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Sulfmyoglobin equilibration from deutero-3-hemin reconstituted myoglobin

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SULFMYOGLOBIN EQUILIBRATION FROM

DEUTERO-3-HEMIN

RECONSTITUTED MYOGLOBIN

A Thesis
Presented to
The Faculty of the Department of Chemistry
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In Partial Fulfillment for the Degree
Master of Science

By
Anh-Tuyet T. Tran
December, 1996

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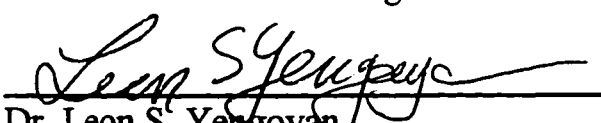

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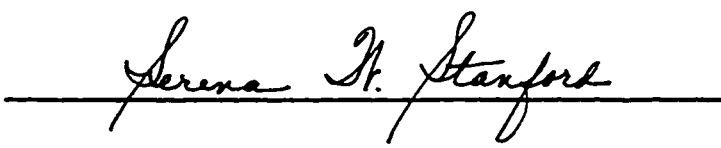

ABSTRACT

Sulfmyoglobin Equilibration from

Deutero-3-Hemin Reconstituted Myoglobin

by Anh-Tuyet T. Tran

The modification of the native red hemin of myoglobin (Mb) to yield the characteristic green color of sulfmyoglobin (SMb) has been attributed to the addition of a sulfur atom into the native protein. This initial unstable complex, termed S_A, spontaneously rearranges to yield another stable green product, termed S_C. However, in case of the deuterohemin reconstituted myoglobin (Mb*), the initial green product S_A equilibrates to two new red sulfmyoglobins, termed S_D and S_E.

In this research project, deutero-3-hemin, an isomer of deuterohemin, was reconstituted into apomyoglobin, and the equilibration of the sulfhemin derived from the deutero-3-myoglobin (Mb**) was studied using paramagnetic nuclear magnetic resonance (NMR) spectroscopy. The results suggest that the initial green product S_A equilibrates to a single red product, termed S_H.

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Last, but not least, I would like to thank God, my parents, and my brothers for helping me grow into what I am today.

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I. INTRODUCTION

Sulfmyoglobin (SMb) is a green heme derivative of myoglobin (Mb) in which the native heme has reacted with a sulfur atom in a manner that leads to partial saturation of the aromatic skeleton.[1-4] SMb is of research interest because it is the analogous complex of sulfhemoglobin (SHb), an abnormal blood pigment that is physiologically inactive.[1,4,5] SHb was first observed by Hoppe-Seyler in 1863 as a green product of the reaction between oxyhemoglobin and hydrogen sulfide.[3,4,6-8] For over 100 years, researchers have been studying this green pigmented protein with much interest to determine its unique properties. The effort to characterize the green blood pigment has been especially intensified when the pathological condition named sulfhemoglobinemia was discovered in the 1930s.[6,9]

Sulfhemoglobin has a substantially lower affinity to bind oxygen than native Hb due to the incorporation of sulfur atom into one or two hemins in the Hb tetramer.[6,10] Consequently, patients with sulfhemoglobinemia have cyanosis, which is characterized by the bluish skin color, along with anemia, which is diagnosed through symptoms of dysphnea, nausea, headache, drowsiness.[6,11,12] Sulfhemoglobin exists in normal blood at concentrations less than 0.4% of the total hemoglobin.[13,14] When the SHb concentrations exceed 2% of the total Hb,[12] signs of sulfhemoglobinemia are observed. In the presence of hydrogen sulfide from intestinal bacterial activity in human body,[15,16] SHb can be induced by various drugs[6,9,12,17-20] such as Dapsone, phenacetin, hydroxylamide, acetanilide, sodium nitrite, phenylazoaminopyridine, sulphonamide. Anesthetic drugs such as lidocain, benzocain also

induce SHb.[6] In addition, SHb can be induced under conditions of chemical air pollution in workplace such as in a coal-chemical industry,[12] or in a rubber manufacturing plant where workers are occupationally exposed to hydrogen sulfide, thiuram, altax, captax, dimethyl amine, diethyl amine, aniline, nitrogen oxide, and sulfur dioxide.[20-22]

Unfortunately, the problems of sulfhemoglobinemia can be treated only through blood transfusion![6,9] Moreover, the formation of SHb is not thought to be reversible, and thus is considered to be of great toxic significance.[2,6] According to Dijkhuizen et al.,[7] the clinical importance of SHb lies more in the diagnostic difficulties it may occasionally cause through its very high absorbance in the red part of the spectrum. That is the reason SHb may cause cyanosis and interfere with the spectrophotometric determination of O_2 saturation as well as of other dysfunctional hemoglobins.

Not until late in the 1980s that the structural as well as the chemical properties of SHb were determined in Timkovich's and in La Mar's laboratories.[2,3,5,23,24] In these laboratories, Mb was used as a simplified model of Hb, and paramagnetic ^{1}H NMR spectroscopy was the primary analytical tool. Thus, in order to understand the significance of the sulfmyoglobin (SMb) protein research, it is appropriate to discuss the relationships between SHb and SMb, paramagnetic ^{1}H NMR spectroscopy, as well as the properties and the newly discovered research of the SMb proteins.

A. Relationships between sulfhemoglobin and sulfmyoglobin

1. Structural and functional similarities of hemoglobin and myoglobin

Both Hb and Mb are oxygen-binding proteins. Hemoglobin is a tetrameric ($\alpha_2\beta_2$) protein of blood with the molecular weight of 64,000 D. Myoglobin is a monomeric protein of muscles with the molecular weight of 17,000 D (Figure 1).[6,11,25] The tertiary structure of the α and β subunits of Hb are remarkably similar both to each other and to that of Mb. Each subunit of Hb or each molecule of Mb noncovalently binds a single heme group through about 80 hydrophobic interactions and a single coordinate bond between the imidazole of the proximal histidine (His F8) and the iron atom.[25,26] It is the heme that is responsible for the characteristic red color of blood and is the site at which each monomer of Hb or each molecule of Mb binds one molecule of oxygen. The conjugated heterocyclic ring system of the heme is an iron-porphyrin derivative. In native Hb and Mb, the heme is protoporphyrin IX which is also called protohemin (Figure 2A). Its periphery has a particular arrangement with four methyl groups at positions 1,3, 5, and 8, two propionates at positions 6 and 7, and two vinyl substituents at positions 2 and 4.

Voet[11] stated that hemoglobin is not just a simple oxygen tank. It is a sophisticated oxygen delivery system that provides the proper amount of oxygen to the tissues under a wide variety of circumstances. Indeed, since the solubility of O_2 in blood plasma under physiological conditions is fairly low (about 10^{-4} M), Hb functions as an oxygen-transporter to transport the oxygenated species throughout the body. Normally, the whole blood in a human body contains about 150 g of Hb per liter of blood, which can carry 0.01M of oxygen.

Myoglobin was originally assumed to function only to store oxygen. Now it is known that its major physiological role is to facilitate oxygen transport in rapid respiring muscles. According to Voet,[11] the rate at which oxygen can diffuse from the capillaries to the tissues and thus, the level of respiration is limited by the low solubility of O_2 in aqueous solution. Mb is assumed to increase the effective solubility of O_2 in muscles by functioning as a kind of molecular bucket brigade to facilitate oxygen diffusion.

2. Identical spectra of sulfhemoglobin (SHb) and sulfmyoglobin (SMb)

Beside the structural and functional similarities of Hb and Mb, Morell and Chang[26] in 1967 found that SMb and SHb have identical and unique visible absorption spectrum with the intense absorbance centered about 620 nm. In addition, the analogous behaviors of SMb and SHb have recently re-confirmed by La Mar and co-workers[27] whose studies showed that SHb and SMb complexes have very similar ^{1}H NMR hyperfine shifted peaks (Figure 3). The ^{1}H NMR spectra of metSHbCN in Figures 3B and 3D exhibit pairs of methyl resonances arising from the nonequivalent subunits α- and β-. However, their chemical shifts are comparable to those of the metSMbCN in Figures 3A and 3C, respectively. Therefore, Mb is generally used as a simplified model of Hb in the sulfheme protein research.

3. Oxidation states and electronic states of the heme iron in Hb and Mb

Under normal physiological conditions, the heme iron atom remains in the ferrous Fe(II) state whether or not the heme binds to O_2. Thus, the oxygenation does not affect the

oxidation state of the heme iron. But it does change the electronic state of the Fe(II)-heme.[4,11,26,28] The iron atom in deoxygenated Hb and Mb is in high-spin state with a $t_{2g}^4 e_g^2$ configuration (S =2). The iron in deoxy-Hb or deoxy-Mb is displaced well out of the porphyrin ring; it has the coordination number 5 formed by square pyramid of five nitrogens: four from the porphyrin and one from the proximal histidine side chain of the protein (Figure 4A). Upon oxygenation, the O_2 binds to the Fe(II) at the sixth ligand position, and causes the Fe(II)-heme to switch to the diamagnetic and low-spin state with a $t_{2g}^6 e_g^0$ configuration (S =0) (Figure 4B). The change in electronic state of the central iron atom is indicated by the color change of blood from the dark purplish hue, characteristic of venous blood, to the brillant scarlet color of arterial blood or blood from a cut finger.[11]

When the Fe(II) of the heme is oxidized to Fe(III) (the ferric oxidation state) , the Hb and Mb in this case are named methemoglobin (metHb) and metmyoglobin (metMb), respectively. These species do not bind O_2, because the sixth liganding position of the central heme Fe(III) is already coordinated with an H_2O molecule. The presence of metHb or metMb is indicated by the brown color of dried blood or of old meat.[11] In the normal blood, there is about 0.4% of metHb,[13] which is spontaneously converted back to Hb by the enzyme methemoglobin-reductase in red blood cells.[9,11] However, if the concentration of metHb exceeds 10% of the total Hb in blood, it causes a pathological condition named methemoglobinemia which is treated with antioxidants such as vitamin C or methylene blue.[9,13,14]

B. Paramagnetic ^{1}H NMR spectroscopy [6,29,30]

1. Chemical shift

Since the chemical shift is dependent on the strength of the applied magnetic field, it is expressed as a function of field strength necessary to achieve the resonance condition:

$$\text{Chemical shift} = \sigma = \frac{H(o) - H(loc)}{H(loc)} = \frac{\Delta H}{H}$$

where H(o) is the applied magnetic field, and H(loc) the local magnetic field of the nucleus.

Resonances of a nucleus in paramagnetic complexes often show large shifts from those in diamagnetic complexes. These shifts result from interaction with unpaired electron(s). Thus, for a proton in a paramagnetic environment, the total chemical shift will arise from the contributions of both the diamagnetic shift, $\Delta H(dia)/H(dia)$, and the paramagnetic or hyperfine shift, $\Delta H(hf)/H(hf)$

$$\text{Chemical shift} = \sigma = \frac{\Delta H(dia)}{H(dia)} + \frac{\Delta H(hf)}{H(hf)}$$

In turn, the paramagnetic contribution is the combination of two components: the contact shift or "through bond" contribution and the dipolar shift or "through space" contribution.

$$\frac{\Delta H(hf)}{H(hf)} = \frac{\Delta H(con)}{H(con)} + \frac{\Delta H(dip)}{H(dip)}$$

The contact shifts result from delocalization of unpaired electron spin density at the resonating nucleus, which is usually transmitted through chemical bonds. Contact shift is sometimes referred to as the Fermi or isotropic contact interaction. For the first-row transition metals, the equation for contact shift is given by

$$\frac{\Delta H(con)}{H(con)} = \frac{-(A)}{(h)} \frac{g\beta S(S+1)}{3kT\gamma_I}$$

where A/h is the hyperfine coupling constant, g is the Lande g factor, T is the absolute temperature, k is the Boltzmann constant, γ_I is the magnetogyric ratio of the hydrogen nuclei, β is the Bohr magneton, and S is the total electron spin.

The dipolar shifts, also called pseudo-contact shifts, are derived from the interaction between the unpaired electron spin and a given nucleus. It is described by

$$\frac{\Delta H(dip)}{H(dip)} = \frac{\beta^2 S(S+1)}{6kTr^3} F'$$

where $F' = (g_z^2 - g^2)(3\cos^2\theta - 1) + (g_x^2 - g_y^2)\sin^2\theta\cos 2\phi$,

and where $g^2 = (g_x^2 + g_y^2 + g_z^2)/3$, ϕ and θ are defined in normal spherical coordinates, $\cos\theta = z/r$, $\sin\theta\cos\phi = x/r$, $\sin\theta\sin\phi = y/r$.

