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Structure of a Stable Form of Sulfheme†

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Received June 20, 1986; Revised Manuscript Received September 3, 1986

ABSTRACT: A stable green heme was extracted from ferric cyanosulfmyoglobin after it had undergone an internal conversion reaction. After iron removal and conversion to the methyl ester, the resulting green porphyrin was purified by high-pressure liquid chromatography. Visible, ^{1}H NMR, and mass spectrometric studies provided evidence to identify the substituents of the porphyrin. Nuclear Overhauser enhancements enabled an assignment of the single modified pyrrole. Substituent positions 1, 2, 5, 6, 7, and 8 have the original protoporphyrin IX substituents. At ring B, the 4-vinyl group has cyclized with a single sulfur atom to form a fifth ring with a 2,5-dihydrothiophene type of structure.

The sulfglobins are unusual derivatives of hemoglobin and myoglobin with the striking characteristic of possessing a strong green color. They are formed as hemoprotein degradation products in vivo and can also be synthesized in high yield in vitro. They have been the subject of intensive biochemical and physiological scrutiny because of their potential pathological/toxicological importance [see the bibliographic literature citations given in Park and Nagel (1984) and Timkovich and Vavra (1985)]. Sulfhemoglobin may be considered by some as the physiologically more important derivative, but because of size it is easier for NMR experiments to be conducted on sulfmyoglobin. Because of the similarity in crystal structures, there seems to have been an implicit assumption that the sulfheme in each protein is the same. This is a reasonable hypothesis, but it must be admitted that the correspondence in visible spectra and synthesis in vitro are about the only evidence to support this. A critical question has been what is the structure that gives rise to the dramatic change in the visible spectrum. Over time various structures (**1**-**6**) have been proposed. The Fe-S ligand structure **1** was ruled out because in model systems this ligand structure was insufficient to cause the visible spectrum actually seen and because the sulfglobins were capable of binding a range of ex-

† Financial support was provided in part by Grant GM37870 from the National Institutes of Health.

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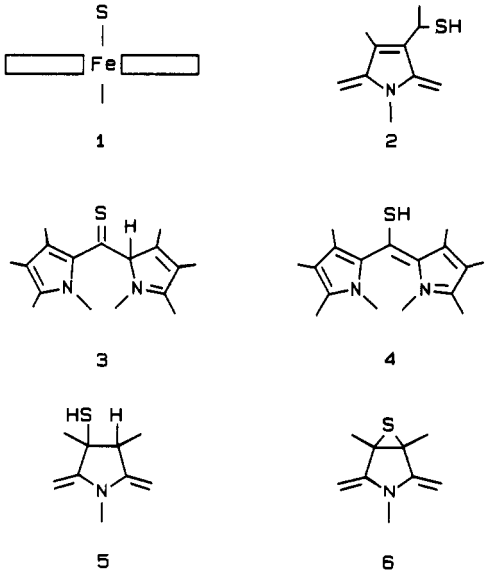


ogenous ligands (cyanide, hydroxide, carbon monoxide) and yet retained the characteristic green color. This type of visible spectrum has been characteristic of disruption of the conjugation system of the inner core π electrons of the porphyrin macrocycle (Smith, 1975). This ruled out modifications of only the substituents, such as **2**, which is a thiol analogue of a hematoporphyrin. Attack at a meso position, giving either **3** or its tautomeric alternative **4**, was also unlikely, because model oxophlorins or thiaphlorins with this core did not have homologous visible spectra to the sulfglobins (Clezy & Smythe, 1968). The visible spectrum of the sulfglobin is most similar to that of an iron chlorin, an iron porphyrin in which one of the pyrroles has been partially saturated at a β-pyrrolic double bond. Resonance Raman studies substantiate this (Andersson et al., 1984). Critical isotope labeling experiments indicated that a single sulfur atom was covalently added to the heme of the sulfoglobins, producing a sulfheme derivative (Berzofsky et al., 1972a). The lack of tritium incorporation seemed to rule out **5**. The major stumbling block to characterization of the sulfheme has been its extreme lability when separated from the protein. Although the sulfglobins are reasonably stable proteins, heme extracts rapidly decompose, in the process regenerating mainly authentic iron protoporphyrin IX (Morell et al., 1967; Berzofsky et al., 1972a). The episulfide structure **6** has been the best available hypothesis to account for the sulfheme, since it is a chlorin, contains a single sulfur, and is presumably a sufficiently strained moiety that external to the protein may revert back to the original porphyrin. A mechanism for its formation has been proposed (Berzofsky et al., 1972a) that involves radical intermediates, which were very reasonable in light of the in vitro reaction sequence for sulfglobin synthesis.

A key breakthrough has been the discovery that sulfmyoglobin exists in at least two distinct chemical forms. This was first observed in the initial NMR study on sulfmyoglobin (Timkovich & Vavra, 1985) and has now been explored in more detail [this paper and Chatfield et al. (1986)]. It appears that the first heme product of sulfmyoglobin formation is the kinetically preferred form, which gradually converts to a more thermodynamically stable form with time. From this form it is possible to extract a stable, covalently modified heme. The purpose of this paper is to describe additional experiments on sulfmyoglobin and the internal conversion reaction and then the extraction, purification, and determination of the structure of the stable form of the sulfheme. From the final deduced structure, it may be possible to realistically hypothesize as to the structure first formed within the protein complex.

