxy Mb* in ^2H_2O, pH 7.1 at 20 °C, is shown in Figure 1A. The trace is very similar to that of native equine deoxy Mb reported earlier,[25] except for the two non-labile proton signals M_5 and M_6, which arise from the 2-H and 4-H positions as determined by deuteration and comparison to the protein reconstituted with hemin possessing only 2-H or 4-H.[37] The *in situ* preparation (*i.e.*, not chromatographed) of deoxy-S_AMb^* at 20 °C at pH 7.1 in ^2H_2O is shown in Figure 1B, which reflects ~40% conversion to deoxy-S_AMb^*, with peaks labeled A_i, with the remainder unreacted or reverted protein; note the absence of the characteristic metS_AMb^* H_2O peaks (see below) indicating that no autoxidation had taken place.[19] The rapid reversion back to deuterohemin (half-life ~5 h at 20 °C) is evidenced by the rapid loss of the intensity of peaks A_5 and A_6 and parallel increase in intensity of peaks M_5 and M_6 (not shown). Preparation of deoxy S_AMb^* in ^1H_2O yields the NMR trace in Figure 1C (~80% deoxy S_AMb^*, 20% unreacted or reverted deoxy Mb*). An

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Table I. Comparison of Chemical Shifts of Characteristic Resonances of the Various Oxidation/Ligation States of the Initially Formed Green Sulfmyoglobin Complexes Derived from Deuterohemin (S_AMb^*) and Protohemin (S_AMb)[a]

		cyano-met		aquo-met		deoxy		carbonmonoxy	
peak i in A_i[b]	assign	S_AMb[c]	S_AMb^*	S_AMb[c]	S_AMb^*	S_AMb[c]	S_AMb^*	S_AMb[c]	S_AMb^*
1	heme methyl	44.75	45.13	122.8	123.9				
2	heme methyl	25.93	25.00	71.6	72.7				
3	heme methyl	*d*		65.2	64.1				
5	pyrrole-H	*3*	−13.42			*b*	47.2		
6	pyrrole-H	*e*				*b*	52.1		
7	meso-H							9.53	9.53
8	meso-H							9.12	~9.1
12	His F8 $N_\delta H$					65.5	66.4		
13	Val E7 $C_\gamma H_3$							−2.06	−1.98

[a] Shifts in ppm from DSS in ^2H_2O at 20 °C. For protoheme containing SMb complexes, the pH is 8.0 except for the metaquo complex where it is 6.3; for the deuterohemin containing SMb* complexes, the pH is 7.1 except for the metaquo derivative, where it is 6.8. [b] A_i as defined in Figures 1 and 3–5. [c] Data taken from ref 10. [d] Blank space indicates unresolved peak or peak not diagnostic for distinguishing between chlorin and porphyrin. [e] Does not contain the pyrrole-H, but vinyl group.

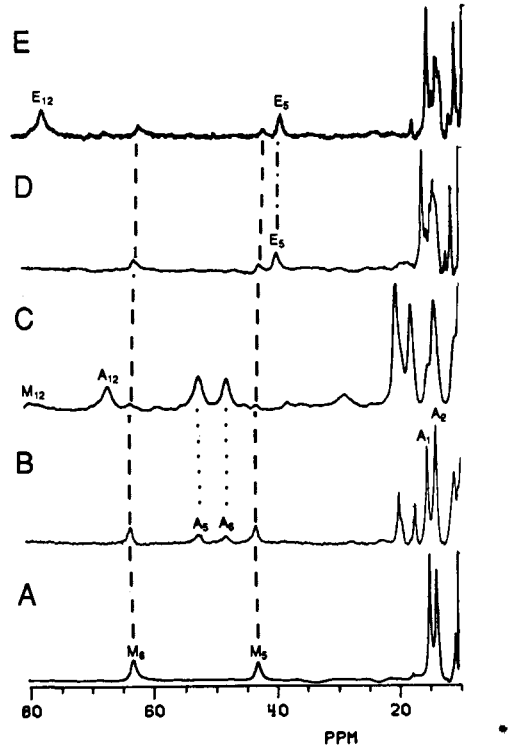


Figure 1. Resolved low-field region of the ^1H NMR spectra of the high-spin ferrous deoxy complexes of sulfMb* at 20 °C. (A) 360-MHz spectrum of unreacted deoxy Mb* in ^2H_2O at pH 7.1; M_5, M_6 arise from the pyrrole 2-H, 4-H. (B) 300-MHz spectrum of deoxy S_AMb^* immediately after *in situ* preparation at pH 7.1 in ^2H_2O. Approximately 40% of the protein species arises from S_AMb^* while 60% is from unreacted Mb*. The peaks labeled A_5, A_6 rise from 2-H, 4-H. (C) 360-MHz spectrum of deoxy S_AMb^* in 90% H_2O/10% ^2H_2O at pH 8.0. Residual deoxy Mb* has been subtracted from the spectrum. The labile proton peak A_{12} arises from the His F8 NδH. (D) 360-MHz spectrum of deoxy S_EMb^* at pH 7.1 in ^2H_2O produced via dithionite reaction of $metS_E$-Mb*CN; the peak labeled E_5 arises from 2-H. (E) 360-MHz spectrum of deoxy S_EMb^* at pH 7.1 in 90% H_2O/10% ^2H_2O produced via dithionite reaction of $metS_EMb^*CN$; the labile proton peak E_{12} arises from the His F8 ring NδH.

additional labile proton signal A_{12} at 66 ppm is identified for deoxy S_AMb^*, which can be unambiguously assigned to the His F8 ring NH.[10,22,33,34] No SMb* complexes in addition to S_AMb^* were observed prior to reversion to Mb*, which is in contrast to native Mb for which the acidic equilibration product S_BMb was identified. The presence of only deoxy S_AMb^* and deoxy Mb* in any of the above solutions was quantitatively confirmed by rapid oxidation with ferricyanide in the presence of cyanide which yielded ^1H spectra identified solely via the better characterized metMb*CN and $metS_AMb^*CN$ species (see below). The chemical shifts for the deoxy S_AMb^* complex are listed in Table I. The optical spectrum for deoxy S_AMb^* is shown in Figure 2A. Comparison of the absorption patterns for deoxy S_AMb^* with protohemin derived S_AMb and earlier SMb* preparations[19] reveals very similar spectra for the green complexes.

