Urry, D. W., Shaw, G., & Prasad, K. U. (1985b) *Biochem. Biophys. Res. Commun. 130*, 50–57.
Venkatachalam, C. M. (1968) *Biopolymers 6*, 1425–1436.
Venkatachalam, C. M., & Urry, D. W. (1981) *Macromolecules 14*, 1225–1229.
Wieland, Th., Heinke, B., & Vogeler, J. (1961) *Justus Liebigs Ann. Chem. 655*, 189–194.
Woody, R. W., (1974) in *Peptides, Polypeptides & Proteins* (Blout, E. R., Bovey, F. A., Goodman, M., & Lotan, N., Eds.) pp 338–350, Wiley, New York.

Proton NMR Spectroscopy of Sulfmyoglobin†

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Received February 7, 1985

ABSTRACT: The ^1H NMR spectra of ferrous sulfmyoglobin, metsulfmyoglobin, and ferric cyanosulfmyoglobin were obtained at 300 MHz. Hyperfine-shifted resonances are observed in the case of metsulfmyoglobin and ferric cyanosulfmyoglobin that have line widths and cover a chemical shift range that are comparable to the corresponding forms of normal myoglobin. Two methyl resonances are observed in the spectrum of ferric cyanosulfmyoglobin at 44.19 and 25.48 ppm (25 °C, pH* 8.3) that have been assigned to heme methyls at the 8- and 5-positions on the basis of pH titration effects homologous to the corresponding methyl resonances in ferric cyanomyoglobin. Examination of aromatic region resonances and the pH titration profiles of histidine resonances lead to the conclusion that the overall conformation of sulfmyoglobin was highly homologous to that of normal myoglobin and afforded assignments of histidine residues of the former. The most likely position for the addition of a sulfur atom to the heme of sulfmyoglobin is pyrrole ring A, with ring B a possible, but less likely, alternative.

Sulfmyoglobin[1] and sulfhemoglobin are unusual derivatives formed from the respective normal globins by the addition of sulfur to the porphyrin skeleton of the prosthetic group heme. This addition leads to conversion of the globins to bright green proteins with characteristic absorption bands in their optical spectra above 600 nm. Although the formation of a sulfglobin was first observed in the 1860s, the precise structure of the modification has never been established.

The existence of sulfglobins is more than a laboratory curiosity. SHb has been reported to be formed in vivo under certain pathological conditions or in the presence of high dosages of some drugs related to common analgesics such as phenacetin [Morell et al. (1967) and references cited therein]. Increased levels of SHb have been correlated with exposure to chemical pollutants (Bucley, 1982; Lambert et al., 1982; Mills et al., 1982). The clinical determination of sulfglobins has remained a major concern (Drabkin & Austin, 1935; Zwart et al., 1984; Tomada et al., 1983).

Several groups have made major contributions to the available structural knowledge of sulfglobins (Michel, 1938a,b; Nicholls, 1961; Morell et al., 1967; Berzofsky et al., 1971a, 1972b) that will be briefly summarized as follows. The unusual green color is not a consequence of the coordination geometry of the central iron but, instead, is due to covalent modification of the porphyrin skeleton. The modification is most probably the reduction of the skeleton to the level of a chlorin. The best available evidence indicates that a single sulfur atom is incorporated, most likely as an episulfide. The in vitro formation of sulfglobins requires the reaction of the Fe(IV) oxidation state with stoichiometric amounts of HS^-. A mechanism has been proposed to account for the in vivo production of sulfglobins on the basis of chemical species known to be present in erythrocytes (Nichol et al., 1968). The sulfglobins are somewhat unstable and can readily decompose. In this process they revert back to apparently unmodified myoglobin. Sulfheme can be extracted from the globin but has a short lifetime, quickly reverting to normal protoheme (Berzofsky et al., 1972b).

The physiochemical properties of the intact green globins have been systematically investigated. The optical spectra of a variety of forms have been reported (Berzofsky et al., 1971a,b, 1972a,b; Nicholls, 1961; Carrico et al., 1978). The infrared (Berzofsky et al., 1972a,b; Carrico et al., 1978) and magnetic circular dichroism (Brittain et al., 1982) spectra have been investigated. Magnetic properties have been charac-