Materials and Methods

Equine heart muscle myoglobin was obtained from Sigma Chemical Co. and used as received. Sulfmyoglobin was prepared by the Berzofsky procedure (Berzofsky et al., 1971). NMR studies were done as described previously (Timkovich & Vavra, 1985) except that the current spectra were obtained at 200 MHz. It has been shown that the peroxide treatment in the procedure for sulfmyoglobin synthesis led to a fraction of high-spin protein that presumably was denatured material (Andersson et al., 1984). It can be removed by ion-exchange chromatography (details provided by a personal communication from Prof. A. G. Mauk). This was generally omitted in this study for practical reasons. It was difficult to prepare samples for NMR this way. The cost of doing the chromatography in deuterium buffers appeared prohibitively expensive. Otherwise, the protein had to be concentrated and protic water exchanged for deuterium oxide. During these manipulations, the sulfmyoglobin reverted in part to myoglobin plus some denatured material, and hence, the benefits of the chromatography step were soon lost. The original denatured material contributed only to the general background level of our optical and NMR spectra and was effectively invisible. The trace of normal myoglobin that was a contaminant in our sulfmyoglobin preparation served well as an NMR internal standard for pH, temperature, and other studies since its resonances did not overlap with unique sulfmyoglobin resonances. For carbon monoxide and cyclopropane binding studies to be discussed, it was possible to ensure that ligand concentrations were appropriate by directly observing the effects on the normal myoglobin resonances.

Attempts were made to prepare sulfmyoglobin-like protein, in any yield, with a reagent other than HS^-. In the standard procedure, CH_3SH, CH_3CH_2SH, C_6H_5SH, and Na_2Se failed to produce optical bands characteristic of a sulfur or selenium adduct, although to variable degrees all acted as a reducing reagent.

Protein NMR samples were typically 3.5 mM in total protein, while optical samples were in the range of 10–100 μM. Prior to the introduction of carbon monoxide or cyclopropane, samples were degassed by alternate cycles of vacuum and argon flushing. The gases were introduced at approximately 1 atm directly into the 5-mm NMR tube or into Thunberg-type curvettes for optical studies, and the sample was gently shaken to promote gas/solution equilibrium. The cells were sealed with ground-glass joints or rubber septa, while the NMR tubes were sealed by glass blowing techniques. The CO complex was prepared by CO addition to reduced sulfmyoglobin which was the redox state at the end of the Berzofsky procedure. For cyclopropane binding, sulfmyoglobin was first converted to the ferric cyano complex with potassium ferricyanide and potassium cyanide as described previously (Timkovich & Vavra, 1985).

Extraction of the heme from freshly prepared $SMbFe^{III}CN$ yielded a green heme with wavelength maxima at 394, 490, 601, and 736 nm in the visible region. This heme rapidly reverted to protoheme and could not be further derivatized or purified. The converted form of sulfmyoglobin that has been designated SMb* was prepared by allowing $SMbFe^{III}CN$ to stand at room temperature for 6 days. This was usually done with no precautions to exclude air, although some experiments to be described were done with storage under an argon atmosphere after vacuum deoxygenation. The resultant heme extracts will be described under Results.

Heme extractions were performed as follows. To 1 volume of SMb*Fe^{III}CN in 0.1 M potassium phosphate buffer with 25 mM potassium cyanide, pH 9.0, was added 5 volumes of 1.0 M glycine hydrochloride (pH 2.8) and then 9 volumes of ethyl acetate. The resulting mixture was centrifuged for 1 min and the upper green organic layer separated. This was examined by visible spectroscopy or further derivatized as described below.

Preparation of the free base, methyl ester form of the green porphyrin typically began with 4.8 mL of 3.5 mM SMb*Fe^{III}CN. The green ethyl acetate heme extract was dried and redissolved in 1.5 mL of glacial acetic acid to which 0.8 mL of ferrous sulfate (10% w/v in concentrated HCl) was added. Within 30 s, the solution was poured into 60 mL of peroxide-free ether, washed with 10 mL of aqueous sodium acetate (20% w/v), and then washed with multiple portions of water. The ether layer was dried with anhydrous magnesium sulfate, filtered, and dried under vacuum. To the dried extract was added slowly a solution of diazomethane in chloroform, prepared from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. This was obtained from Aldrich Chemical Co. and treated to produce the diazomethane according to their standard instructions. Excess diazomethane was eliminated with acetic acid. Some esterifications were performed by room temperature incubation for 12 h in anhydrous methanol with 5% sulfuric acid. The ester was recovered by extraction into benzene that was then washed with aqueous sodium bicarbonate. This solution or the dichloromethane solution in the case of diazomethane esterification was concentrated under vacuum and applied to a short silica gel column (0.5 × 6 cm) and eluted with chloroform. The green fraction was dried under vacuum, redissolved in a minimal volume of high-pressure liquid chromatography (HPLC) buffer, and chromatographed by HPLC (conditions: 10-μm silica gel column, 4.6 × 250 mm, 1 mL/min, 1:1 chloroform–hexane containing 0.025% pyridine, detected optically at 400 or 662 nm). The green porphyrin eluted with a capacity factor of 2.12 but was still contaminated with some protoporphyrin IX dimethyl ester. It was rechromatographed in 2:3 chloroform–hexane containing 0.025% pyridine where it eluted with a capacity factor of 4.87 and was free of protoporphyin IX. In these chromatography steps, the diazomethane-esterified material gave only one green porphyrin and traces of protoporphyrin IX dimethyl ester. The material esterified by methanol/mineral acid also gave a second minor green porphyrin that will be discussed. The main green porphyrin was dried under vacuum and subsequently dissolved in the appropriate solvent for NMR, visible, or mass spectrometry. The sample was relatively stable when stored dry, although not as stable as protoporphyrin IX or other fully aromatic porphyrins. It was susceptible to decomposition when kept in solution. Methyl, vinyl containing porphyrins are well-known to be sensitive to photochemical oxidation (DiNello & Chang, 1979). During the decomposition it was possible to detect the appearance of new optical bands at 654, 646, and 630 nm, before the visible spectrum gradually faded to a featureless sloping base line. The lifetimes and the amounts of these decomposition products were not sufficient to allow further characterization.

NMR spectra of porphyrins were obtained in deuteriated dichloromethane or benzene (Gold Label grade, from Aldrich, nominal 100 atom % deuterium) at 200 MHz on a Nicolet spectrometer. Nuclear Overhauser enhancements (NOE's) to determine nearest-neighbor substituents were measured as described previously (Timkovich et al., 1984). Fast atom bombardment (FAB) mass spectra were obtained in a dithioerythritol matrix at the Midwest Center for Mass Spectrometry, a National Science Foundation Regional Facility (Grant CHE 8211164). Low-resolution mass spectra were obtained on a Hewlett-Packard 5985A mass spectrometer.