In the case of axial symmetry, $g(x) = g(y) = g(\perp)$ and $g(z) = g(/\!/)$, the dipolar shift formula is simplified to

$$\frac{\Delta H(dip)}{H(dip)} = \frac{\beta^2 S(S+1)}{9kTr^3} (g(/\!/) - g(\perp))(1 - 3\cos^2\theta)$$

Since the g tensor for low-spin systems is more anisotropic than that for high-spin ferric states, the spectrum for the low-spin systems shows larger dipolar contribution than the one for the high-spin complexes. This explains for the greater spectral resolution of the low-spin metcyano complexes compared to the high-spin metaquo complexes.

2. Nuclear relaxation

In addition to large chemical shifts, the unpaired electrons on paramagnetic species provides a very efficient relaxation mechanism. They are so because the magnetic moments of unpaired electrons are about 10^3-fold greater than the nuclear magnetic moments. As a result, the local fields generated are much greater. These in turn lead to a decrease of relaxation times of the nuclei bound near the paramagnetic center.

C. Previous sulfmyoglobin protein research

1. Previous studies of sulfmyoglobin preparation

In 1961, the preparation of metsulfmyoglobin was formulated by Nicholls[31] as the sequential addition of hydrogen peroxide, catalase, and hydrogen sulfide to metMb.

The addition of hydrogen peroxide to metMb results in the formation of ferrylMb, in which the heme iron is oxidized from the ferric state (Fe^{3+}) to the ferryl state (FeO^{2+} or Fe^{4+}).

$$\text{metMb} + O_2^{2-} \longrightarrow \text{FerrylMb}$$

The hypervalent state of Mb (or Hb), first described in 1935 by Haurowitz [32] and by Keilin and Hartree,[33] is well known as the product of the reaction of ferric Mb with hydrogen peroxide,[34-39] or with other strong oxidants.[37,40-48] They showed that the UV-VIS spectrum for the ferryl ion state has two unique absorption bands at 545 and 580 nm, whereas that for the ferrous state are at 540 and 577 nm, and for the ferric state is at 630 nm.

In the second step of SMb preparation, the excess of hydrogen peroxide needs to be removed by catalase, since spectrophotometric studies have shown that hydrogen peroxide destroys the SMb produced in the third step,[31] by the following reaction

$$MbSFe^{2+} + H_2O_2 \longrightarrow MbFe^{2+}O_2 + [H_2S](?)$$

Sulfmyoglobin Oxymyoglobin

Then, the addition of hydrogen sulfide to the ferrylMb results in a rapid formation of a mixture of metSMb, i.e., $MbSFe^{3+}$, and small quantities of SMb, i.e., $MbSFe^{2+}$. SMb was recognized by its intense absorption band at 617 nm, and metSMb by the bands at 595 and 715 nm.

$$MbFeO^{2+} + H_2S \longrightarrow \begin{cases} MbSFe^{2+} + H_2O & \text{(a)} \\ MbSFe^{3+} + H_2O + e^- & \text{(b)} \end{cases}$$

The metSMb species produced in the pathway (b) is reduced to SMb by the excess of hydrogen sulfide

$$MbSFe^{3+} + H_2S \longrightarrow MbSFe^{2+} + H^+ + SH^\bullet$$

Thus, the major overall reaction happened in the conversion of ferrylMb to SMb is as follows

$$MbFeO^{2+} + H_2S \longrightarrow MbSFe^{2+} + H_2O + [H^{\bullet}] + [SH^{\bullet}]$$

in which the heme iron is reduced from the ferryl state to the ferrous state.

(It is noted that the fate of the extra reducing equivalent H is still unknown.[31])

Then the SMb is converted to metSMb by the addition of an oxidizing agent such as ferricyanide or molecular oxygen

$$SMbFe^{2+} + Fe(CN)_6^{3-} \longrightarrow SMbFe^{3+} + Fe(CN)_6^{2-}$$

In 1971, Berzofsky et al.[1] optimized the conditions of the above reaction scheme by determining the optimal molar ratios of 1:4 for Mb:H_2O_2 and 1:1.5 for Mb: $(NH_4)_2S$. Also in this modified procedure, ammonium sulfide is used, instead of hydrogen sulfide. It is so because, according to Nicholls,[31] in order to generate the hydrogen sulfide content of neutralized solutions, both sodium sulfide and ammonium sulfide should be added to a known excess of iodine solution in dilute sulfuric acid. Then, the remaining iodine was titrated against standard sodium thiosulfate, with starch as indicator at the end point. The use of ammonium sulfide makes the procedure much simpler, because the hydrogen sulfide needs not to be generated. Also according to Nicholls,[31] ammonium sulfide is preferred for spectrophotometric experiments since the sodium sulfide, required for the formation of hydrogen sulfide, contains small quantities of polysulfides which tend to precipitate at neutral pH.

1. Structural properties of SMb

In 1961, Nicholls[31] found that upon the addition of ferricyanide, SMb is oxidized to metSMb. The product metSMb behaves like metMb, since the heme iron atom in metSMb is also coordinated with H_2O molecule that is dissociated under alkaline conditions.

In 1967, Morell and Chang[17] found that the additional sulfur atom in SMb is not attached to the heme iron atom. Instead, it must be bound so as to affect the porphyrin conjugation and to yield such unusual spectrum characterized by a strong band at 617 nm (ϵ_{mM} = 17.5). By comparison of the spectra of SMb derivatives with those of the corresponding derivatives of ferromesochloringlobin, a model compound prepared from Mb and ferric mesochlorin, Morell and Chang concluded that the heme of intact SMb has a chlorin-type conjugation, i.e. a partially saturated porphyrin.

Then in the late of 1980s, the molecular structure of SMb was completely determined in La Mar's laboratory [2,3] as well as in Timkovich's laboratory,[2,5,23] using ^{1}H NMR spectroscopy (Figure 5). Speculative assignments on the ^{1}H NMR spectra of the SMb complexes were based on the isotope labeling at individual methyl positions and at other useful structural probes such as the meso and propionate protons. Figure 5A displays the spectrum of metMbCN with three low-field shifted methyl peaks (M_1 to M_3) while the fourth one resonates in the crowded diamagnetic envelope. Figure 5B of the metS_AMbCN complex shows only two methyl peaks (A_1 and A_2), compared to the three methyl peaks of metMbCN. The fact that only one apparent heme methyl exhibits a considerably reduced contact shift supports a model where only a single pyrrole is saturated in each SMb isomer. Isotope

labeling of the vinyl positions as well as the decoupling of the hemin spectrum indicates the formation of a cyclic thioether on the saturated pyrrole II. Thus, the ^{1}H NMR analysis of the SMb complexes confirmed Morell's and Chang's[17] observations as follows: (1) The sulfur atom is not attached to the iron atom but it binds to the heme periphery and produces an episulfide across a pyrrole β-β bond, (2) the pyrrole where the sulfur atom binds is saturated and the conjugation throughout the heme is disrupted, (3) for reasons that are still unknown, the sulfur atom only attaches to the pyrrole II of the heme.

2. Characterization of SMb

La Mar and coworkers[2,3,4,6] discovered that SMbs are heterogeneous. This means that in any SMb samples there exist more than one species (Figure 5). The equilibration of the initial green product gives rise to several other species, including a major terminal product, with a thioether ring, termed S_CMb (Figure 5 F). This final product is stable for years in alkaline pH at 4^{0}C. Its molecular structure was completely determined by the unambiguous peak assignments in its spectrum. Based on the spectral information previously acquired as well as the molecular structure of the final product S_CMb, the initial green product was deduced as S_AMb (Figure 6). Besides, it is noted that another species named S_BMb is found to be present in every SMb preparation.[2,3] It is stable in acidic pH but exists only in small amounts in alkaline pH. In addition, studies show that this product does not equilibrate to the terminal product S_CMb. Thus, S_BMb is considered as a side product. Structure of the sulfhemin B has not been determined. But based on the fact that it is stable in acidic pH, its

structure is suggested as the one shown in Scheme 1.

The formation pathway of the green SMb[27] is summarized in Scheme 1. Following the standard procedure for the preparation of SMb, the experiment yields an initial green product which is S_AMb. About a week later under the condition of alkaline pH at 4 ^{0}C, the unstable S_AMb rearranges to form the terminal green product S_CMb.

In addition, La Mar and coworkers[2-4,6] also found that S_CMb could only be prepared in the presence of a vinyl group at positon 4 on the heme periphery. If the vinyl groups at positions 2 and 4 of the heme are synthetically replaced by protons, the native heme becomes the deuterohemin (Figure 2B). In this case, it is believed that the initial green product would equilibrate to another set of two new SMb derivatives. Unlike the green S_CMb, these new SMbs are red pigmented species. Their UV-VIS optical spectra are very similar to those of native Mb, due to the red color of these proteins. However, the features of their spectra (Figure 7) are quite distinct from those of native Mb, indicating different structures of SMbs from that of the native Mb.

In La Mar's lab, Scharberg [4,6,24] completely characterized these two red sulfhemins, by studying the SMbs derived from this synthetic deuterohemin reconstituted with apomyoglobin. They were named S_DMb* and S_EMb* (Note that the asterik followed these names is to indicate that these are not native hemin). Figure 8B of the green initial product, metS_AMb*CN, shows two methyl peaks (A_1 and A_2) comparable to those of metS_AMbCN. This confirms the presence of the episulfide on the saturated heme pyrrole II in metS_AMb*CN. However, both spectra of metS_DMb*CN (Figure 8C) and metS_EMb*CN

(Figure 8D) depict three methyl peaks (D_1 to D_3, and E_1 to E_3, respectively) that are more similar to the three methyl peaks of metMb*CN (Figure 8A). This feature indicates that the pyrrole II in sulfhemin D and sulfhemin E regains its conjugation state. Of these two red sulfhemins, only S_E Mb* is stable for years and endures the acidic extraction so that its structure has been completely determined. The other species, S_D Mb*, is stable for only several weeks but unstable to acidic extraction. At 20 ^{0}C and pH 7.0, S_DMb* equilibrates to S_E Mb* within 4 weeks. Therefore, S_D Mb* is considered as an intermediate species on the pathway to convert S_AMb* to S_E Mb*.

The mechanism of SMb* formation, therefore, is suggested in Scheme 2.[4,6,24] By the standard preparation, the initial green product S_AMb* is formed. It is unstable and equilibrates to the intermediate red product S_DMb* at 20 ^{0}C, pH 7.0. The heme of the intermediate S_D Mb* contains a thiol group at position 4, which is easily oxidized to the unstable sulfenic acid group. In the presence of a strong nucleophile X^-, such as cyanide or azide, the sulfenic acid group undergoes a nucleophilic substitution reaction[4,6,24,40] to yield an -SX group (thiocyanide or thioazide) attached at position 4 of the terminal S_E Mb* hemin. Again, the heme in these SMb* species regains the complete conjugation and thus they appear as red-pigmented proteins.

D. Goal of this Project

In comparing the final products from the studies mentioned above (Schemes 1 and 2), it is clear that the sulfur atom attacks the pyrrole II of the heme in native Mb as well as in

deuterohemin reconstituted Mb*. Also, the sulfur atom is retained during equilibration in both cases. However, the sulfur atom is found at position 3 in S_CMb, as part of a 5-membered thioether ring, whereas it is incorporated into a thiocyanate substituent at position 4 in S_E Mb*. [2-4,6,24] In addition, the pyrrole II in S_CMb is saturated, but it regains the conjugation in S_E Mb*.

These structural differences between S_CMb and S_E Mb* give rise to a question. How would the sulfur atom behave in the case of Mb reconstituted with deutero-3-hemin? Note that deutero-3-hemin (Figure 2C), with the protons at positions 2 and 3 and the methyl groups at positions 1 and 4, is an isomer of deuterohemin.

To answer that question is the goal of this project. In other words, deutero-3-hemin was reconstituted with apomyoglobin to form Mb** (the double asterik followed this name is to distinguish it from the native Mb and from the deuterohemin reconstituted Mb* species). Then, the possible initial SMb** derivative was prepared and its equilibration monitored by ^{1}H NMR spectroscopy. The low spin metcyanoSMb** complex is studied in this project, based on the fact that low-spin metcyano complexes exhibit greater spectral resolution, compared to the high-spin metaquo complexes.[23] Paramagnetic ^{1}H NMR was the primary analytical tool, since this method has been successfully used to characterize the SMb products of the native Mb and those of the deuterohemin reconstituted Mb* as well. The ^{1}H NMR spectra of these SMb** species will provide information about: (1) on which pyrrole the sulfur atom would bind to the heme, (2) at which position on that pyrrole the sulfur atom would attack, and (3) whether or not the sulfur atom would be retained during equilibration.