† Financial support was provided in part by Grant GM23869 from the National Institutes of Health. The NMR facility was supported by a grant from the NIGMS Shared Instrumentation Program of NIH, GM26071-02S1.
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[1] Heme nomenclature and abbreviations are as follows: The structure of the heme prosthetic group of hemoglobins and myoglobins is shown in Figure 1 with the substituent positions labeled according to a conventional scheme. This heme is commonly referred to as protoheme, heme *b*, or iron protoporphyrin IX. The organic macrocycle is a porphyrin when fully aromatic and is called a chlorin when one of the pyrrole rings has been saturated at the β carbons to acquire two extra substituents. The β carbons are the outermost of the pyrrole rings and bear the substituents. An episulfide is the sulfur analogue of an epoxide. In the proposed substructure for the sulfglobins, the covalently added sulfur bridges two adjacent β-pyrrolic carbon atoms forming a three-atom ring. The term "meso" refers to the methine bridge carbons linking the four pyrroles. The term heme will be used in this paper to denote the iron porphyrin complex without regard to oxidation state of the iron or ligands at the fifth and sixth axial coordination sites. The term sulfheme will be used to refer to the product of covalent addition of the sulfur moiety. The abbreviations Hb and Mb will respectively represent hemoglobin and myoglobin, and SHb and SMb will represent the corresponding sulfur-modified forms. When so used, the context will be general, and nothing is implied about the oxidation or ligation states. When necessary to specify these, the following abbreviations will be used: $SMbFe^{III}$, the oxidized or met form; $SMbFe^{II}$, the ferrous deoxy form; $SMBFe^{III\text{ or }II}X$, the given oxidation state with X as a ligand. For example, $SMbFe^{III}CN$ is the ferric cyano complex of sulfmyoglobin.

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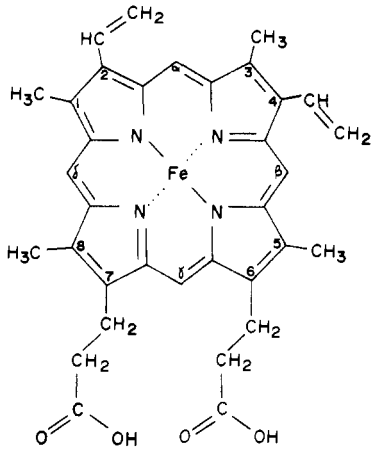


FIGURE 1: Structure and conventional labeling scheme for protoheme, the normal prosthetic group globins. The top-right pyrrole ring with the 1,2-substituents is termed the A ring, and the others are, in the clockwise sense, B–D. In some labelng schemes, these have also been termed respectively I–IV.

terized at cryogenic temperatures by EPR (Berzofsky et al., 1971a; Carrico et al., 1978). Ligand binding of oxygen (Berzofsky et al., 1971b) and carbon monoxide (Berzofsky et al., 1972a) have been measured and shown to occur with much reduced affinities compared to the normal globins. Kinetics of ligand association and dissociation have been determined (Brittain et al., 1982; Brittain & Greenwood, 1982).

The purpose of the present paper is to report the ^1H NMR spectra of various forms of sulfmyoglobin. Sulfmyoglobin was chosen for study over sulfhemoglobin in this initial paper because the lower molecular weight makes spectroscopic resolution, analysis, and interpretation more tractable. However, the similarity of SMb and SHb in other properties ensures that general results on SMb are readily applicable to SHb. ^1H NMR spectroscopy probes the magnetic properties of the sulfglobin under physiological conditions. It is also capable of addressing the question of conformational similarity between the native and sulf forms. Data will be presented that for the first time provide evidence as to which porphyrin pyrrole ring is the site of covalent addition of the sulfur.

Materials and Methods

Equine and sperm whale myoglobin were purchased from Sigma Chemical Co. and used as received. SMb was prepared by the procedure of Berzofsky et al. (1971a), which initially yields the ferrous form of sulfmyoglobin. The procedure was found to work equally well when performed in deuterium oxide buffers using reagents diluted from concentrated stock solutions into deuterium oxide buffers to the working concentrations. $SMbFe^{III}$ was prepared by the addition of a 5-fold molar excess of ferricyanide. Residual reagents were generally removed by passage through a Sehadex G-25 column preequilibrated with deuterium oxide buffers corresponding to the buffer desired for the NMR experiment. However, in the latter stages of this work it was discovered that residual catalase and residual ferricyanide had no observable effects upon the NMR spectra. The ferric cyano complex of SMb was formed by the addition of potassium cyanide from concentrated stock solutions in deuterium oxide at the same pH as the protein to a final concentration of 25 mM. Optical and NMR spectra were not sensitive to the precise cyanide concentration, as long as it was in excess. Optical spectra of the initial ferrous SMb, the met form, and the ferric cyano complex agreed with literature reports (Berzofsky et al., 1971a, 1972b). We were not always able to prepare the initial ferrous SMb in greater than 90% yield as reported by Berzofsky et al. (1971a), but our preparations were typically in excess of an 80% yield with an average of 85%. NMR spectra clearly indicated that the remainder was unmodified myoglobin. Percent conversion calculated from peak intensities in the NMR spectra of $SMbFe^{III}$ CN agreed with yields calculated from the optical criteria of Berzofsky et al. (1971a).