(b) S_AMb^*CO. The resolved portions of the 500-MHz ^1H NMR of Mb*CO in ^2H_2O at pH 7.0 and 20 °C are displayed in Figure 3A; in analogy with native MbCO, the three low-field single-proton peaks M_7–M_9 arise from meso-H's, and the upfield methyl, M_{13}, at −2.3 ppm from E7 Val CγH_3; the shifts are essentially the same as for native MbCO.[30] Addition of CO to a solution of 80% deoxy S_A*Mb and 20% deoxy Mb* yields a sample with ^1H NMR spectra shown in Figure 3B; the upfield E7 Val methyl, which shows a significantly diminished upfield shift (−1.8 ppm), is labeled A_{13}, and only a single less strongly low field shifted meso-H (peak A_7) is clearly resolved. The chemical shifts for the S_AMb^*CO complex are included in Table I.

(c) $metS_AMb^*H_2O$. The 360-MHz ^1H NMR reference spectrum of unreacted $metMb^*H_2O$ in ^2H_2O at pH 6.8 and 20 °C is illustrated in Figure 4A. The trace is essentially the same as that for native equine $metMbH_2O$ and sperm whale $metMbH_2O$,[23] for which isotope labeling has identified the four methyl peaks labeled M_1–M_4.[35] Oxidation of a freshly prepared and chromatographed deoxy S_AMb^* sample in ^2H_2O yields the ^1H NMR trace shown in Figure 4B; ~40% is unreacted or reverted $metMb^*H_2O$. Only three of the peaks in the 20–100-ppm window for $metS_AMb^*H_2O$ have the intensity of methyl peaks, and these are labeled A_1–A_3. The intensity of the A_i peaks decreases with time with concomitant increase in intensity of M_i resonances; no additional resonances were observed for another SMb* derivative. This was quantitatively confirmed by trapping the complex at various times with cyanide to yield the better resolved and characterized spectra of the cyano-met complexes (see below). The area loss of peaks A_i with time yields an estimate of the half-life for reversion of ~12 h at pH 6.8 and 20 °C. Reduction of the $metS_AMb^*H_2O$ sample with dithionite yielded an ^1H NMR spectrum with only the resonance from deoxy S_AMb^* and deoxy Mb* (not shown), indicating that neither ferricyanide oxidation nor dithionite reduction altered the functionality of the prosthetic group in S_AMb^*. The chemical shifts for $metS_AMb^*H_2O$ are listed in Table I. The optical spectrum for $metS_AMb^*H_2O$ displayed in Figure 2B possesses absorption maxima very similar to those of the green $metS_AMbH_2O$ complex derived from protohemin.[10]

(d) $metS_AMb^*CN$. The resolved portions of the 360-MHz ^1H NMR spectrum of metMb*CN in ^2H_2O at pH 7.1 and 20 °C are shown in Figure 5A. The spectrum is essentially identical to that of the deuterohemin reconstituted cyano-met complex of sperm whale Mb for which detailed studies have been provided.[36] The low-field signals, M_1–M_3, arise from three heme methyls, while the upfield signals, M_5, M_6, and M_7, arise from heme, 2-H, 4-H, and Ile 99(FG5) CγH, respectively. Oxidation in the presence of cyanide of chromatographed deoxy S_AMb^* yields the ^1H NMR spectrum shown in Figure 5B; the major set of peaks attributed

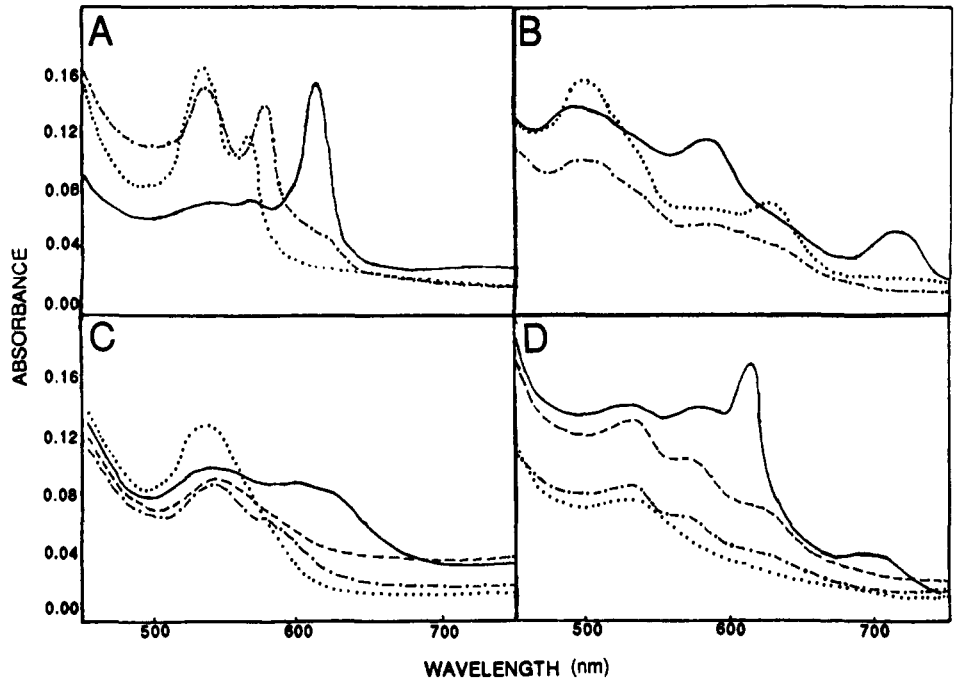


Figure 2. (A) Optical spectra of unreacted and sulfmyoglobin complexes of equine myoglobin reconstituted with deuterohemin complexes at pH 7.1 in ^1H_2O at 22 °C; unreacted deoxy Mb* (···); 75% deoxy S_AMb^* and 25% unreacted deoxy Mb* (—); 75% deoxy S_BMb^* and 25% unreacted deoxy Mb* (–·–). (B) Met-aquo complexes at pH 6.8: unreacted $metMb^*H_2O$ (···); 75% $metS_AMb^*H_2O$ and 25% unreacted $metMb^*H_2O$ (—); 75% $metS_BMb^*H_2O$ and 25% unreacted $metMb^*H_2O$ (– · –). (C) Met-cyano complexes at pH 7.1: unreacted metMb*CN (···); 75% $metS_AMb^*CN$ and 25% unreacted metMb*CN (—); 75% $metS_DMb^*CN$ and 25% unreacted metMb*CN (– – –); 75% $metS_BMb^*CN$ and 25% unreacted metMb*CN (–·–). (D) Met-azide complexes at pH 7.1: unreacted $metMb^*N_3$ (···); 75% $metS_AMb^*N_3$ and 25% unreacted $metMb^*N_3$ (—); 75% $metS_DMb^*N_3$ and 25% unreacted $metMb^*N_3$ (– – –); 75% $metS_FMb^*N_3$ and 25% unreacted $metMb^*N_3$ (– · –).