II. EXPERIMENTAL SECTION

A. Materials and Standard Solutions

All the solutions were prepared as described by Berzofsky et al.[1], and were used within several hours of preparation.

1. Horse heart myoglobin was purchased from Sigma Chemical Co. as a lyophilized salt-free powder of metMb. It was used without further purification.
2. Deutero-3-hemin was obtained from Dr. Kevin M. Smith, Department of Chemistry, University of California at Davis.
3. The dilute hydrogen peroxide solution was prepared by adding 420 μL of the 0.1M potassium phosphate (KPi) buffer pH 8.0, into 10 μL of the certified A.C.S. hydrogen peroxide 30 %.
4. The catalase dilute solution was prepared by dissolving 5 mg of the lyophilized powder of bovine liver catalase (purchased from Sigma Chemical Co.) in 200 μL of 0.1 M buffer KPi, pH 8.0.
5. The dilute ammonium sulfide was prepared by adding 370 μL of 0.1 M buffer KPi, pH 8.0 to 10 μL of the certified Fisher light solution of ammonium sulfide.
6. The aliquot of potassium ferricyanide was prepared by dissolving 30 mg of potassium ferricyanide (in crystal form) into 500 μL of 0.1 M buffer KPi in D_2O, pH 7.0.
7. The aliquot of potassium cyanide was prepared by dissolving 32 mg of the potassium cyanide crystals in 500 μL of 0.1 M buffer KPi in D_2O, pH 7.0.

8. The aliquot of iodoacetamide was prepared, following Scharberg's description[4,6,24], by dissolving 9.25 mg of iodoacetamide powder in 50 μL of 0.1 M buffer KPi in D_2O, pH 7.0.

B. Experiments

All experiments were carried out at 0-4 ^{0}C.

1. Preparation of apo-Myoglobin (apoMb)

Following La Mar and coworkers' preparation of apo-Myoglobin, [2,3,6,24,49-54] a solution of 1 g of horse heart metMb in 100 mL of cold water was acidified to pH 2.5 with a cold solution of 0.1 M HCl. Then it was treated 3 to 4 times with cold methylethylketone to extract the released hemin. The clear aqueous apoMb solution was dialyzed exhaustively against chilled water until the odor of ketone could no longer be detected. The protein solution was then dialyzed against a chilled 50 mM NaPi buffer pH 7.0 to adjust the pH without precipitation of the protein. Next, a final dialysis against chilled water was done to remove the buffer salts prior to lyophilization. The lyophilized apoMb was stored at 4 ^{0}C for later use.

The aliquots of apoMb used in the reconstitution experiments were prepared by dissolving 40 mg of lyophilized apoMb in 500 μL of 0.1 M KPi buffer pH 8.0. Prior to reconstitution, any precipitate was removed by centrifugation.

2. Reconstitution of apoMb and deutero-3-hemin

Following La Mar's reconstitution method,[4-6,24,51-53] deutero-3-hemin (1 mg) was dissolved in 100 μL of 0.2 M NaOH. The heme solution was added gradually to the apoMb aliquot. Reconstitution of deutero-3-hemin into apoMb took place in phosphate buffer pH 8.0.[4,6,24,49] Then, the solution was chromatographed at 4 ^{0}C on a G-25 fine Sephadex column (1.5 cm x 36 cm) equilibrated with 0.1 M KPi buffer pH 8.0. The eluting solution was allowed to equilibrate for at least 24 hours at room temperature to remove the heme disorder.[49] Next, it was concentrated and exchanged with 0.1 M buffer KPi, pH 8.0 by ultrafiltration on an Amicon 8MC equipped with a YM5 membrane. Then the sample of reconstituted Mb was centrifuged before its pH was confirmed at pH 8.0 using a Beckman model 3550 pH-meter equipped with an Ingold microcombination electrode.

3. Preparation of metMbCN sample**

The conditions in which the metMb**CN sample was prepared were exactly the same as those of metSMb**CN preparation. To the aliquot of deutero-3-hemin reconstituted Mb** was added 5 μL of a freshly prepared solution of KCN. The sample turns from the brown color of metaquoMb to the bright red color of metcyanoMb. It was exchanged into 0.1 M buffer KPi in D_2O, pH 7.0 and then transferred into a 5 mm NMR tube.

4. Preparation of metSMbCN samples**

It was carried out following the modified method described by Berzofsky et al.[1]

To the aliquot of deutero-3-hemin reconstituted Mb** (approximately 3 mM solution in 500 μL of 0.1 M KPi buffer pH 8.0), was added 35 μL of a freshly diluted hydrogen peroxide solution. The color of the solution immediately changed from brown to red. This change of color is characteristic of the formation of the ferryl derivative.[1,2] About a minute later, 10 μL of the dilute catalase solution was added. Bubbles appeared as the oxygen gas was released from the catalase digestion of the excess hydrogen peroxide. One minute was allowed for the catalase to destroy the residual hydrogen peroxide, and then 35 μL of ammonium sulfide was added, producing an immediate change to the dark green color of ferrous SMb. Within 2 or 3 minutes, the green solution was placed onto a column (1.5 cm x 28 cm) of fine Sephadex G-25 equilibrated with 0.1 M KPi buffer pH 8.0, at 4 ^{0}C. The green material was eluted within 25 to 30 minutes with the same buffer.

To the ferrous SMb** solution that was just eluting from the column, was added 30 μL of a fresh solution of potassium ferricyanide. The solution turned to the brown color of ferric Mb. It was converted into metcyano SMb** by the addition of 3 equivalents (5 μL) of a fresh potassium cyanide solution. Finally, the excess of potassium ferricyanide was removed by ultrafiltration on an Amicon 8MC with a YM5 membrane, while the sample was undergone 5 times of D_2O-exchange into 0.1 M KPi buffer in D_2O, pH 7.0. Due to the low yield of SMb**, two samples of SMb**CN were combined to generate a sample of appropriate concentration for paramagnetic ^{1}H NMR studies. After being concentrated to 0.5 mL (approximately 4 mM), the sample was transferred into a 5-mm NMR tube and stored at 4^{0}C.

5. Preparation of the thiol-trapping sulf-iodoacetamide.

Excess cyanide was removed from the solution of metS_HMb**CN in D_2O, pH 7.0 via ultrafiltration. Then the S_HMb**CN was equilibrated 25 days at 4^0C with 8 equivalents (4 μL) of iodoacetamide, ICH_2CONH_2, before being analyzed by NMR.

5. NMR measurements

^{1}H NMR spectra were recorded at 20^0 C on a GE NMR-QE PLUS spectrometer operating in the quadrature mode at 300 MHz. Typical spectra were collected over 30-KHz bandwidths with 16,000 datapoints and a recycling time of 0.75 second. All chemical shifts are given in ppm from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) referenced by the HOD resonance. Resonances are labeled as previously [4-6,24,49-53] with M_i, A_i, H_i, I_i designating resonances of metMb**CN, metS_AMb**CN, metS_HMb**CN, metS_IMb**CN, respectively. Peak assignments were completed by comparison to those of the native Mb and the deutero Mb*.

III. RESULTS AND DISCUSSION

The preliminary inspection of the NMR spectra shows that none of the prepared samples exhibited peaks of heme disorder. (For reference, figures 9 and 10) are included to display the spectral pattern of heme disorder in native Mb and in deuterohemin myoglobin, Mb*). It is so because the deutero-3-myoglobin, Mb**, samples were allowed to equilibrate for at least 24 hours at room temperature so that the heme reorientation took place before the samples underwent any further modification. Thus, all the peaks appear in these presented spectra arise from metMb**CN or metSMb**CN.

It is also noted that like any other SMb species, metSMb**CN is heterogeneous. Specifically, four species has been identified in these samples: metMb**CN, metS_AMb**CN, metS_HMb**CN, and metS_IMb**CN. For a rough estimation, the total yield was about 45% for all SMb** products and 55% for the unreacted Mb**.

1. MetMb**CN

The resolved portions of the 300-MHz ^{1}H NMR of metMb**CN in D_2O at pH 7.0 and 4 ^{0}C are displayed in Figure 11A. The chemical shifts for metMb**CN are shown in Table 1. In analogy with those of native metMbCN (Figure 5A) and of deutero-metMb*CN (Figure 8A), the spectrum of metMb**CN shows three low-field shifted methyl peaks (M_1-M_3). This indicates that deutero-3-hemin was actually incorporated into apoMb to yield the reconstituted Mb**. All the peaks M_i of the metMb**CN complex are single peaks. They

do not show pairs of peaks similar to those of heme disorder shown in Figure 10. Thus, the heme in metMb**CN was in its original orientation, and all the peaks in its spectrum are real signals for the metMb**CN species.

2. MetS_AMb**CN

The resolved portions of the 300-MHz ^1H NMR reference spectrum of metS_AMb**CN in D_2O at pH 7.0 and 4 ^0C, is illustrated in Figure 11B, and its chemical shifts are shown in Table 2. It was taken immediately after the sulf-preparation. The trace is essentially identical to those of the native metS_AMbCN (Figure 5B) and of the deutero-metS_AMb*CN (Figure 8B). All of these traces show only two peaks of the low-field signals, A_1 and A_2, arising from the two heme methyls, compared to the three signals, M_1 to M_3, in any met MbCN species (Figures 5A, 8A, 11A). This feature confirms that the prosthetic group in met S_AMb**CN is an iron-chlorin, and explains for the green color of this initial S_A Mb** species. Thus, S_AMb**CN is deduced to have a sulfur atom incorporated into the heme pyrrole II like the sulfhemin A in native Mb and in deuteroMb*.

3. MetS_HMb**CN

Within an hour after its preparation at 4 ^0C and pH 7.0, the initial green product S_AMb** equilibrated to a red species termed S_HMb**. Figure 11C depicts the 300-MHz ^1H NMR reference spectrum of the sample in trace 11B after a week of equilibration in D_2O at pH 7.0 and 4^0C, and Table III includes the chemical shifts for this species. The trace shows

three, rather than two, low-field heme methyl peaks with shifts more similar to those of the unreacted metMb**CN (Figure11A) than those of the green chlorin containing metS_AMb**CN (Figure 11B). It unambiguously indicates that the prosthetic group in metS_HMb**CN is a peripherically functionalyzed hemin rather than an iron chlorin.

In comparing to metS_DMb*CN, metS_HMb**CN shows identical spectral pattern (Figures 8C, 11C, and Table 3). However, while the sulfhemin D equilibrates to sulfhemin E in 3-4 weeks at 20 ^{0}C and pH 7.0, the sulfhemin H did not. Priliminary studies indicated that metS_HMb**CN decomposed after two weeks of equilibration at 20 ^{0}C and pH 7.0, without giving rise to any other species (Figure 12B). And even when equilibrating at 4 ^{0}C, pH 7.0 the sulfhemin did not give rise to any new product. Figures 13B and 13C were from the same sample of metS_HMb**CN, but the spectrum in Figure 13C was recorded a week later than the one in Figure 13B. Both of them reveal the presence of the sulfhemin H and the unreacted deutero-3-Mb**. The peak ratios for H_1/M_1 is 0.82/1 in Figure 13B, and 0.64/1 in Figure 13C. Thus, the peak intensity for the metS_HMb**CN is lesser in trace C than it is in trace B. Yet no signals of a new species arise in trace C. This fact indicates that the sulfhemin H gradually decomposed without equilibrating to any further product at 4 ^{0}C, pH 7.0. Therefore, from the results of the SMb** equilibration at both 20 ^{0}C and 4 ^{0}C, pH 7.0, it is conclusive that the sulfhemin H must be the terminal product in the equilibration of the deutero-3-sulfmyoglobin.

4. Characterization of the sulfhemin H

Since metS_HMb**CN gives identical ^{1}H NMR spectrum to that of metS_DMb*CN, and since the sulfhemin D contains a thiol group, it is proposed that the sulfhemin H also have a thiol group. In order to confirm the thiol group in the sulfhemin H, an experiment, namely the iodoacetamide reaction, was carried out. Iodoacetamide, ICH_2CONH_2, is a reagent commonly used to protect thiol groups.[4,6,24,57] It was successfully used to characterize the prosthetic group in S_DMb* as the thioldeuterohemin, based upon 1-D, 2-D NMR and mass spectra studies of the extracted thiol-trapped heme.