Samples for NMR spectroscopy were 3–7 mM in protein in deuterium oxide buffered with 50 mM potassium phosphate to the appropriate pH. Spectra were insensitive to added NaCl. Values of pH reported as pH* represent the direct reading of a glass combination electrode standardized against protic reference solutions. When necessary, the value of pH* was adjusted by additions of ^2HCl or NaO^2H. Titration experiments were generally performed on samples starting at high values of pH* and adding acid to lower the pH*. This was necessary because SMb is more stable at alkaline pH* and degrades rapidly below pH* 6. In some cases, fresh independent samples were prepared and adjusted to the appropriate pH*, and the spectra were recorded. We were unable to observe the spectrum of any form of SMb at values of pH* below 5 because of rapid degradation. Chemical shifts are reported in parts per million from internal sodium 4,4-dimethyl-4-silapentanesulfonate (DSS). In some cases internal dioxane (3.746 ppm) was used as the shift reference. Spectra were obtained by a Nicolet spectrometer operating at 300 MHz for protons and equipped with a temperature-controlled probe. Aromatic-region spectra to be shown were calculated by multiplying the observed free induction decay with a trapezoidal weighting function in order to enhance the appearance of sharp singlets. Unfortunately, this also increases the noise level, introduces false negative dips, and somewhat distorts the apparent intensities. Chemical shifts vs. pH* data for histidine aromatic resonances were fit by a nonlinear least-squares algorithm to extract the statistically best value of pK_a. Some resonances have not reached the acidic region plateau chemical shift by pH* 5, and the program also fit the data to obtain the acid-limit shift. Standard deviations for best fit pK_a values from a single series of data points were on the order of ±0.04, but replicate independent experiments indicated that ±0.1 was a more realistic indication of the standard deviation. Line-broadening experiments with Mn^{2+} were performed by preparing a fresh 0.1 M solution of analytical grade $MnSO_4$ in deuterium oxide. Aliquots of the stock Mn^{2+} reagent were added to samples of $SMbFe^{III}$ CN, and the final solution was adjusted to pH* 7.0, after which the spectra were immediately recorded. We attempted to do these experiments as titrations in which the Mn^{2+} concentration was systematically increased from 0.5 to 13 mM, and we sought a correlated successive increase in line-broadening effects. However, aqueous Mn^{2+} is autooxidizable in all but acidic media and forms the insoluble precipiate MnO_4 with time. Therefore, the actual Mn^{2+} concentration was unknown, variable, and dynamically changing from experiment to experiment. Typical line broadening observed will be reported at what will be called nominal millimolar concentrations of Mn^{2+}. It must be stressed that, for the present purposes, what is important is the differential line-broadening effects observed within a single sample tube and that, therefore, the ambiguity over concentrations does not invalidate the conclusions.

Results

Freshly prepared SMb is in the ferrous oxidation state. Although the solution contains oxygen [from the catalase decomposition of hydrogen peroixde; see Berzofsky et al. (1971a)], the affinity of ferrous SMb for oxygen is apparently

Table I: Chemical Shifts of Select Resonances for Sulfmyoglobin[a]

high-temp form of equine ferric cyanosulf-myoglobin[b]	equine metsulf-myoglo-bin[c]	ferrous equine sulfmyoglobin[d]	ferrous whale sulfmyoglobin
45.35 (M)	66.0	14.2	13.8
34.38	60.6	11.7	12.1
28.56	49.0	11.2	11.7
26.18	33.7	10.9	11.1
25.28	21.9		
20.40 (M)	12.8		
17.72	11.1		
17.32			
14.83			
13.92			
−1.03			
−1.51			
−4.61			
−5.67			

[a] Chemical shifts in ppm from internal DSS. [b] High-temperature form observed at pH* 8.3, 55 °C. Peaks labeled M are believed to represent methyl groups from their intensity relative to other high temperature form resonances. [c] The oxidized form of sulfmyoglobin at pH* 8.0 and 25 °C produced by oxidation of a fresh preparation of ferrous sulfmyoglobin with excess ferricyanide. [d] Possible hyperfine-shifted resonance observed in fresh preparations of ferrous sulfmyoglobin at pH* 7.96, 20 °C.

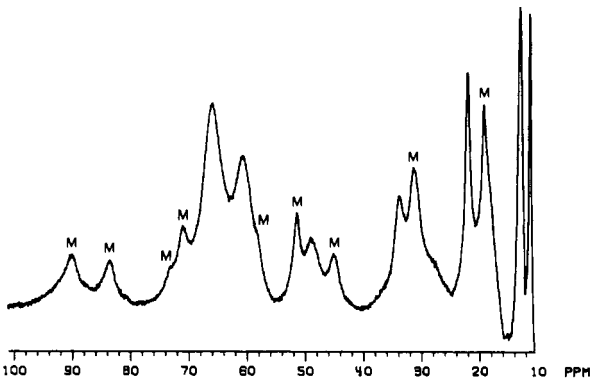


FIGURE 2: Downfield hyperfine-shifted ^{1}H NMR spectrum at 300 MHz, pH* 7.96, 25 °C, of equine metsulfmyoglobin. Peaks labeled M correspond to residual normal metmyoglobin in this sample.