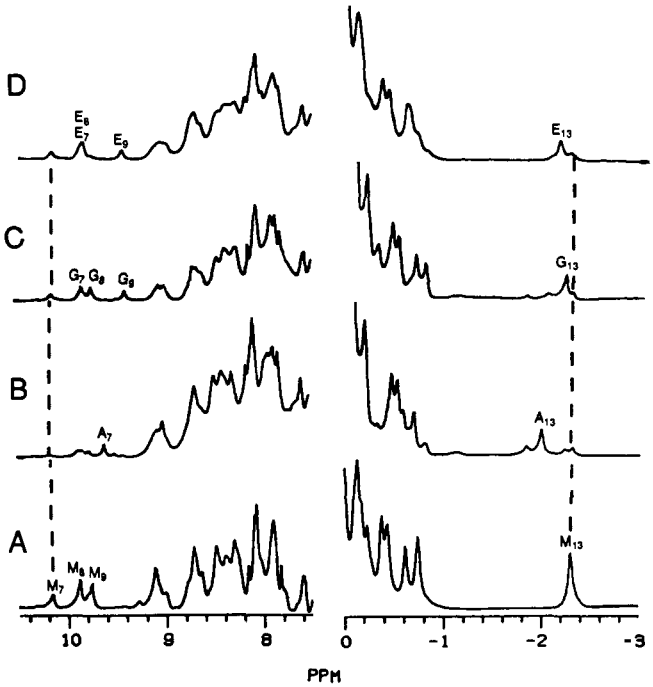


Figure 3. Resolved regions of the 500-MHz ^1H NMR spectra of diamagetic carbonmonoxy complexes of SMb* at 20 °C and pH 7.1 in ^2H_2O. (A) Reference spectrum of unreacted Mb*CO with three resolved meso-H signals labeled M_7–M_9 and the upfield Val E7 $C\gamma H$ peak labeled M_{13}. (B) Spectrum of S_AMb^*CO immediately after preparation. (C) Spectrum of S_GMb^*CO, after 24 h of equilibration of S_AMb^*CO, with meso-H peaks G_7–G_9 and Val E7 $C\gamma H_3$ peak G_{13}. (D) Spectrum of S_BMb^*CO immediately after flushing deoxy S_BMb^* with CO(g) at 0 °C, with meso-H peaks E_7–E_9 and Val E7 $C\gamma H_3$ peak E_{13}.

to $metS_AMb^*CN$ are labeled A_i with only two low-field methyl peaks A_1 and A_2, and only a single narrow upfield peak A_5; the chemical shifts are listed in Table I. The optical spectrum illustrated in Figure 2C is very similar to that of $metS_AMbCN$ reported earlier.[10]

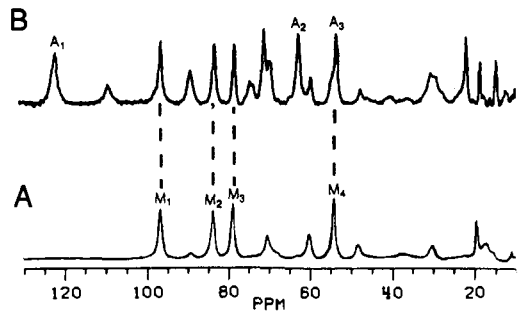


Figure 4. Resolved low-field region of the 360-MHz ^1H NMR spectra of the met-aquo complexes at 20 °C in ^2H_2O. (A) Unreacted $metMb^*H_2O$, pH 6.8, with heme methyl peak M_1–M_4 and (B) $metS_A$-Mb^*H_2O immediately after preparation and oxidation at pH 6.8, with heme methyl peaks A_1–A_3; approximately 60% of the protein species arises from S_AMb^* and 40% from the unreacted Mb* species.

A set of peaks, labeled B_1, B_2 from a minor product (~5%), is present in the NMR spectrum of $metS_AMb^*CN$ in Figure 5B. The intensity of these peaks is increased to 15% at pH ~6 (not shown). The resonance positions of B_1 (40.1 ppm) and B_2 (33.4 ppm) are very similar to those of the low-field methyl, 40.6, 33.5 ppm, of the previously characterized acid equilibration product,[10] and hence we identify peak B_1, B_2 with the analogous species $metS_BMb^*CN$. No further characterization of this complex was carried out.

(e) $metS_AMbN_3$. The low-field resolved portion of the 360-MHz ^1H NMR trace of unreacted $metMb^*N_3$ is given in Figure 6A, with methyl peaks labeled M_1–M_3; the spectrum is very similar to that of native sperm whale $metMbN_3$ which has been assigned by isotope labeling.[38] The spectrum of a sample of chromatographed deoxy S_AMb^* oxidized in the presence of azide is shown in Figure 6B. The methyl resonances of $metS_AMb^*N_3$ (~70%) are labeled A_1–A_3, and their shifts are listed in Table I; the optical spectrum is displayed in Figure 2D. The interconversion among

(38) La Mar, G. N.; Budd, D. L.; Smith, K. M. *Biochim. Biophys. Acta* **1980**, *627*, 210–218.