The 300-MHz ^{1}H NMR reference spectra of metS_HMb**CN in equilibrating with iodoacetamide at 4 ^{0}C, pH 7.0 are displayed in Figure 14, and its chemical shifts are shown in Table 4 . Figure 14A was taken two days after iodoacetamide addition. The trace shows the existence of two species, metMb**CN and metS_HMb**CN, but no peaks of iodoacetamide adduct, like those depicted in Figure 8E of the iodoacetamide trapped metS_DMb*CN. It suggests that within the first two days, the sulfhemin H did not react with iodoacetamide.

However, after 25 days since the addition of iodoacetamide at 4 ^{0}C and pH 7.0, the trace in Figure 14B indicates the presence of a new red species, termed S_IMb**, that is possibly the iodoacetamide adduct of the sulfhemin H. But the peaks of the S_IMb** complex shifted far upfield compared to those of the iodoacetamide adduct S_DMb* shown in Figure 8E. Due to the uniqueness of the spectrum for the sulfhemin I, the peak assignments could not be easily completed by comparison to those of any native SMb nor of any deutero SMb*

complexes.

The presence of the sulfhemin I, though it has not been fully characterized, provides more support for the hypothesis that the prosthetic group in S_HMb** is the thiol deutero-3-hemin. This thiol group may be at position 3 of the pyrrole II, and the heme pocket at this position may have some factors that hinder the interaction between the thiol group and iodoacetamide. For an inspection of the protein pocket, Figure 15 shows the orientation of the native hemin in the protein matrix. In comparing to those shown in Figure 15, Figure 16 depicts the proposed orientation of the sulfhemin H in the protein cavity. It shows that the thiol group at the heme position 3 is surrounded by the nonpolar side chain of the residues Ile 99, Ile 107, and Leu 104. Indeed in this environment, such a large and polar molecule of iodoacetamide could have difficulties in reacting with the thiol group of the sulfhemin H. However, this explanation is only a hypothesis that requires a 2-D NMR analysis of the heme-protein interaction to test for its validity.

CONCLUSION

The SMb** complex, derived from the deutero-3-hemin reconstituted myoglobin, was prepared and its equilibration has been studied. Deutero-3-sulfmyoglobin (SMb**) appears to be less stable than deuterosulfmyoglobin (SMb*). The total yield of SMb** was only 45%, compared to the reported yields of 90% for the native SMb[1-3] and of 75% for the deuteroSMb*.[4,6,24] Furthermore, the instability of Mb** is revealed by the hasty equilibration of the initial green product S_AMb** to the terminal red product S_HMb** within an hour after preparation.

The ^{1}H NMR studies of SMb** suggest that the initial green product has similar structure to those of the native S_AMb and the deutero S_AMb*. This result seems to readily confirm that, despite the differences in structural details of the prosthetic groups, the initial sulfhemin is invariably the green S_A containing an episulfide across the β-β bond of the pyrrole II. The existence of another SMb** species, the sulfhemin H, unambiguously states that the sulfur atom is retained during the equilibration of the initial sulfhemin A. Besides, since the structure of S_HMb** is more similar to the deutero S_DMb* than to the native S_CMb, the results of this project confirms La Mar and coworkers' observation[2-4,6,24] that the sulfhemin C could only be prepared in the presence of a vinyl group at position 4 on the heme periphery. The sulfhemin H gave identical spectral lines to those of the sulfhemin D. But unlike the sulfhemin D that equilibrates to the sulfhemin E at 20 ^{0}C, pH 7.0, the sulfhemin H decomposed under such conditions. Even at 4 ^{0}C, pH 7.0, the sulfhemin H gradually

decomposed without equilibrating to any further species. Thus, the sulfhemin H is considered to be the terminal product of the deutero-3-SMb** equilibration.

Also due to the identical spectrum of the sulfhemin H and the sulfhemin D, the prosthetic group in the sulfhemin H is proposed to be the thiol deutero-3-hemin. However, the reaction between the sulfhemin H and iodoacetamide occurred very slowly, and the possible iodoacetamide adduct S_IMb** shows ^{1}H NMR peaks shifted far upfield compared to those of the iodoacetamide adduct S_D·Mb*. Thus, to satisfactorily characterize the red S_IMb** species, 2-D NMR and mass spectra studies should be done as they were successfully used in the characterization of S_D·Mb*.

Based on the very slow reaction of the sulfhemin H with iodoacetamide, the thiol group in S_HMb** is hypothesized to be at position 3 of the heme periphery. Inspection of the heme pocket suggests that the thiol group in the sulfhemin H is positioned in such a crowded environment of nonpolar residues that the big and polar molecule of iodoacetamide could not easily enter and react with it. However, a 2-D NMR analysis of the heme-protein interaction should be carried out to verify this hypothesis.

Thus, the reconstituted Mb** is much less stable than the native Mb or the reconstituted Mb*. For all these three species, the initial sulfmyoglobin product is invariably the green S_A containing an episulfide across the β-β double bond of the pyrrole II. Like the native SMb, the equilibration of SMb** took place at 4 ^{0}C, pH 7.0. And like the deutero SMb*, the initial green product S_AMb** equilibrated to the terminal species, the red sulfhemin H, that possibly contains a thiol group at position 3.

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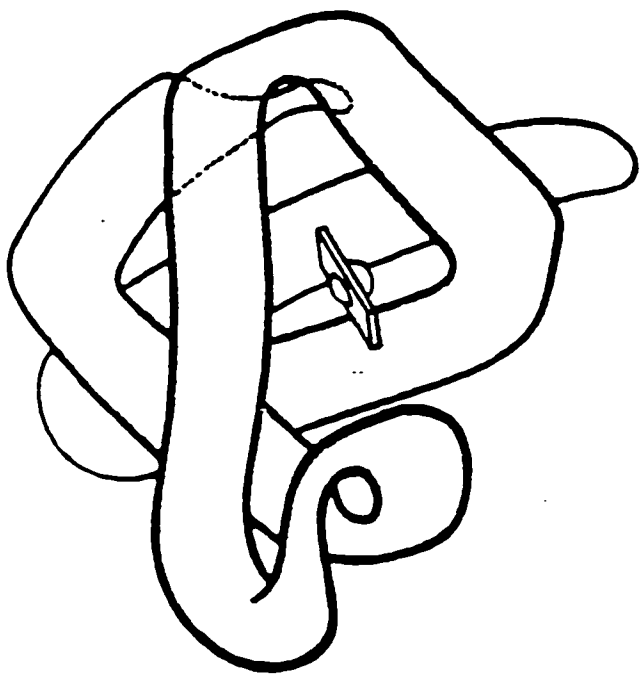
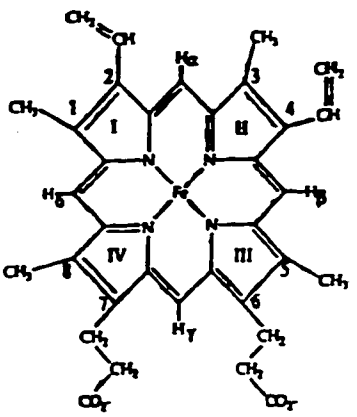
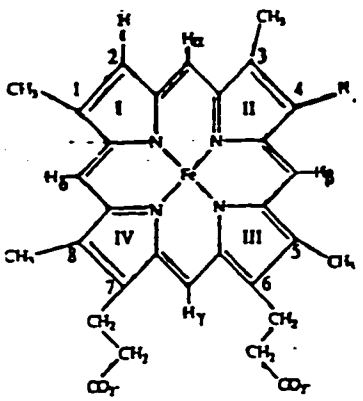


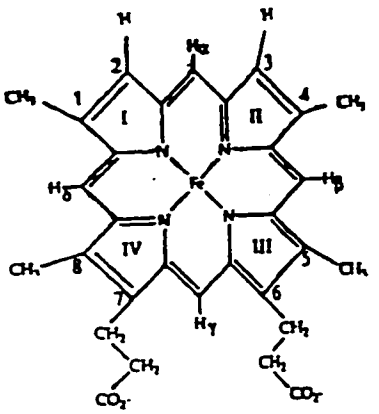
Figure 1: Schematic structure of Mb



Native Hemin
Figure A



Deuterohemin
Figure B



Deutero-3-hemin
Figure C

Figure 2: Structural diagram of (A) native hemin in Hb and Mb. (B) deuterohemin ($R_2 = R_4 = H$). (C) deutero-3-hemin ($R_2 = R_3 = H$, $R_1 = R_4 = CH_3$).

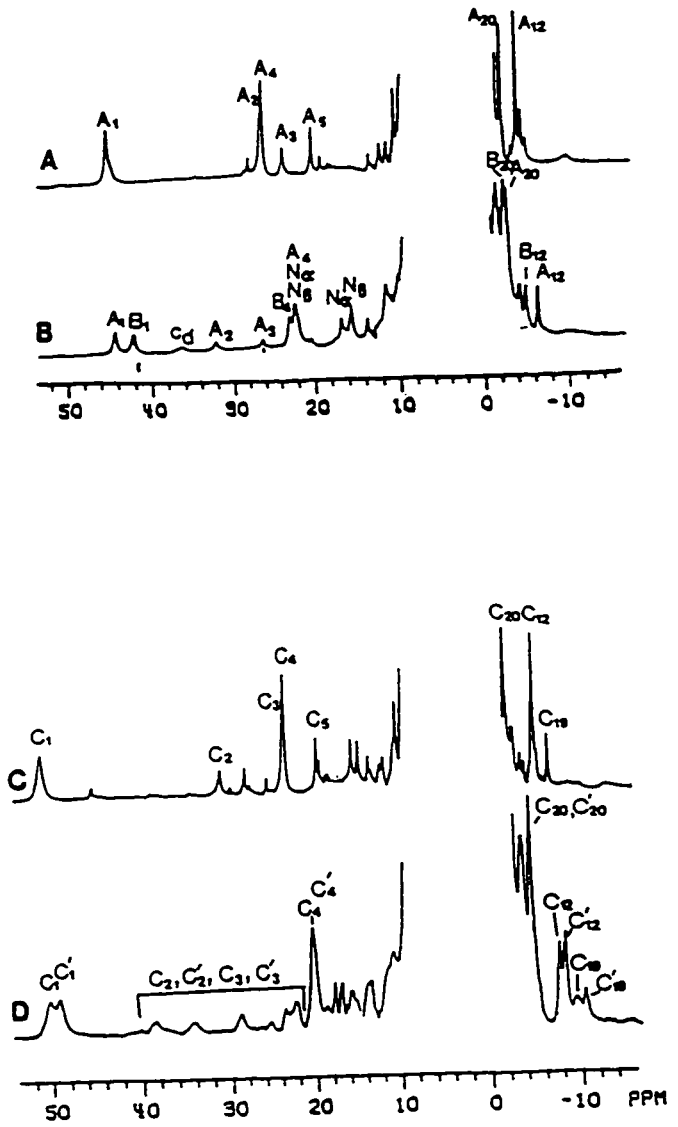


Figure 3: The 360-MHz ^{1}H NMR reference spectra of (A) metS_AMbCN, (B) metS_AHbCN, (C) metS_CMbCN, (D) metS_CHbCN, respectively, at pH 8.0, 20^{0}C, in D_2O. (From reference 27. pp.292-293. Reprinted with permission).