too low to lead to binding under these conditions (Berzofsky et al., 1971b). ^{1}H NMR spectra were obtained on samples of SMb immediately after preparation (within 1 h, where most of the time was for signal averaging) at 20 °C. No clear hyperfine-shifted resonances were observed with the exception of some broad, weak resonances between 10 and 14 ppm as listed in Table I. These features were not present in the control spectra of normal $MbFe^{II}$ O_2, which is known to be the main contaminant in fresh preparations of SMb (Berzofsky et al., 1971a). As noted in Table I, slight differences were observed between equine and whale myoglobins. Line widths in the general aromatic and aliphatic regions were in general broader than those for $MbFe^{II}$ O_2 or for any of the cyano complexes to be discussed. In this form it was observed that SMb autooxidized and reverted to unmodified myoglobin, as reported previously (Morell et al., 1967; Berzofsky et al., 1971a).

$SMbFe^{III}$, prepared by ferricyanide oxidation of freshly prepared SMb, afforded the hyperfine-shifted spectrum shown in Figure 2 and summarized in Table I. All the observed resonances shifted upfield with increasing temperature, consistent with a Curie's law type of behavior. This form of SMb was more stable than ferrous SMb, but less than the ferric cyano complex to be discussed. The appearance of normal metmyoglobin resonances in the spectrum (labeled M) was

Table II: Main Hyperfine-Shifted Resonances in Ferric Cyanosulfmyoglobin

label[a]	shift[b]	intensity[c]	temp coeff[d] a	temp coeff[d] $b \times 10^{-3}$
a	44.90	3	−2.45	13.84
b	25.99	1	8.70	5.13
c	25.48	3	−0.29	7.64
d	24.42	1	11.29	3.90
e	20.09	1	7.93	3.61
f	11.95	1	2.39	2.83
g	10.88	1	5.32	1.65
h	9.87		6.34	1.04
i	9.51	3	5.58	1.15
j	9.26			
k	−0.31	5 (?)	2.37	−0.74
l	−0.71		5.87	−1.96
m	−1.73	4 (?)	2.39	−1.22
n	−2.34	2 (?)	2.63	−1.48
o	−4.22	2 (?)	2.21	−1.91
p	−4.53	2	8.64	−3.91
q	−4.83			

[a] Peak label corresponding to the spectrum displayed. [b] Chemical shift in ppm at 25 °C and pH* 8.3. [c] Intensity in terms of protons per molecule of protein taken from observed relative areas rounded to the nearest whole integer. The symbol (?) implies ambiguity about the intensity, usually due to overlap with other resonances. Because of overlap, certain resonances have been grouped together. [d] Chemical shifts of resonances with respect to temperature were fit to ppm(obsd) = $a + b/T$, where the shift is in ppm and T is in Kelvin. a and b represent the linear intercept and slope. Such plots were apparently linear, but because of the limited temperature range accessible (276–328 K), the parameters a and b should be considered only empirical parameters that allow a report of experimental shifts. Resonances follow Curie's law if the slope b is positive and the chemical shift is downfield of DSS, i.e., positive, or the slope is negative and the shift is upfield of DSS.

higher than for the ferric cyano complexes and continued to grow with time. Attempts to reduce $SMbFe^{III}$ with dithionite or sulfhydryl reagents catalyzed the reversion to normal $MbFe^{II}$. The main conclusions to be drawn from the spectrum are qualitative. From the peak widths and chemical shifts, it can be inferred that the electronic relaxation time of the iron in $SMbFe^{III}$ is comparable to that of $MbFe^{III}$. From the chemical shift differences, it can be inferred that the g-tensor anisotropy and/or the unpaired electron spin density on the heme has changed.

$SMbFe^{III}CN$ provided a strong hyperfine-shifted spectrum as shown in Figures 3 and 4 and summarized in Table II. The hyperfine-shifted spectra of equine and whale $SMbFe^{III}CN$ were highly homologous. The line widths of the strong SMb peaks a and c were respectively 113 and 63 Hz at pH* 8.3, 25 °C. Heme methyl resonances in normal myoglobin have line widths of 40, 28, and 24 Hz under the same conditions as determined from control spectra. Since the unpaired electron spin on the ferric iron is a major (if not the major) factor in determining the NMR line width, one can conclude that the electron-spin relaxation time in the SMb complex is only 2–3-fold slower than that in the normal Mb complex. The result was surprising in light of previous EPR studies. At 1.5 K, the ferric cyano complexes of sulfglobins give sharp EPR spectra (Berzofsky et al., 1971a; Carrico et al., 1978), implying long electronic relaxation times. under comparable conditions, normal myoglobins do not give observable spectra. This is attributable to broad line widths because of short electronic relaxation times. However, Berzofsky et al. (1971a) alluded to a strong temperature dependence for the electronic spin of ferric iron in SMb. Evidently, this must be the case, because by room temperature the NMR spectra indicate that the spin states and relaxation times are comparable.