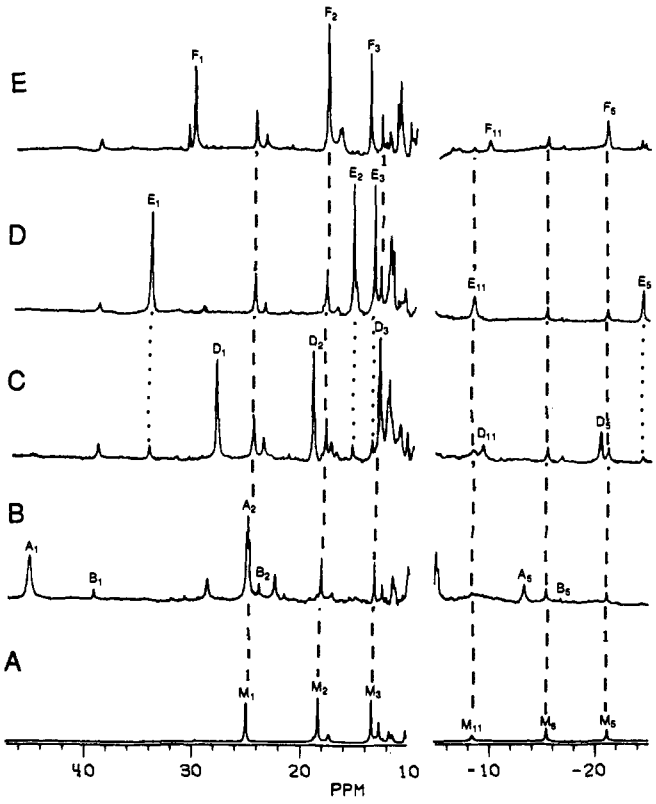


Figure 5. Resolved portions of the ^1H NMR spectra of the met-cyano complexes of sulfMb* at 20 °C in ^2H_2O. (A) 360-MHz reference spectrum of unreacted metMb*CN at pH 7.1 with heme methyl peak M_1–M_3, pyrrole 2-H, 2–4 peak M_5, M_6, and Ile 99 (FG5) CγH peak M_{11}. (B) 360-MHz spectrum of metS_AMb*CN immediately after preparation, oxidation, and cyanide addition at pH 7.1, with heme methyl peaks A_1, A_2 and pyrrole 2-H signal A_5; approximately 75% of the protein species arise from metS_AMb*CN, 20% from unreacted metMb*CN, and ~5% from metS_BMb*CN (with methyl peaks B_1, B_2 and pyrrole 2-H peak B_5). (C) 360-MHz spectrum of metS_DMb*CN from the 3-day 22 °C equilibration of metS_AMb*CN, with heme methyl peak D_1–D_3, pyrrole 2-H peak D_5, and Ile 99 (FG5) CγH peak D_{11}. (D) 360-MHz spectrum of metS_EMb*CN from the 22 °C 3-week equilibration in the presence of excess CN^- of metS_DMb*CN, with heme methyl peaks E_1–E_3, pyrrole 2-H peak E_5, and Ile 99 (FG5) CγH peak E_{11}. (E) 500-MHz spectrum of metS_FMb*CN immediately after cyanide addition to metS_FMb*N_3 with heme methyl peak F_1–F_3, pyrrole 2-H peak F_5, and Ile 99 (FG5) CγH peak F_{11}.

the oxidation/ligation states of S_AMb* is summarized above **4** in the flow chart in Scheme I.

Intermediate Sulfmyoglobin Complex (S_DMb*). Neither the ferric nor ferrous high-spin S_AMb* species produced with time any other major SMb* species except for reversion to the native protein. However, all three strong field ligated complexes, which reverted only slowly to native protein, spontaneously produced a major new species which we define as S_DMb*, as shown by the conversion **4** → **5** in Scheme I.

(a) Conversion from metS_AMb*CN. An aerobic ^2H_2O solution of a metS_AMb*CN sample converted smoothly to a major new species, metS_DMb*CN, which is a red protein complex with optical spectrum as displayed in Figure 2C and NMR spectrum as shown in Figure 5C; the low-field methyls are labeled D_1–D_3, an upfield narrow single peak attributed to a pyrrole-H is labeled D_5, and the rapidly relaxed CγH signal of Ile 99(FG5) at –8 ppm is labeled D_{11}. Another minor new species also can be observed, whose peaks we label E_t for a complex designated metS_EMb*CN (see below). The formation of metS_DMb*CN in excess cyanide was found to have a half-life of ~20 h at 20 °C and pH 7; this rate was not significantly influenced by either CN^- concentration (1 to 10 equiv) or pH (7–9). Morever, neither the properties of the product nor the rate of formation of metS_DMb*CN was influenced by whether the conversion was allowed to proceed in the absence or presence of atmospheric oxygen. Only a very small increase in the mole fraction of unreacted metMb*CN was observed during the conversion of metS_AMb*CN to metS_DMb*CN, indicating that the reversion of metS_AMb*CN to native protein is much slower than the conversion to metS_DMb*CN. Comparison of a ^1H_2O and ^2H_2O trace exhibited the same labile proton peaks as observed for unreacted metMb*CN[24] and revealed no new labile proton unique for metS_DMb*CN (not shown). A pH titration of metS_DMb*CN in the range 5–10 yielded a pH profile essentially identical to that of unreacted metMb*CN[39] (not shown).

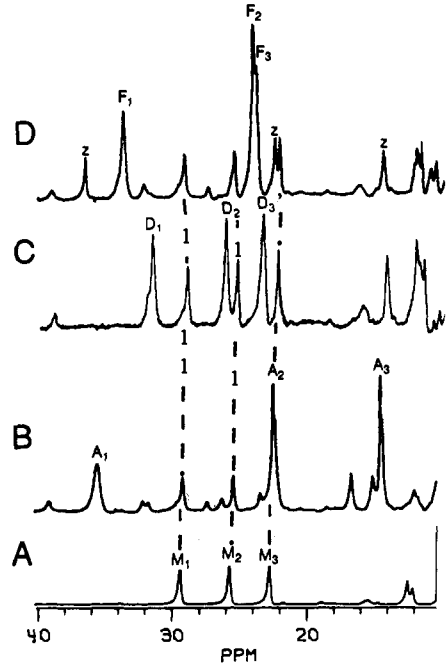


Figure 6. Resolved low-field portion of the 500-MHz ^1H NMR spectra of met-azido complexes at pH 7.1 and 20 °C in ^2H_2O. (A) Spectrum of unreacted metMb*N_3 with heme methyl peaks M_1–M_3. (B) Spectrum of metS_AMb*N_3 with heme methyl peak A_1–A_3; approximately 75% of the protein species arises from metS_AMb*N_3 and the remaining 25% is from the unreacted Mb* and other unidentified minor products. (C) Spectrum of approximately 65% metS_DMb*N_3 (with heme methyl peaks D_1–D_3), 30% unreacted metMb*N_3, and 5% unidentified products. (D) Sample of 55% metS_FMb*N_3, 30% unreacted metMb*N_3 (with heme methyl peaks F_1–F_3), and 15% of a minor met-azide species with apparent methyl peaks labeled *z*.