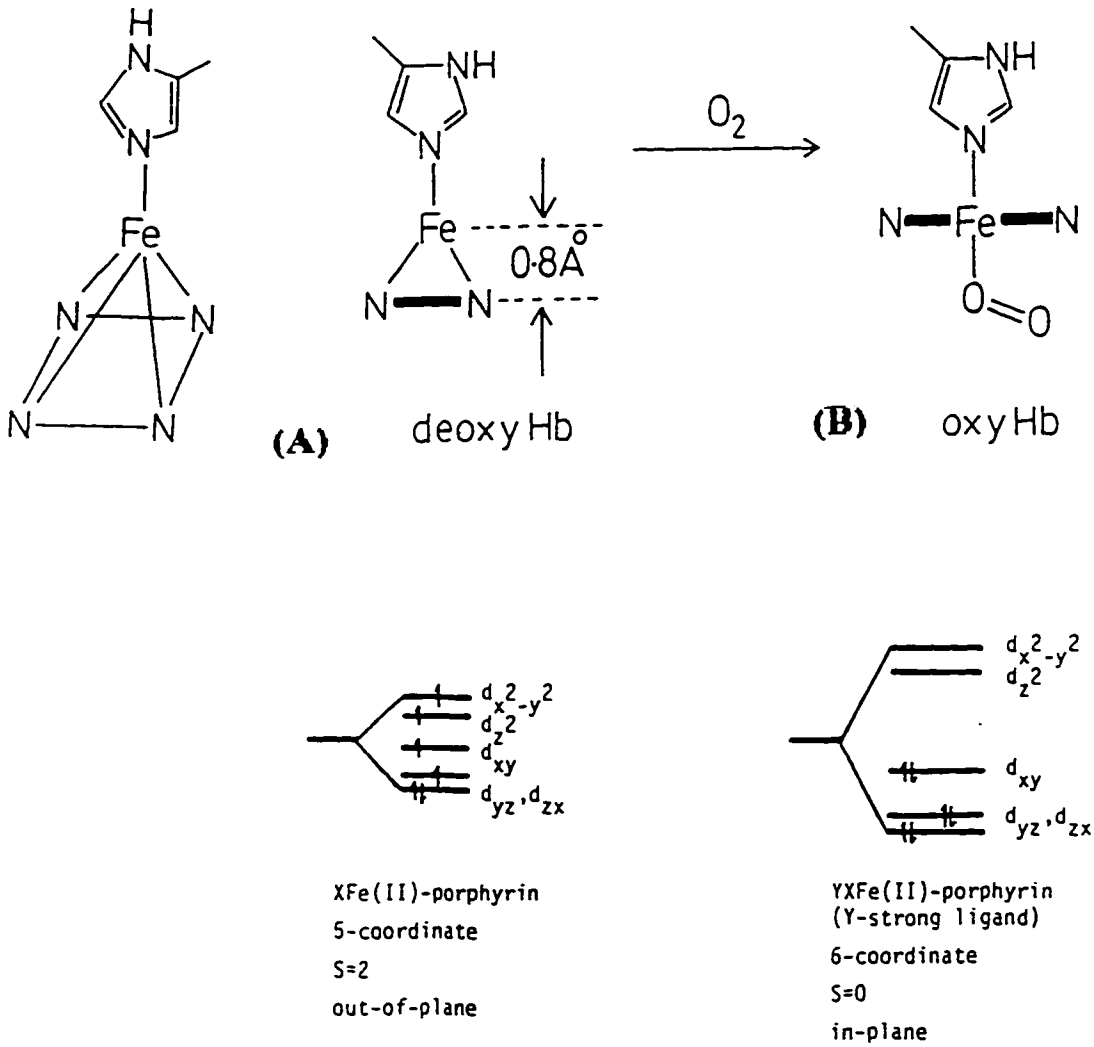


Figure 4: Structural diagram of the heme and the electronic states of the central iron atom in (A) deoxy Hb, (B) oxy Hb.

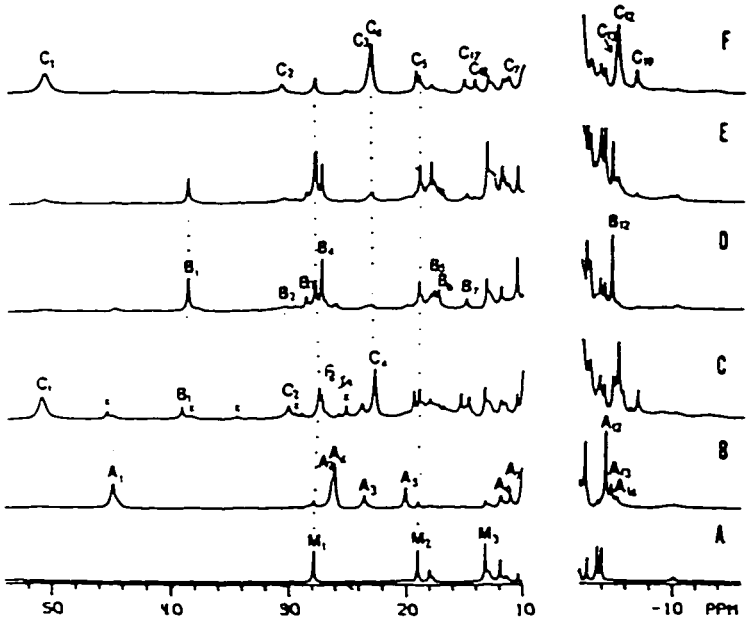


Figure 5: 360-MHz ^{1}H NMR spectra of the met-cyano SMb complexes at 20 °C in D_2O. (A) Native metMbCN, pH7.1. (B) Chromatographed metS_A MbCN, pH 7.1; an identical spectrum was observed prior to chromatography. (C) Products of chromatographing metS_A MbCN at pH6.0 and storing at 4 °C for 2 months. (D) MetS_BMbCN made by ligating with cyanide pH7.1. (E) Sample from trace D following 7 days at 22 °C; increased metS_BMbCN is observed. (F) MetS_CMbCN resulting from oxidizing and liganding the sample of metS_AMbCO, pH7.1. Peaks of metS_AMbCN, metS_BMbCN, metS_CMbCN, and native metMbCN are labeled A_i, B_i, C_i, and M_i, respectively; impurities are labeled x. (From reference 3, p. 6941). (Reprinted with permission).

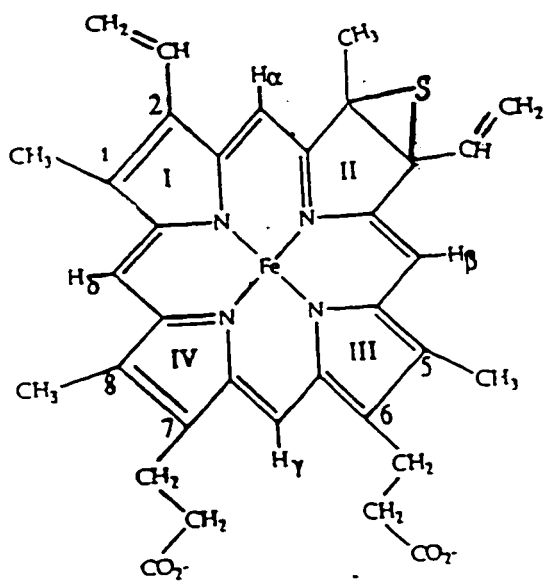


Figure 6 · : Structural diagram of the sulfhemin in SMb.

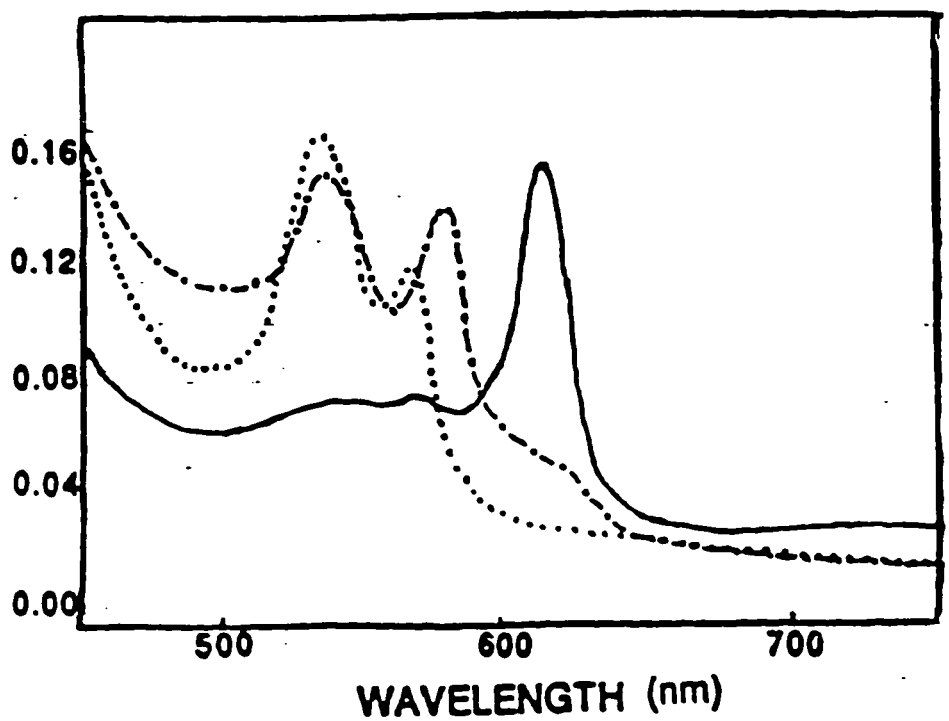


Figure 7 : Optical spectra of SMb*. (A) Optical spectra of unreacted and SMb*complexes of equine Mb reconstituted with deuterohemin in 0.1 M phosphate buffer pH 7.1 at 22 °C; 75% deoxy S_AMb* and 25% unreacted deoxyMb* (—); 75% deoxy S_EMb* and 25% unreacted deoxyMb* (-·-·-); unreacted deoxy Mb* (...). (From reference 4, p.6517. Reprinted with permission).

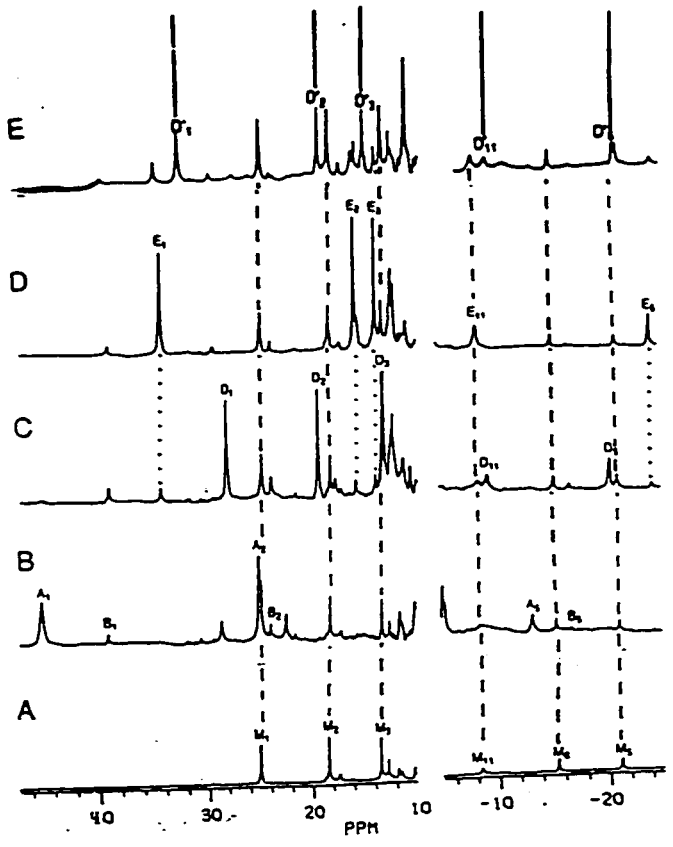


Figure 8 : The 360-MHz reference ^{1}H NMR spectra of the metcyano SMb* complexes at 20 °C and pH 7.1 in D_2O. (A) Unreacted metMb*CN with heme methyl peak M_1 to M_3, pyrrole 2-H, 4-H, and Ile 99 (FG5) CγH peaks M_5, M_6, and M_{11}, respectively. (B) $MetS_AMb^*CN$ immediately after preparation with heme methyl peaks A_1, A_2 and pyrrole 2-H signal A_5. Peaks B_1, B_2, B_5 are from about 5% of $metS_B$ *CN present in the sample. (C) $MetS_DMb^*CN$ 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 (FG%) CγH peak D_{11}. (D) $MetS_EMb^*CN$ from the 22 °C 3-week equilibration in the presence of excess cyanide 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) Met S_D'Mb*CN resulting from reacting $metS_DMb^*CN$ with iodoacetamide. (From reference 4, p. 6518. Reprinted with permission).