RESULTS

An ^{1}H NMR study of metsulfmyoglobin and the ferric cyano complex had provided evidence that either pyrrole ring A or B was the site of sulfur addition (Timkovich & Vavra, 1985). Additional NMR experiments on sulfmyoglobin support the hypothesis. A resolved three-proton resonance at −1.99 ppm was observed in the NMR spectrum of SMbFe^{II}CO. In normal MbFe^{II}CO, a resonance has been observed at −2.32 ppm and has been assigned to a methyl of Val-68 (Bradbury & Carver, 1984). Its significant upfield chemical shift may be ascribed to its position with respect to the heme plane and the attendant ring current. The residue is located within 4 Å of the heme, over the macrocycle plane, near pyrrole rings A and B [Takano (1977), especially Figure 7]. In a chlorin, the ring current is diminished (Scheer & Katz, 1975). The fact that this resonance moved back toward a more usual methyl chemical shift may be interpreted as due to a decrease in the ring current above rings A and B and so was supportive of the assignment of one as the site of sulfur addition.

It has been suggested that an unusual cavity, or hydrophobic hole, in the interior of myoglobin (Mb) near pyrrole ring A was responsible for creating a special microenvironment that promoted the sulfur addition (Timkovich & Vavra, 1985). This hole is a binding site for xenon (Schoenborn et al., 1965; Triton & Kuntz, 1982) and cyclopropane (Shulman et al., 1969). It has been shown that binding perturbed the hyperfine-shifted resonances of MbFe^{III}CN. The effects of cyclopropane on the hyperfine-shifted resonances of SMbFe^{III}CN were investigated. In control experiments on MbFe^{III}CN at 20 °C and pH 9.1, 5-methyl shifted −0.05 ppm, 1-methyl +0.07 ppm, and 8-methyl −0.94 ppm, where a negative value means the resonance shifted upfield in the presence of cyclopropanes. Mayer et al. (1974) reported respective shifts of 0.0, +0.19, and −1.15 ppm at pH 9 and 31 °C. For SMbFe^{III}CN at pH 9.1, 20 °C, 8-methyl shifted +0.13 ppm and 5-methyl −0.29 ppm. Although the magnitudes have not been interpreted quantitatively, the observed perturbations on the heme methyl resonances of SMbFe^{III}CN were good evidence that cyclopropane bound. Therefore, the sulfheme modification must leave the hydrophobic hole largely intact. Of the two observable, resolved ring methyls, cyclopropane binding most strongly affected 8-methyl. The mechanism of the transmission through the electron density of the heme to affect the 8-substituent chemical shift is not known. Mayer et al. (1974) originally addressed this point. It was also 8-methyl in SMbFe^{III}CN that had the greatest shift with respect to its position in MbFe^{III}CN. 5-Methyl shifted ca. 2 ppm. 1-Methyl was not observed in SMbFe^{III}CN presumably because it had been shifted into the main protein envelope between 0 and 10 ppm. This sets an upper limit of 18 ppm for its shift difference between SMbFe^{III}CN and MbFe^{III}CN. 3-Methyl was not resolved from the main envelope in either case, thus setting a limit of about 10 ppm for any possible shift difference. The shift difference for 8-methyl was ca. 32 ppm.

It was possible to synthesize sulfmyoglobin under a strict atmosphere of cyclopropane with no observable decrease in its rate of formation or yield. We have interpreted this plus the NMR-detectable binding to preformed SMbFe^{III}CN as weak but negative evidence against ring A as the site of addition.

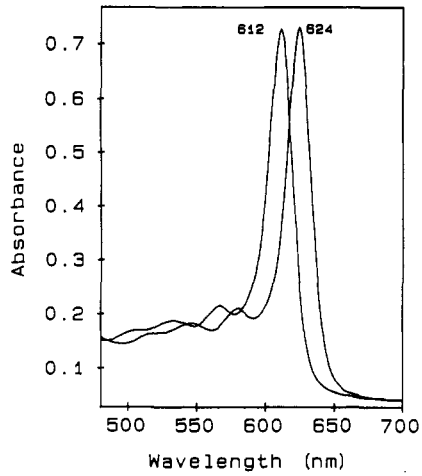


FIGURE 1: Optical absorption spectrum in the visible region at pH 8 of the ferrous carbonyl complex of freshly prepared equine sulfmyoglobin (peak maximum at 612 nm) and after 18 h at room temperature (peak maximum at 624 nm). The vertical scales of the spectra are not the same. The intensity of the 624-nm peak is actually 74% of the starting 612-nm peak.

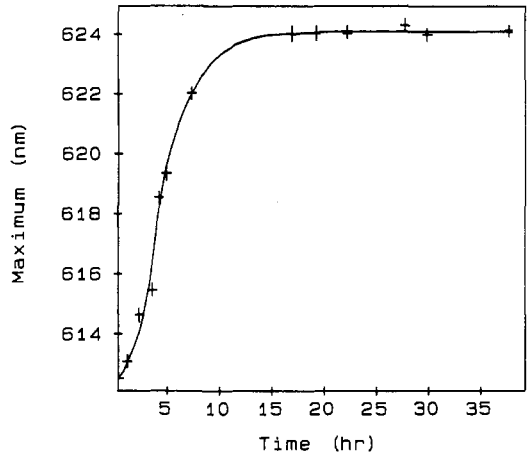


FIGURE 2: Time course of transformation of the ferrous carbonyl complex of equine sulfmyoglobin. The observed apparent wavelength maximum of the major visible band is plotted as a function of time. This band is sufficiently wide in both the 612-nm form and the 624-nm form compared to the separation that a mixture of the two species does not show resolved maxima but an apparent maximum due to the superposition of the two components that shifts from one extreme to the other as the relative populations change.