(b) Conversion from metS_AMb*N_3. A sample of 60% metS_AMb*N_3 in ^2H_2O at pH 7.0 and 20 °C in the presence of 4 equiv of azide converted within 24 h to a new red complex (half-life ~6 h) with the ^1H NMR spectrum shown in Figure 6C (methyl peaks D_1–D_3) and the optical spectrum shown in Figure 2D. Addition of excess cyanide to this solution instantly displaced the coordinated N_3^- to yield a complex with an ^1H NMR spectrum indistinguishable from that of metS_DMb*CN shown in Figure 5C. Hence the reaction product of metS_AMb*N_3 can be labeled metS_DMb*N_3. The reaction rate of the azide complex is independent of the amount of excess azide in solution. Moreover, the rate of reaction of the metS_AMb*CN complex in the presence of only 1 equiv of CN^- to bind the heme (confirmed by the ^1H NMR spectrum) and 4 equiv of excess N_3^- is the same as when only 4 equiv of CN^- is present. This dictates that the different half-lives for equilibration of S_AMb* to S_DMb* for the azide (6 h) and cyanide (20 h) complexes noted above are due to an electronic effect of the coordinated ligand and not to an influence of the ions in solution. The chemical shifts for metS_DMb*N_3 are listed in the supplementary material.

(c) Conversion from S_AMb*CO. A ~70% S_AMb*CO sample in ^2H_2O converted to a major new diamagnetic complex with ^1H

(39) Krishnamoorthi, R.; La Mar, G. N. *Eur. J. Biochem.* **1984**, *138*, 135–143.

Scheme I

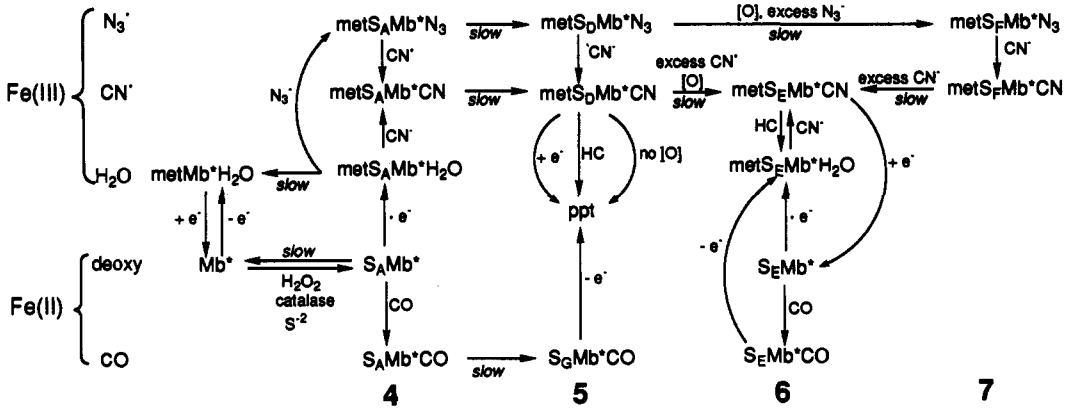


Table II. Chemical Shifts of the Met-Cyano Complex of Red Sulfmyoglobin Reaction Products Derived from Deuterohemin[a]

peak[b]	assign	Mb*	S_DMb^*	$S_D'Mb^*$ [c]	S_EMb^*	S_FMb^*
χ_1	heme methyl	24.88	28.15	32.27	34.47	31.11
X_2	heme methyl	18.22	19.35	19.15	15.77	18.28
χ_3	heme methyl	13.27	13.13	14.88	13.93	14.34
χ_5	2-H	-21.27	-20.47	-21.20	-24.51	-21.11
χ_6	4-H	-15.54	*d*	*d*	*d*	*d*
χ_{11}	Ile 99(FG5) $C_\gamma H$	-8.56	-9.40	-9.77	-8.83	-9.98

[a] Shifts in ppm from DSS in ^2H_2O at 20 °C and pH 7.1. [b] Peak χ_i with χ = D, D', E, F as labeled in Figure 5. [c] Shifts for the iodoacetamide complex of S_DMb^*, as taken from ref 20. [d] This proton is eliminated in forming S_DMb^*, S_EMb^*, and S_FMb^*.

Table III. Chemical Shifts of the Carbonyl Complex of Red Sulfmyoglobin Reaction Products Derived from Deuterohemin[a]

peak[b]	assign	Mb*CO	S_GMb^*CO	S_EMb^*CO
χ_7	meso-H	10.15	9.84	9.85
χ_8	meso-H	9.87	9.74	9.85
χ_9	meso-H	9.75	9.40	9.44
χ_{13}	Val 68(E11) $C_\gamma H_3$	-2.32	-2.33	-2.13

[a] Chemical Shift in ppm from DSS in ^2H_2O at 20 °C and pH 7.0. [b] χ_i for χ = M, G, E as labeled in Figure 3.

NMR spectra shown in Figure 3C which is distinct from that of both S_AMb^*CO and unreacted Mb*CO, although the shifts for both the meso-H's and the E11 Val methyl are much closer to that of the latter; we label this complex S_G*MbCO with meso-H peaks G_7–G_9 and Val E7 $C\gamma H_3$ peak G_{13}. Treatment of the S_GMb^*CO with ferricyanide and cyanide yields the spectrum of a dilute solution of unreacted metMb*CN and a trace of $metS_D$-Mb*CN, indicating that the prosthetic group of S_GMb^* is largely destroyed by the oxidizing agent. Hence it could not be definitively established that the prosthetic groups of S_DMb^* and S_GMb^* are identical, although this is most likely the case (see below). The chemical shifts for S_GMb^*CO are listed in Table II.

(d) Stability of $metS_DMb^*CN$. Reduction of anaerobic $metS_D$-Mb*CN with dithionite in the presence of CO yielded an optical spectrum (not shown) suggested for the formation of the CO complex. However, the spectrum quickly (5 min) reverted back to the $metS_DMb^*CN$ spectrum with significant precipitation; the life-time of this unstable reduced species was insufficient to yield an ^1H NMR spectrum. Abstraction of the bound CN^- by hydroxycobalamine[10] from an anaerobic $metS_DMb^*CN$ sample to form the $metS_DMb^*H_2O$ species resulted in the appearance of numerous broad resonances in the low-field 100–40-ppm window but significant precipitation that resulted in the loss of these broad resonances invariably followed (spectrum not shown). Hence we conclude that the high-spin forms of S_DMb^* are unstable with respect to some reaction(s) that cause protein precipitation within minutes of preparation.