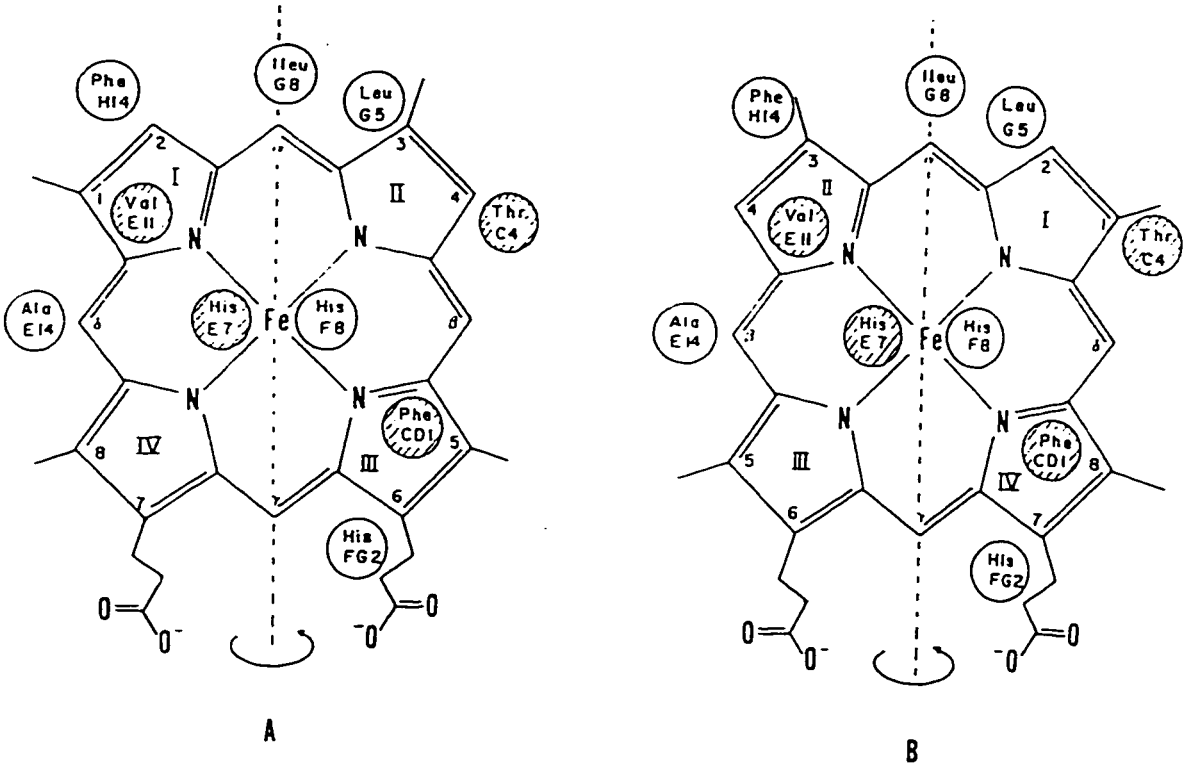


Figure 9 : Heme-apoprotein contacts for protoporphyrin in the pocket of sperm whale Mb. Open circles indicate contacts on the proximal side; shaded circles represent contacts on the distal side of the heme. Only the methyl group and the propionic acid substitutents are included to make the arguments applicable to deuteroporphyrin. (A) Normal orientation as found in the native protein; (B) Reversed orientation with the porphyrin rotated 180 ^{0} in the heme pocket about the α -γ meso axis. (From reference 57, p. 5756. Reprinted with permission).

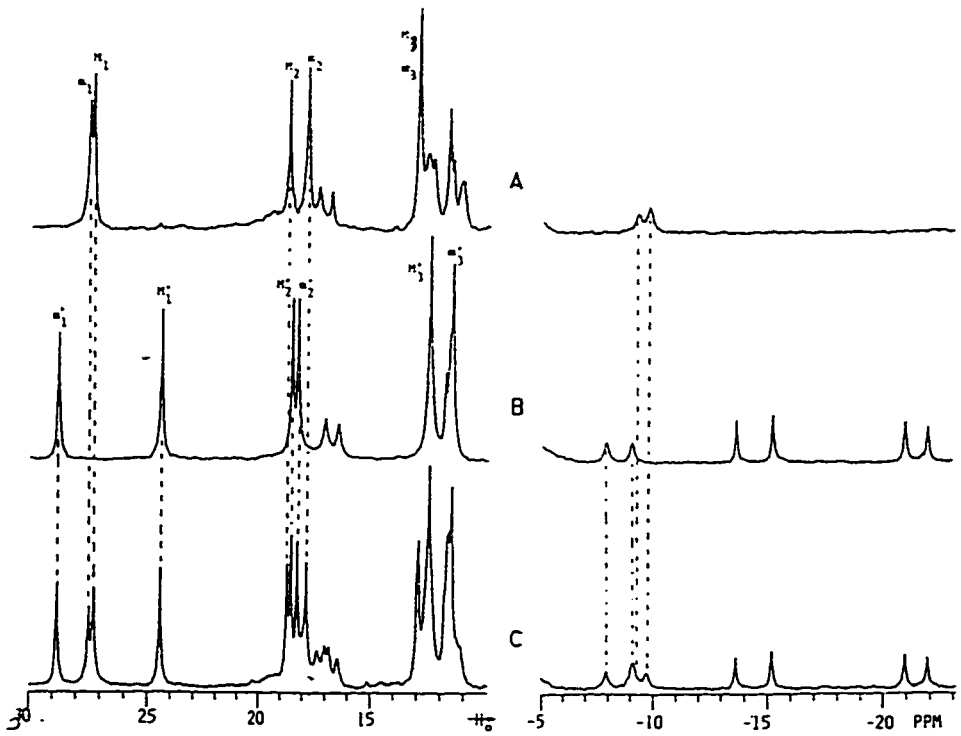


Figure 10: The 360-MHz ^{1}H NMR spectra of the initial products of metMbCN of (A) protohemin, (B) deuterohemin, and (C) protohemin and deuterohemin in D_2O at 25 ^{0}C, pH 7.2. The heme-methyl peaks for native metMbCN are designated M_i and m_i for the heme orientations A and B in Figure 14, respectively; the same signals are designated M_i' and m_i' for deuterohemin-metMb*CN. (From reference 49, p. 6399. **Reprinted with permission**).

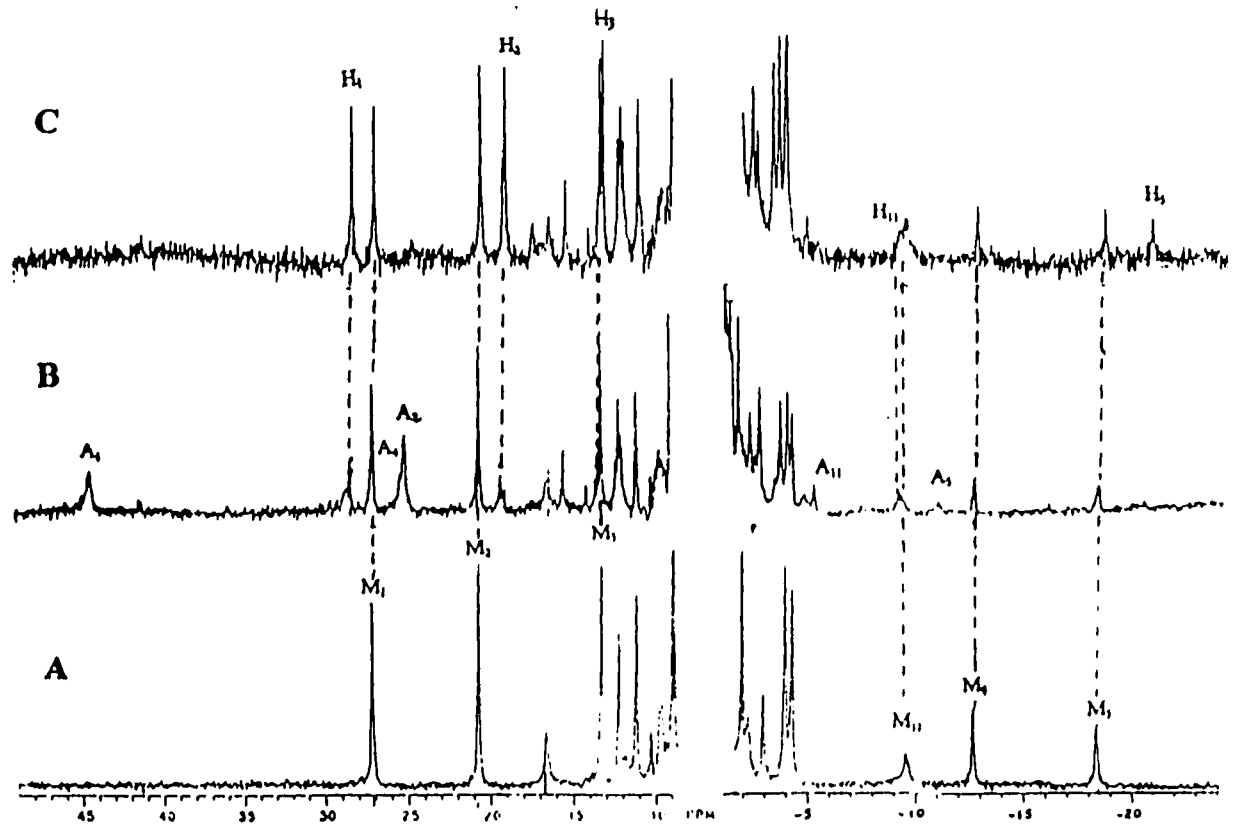


Figure 11: The 300-MHz ^{1}H NMR spectra of metSMb**CN complexes at 4 ^{0}C, pH 7.0. (A) Unreacted metMb**CN. (B) MetS_AMb**CN immediately after preparation; 30% of metS_AMb**CN, 15% of metS_HMb**CN, and 55% of metMb**CN are present in the sample. (C) MetS_HMb**CN four days of equilibration at 4 ^{0}C, pH 7.0 in the presence of excess cyanide; 45% of metS_HMb**CN and 55% of metMb**CN are present. Peaks of metMb**CN, metS_AMb**CN, metS_HMb**CN are labeled M_i, A_i, H_i, respectively.

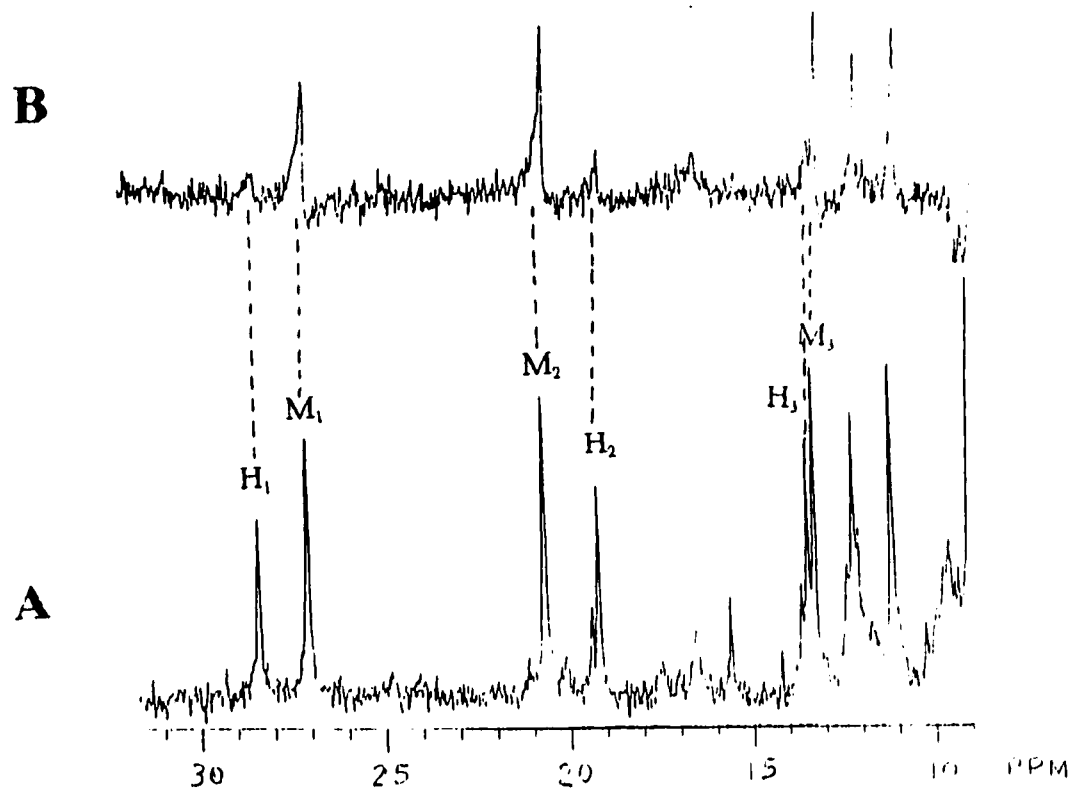


Figure 12: Equilibration of metS_HMb**CN at 20 °C, pH 7.0. (A) The 300-MHz ^{1}H NMR spectrum of metSMb**CN complexes four days of equilibration at 4 °C, pH 7.0 in the presence of excess cyanide. (B) The sample of trace A after two weeks of equilibration at 20 °C, pH 7.0. Peaks of metMb**CN, metS_HMb**CN are labeled M_1, H_1, respectively.