Both SMbFe^{II}CO and SMbFe^{III}CN underwent a slow internal conversion reaction that was first noted for the ferric cyano case (Timkovich & Vavra, 1985; Chatfield et al., 1986). Freshly prepared SMbFe^{II}CO had a visible band maximum at 612 nm (Figure 1), as described previously (Berzofsky et al., 1972a). As a function of time, this apparent maximum shifted and decreased in intensity to produce a species with an absorbance band at 624 nm (Figure 1). If the peak separation of homologous absorbance bands between two distinct species is small compared to their band widths, then a transformation from one to the other involving increasing fractions of the new form can appear as a gradual change in the observed maximum. The form with the 624-nm band has been designated SMb*Fe^{II}CO. The half-time for the conversion is on the order of days at cold temperatures but decreases to about 5 h at room temperature and pH 8 as shown in Figure 2.

In the case of SMbFe^{III}CN, a conversion to a species that has been named SMb*Fe^{III}CN could be followed in visible spectra by a similar shift of the absorbance maximum at 591 nm in freshly prepared protein to about 598 nm. This was difficult to follow because the absorbance band was broad. However, the change was easily observed by the appearance of resolved, distinct NMR resonances at 47.5, 29, 21.5, 18.2, and 14 ppm (Figure 3) as previously reported (Timkovich & Vavra, 1985).

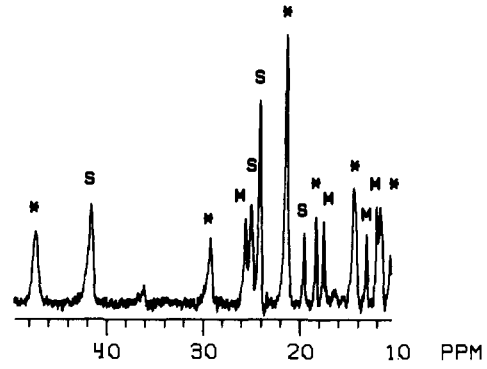
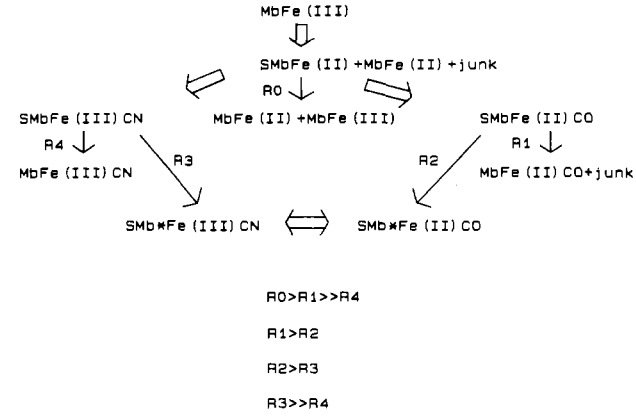


FIGURE 3: Downfield region of hyperfine-shifted ^{1}H NMR spectrum of SMb*Fe^{II}CO *after* it had been converted to SMb*Fe^{III}CN by oxidation by ferricyanide and addition of potassium cyanide. Peaks marked with (*) had been previously identified during the slow transformation of SMbFe^{III}CN to SMb*Fe^{III}CN. Peaks marked with S correspond to resonances observed in freshly prepared SMbFe^{III}CN. Peaks marked with M are resonances of normal myoglobin. The sample was at 20 °C and pH* 9.1.

Scheme I



Both internal conversions represented the same chemical transformation on the porphyrin skeleton itself. It was possible to interconvert SMb*Fe^{III}CN and SMb*Fe^{II}CO, rapidly, by changing the oxidation state and exogenous ligand. SMb*Fe^{II}CO was prepared by allowing the slow, natural transformation. CO was removed by vacuum degassing, the sample was oxidized with ferricyanide, and potassium cyanide was added to produce the ferric cyano complex. Within 15 min, ^{1}H NMR spectra such as shown in Figure 3 were obtained. The spectrum is a mixture of three species, MbFe^{III}CN (due to incomplete original reaction and reversion), SMbFe^{III}CN, and SMb*Fe^{III}CN, as would be produced from the slow conversion of SMbFe^{III}CN. In the converse experiment, SMb*Fe^{III}CN was deoxygenated, equilibrated with 1 atm of CO, and reduced by the addition of a small excess of solid sodium dithionite. The visible absorption maximum was at 623 nm, matching the spectrum of SMb*Fe^{II}CO. The experiments strongly suggested that neither cyanide nor carbon monoxide was directly involved in the conversion, although the presence of a strong field ligand may somehow have facilitated it.

Scheme I clarifies our present interpretation and summarizes in a qualitative sense the relative rates of the reactions that

Table I: ^{1}H NMR Assignments for S662[a]

assignment	chemical shift[b]	protons[c]	coupling[d]
meso	9.93 (9.86)	1	s
	9.90 (9.80)	1	s
	9.37 (9.34)	1	s
	9.08 (9.03)	1	s
2-vinyl			
H_X	8.14 (8.16)	1	J (A, X) = 17.7
H_A	6.33 (6.34)	1	J (B, X) = 11.8
H_B	6.15 (6.13)	1	J (A, B) = 1.3
6,7-propionates			
$-CH_2CH_2COOCH_3$	4.35, 4.20 (4.37, 4.18)	2, 2	t, J = 7.5
$CH_2CH_2COOCH_3$	3.23, 3.15 (3.23, 3.18)	2, 2	t, J = 7.5
ring, ester $-CH_3$			
3-CH_3	2.12 (2.12)	3	s
	3.65, 3.64, 3.63 (3.68)	9	s
	3.50 (3.51)	3	s
	3.44 (3.44)	3	s
s ring			
H_X	6.96 (6.92)	1	dd, J (A, X) = 4.3
H_M	4.91 (4.89)	1	dd, J(M, X) = 1.9
H_A	4.18 (4.15)	1	dd, J (A, M) = 15.8
NH	−2.6 (−2.6)	2	s, br