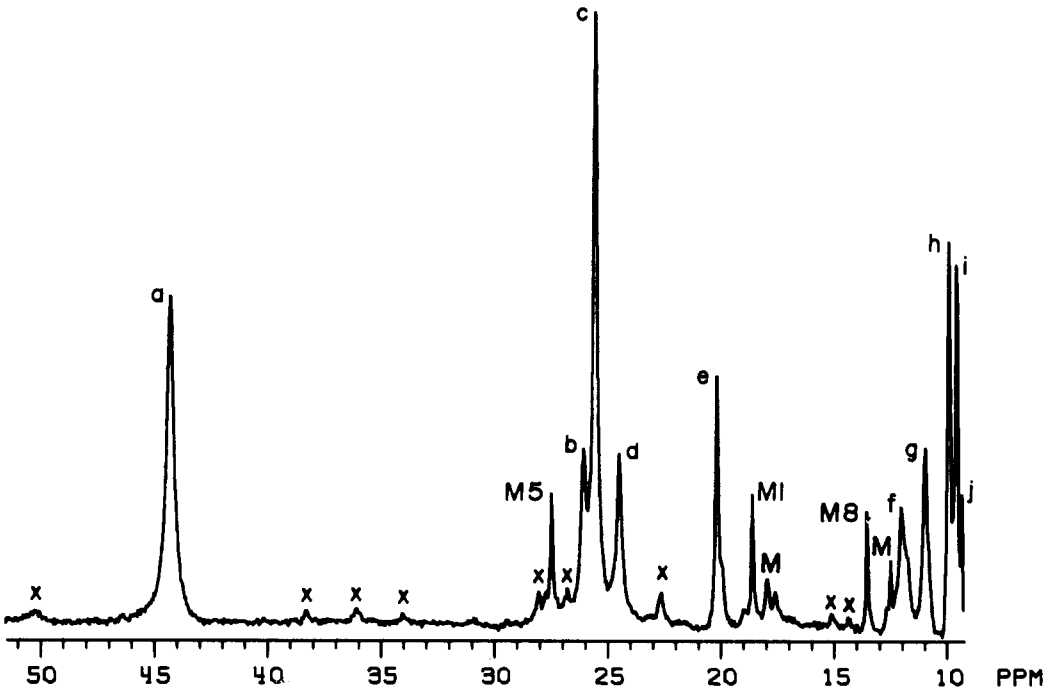


FIGURE 3: Downfield hyperfine-shifted ^{1}H NMR spectrum at 300 MHz, pH* 8.3, 25 °C, of equine ferric cyanosulfmyoglobin. Peaks labeled a–j are the main sulfmyoglobin resonances in conformity with Table II. Peaks labeled M are due to residual normal myoglobin. Minor peaks labeled X correspond to traces of the high-temperature form of sulfmyoglobin as listed in Table I and discussed in the text. In this spectrum, the intensities of peaks h and i appear abnormally high because of mutual overlap and overlap with unidentified broad, minor resonances.

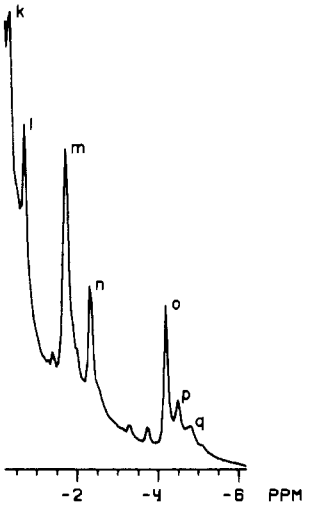
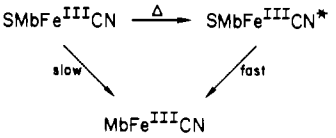


FIGURE 4: Upfield hyperfine-shifted ^{1}H NMR spectrum at 300 MHz of equine ferric cyanosulfmyoglobin. Conditions were as discussed for Figure 3. Note that the horizontal and vertical scales have been adjusted for clarity of presentation and are different from those of Figure 3.

The temperature behavior of the SMbFe^{III}CN NMR spectrum was studied over the range of 3–55 °C. All SMb hyperfine-shifted resonances appeared to follow a Curie's law type of behavior as summarized in Table II. No dramatic change in line widths occurred over this range. The average line widths for SMb resonances decreased by 2-fold on going from 3 to 55 °C, but this was similar to the sharpening observed for MbFe^{III}CN hyperfine-shifted resonances over the same range.