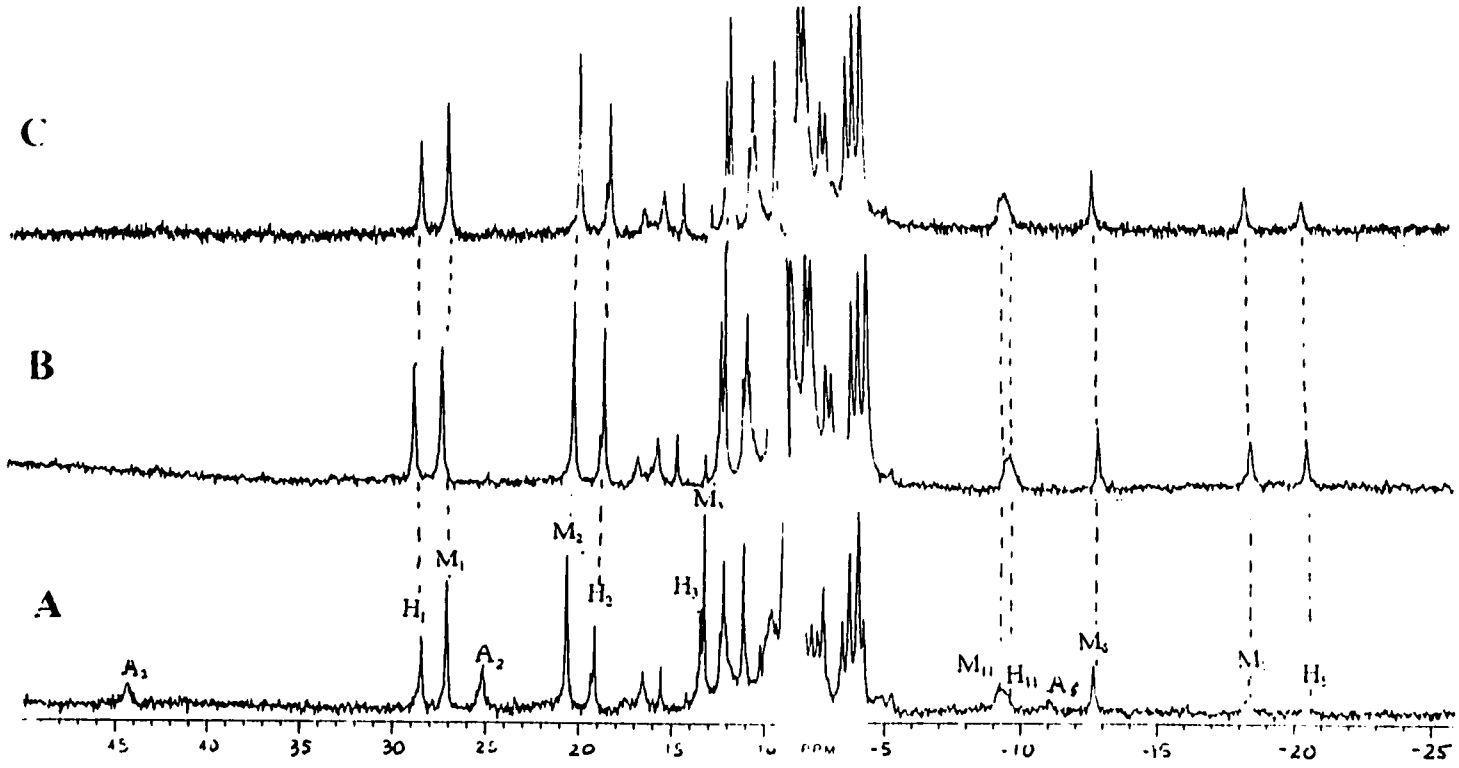


Figure 13 : The 300-MHz ^{1}H NMR spectra of MetSMb**CN in the prosence of excess cyanide at 4 ^{0}C, pH 7.0: (A) 2 days after preparation, (B) 9 days after preparation, (C)16 days after preparation. Peaks of metMb**CN, metS_AMb**CN, metS_HMb**CN are labeled M_i, A_i, H_i, respectively.

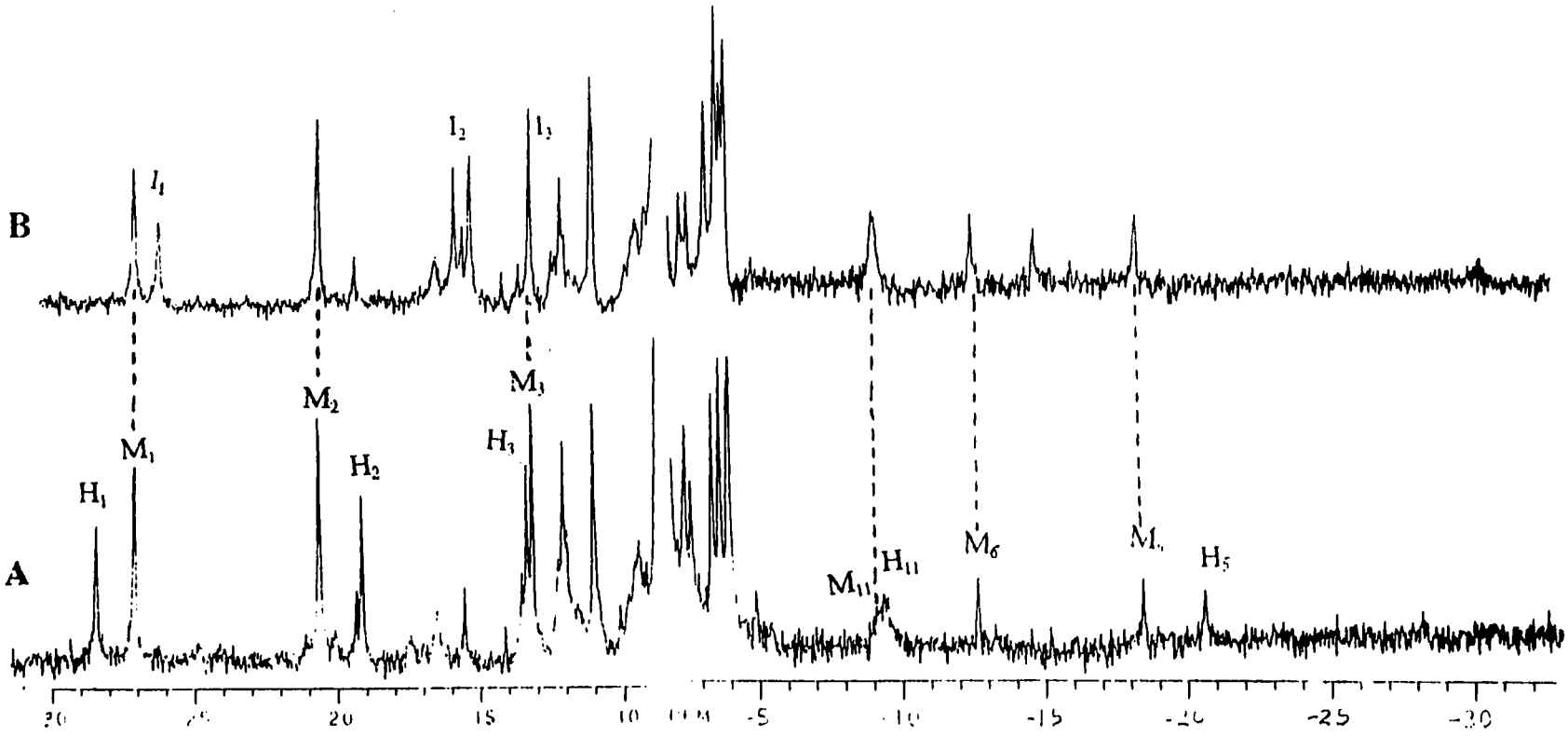


Figure 14: The 300-MHz ^{1}H NMR spectra of the thiol trapped metS_{II}Mb**CN at 4 ^{0}C, pH 7.0. (A) 2 days after iodoacetamide addition. (B) 25 days after iodoacetamide addition. Peaks of metMb**CN, metS_{II}Mb**CN, metS_IMb**CN are labeled M_i, H_i, I_i, respectively.

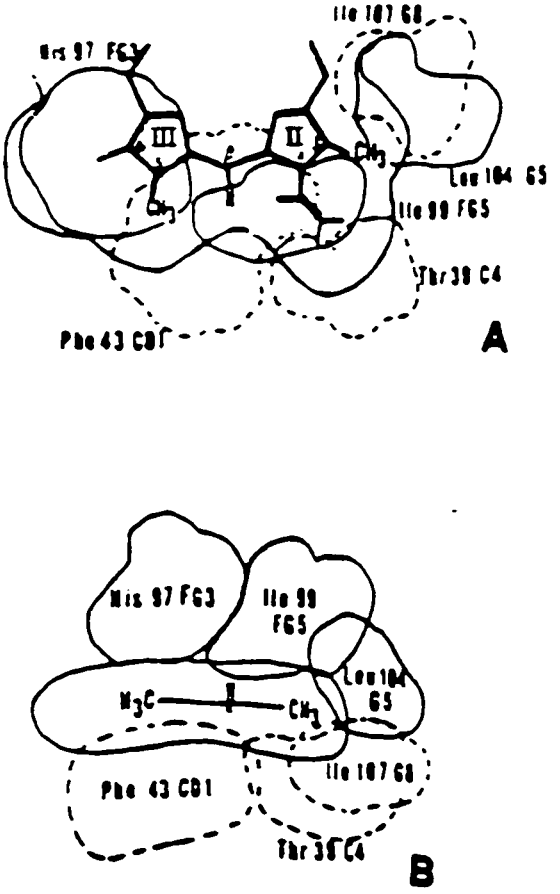


Figure 15: Orientation of residues surrounding pyrrole rings II and III of the native hemin. (A) from the front-view, and (B) from the side-view of (A) looking at the heme along the β-δ vector for the side of the β-meso-H. The proximal side is above the plane of the page (solid lines); the distal side is below (broken lines). (From reference 58, p. 1518.. Reprinted with permission).

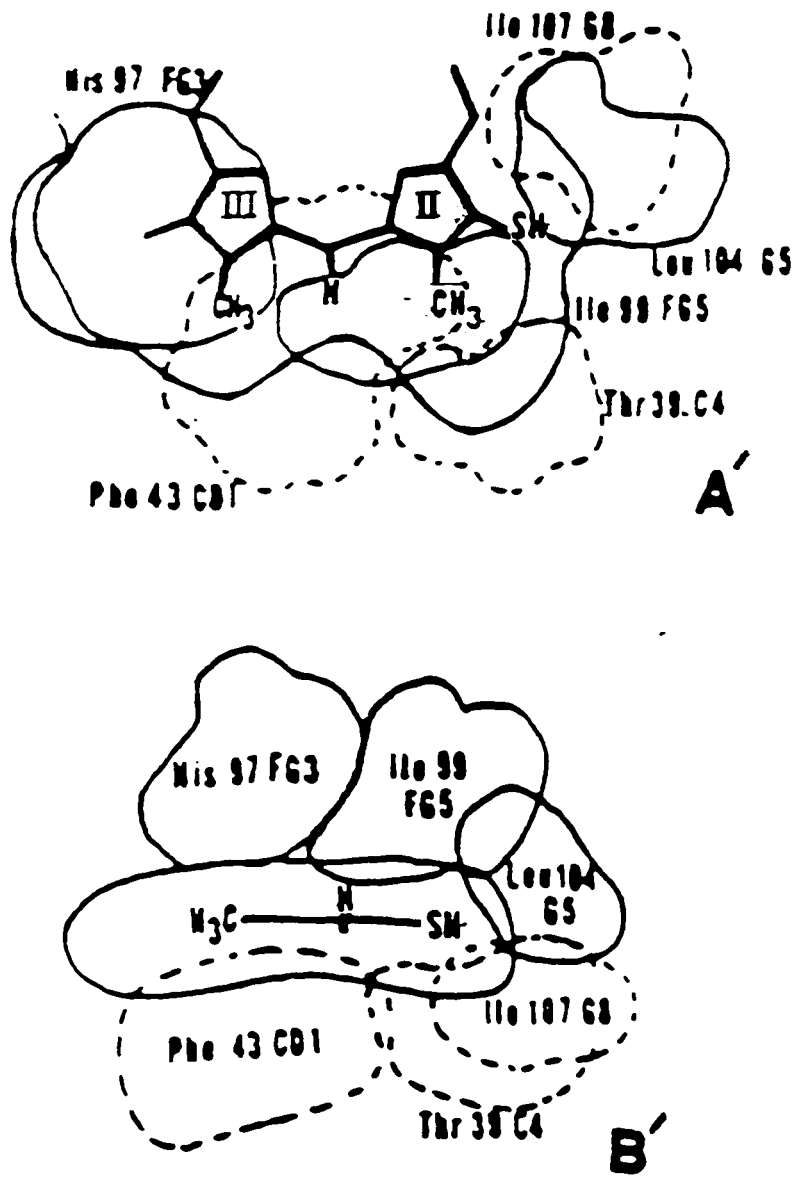


Figure 16 : Proposed orientation of residues surrounding pyrrole rings II and III of the sulfhemin H (A') from the front view, (B') from the side view.