[a] At 20 °C in dichloromethane. [b] In parts per million, nominally with respect to tetramethylsilane. The residual proton signal from dichloromethane was used as an internal standard and assigned a chemical shift of 5.32 ppm. The numbers without parentheses are for HPLC-pure S662; those with are for a mixture of S662 and approximately 30% protoporphyrin IX dimethyl ester. [c] The average integrated intensity rounded to the nearest whole integer, assuming that any meso proton area is one proton. [d] Coupling constants are reported in Hz. The single vinyl group appears as an ABX second-order subspectrum that was simulated to give the reported shifts and coupling. Other coupling constants are directly taken from the spectrum. Abbreviations: s, singlet; t, triplet; dd, doublet of doublets; br, broad.

can occur. Synthetic transformations are represented by the double arrows, while conversions that occur naturally with time are indicated with single arrows, and R_0 to R_4 will be used to qualitatively compare their rates. The initial reaction of Mb with hydrogen peroxide/sulfide produced mostly SMbFe^{II} (>80%), some unreacted Mb, and some denatured protein that remained invisible in our spectra. The reversion of SMb back to Mb occurred with a rate (R_0) that, depending upon conditions, took hours or a few days. The addition of a strong field ligand to either the ferrous state or the ferric state (produced by ferricyanide oxidation) conferred a certain stability to the sulfheme so that the rate of reversion (R_1) was less in the ferrous CO complex and was still less, minimal, in the case (R_4) of the ferric cyano complex. Perhaps because of the added stability toward reversion, the strong field ligand complexes had sufficient lifetimes so that the slow internal conversion (R_2 and R_3) to the SMb* species could take place. The heme crevice geometry may also have facilitated the transformation. For the CO complex, the rate of reversion (R_1) was still significant compared to the rate of conversion (R_2), while for the ferric cyano complex the conversion (R_3) was dominant. All the rates were strongly temperature dependent, increasing at higher temperatures, but the rate increases qualitatively did not seem uniform.

Sulfhemoglobin is similar to SMb in many of its spectroscopic parameters. However, it did not appear to undergo the internal conversion reaction. The ferrous carbonyl complex of sulfhemoglobin was monitored for 4 weeks, and there was no detectable shift of the 612-nm visible maximum.

Further evidence that the conversion reaction of sulfmyoglobin involved a covalent rearrangement of the heme skeleton came from heme extraction studies of the ferric cyano complex. Heme extracted from freshly prepared SMb had a visible band maximum in the range of 601–603 nm. After storage of the protein as the ferric cyano complex at pH 9, this band maximum for the extract shifted gradually to 612–613 nm, either in the presence or absence of oxygen. In both cases the half-time was 3.5 days at 20 °C and pH 9. As in the CO case, the apparent shifting of the maximum is presumably due to an increasing proportion of the final 612-nm product over the initial 601-nm form where the width of the bands precludes resolution of the individual peaks.

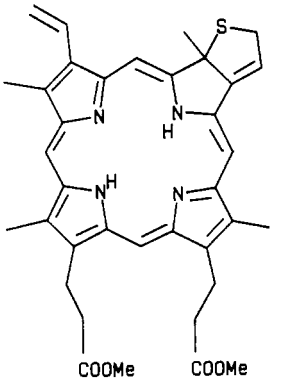


FIGURE 4: Proposed structure for stable green porphyrin isolated from aged ferric cyanosulfmyoglobin.

The sulfheme with the wavelength maximum at 612 nm was appreciably more stable than the initial sulfheme extract, although not as stable as protoheme, and it proved possible to convert it into a metal-free sulfporphyrin as the dimethyl ester. After HPLC purification, this form, which will be abbreviated S662, was suitable for mass spectrometric and NMR structural analyses. The structure proposed is presented in Figure 4 to aid in the following discussion.

The visible absorption spectrum of the S-porphyrin S662 is shown in Figure 5. The pronounced red shift of the highest visible band is characteristic of a porphyrin partially saturated to the level of a chlorin. Typical chlorins have an absorption maximum at 650–654 nm (Chang, 1985; Timkovich et al., 1985), while the S-chlorin here has a maximum at 662 nm. This shift is comparable to that in the photoporphyrins at 668 and 671 nm (DiNello & Chang, 1979). In both cases, the extra shift may be due in part to the presence of a double bond at a β-pyrrolic carbon of the partially saturated ring.

The ^{1}H NMR spectrum of S662 provided strong evidence for the assigned structure. The spectrum is shown in Figure 6 and shift and coupling data are provided in Table I. The spectra of S662 showed some unusual behavior that may be

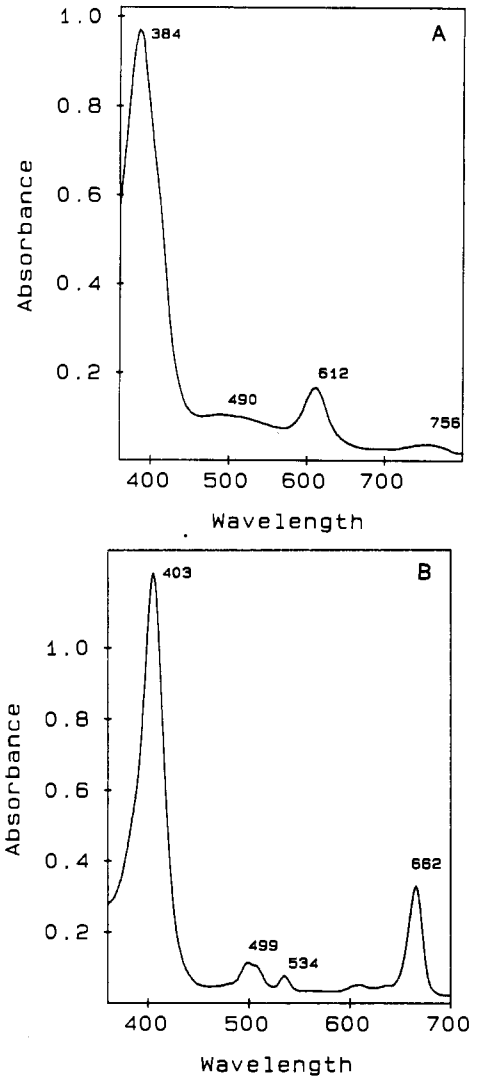


FIGURE 5: (A) Visible spectrum of the green heme in ethyl acetate isolated from aged ferric cyanosulfmyoglobin. (B) Visible spectrum of the green porphyrin in chloroform prepared from the green heme by iron removal and esterification.