For a $metS_DMb^*CN$ (or $metS_DMb^*N_3$) complex in the presence of excess anion and *complete absence of molecular oxygen*, the sample, over a period of several weeks, exhibits an increasing amount of precipitate and a gradual weakening of the red color. The ^1H NMR spectra for centrifuged samples over a period of time revealed the gradual loss of the resonance of $metS_DMb^*CN$ without either an increase in the intensity of the resonance of unreacted or reverted metMb*CN or the appearance of any new resonances indicative of the formation of yet another SMb* species. Hence we conclude that S_DMb^* is an unstable terminal complex in an anaerobic environment, with the low-spin ferric complexes significantly more stable than the high-spin derivatives. In the presence of molecular oxygen, however, both $metS_DMb^*CN$ and $metS_DMb^*N_3$ react further to yield new complexes as described in the next section (see below). The characterized quantitative ligation state interconversions of S_D-Mb* are summarized above **5** in Scheme I.

Formation of Terminal Stable SMb* Complexes. (a) Conversion from $metS_DMb^*CN$. *In the presence of molecular oxygen and excess CN^-*, a solution of 70% $metS_DMb^*CN$, 30% metMb*CN in ^2H_2O at pH 7.0 and 20 °C converts the former complex essential quantitatively into a new red complex we define as $metS_EMb^*CN$. The ^1H NMR spectrum with resonances labeled E_i is shown in Figure 5D, and the optical spectrum is given in Figure 2C. Three prominent low-field methyl peaks, E_1–E_3, a single narrow upfield single proton peak, E_5, and a rapidly relaxed upfield peak, E_{11}, are observed by ^1H NMR; the latter peak yields an NOE pattern diagnostic[28] of Ile 99 (FG5) $C\gamma H$ (not shown). The rate of the conversion of $metS_DMb^*CN$ to $metS_EMb^*CN$ depends on the total CN^- concentration, with a half-life for conversion of 1 week, 3 days, and 1 day for solutions containing 4, 6, and 10 equivs of CN^- per protein (spectra not shown). The dependence of the rate of $metS_EMb^*CN$ formation on $[CN^-]$ indicates that the conversion of $metS_DMb^*CN$ to $metS_E$-Mb*CN requires not only the presence of oxygen *but also the direct involvement of cyanide in the reaction.* This suggests that the conversion of S_DMb^* to S_EMb^* involves the incorporation of CN^- into the prosthetic group. ^{13}C NMR studies of this conversion using $^{13}CN^-$ in solution yield a new resonance at 126 ppm with a line width <20 Hz not observed when using unlabeled CN^-, and whose intensity increases with time parallel to the conversion of the peaks D_i of $metS_DMb^*CN$ to the peak E_i of $metS_EMb^*CN$ (not shown). The hyperfine shifted but weakly relaxed ^{13}C signal must arise from the incorporation of the label into the prosthetic group, since the iron-ligated $^{13}CN^-$ signal would be too broad to detect. The conclusion is confirmed by detailed ^{13}C and ^1H NMR, as well as mass spectrometeric studies of the extracted chromophore.[20] The ^1H chemical shifts for $metS_EMb^*CN$ are listed in Table II. The conversion $S_DMb^* \rightarrow S_EMb^*$ is summarized as **5** → **6** in Scheme I.

(b) Oxidation/Ligation States of S_EMb^*. Reduction of $metS_E$-Mb*CN in ^2H_2O with dithionite yields the ^1H NMR spectra of deoxy S_EMb^* shown in Figure 1D; with only one low-field signal, E_5 at 39.2 ppm, in the window where the 2,4-H resonance is expected.[22,37] Preparation of the same sample in ^1H_2O yields the characteristic[34] His F8 ring NH peak E_{12} at 78.8 ppm (Figure

1E); the latter shift is essentially the same as for unreacted deoxy-Mb* at 79.0 ppm. Saturating a deoxy-S_EMb* sample with CO yields the ^{1}H NMR spectra of diamagnetic S_EMb*CO (Figure 3D; shifts are listed in Table III); reoxidation with ferricyanide and addition of excess CN^- regenerated the metS_EMb*CN spectrum (not shown). Addition of hydroxycobalamine[10] (designated HC in Scheme I) to a metS_EMb*CN solution led to the loss of the low-spin peaks with the concomitant appearance of broad low-field peaks indicative of the metS_EMb*H_2O species of which the best resolved resonances are at 98 and 89 ppm. The same set of peaks indicative of the metS_EMb*H_2O species is observed upon $Fe(CN)_6^{3-}$ oxidation of S_EMb*CO in the absence of CN^- (not shown). The interconversions of S_EMb* are summarized above **6** in Scheme I. Hence the prosthetic group in S_EMb* is stable to the same agents that interconvert between the ferric and ferrous states of both S_AMb* and unreacted Mb*, as well as S_CMb.[10]

(c) Conversion from metS_DMb*N_3. An aerobic sample of ~55% metS_DMb*N_3 in the presence of 4 equiv of excess azide reacted smoothly to yield a major red component labeled metS_FMb*N_3 with a ^{1}H NMR spectrum (peaks labeled F_i) as shown in Figure 6D and an optical spectrum as shown in Figure 2D. That the prosthetic group of S_FMb* is different from that of S_EMb* is evidenced by the addition of excess CN^- to metS_FMb*N_3 to displace the azide, which yields metS_FMb*CN with the ^{1}H NMR spectrum shown in Figure 5F (which peaks F_i) that is clearly distinct from that of metS_EMb*CN shown in Figure 5F. However, after 1 week in the presence of excess CN^-, the metS_FMb*CN sample converted to metS_EMb*CN with the ^{1}H NMR spectrum identical to that shown in Figure 5E. The conclusion reached on the basis of the above experiments is that the reaction of aerobic metS_DMb* in the presence of either CN^- or N_3^- incorporates the anion into the prosthetic group. The incorporated azide, however, is readily displaced by the stronger nucleophile, cyanide. The chemical shifts for metS_FMb*CN are included in Table II, while the chemical shifts for metS_FMb*N_3 are listed in the supplementary material. The reaction of S_D-Mb* to yield S_FMb* is shown as **5** → **7**, and the conversion of S_FMb* to S_EMb* is shown as **7** → **6** in Scheme I. Studies on the interconversion among different oxidation/ligation states of S_FMb* were not pursued.