Table 1. ^{1}H NMR Chemical Shift Data, in ppm, for the Metcyano Myoglobin Complexes Derived from Native Hemin (MetMbCN)[2,3], Deuterohemin (MetMb*CN)[4,6], and Deutero-3-hemin (MetMbCN)**

Peak label	MetMbCN	MetMb*CN	MetMb**CN
M_1 (-CH_3)	27.7	24.9	27.2
M_2 (-CH_3)	18.9	18.2	20.7
M_3 (-CH_3)	13.1	13.3	13.2
M_5 (-H)		-213	- 17.4
M_6 (-H)		-155	-12.4
M_{11} (Ile 99 C_γH)		-8.56	-9.37

Table 2 . ^{1}H NMR Chemical Shift Data, in ppm, for the Initial Green Metcyano Sulfmyoglobin Complexes Derived from Native Hemin ($MetS_AMbCN$)[2,3], Deuterohemin ($MetS_AMb^*CN$)[4,6], and Deutero-3-hemin ($MetS_AMb^{**}CN$)

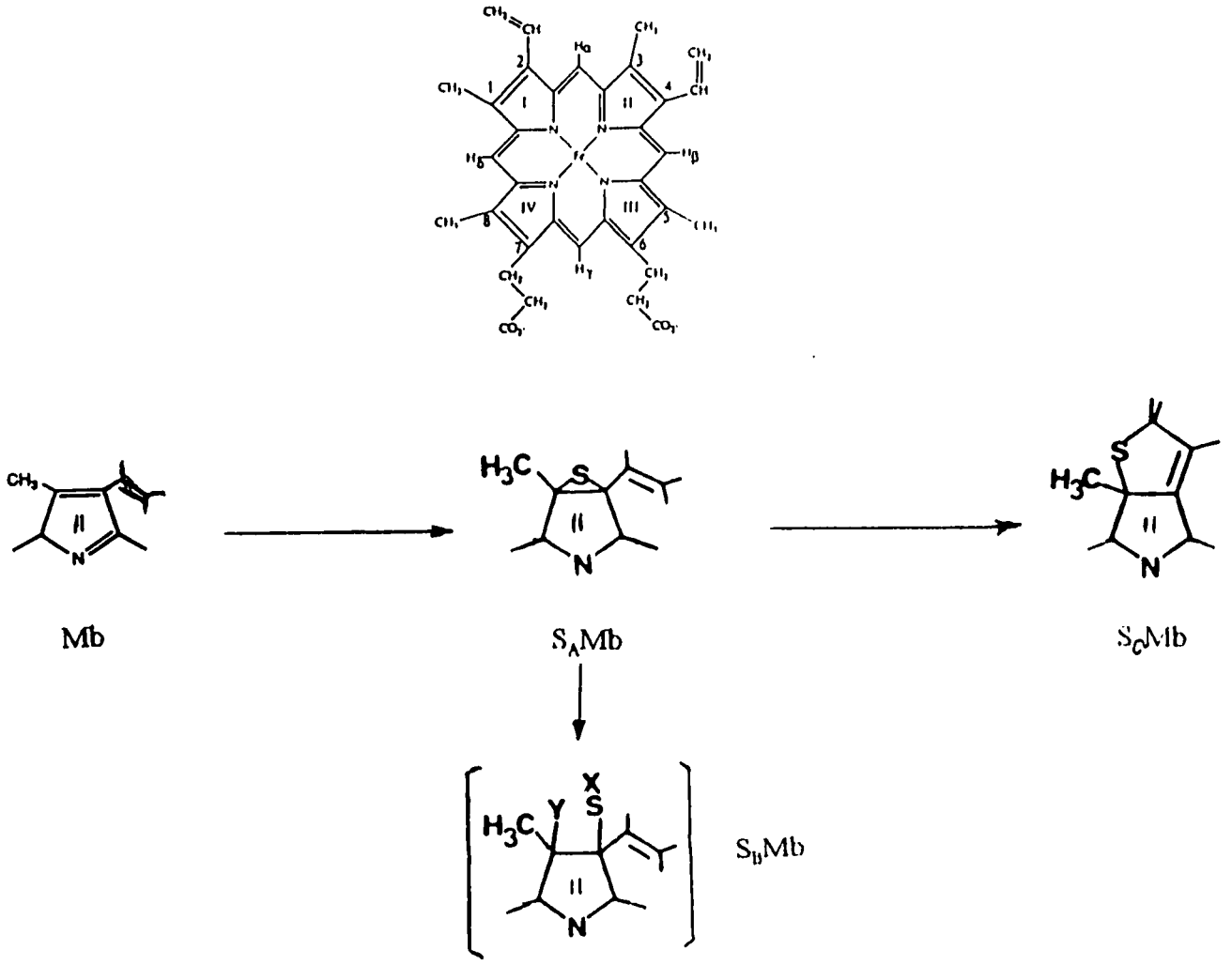
Peak label	$MetS_AMbCN$	$MetS_AMb^*CN$	$MetS_AMb^{**}CN$
A_1 ($-CH_3$)	44.7	45.1	44.3
A_2 ($-CH_3$)	25.9	25.0	25.2
A_5 (-H)		-13.4	11.1
A_{11} (Ile 99 $C_\gamma H$)			-5.33

Table 3 :. ^{1}H NMR Chemical Shift Data, in ppm, for the Red Metcyano Sulfmyoglobin Complexes Derived from Deuterohemin ($MetS_DMb^*CN$)[4,6] and Deutero-3-hemin ($MetS_HMb^{**}CN$)

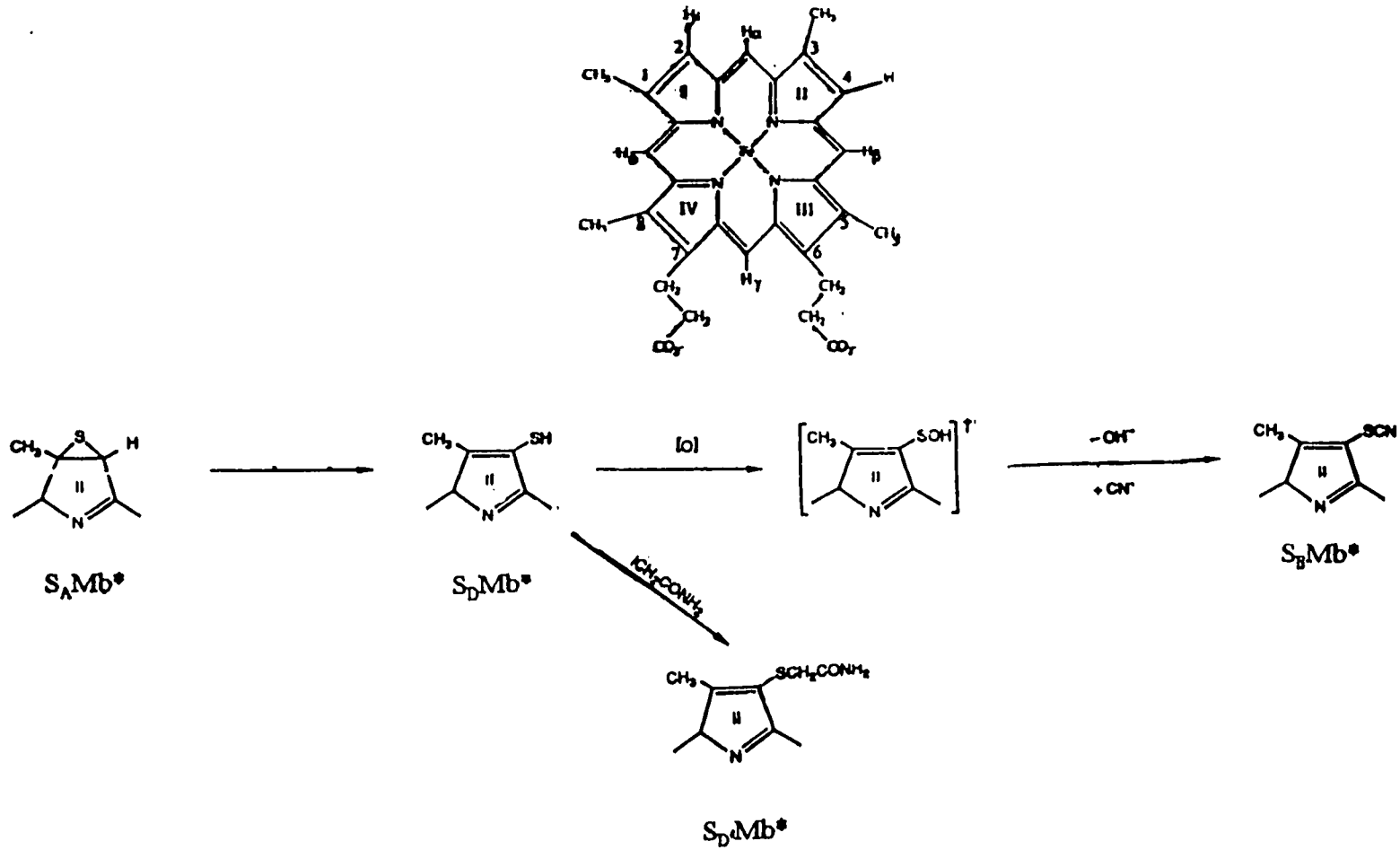
Peak labeled I	$MetS_DMb^*CN$	$MetS_HMb^{**}CN$
1 ($-CH_3$)	28.2	28.5
2 ($-CH_3$)	19.4	19.2
3 ($-CH_3$)	13.1	13.5
5 (-H)	-20.5	-20.7
11 (Ile 99 $C_\gamma H$)	-9.40	-9.44

Table 4 . ^{1}H NMR Chemical Shift Data, in ppm, for the Iodoacetamide Trapped Metcyano Sulfmyoglobin complexes Derived from Deuterohemin ($MetS_D$,Mb*CN)[4,6] and Deutero-3-hemin ($MetS_I$MbCN)**

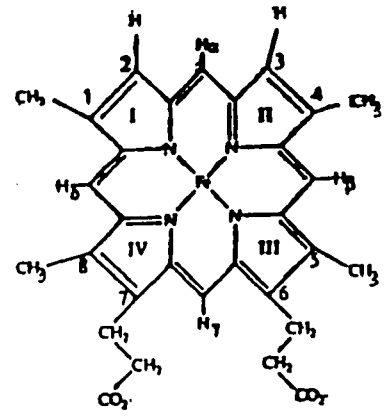
Peak labeled I	$MetS_D$,Mb*CN	$MetS_I$Mb**CN
1 (-CH_3)	32.3	26.3
2 (-CH_3)	19.2	15.8
3 (-CH_3)	14.9	13.2
5 (-H)	-21.2	
11 (Ile 99 C_γH)	-9.77	

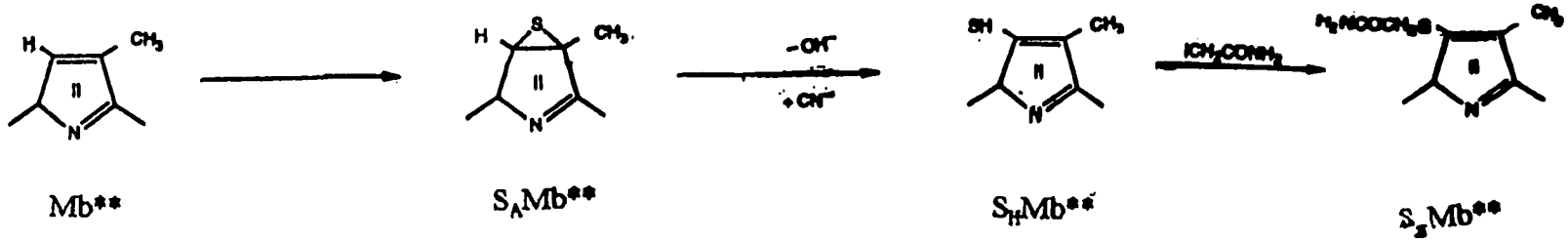


. Scheme 1; Summary of the formation of the SMb complexes



Scheme 2: Summary of the formation of the SMb* complexes.





Scheme 3: Summary of the formation of the SMb** complexes.