At temperatures above 25 °C, a new set of hyperfine-shifted resonances appeared as listed in Table I. These grew in intensity at the expense of the usual SMb resonances and are believed to represent an alternative form of sulfmyoglobin that will be designated SMb*. This form was much more labile than SMb and rapidly reverted to normal MbFe^{III}CN. Because of its limited lifetime, it proved impossible to obtain a spectrum of SMb* alone. The conversion of SMb to SMb* appeared to be irreversible. When a heat-treated sample was cooled and the spectrum was recorded again, the SMb* resonances persisted, although they still rapidly were replaced by normal Mb resonances. At no temperature was there any indication of chemical exchange induced line broadening. These observations support the following scheme:



The conversion may represent a change in the iron electronic properties, perhaps due to a coordination sphere rearrangement. However, there is no gross spin-state change. The new resonances that are the best candidates for methyls (see Table I) have hyperfine shifts comparable to strong resonances in SMb. The conversion may represent a conformational change in the heme pocket. We point out that the saturated pyrrole in chlorins is puckered and may readily undergo a local ring flipping motion.

Resonances a and c in SMbFe^{III}CN are assigned as heme methyls on the basis of their intensity and large downfield shift. Heme methyls are routinely among the farthest downfield hyperfine-shifted peaks in low-spin ferric heme proteins because of large contact and pseudocontact shift contributions. It is intriguing that only two of the four possible heme methyls are observed resolved from the 0–10 ppm range. In MbFE^{III}CN, three methyls are observed and have been assigned on the basis of selective deuteration (Sheard et al., 1970; Mayer et al., 1974). The three resolved heme methyls in normal Mb have distinctive chemical shift dependences upon pH* (Sheard et al., 1970; Carver & Bradbury, 1984; Bradbury & Carver, 1984). The pH* effects have been attributed to ionization of groups in the heme pocket, especially His-97 (Carver & Bradbury, 1984). If the surrounding heme pocket

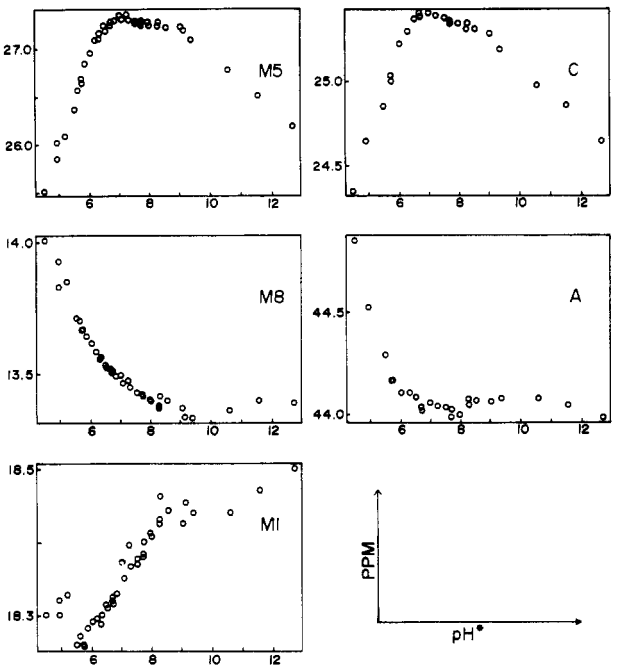


FIGURE 5: Titration profiles of chemical shift vs. pH* for the heme methyl resonances of equine ferric cyanomyoglobin (M1, M5, and M8) and cyanosulfmyoglobin (A and C). Note the very expanded chemical shift scale for M1. The chemical shift differences between the highest and lowest points are as follows: M5, 1.8 ppm; C, 1.1 ppm; M8, 0.7 ppm; A, 0.9 ppm; M1, 0.24 ppm.

in SMb remains homologous to that in Mb, it is likely that similar pH* effects would be seen, *even though the root chemical shifts of SMb resonances are radically different because of changes in the contact and pseudocontanct contributions*. Figure 5 shows the effects of pH* on SMbFe^{III}CN heme methyl chemical shifts and compares them with the behavior of MbFe^{III}CN heme methyl resonances. The normal Mb shifts were measured on the 10–20% resdual normal Mb in our SMb preparations, and so were rigorously determined under identical conditions. The pH* profiles for the normal Mb resonances agree well with previously published profiles for sperm whale MbFe^{III}CN (Sheard et al., 1970; Carver & Bradbury, 1984). It can be seen that the profile for resonance a (at 44 ppm) in SMb matches that for the 8-methyl (at 13.5 ppm), while resonance c (at 25.5 ppm) matches the 5-methyl (at 28 ppm) both in general shape and in total excursion.