qualitatively described. The chemical shifts and line widths of some of the resonances were concentration-dependent. The two furthest downfield meso protons were most seriously affected. The resonances of the assigned S ring were less sensitive. Interestingly, the sharpest lines were obtained when the S662 sample was contaminated with some protoporphyrin IX dimethyl ester, such as, the fraction after the first but prior to the second HPLC chromatographic step. Table I contains shifts for such a mixture to establish the limits of variability observed. A spectrum of such a mixture has been provided as supplementary material (see paragraph at end of paper regarding supplementary material). It may be that S662 aggregates strongly in dichloromethane, and perhaps the aggregates can be disrupted by protoporphyrin IX. Similar aggregation behavior has been observed for the relatively saturated photoporphyrins (Padavanija & Timkovich, 1986).

Most of the resonances of S662 may be readily assigned to the usual substituents of protoporphyrin IX. The upfield shifts of these resonances are similar to other partially saturated porphyrins such as chlorin *d* (Timkovich et al., 1985) and the photoporphyrins (Padavanija & Timkovich, 1986) that contain many of the protoporphyrin IX substituents. The singlet at 2 ppm is characteristic of a methyl substituent on a partially saturated pyrrole. The NH resonance at −2.6 ppm is also highly diagnostic of a porphyrin skeleton saturated to the level

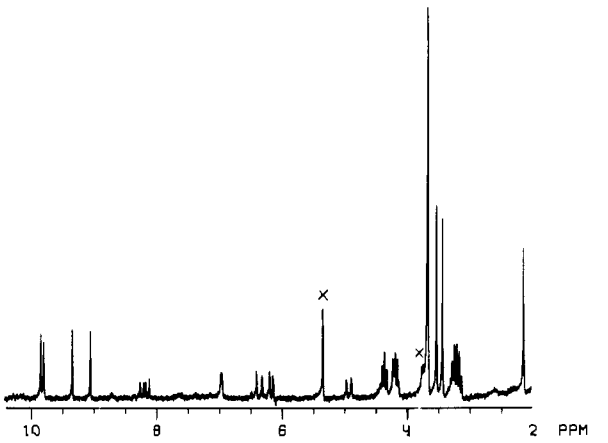


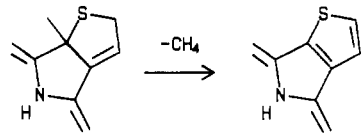
FIGURE 6: The ^1H NMR spectrum of the green porphyrin dimethyl ester at 20 °C in dichloromethane. Residual impurities are marked with X.

of a chlorin. In further analogy to these systems, the partial saturation of a single pyrrole breaks the pseudo 2-fold symmetry of the macrocycle. In S662, the propionate triplets on adjacent rings are resolvable, even at 200 MHz. The presence of two propionates and one vinyl substituent on unsaturated pyrroles makes it clear that the site of partial saturation must be the other methyl, vinyl bearing pyrrole.

Novel resonances are the three single proton doublet-of-doublets that are mutually spin coupled at 6.9, 4.9, and 4.2 ppm. These were assigned on the basis of the following considerations. The proton at 6.9 ppm is at a shift expected for a vinylic proton attached to a carbon one bond away from a partially saturated pyrrole. In normal protoporphyrin IX this type of proton appears at approximately 8.1 ppm. If the pyrrole ring were to be partially saturated, an upfield shift would occur because of a decrease in the macrocyclic ring current. In the photoporphyrins, the analogous ethylidene proton is at 7.8 ppm (Padavanija & Timkovich, 1986), but this final shift contains a new downfield contribution from the adjacent electronegative aldehyde group. Therefore, 6.9 is reasonable for an ethylidine proton adjacent to a less electronegative group. Bacteriochlorophyll *b* (Scheer & Katz, 1975) contains at the 4-position a substituent $=CHCH_3$ where the proton is found at 6.84 ppm. The methyl resonance of this substituent is found at 2.01 ppm. If this is taken as a base shift for a methyl proton in such a position, one could add +0.4 ppm as an increment for going from $-CH_3$ to $-CH_2R$ and add +1.4 to +2.2 ppm as an increment for going from $-CH_2R$ to $-CH_2OR$ or $-CH_2SR$ (Silverstein et al., 1981). This would predict that protons of the proposed structure of S662 would fall in the range of 3.8 to 4.6 ppm. The methylene protons are diastereotopic and are not expected to have identical chemical shifts. The presence of the sulfur and possible ring puckering could separate the methylene protons considerably [for example, see Timkovich et al. (1985)]. The large coupling constant between these protons is consistent with geminal coupling. Models indicate that the sulfur-containing ring is not likely to be planar. A modest twist along the $=CH-CH_2-$ single bond could lead to dihedral angles of approximately 40° and 60° between the $=CH-$ and the methylene protons, respectively. Standard Karplus vicinal coupling predicts constants of 2.0 and 4.8 Hz for this geometry, in good accord with observations.

The assignment of ring B as the site of partial saturation was accomplished by NOE measurements. Irradiation of the methyl resonance at 2 ppm gave a positive 8% NOE to the 9.08 ppm meso proton as the only meso proton to show an

Scheme II



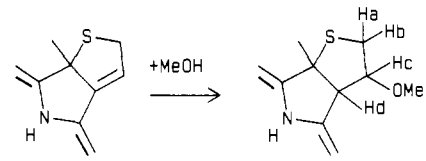
enhancement. This established these as adjacent substituents. Irradiation of the 9.08 ppm meso gave a weak but detectable 2% NOE back to the 2 ppm methyl and an additional 6% NOE to the vinyl proton at 8.14 ppm. This established a pattern of adjacent neighbors as methyl, meso, and then vinyl. This is only consistent with ring B. If ring A had been the saturation site, the pattern would have been upfield methyl, then meso, and then downfield methyl.