Discussion

Characterization of Heterogeneity. For the present SMb complexes containing deuterohemin (**3**: $R_2 = R_4 =$ H), many samples contain as many as six identifiable chemically distinct species, all but one of which can exist as the major molecular species under the appropriate conditions: the red unreacted heme-containing protein, Mb*, the two green chlorin containing SMb products, S_AMb* and S_BMb*, and the three red heme-containing SMb complexes, S_DMb*, S_EMb*, and S_FMb*. In each case, ^{1}H NMR is able to provide quantitative information on both the amount and identity of the species. The two green pigmented products, S_AMb* and S_BMb*, like those based on protohemin,[10] are marginally distinguishable optically. Similarly, the three red pigmented species, S_DMb*, S_EMb*, and S_FMb*, are difficult to distinguish optically from each other or from the unreacted proteins (Figure 2). With the presently determined conditions for their optimal preparation and the use of ^{1}H NMR to define composition, it should be possible to pursue characterization of each of the SMb complexes by other spectroscopic techniques.

Properties of S_AMb*. This complex is clearly a chlorin based on its characteristic absorption[3-7,10] at 700 nm in primarily high-spin complexes, the appearance of only three low-field methyls in the high-spin metS_AMb*H_2O ^{1}H NMR spectrum[15,21] (Figure 4B), the resolution of only two heme methyl peaks in metS_A-Mb*CN and the absence of characteristic upfield signal χ_{11} for Ile 99(FG5),[16] the strong upfield basis of the His F8 ring NH contact shift in deoxy S_AMb* relative to deoxy Mb*,[10,22,33] and the reduced low-field meso-H and upfield Val E11 CγH_3 ring current shifts[10,31,32] in S_AMb*CO (Table I). In fact, comparison of the hyperfine shifts of the deuterohemin containing S_AMb* with protohemin containing S_AMb[10] complexes in the same oxidation/ligation state (Table I) reveals sufficiently striking similarities to allow the conclusion that the functionalization in forming the SMb from the unreacted protein is the same for deuterohemin and protohemin.

A characteristic difference between the S_AMb complexes of protohemin (**3**: $R_2 = R_4 =$ vinyl) and deuterohemin (**3**: $R_2 = R_4 =$ H) is their stability with respect to reversion to native protein, and this is strongly reflected in the percent conversion to S_AMb that can be effected under any fixed set of conditions. Thus, while native protohemin allows formation of >95% S_AMb, deuterohemin allows only ≤80% conversion under the same conditions. The rate of loss of ^{1}H peak intensity with time for the S_AMb and S_AMb* complexes as either deoxy or met-aquo complexes demonstrates directly that the rates of reversion differ markedly, with the rates much faster for 2,4-H than 2,4-vinyl. For both hemins, the rate of reversion is also much greater in either oxidized or reduced high-spin than low-spin forms of the proteins. Optical studies of deuteroheme deoxy S_AMb* had previously reported[19] a similarly enhanced reversion to Mb* as compared to protoheme containing S_AMb.

The low-spin metS_AMb*CN at low pH reacted to form small amounts of another green species with two low-field methyl signals characteristic of a chlorin.[10,15] The hyperfine shift pattern is very similar to that for metS_BMbCN for protohemin,[11] with the lowest-field methyl near 40 ppm (Figure 5B). Only a small amount of metS_BMb*CN is formed, since at acidic pH, reversion to metMb*CN is much faster than metS_BMb*CN formation, and the neutral to alkaline pH conversion to S_DMb* is faster than that to S_BMb*.

Rearrangement of S_AMb* to S_DMb*. Both the optical (Figure 2) and ^{1}H NMR spectral data (Table II) dictate that the prosthetic group of S_DMb* is a peripherally functionalized hemin rather than an iron chlorin. Thus metS_DMb*CN exhibits three rather than two low-field heme methyl peaks[15] with shifts more similar to unreacted metMb*CN[36] than the green chlorin containing metS_AMb*CN. Moreover, the complex exhibits the Ile 99 (FG5) CγH peak in the ca. −9 ppm window highly characteristic of the dipolar shifts and magnetic anisotropy of a hemin.[15,28] The molecular structure of the iodoacetamide trap of the sulfhemin-D extract from S_DMb*, described in detail in our companion report,[20] revealed that the prosthetic group is 4-thiol-deuterohemin (**3**: $R_2 =$ H, $R_4 =$ SH). The chromophore of S_DMb* was not stable to extraction prior to reaction with iodoacetamide. The expected very reactive nature of the 4-thiol group[40] is likely responsible for the instability of the metS_DMb*CN complex. The strong tendency for the S_DMb* complex to precipitate in an anaerobic solution may be due to reaction of the 4-thiol group with protein side chains. The absence of an additional pK attributable to the 4-thiol group in the pH range 5–10 for metS_DMb*CN, as compared to unreacted metMb*CN,[39] dictates that the 4-thiol pK is either <5 or >10. Previous studies with polypropionic acid hemins reconstituted into Mb have revealed[41] that a propionic acid pK is elevated by 4–6 pH units in the hydrophobic environment of pyrrole II; such an elevation of a normal thiol[40] pK (8–10) would place it outside the studied pH range.

The proposed mechanism for the rearrangement of the green chlorin-containing S_AMb* as an episulfide to a red, heme-containing S_DMb* by a first-order reaction is given in Figure 7. The rearrangement has precedence in the isomerization of 1,4-

(40) Wolman, Y. In *The Chemistry of the Thiol Group*, Part 2; Patai, S., Ed.; 1974; pp 675–676.
(41) Hauksson, J. B.; La Mar, G. N.; Pandey, R. K.; Rezzano, I. N.; Smith, K. M. *J. Am. Chem. Soc.* **1990**, *112*, 8315–8323.