Further evidence for the assignment of the SMb heme methyls was obtained by determining the differential line-broadening effects of added Mn^{2+} as a selective relaxation reagent. The heme group in myoglobin is oriented so that the heme methyls 5 and 8 are closer to the protein surface and more solvent exposed than the buried methyls 1 and 3. It is not known whether Mn^{2+} binds to any specific site on myoglobin. Some divalent cations such as Cu^{2+} or Zn^{2+} have been implicated in binding (Rifkind, 1981; Rifkind, 1983), and so specific binding is possible. In any case, it is most reasonable to assume that any specific or nonspecific interaction is directed toward the surface of the protein. Then, paramagnetic Mn^{2+} should broaden the more exposed heme methyls because of an average closer distance of approach for all but a few cases involving possible associations on the hemisphere opposite to the heme crevice. Since in the majority of experiments Mn^{2+} was in stoichiometric excess, nonspecifically binding Mn^{2+} may still be most important. At any rate, it is the comparison between normal and sulfmyoglobin that is most critical for the present purpose. At nominal millimolar concentrations of Mn^{2+} the average excess line widths (with Mn^{2+} minus an initial control spectrum without) for normal MbFe^{III}CN methyl resonances were in the order

$$5\ (52\ Hz) > 8\ (37\ Hz) > 1\ (17\ Hz)$$

while for SMbFe^{III}CN the average excess line widths were in the order

$$c\ (57\ Hz) > a\ (50\ Hz)$$

The magnitude of the effects and the relative order are consistent with the assignment of c as the 5-methyl and a as the 8-methyl.

Broad hyperfine-shifted resonances have been reported in spectra of MbFe^{III}CN taken in ^1H_2O that are absent in spectra where ^2H_2O is the solvent (Sheard et al., 1970). These resonances are of extreme interest because of their possible relation to exchangable NH protons on the distal and/or proximal histidines. Spectra of SMbFe^{III}CN were recorded in solvents 90% ^1H_2O and 10% ^2H_2O (necessary to maintain spectrometer lock), employing a water presaturation pulse. Unfortunately, no additional hyperfine-shifted resonances were observed.

In order to assess the overall conformational similarity of SMb and Mb, potential histidine protons were studied in the aromatic regions of the respective ferric cyano complexes. Equine myoglobin was used. Histidine protons had not been studied in equine ferric cyanomyoglobin, although they have been assigned in the closely homologous sperm whale ferric cyanomyoglobin (Carver & Bradbury, 1984). His protons have been observed and assigned in many species and forms of myoglobin in extensive studies by independent groups (Bothelho & Gurd, 1978a,b; Ohms et al., 1979; Carver & Bradbury, 1984; Bradbury & Carver, 1984). Myoglobins possess a large number of His residues; many of these are in environments where there is sufficient conformational flexibility to give rise to sharp singlets for the nonexchangeable C2 and C4 protons. The local microenvironments are unique, and the various residues have distinctive pK_a's. The associated resonances also shift by distinctive amounts when going from alkaline to acid pH*. Previous workers have assigned many such His protons by comparing chemical shifts, pK_a values, and the total shift change on protonation among homologous myoglobins from different species that nevertheless have a common three-dimensional conformation. Similarities in the above three areas have been used to mark common residues, and their absence in some primary sequences has led to unambiguous assignments.

In the case of SMb, potential His resonances were sought for comparison to normal Mb. Figure 6 displays the resolution-enhanced aromatic region of equine SMbFe^{III}CN and MbFe^{III}CN. The differences in appearance of the spectra are about what one observes when comparing myoglobins from two different species. The sharp resonances labeled in Figure 6 and tabulated in Table III displayed pH*-sensitive chemical shifts that could be accounted for by one-proton titration curves. Typical curves are shown in Figure 7 for select C2 resonances. The pH* behavior is summarized in Table III in a now conventional format. By considering shift, pK_a, and total jump, it is possible to align homologous resonances between SMb and Mb, even when there are substantial differences between the root chemical shifts. These root differences presumably arise from changes in the pseudocontact contribution due to the presence of the paramagnetic iron, although very minor conformational perturbations cannot be strictly ruled out.

The similarity of His resonances is very strong evidence that the total protein conformation of SMb is homologous to that