Mass spectrometry provided evidence that sulfur was covalently bonded to S662. Low-resolution spectra with electron-impact ionization gave the highest mass peak at 622, a weak peak at 606, and extensive fragment ions at lower masses. High-resolution FAB mass spectrometry was done in a matrix that consistently gives the $(M + H)^+$ ion for porphyrins. The observed peak for S662 had a mass of 623.2679 corresponding to $C_{36}H_{39}N_4O_4S$ (calcd 623.2683). Protoporphyrin IX dimethyl ester is susceptible toward photochemical oxidation to produce the chlorins known as photoporphyrins (DiNello & Chang, 1979), which have an integral mass of 622. However, the high-resolution mass for the $(M + H)^+$ ion (calcd 623.2860) is 29 ppm different from the observed mass and is readily ruled out. Furthermore, although S662 has analogous chemical shifts for some resonances, the precise NMR spectra are quite distinctive (Padavanija & Timkovich, 1986). The high-resolution FAB mass spectra showed a weak feature at 607.2386 corresponding to $C_{35}H_{35}N_4O_4S$ (calcd 607.2371) or the loss of a CH_4 fragment. This could correspond to a fragmentation such as shown in Scheme II. The loss of a formal methane unit could be driven by the formation of the aromatic thiophene ring and the full aromatic stabilization of the porphyrin.

When the demetallized S-porphyrin was esterified in methanol/sulfuric acid, a minor green porphyrin was obtained that has been designated S651, in addition to the main S662 porphyrin. In the 2:3 chloroform–hexane HPLC solvent system, it eluted with a capacity factor of 24.0. Its visible spectrum in chloroform showed band maxima at 394 (100), 496 (12), 596 (6), and 651 (32) nm, where the values in parentheses are the relative heights. Mass spectrometry gave a molecular ion at a mass of 654. In deuteriated dichloromethane the NMR spectrum gave the following shifts in parts per million: −2.60 (2 H, br s, NH), 2.57 (3 H, s, saturated ring CH_3), 3.43, 3.48, and 3.65 (3 H each, s, ring CH_3), 3.63 and 3.64 (3 H each, s, propionate ester CH_3), 3.78 (3 H, s, OCH_3), 3.17 and 3.21 (2 H each, t, propionate $-CH_2-$), 4.18 and 4.32 (2 H each, t, propionate$-CH_2-$), 2.67 (1 H, dd, H_a), 3.31 (1 H, dd, H_b), 5.05 (1 H, dt, H_c), 5.32 (1 H, d, H_d), 6.14, 6.35, and 8.20 (1 H each, ABX type, vinyl H's), and 9.00, 9.19, 9.77, and 9.83 (1 H each, s, meso H's). In deuteriated benzene the shifts were (ppm) −1.96 (NH), 2.44 (saturated ring CH_3), 3.15, 3.18, and 3.24 (ring CH_3), 3.21 and 3.23 (propionate ester CH_3), 3.26 (OCH_3), 3.06 and 3.12 (propionate $-CH_2-$), 4.20 and 4.21 (propionate $-CH_2-$), 2.38 (H_a), 2.97 (H_b), 4.63 (H_c), 5.13 (H_d), 5.86, 6.15, and 7.97 (vinyl H's), and 8.86, 9.41, 9.74, and 10.07 (meso H's). The chlorin S651 has been assigned as the product of methanol addition to S662 as shown in Scheme III on the basis of the following arguments.

The porphyrin S651 has a visible spectrum more typical of chlorins because the extra conjugation of S662 has been removed. Its molecular mass, 654, corresponds to the addition of one CH_3OH to S662. The new OCH_3 singlet appears in the NMR spectrum at 3.78 ppm (dichloromethane). Most of the remaining resonances are immediately assignable upon comparison to S662. However, the thiophene-like protons in S662 at 4.2, 4.9, and 6.9 ppm are absent and new resonances appear at 2.67, 3.31, 5.05, and 5.32 ppm. The resonances at 2.67 (H_a) and 3.31 ppm (H_b) agree well with the 2.8 ppm shift of the methylene protons next to the sulfur in tetrahydrothiophene. In the chlorin case they are adjacent to a chiral center and are not magnetically equivalent. They are spin-coupled with a 12.5-Hz geminal coupling constant. Methylene protons one bond removed from the pyrrole ring are observed at 2.2 ppm with octaethylchlorin as a model. H_c is a methine proton, not a methylene, and this predicts a further +0.4 ppm shift. The neighboring methoxy group would also cause an approximate 2 ppm further downfield shift. Thus, the predicted shift for the H_c proton is 4.6 ppm, while that observed is 5.05 ppm. This is reasonable, considering that the rigidity of the ring or some chlorin macrocycle ring current could account for the remaining downfield shift. H_c is spin-coupled to H_a and H_b with constants of 2.4 and 4 Hz, respectively, and to H_d with a constant of 2.4 Hz. Protons directly attached to the β-pyrrolic position of a chlorin are observed as far downfield as 4.93 ppm. The observed shift of H_d at 5.32 ppm is therefore reasonable. The pattern of coupling constants is completely consistent with the assigned structure, and the magnitudes can be rationalized by appropriate dihedral angles in the rigid saturated thiophene-like ring. The resonances H_c and H_d may have been partially split due to the presence of diastereomers, but at 200 MHz this was uncertain.

Scheme III

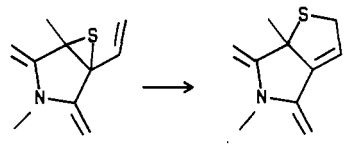


Discussion

The evidence for the assigned structure of S662 rests on the visible spectrum, the NMR spectrum, the mass spectra, and the NOE experiments all performed on the free base. A full pattern of NOE's was not determined as in past studies (Timkovich et al., 1984, 1985) because in this case the starting configuration, that of protoporphyrin IX, is clearly known. The high-resolution mass appears to be definitive evidence that S662 is produced by the addition of only a single sulfur atom, consistent with the previous isotope work. The NMR spectrum is readily interpretable, except for the new resonances of the sulfur ring. However, the argument to their assignment is reasonably straightforward. The minor isolated and characterized product S651 is important in this regard. It is a derivative related by a clear chemical transformation to the main product S662. Methanol has added across the double bond of the sulfur-containing fifth ring of S662. The addition is anti-Markovnikov because of the electron-withdrawing properties of the rest of the chlorin ring system. The fact that the unusual spectroscopic features of both compounds can be explained in terms of related structures adds high confidence that the interpretation is correct. The identification of pyrrole ring B as the site of addition is consistent with the studies on the protein SMb where either ring A or B was the candidate.