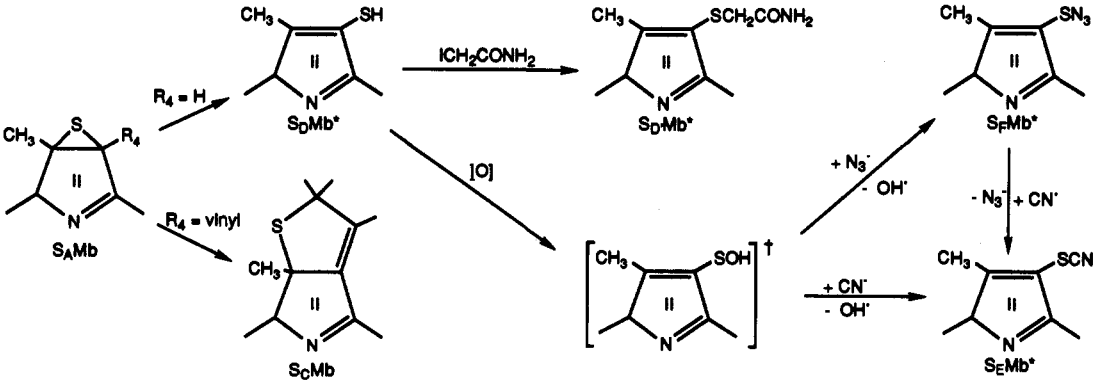
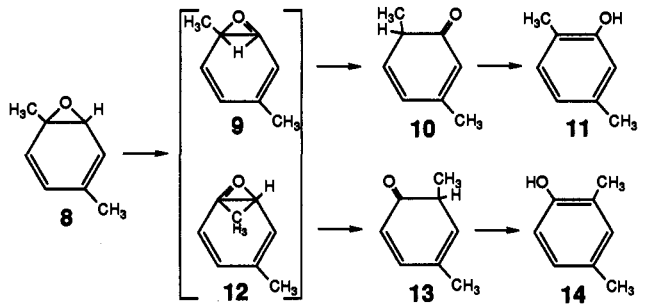


Figure 7. Summary of the reaction mechanisms that produce the structurally characterized sulfmyoglobin complexes for myoglobin containing native protohemin (Mb) and synthetic deuterohemin (Mb*). Only the affected pyrrole II is illustrated. The sulfheme complex given in brackets is not detected but inferred by the conditions for its formation and the products formed.

Scheme II

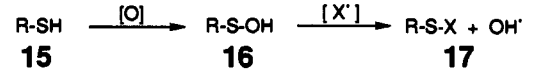


dimethylbenzene oxide,[42] which can be considered an oxygen analog for S_AMb, as shown in Scheme II. At pH > 6, a pH-dependent rearrangement occurs for **8** that can result in both hydrogen (**8** → **9** → **10** → **11**) and methyl (**8** → **12** → **13** → **14**) migration to yield the two products **11**,**14** analogous to sulfhemin-D. We detect only the analog of **11**, in that the hydrogen has migrated in the major product, suggesting that the steric constraints of the protein matrix may deter the methyl migration. It is possible that a S_DMb^* product with a migrated methyl ring comprises one of the uncharacterized minor products in the reaction. Nevertheless, the rearrangement $S_AMb^* \rightarrow S_DMb^*$ is concluded to be completely consistent with the expectation of a pyrrole II 3,4-episulfide rearranging by hydrogen migration to yield a pyrrole II 4-thiol derivative, as shown in Figure 7.

Conversion of S_DMb^* to the Terminal S_EMb^* and S_FMb^* Complexes. The optical bands of all S_EMb^* complexes resemble more those of a hemin than a chlorin (Figure 2). Moreover, the ^1H NMR data for a variety of complexes clearly dictate that the prosthetic group of S_EMb^* is a hemin; the metS_EMb^*CN exhibits three low-field heme methyl signals and the highly characteristic[28] Ile 99 (FG5) CγH peak at ca. −9 ppm (Table II), deoxy S_EMb^* exhibits a His F8 NδH shift at ~80 ppm[34] rather than 65 ppm,[10,22,33] and S_EMb^*CO displays meso-H and E7 Val CγH shifts of ring current indicative of a porphyrin[30] rather than chlorins[10,31,32] (Table III). Studies of the extracted prosthetic group of S_EMb^* show[20] that the 4-substituent is –SCN, so that its structure is 4-thiocyanatodeuterohemin, as shown in Figure 7. The conversion of the 4-thiol group of S_DMb^* to the 4-thiocyanato group of S_EMb^* is dependent on the presence of molecular oxygen. Thiol groups, **15**, are easily oxidized to unstable

(42) Kasperek, G. J.; Bruice, T. C.; Yagi, H.; Kaubisch, N.; Jerina, D. M. *J. Am. Chem. Soc.* **1972**, *94*, 7876–7882.

Scheme III



sulfenic acid groups,[43] **16**, according to Scheme III. The unstable sulfenic acid, **16**, in the presence of a strong nucleophile, X^-, such as cyanide or azide, will rapidly lead to the replacement[44] of OH^- by X^- to yield –SX, **17**. Within the protein matrix, the stronger nucleophile, cyanide, will displace the azide to yield the stable 4-thiocyanato group of S_EMb^* that can be readily extracted and characterized.

The Structure of S_AMb Complexes. The mechanisms of reactions of a common episulfide structure for the initial complex (S_AMb, S_AMb^*) for protohemin (**3**: $R_2 = R_4$ = vinyl) and deuterohemin (**3**: $R_2 = R_4 = H$) to form the various structurally characterized products are summarized in Figure 7. Subsequent to the proposed rearrangement of S_AMb to yield S_CMb for protohemin,[10] the oxo analog to sulfhemin-A was prepared and shown[45] to spontaneously rearrange to the oxo analog of sulfhemin-C. The fact that an identically functionalized initial S_AMb complex for both protohemin and deuterohemin can rearrange to yield products with the sulfur retained at the 3- and 4-positions, respectively, provides compelling evidence that the initial SMb complex indeed has the episulfide across the β–β bond of pyrrole II. ^1H NMR assignment of the hyperfine shifted resonance of the individual subunits of the cyano-met complex of the initially formed sulfhemoglobin complex provides evidence[33] for an electronic/molecular structure of the altered chromophore that is the same as in S_AMb.

Acknowledgment. The authors are indebted to A. L. Balch, J. Takahashi, W. K. Musker, and K. M. Smith for valuable discussions. This research was supported by a grant from the National Institutes of Health (GM 26226). The GE Ω-500 and Ω-300 NMR instruments were purchased, in part, with funds provided by the National Institutes of Health (RR-04795) and the National Science Foundation (BBS-88-04739 and DIR-90-16484).

Supplementary Material Available: Tables of chemical shifts for met$S_DMb^*N_3$ and met$S_FMb^*N_3$ and the position of the absorption bands for spectra in Figure 2 (1 page). Ordering information is given on any current masthead page.

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