Taking the assigned structure of S662 as correct, then there still remain questions of the structure of the sulfheme in the

Scheme IV



sulfglobins. First, is the modification pyrrole specific, or may more than one pyrrole be attacked randomly? Second, does S662 correspond to the heme of SMb*? Third, how does the heme of SMb* relate to that of SMb?

During the conversion of SMbFe^{III}CN to SMb*Fe^{III}CN, new peaks appeared in the hyperfine-shifted spectrum, but a reasonably small number, about equivalent to the starting spectrum. If the sulfur were migrating randomly among the pyrroles, a greater multiplicity of peaks should have appeared. This does not rule out a specific one to one shift. We do not feel this is likely but mention it for the sake of completeness. If it were to occur, the protein NMR data would confine any switch to the A and B rings.

Possible rearrangements during or after the extraction must be considered. Major losses occur in purifying S662 from SMb* to the state where it is suitable for chemical spectroscopy. The extraction of green heme from SMb* is not complete, because the protein left behind retains some green color. Large losses occur during Fe removal, producing material that remains tightly bound to silica gel and can only be removed with strong (e.g., acidic methanol) solvents. This has a generally featureless visible spectrum and has also always been produced even in a workup of protoheme. Losses during esterification or chromatography are not severe, but do occur. None of these steps should discriminate against or in favor of a porphyrin modified at one specific pyrrole. Yet, at the end a single compound modified at a single pyrrole has been isolated.

The 662-nm maximum is observable after Fe removal, so it seems unlikely that any structural rearrangement occurs after this step. None of the present data preclude a rearrangement confined to a single pyrrole during or prior to this step, but at the present time neither is there any indication that such occurred. For the present it will be assumed that S662 is the chlorin skeleton for the heme of SMb*. Ultimately, the question must be confirmed by independent techniques.

The question of the change from SMb to SMb* requires speculation but must be addressed because these are related forms. At the present time only a reasonable hypothesis may be given that is chemically feasible and consistent with the data so far. A possibility is presented in Scheme IV. If the sulfur in SMb is incorporated initially as an episulfide because this is the kinetically favored form, then it resides on a methylvinylpyrrole. The episulfide–vinyl substructure is the sulfur analogue of an epoxide–vinyl. Although we were unable to find precedents for chemistry of the former, the epoxide–vinyl case has been studied. It undergoes thermal rearrangement to a five-membered ring that has a dihydrofuran type of structure (Vogel & Gunther, 1967; Paladini & Chuche, 1971). It is reasonable to suppose that the episulfide would similarly rearrange. The slowness of the reaction in the sulfur case may be due to extreme steric hindrance imposed by the protein. The SMb to SMb* conversion was followed in the ferrous carbon monoxide and the ferric cyano cases by shifts of the main visible band to a higher wavelength, and the extracted heme also showed a shift to higher wavelength. In the hypothetical episulfide–vinyl pyrrole, the double bond is isolated from the rest of the aromatic system, while in the case of S662 some π electron delocalization is possible. In the more delocalized system, a shift of optical bands to higher wavelengths would be expected.

Sulfhemoglobin does not undergo the internal conversion reaction. This raises an issue of whether the sulfheme is the same as in sulfmyoglobin. The conversion may not take place because the sulfur does not reside on a pyrrole with a vinyl substituent or because in the Hb case there is insufficient internal flexibility to permit the rearrangement. None of our data presently resolve this point. We prefer the simpler hypothesis that they are the same for the following reasons. The heme crevice region of the two proteins is similar. The reactivity of the two toward in vitro modification is similar. The visible spectrum of the freshly extracted heme is similar. None of these is by itself sufficient to prove identity, but accumulation of evidence is difficult to ignore. The episulfide hypothesis would account for the reports of Michel (1938a,b), who found that hemoglobin reconstituted with hematoheme (vinyls replaced by hydroxyethyls) still formed a sulf derivative. The vinyl group is not required for the initial formation of the kinetically favored product. A globin reconstituted with a heme lacking a vinyl group might still be able to form the episulfide, but the present results predict that the internal conversion would not take place.

The major difficulty with the episulfide hypothesis concerns the rapid reversion of the first sulfheme formed back to protoheme upon extraction. The steric hindrance argument for the slowness of the conversion could imply that upon extraction from the heme crevice the conversion to a dihydrothiophene should be facile. It may be the case that the reaction of an episulfide–vinyl is pH-dependent. Extractions are done under acidic conditions, while the conversion reaction took place under mildly basic conditions. Storage of sulfmyoglobin at a pH below 7 inevitably led to reversion or precipitation rather than conversion. It also may be the case that some unrecognized feature of the heme crevice promotes the conversion.

Of the four pyrrole rings in the prosthetic group of myoglobin, ring B is the least accessible to solvent. It was quite surprising to find that the sulfur ended up here. There is an accumulating body of evidence that the heme crevice in globins has considerable flexibility [see Karplus and McGammon (1983) and references cited therein]. This may allow attack at the B ring while at the same time creating the special environment required for this unusual reaction.

Acknowledgments

We are indebted to Z. Yun for technical assistance in some of these experiments, to Prof. Grant Mauk for stimulating discussions of his own work in this area, and to Prof. P. Y. Johnson for correctly guessing, prior to any data, that a thiophene-type ring was involved.

Supplementary Material Available

An additional NMR spectrum of S662 mixed with protoporphyrin IX dimethyl ester (4 pages). Ordering information is given on any current masthead page.

Registry No. S662, 105253-67-6.

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