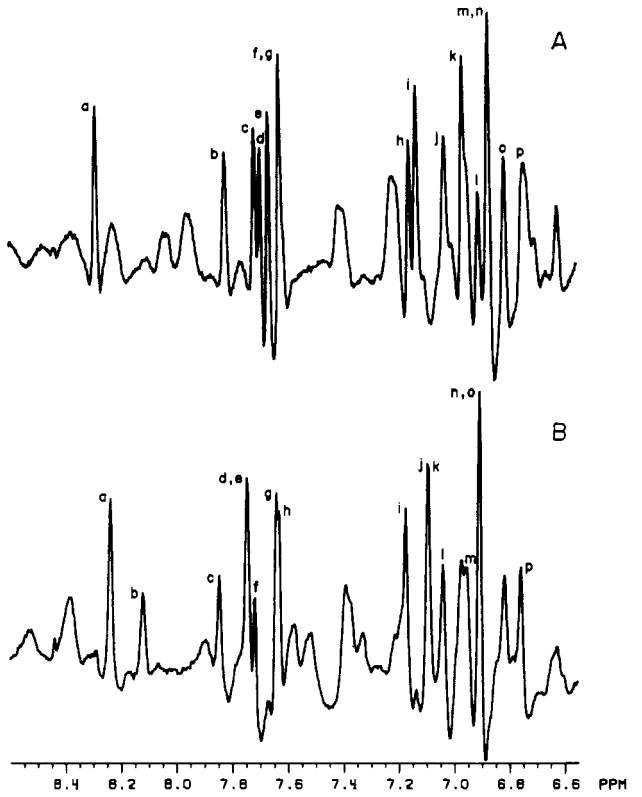


FIGURE 6: Aromatic regions of equine ferric cyanomyoglobin and cyanosulfmyoglobin at 25 °C. The spectra were computed after application of a trapezoidal weighting function to the free induction decay to emphasize the appearance of potential sharp singlets. Peaks are labeled in conformity to Table III. (A) Ferric cyanomyoglobin at pH* 9.26; (b) ferric cyanosulfmyoglobin at pH* 9.07.

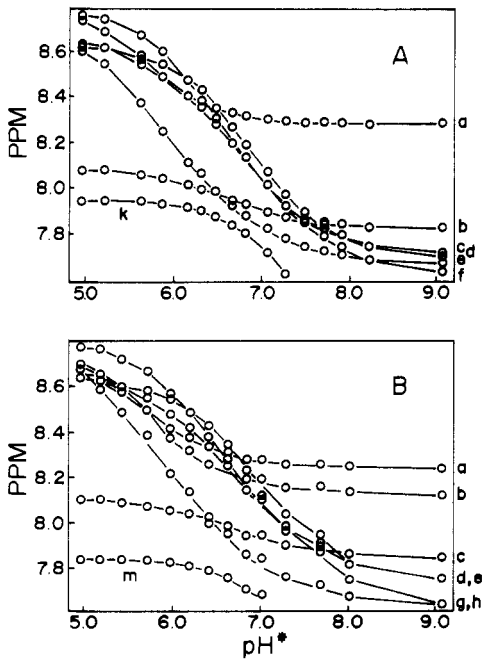


FIGURE 7: Titration profiles of chemical shift vs. pH* for select His C2 protons at 25 °C for ferric cyanomyoglobin (A) and cyanosulfmyoglobin (B).

of Mb. The sulfur modification must alter only the immediate vicinity without other major perturbations. The His data add confidence to the previously discussed heme methyl pH* titrations and interpretations and make it highly unlikely that the similarity in pH* profiles that led to those assignments was coincidental.

Further comparison of the His resonances to literature data for whale myoglobin led to the assignments proposed in Table III. Certain resonances are so distinctive that the assignments can be made with confidence. These include His-119 C2 and C4, His-36 C2, and His-116 C4. At the other extreme, His-81, -48, and -116 C2 resonances are sufficiently similar that interchanging the assignments would not be totally unreasonable, only less convincing. Certain differences are evident in the table. SMb resonance c and Mb resonance c were assigned to His-81 C4 on the basis of a distinctive downfield shift and the size of the pH* jump, but in whale Mb the observed pK_a is significantly lower. Resonances o (SMb), 1 (Mb), and 19 (whale Mb) certainly seem homologous, but the previous literature assignment to a His-36 C4 proton seems questionable because of the large pK_a difference as compared to the C2 protons. In whale MbFe^{III}CN, the C2 resonance of His-97 (peak 15) is somewhat broad and difficult to observe at 20 °C [see Carver & Bradbury (1984); Figure 7). In our data for the equine myoglobins, it was not possible to resolve and follow an analogue with confidence, and we were precluded from doing the titration studies at higher temperatures by the temperature lability of SMb. SMb appeared to possess a unique singlet, resonance *b* in SMb. Lack of a homolgue makes assignment difficult.

Discussion

On the basis of the ^{1}H NMR spectra of SMbFe^{III}CN, it is proposed that pyrrole ring A, bearing the 1-methyl and 2-vinyl substituents in normal protoheme, is the site of sulfur addition in sulfglobins. A less likely possibility is ring B. The data rule out ring C or D. The rationalization for this interpretation may be summarized as follows.

The homology in aromatics between SMb and Mb is prime evidence for equivalent conformations in the overall polypeptide folding. Sulfur modification does not lead to a major reorganization. Local perturbations are of course possible and expected, but by and large, the additional sulfur moiety must be accommodated within the normal heme pocket. On the basis of the chemical shifts and line widths of hyperfine-shifted resonances, the electronic spin state and relaxation time of the paramagnetic iron in SMb remain comparable to those of Mb in the ferrous form, the met form, and the ferric cyano complex. This argues that the coordination geometry is similar in each case. Chemical shift differences that have occurred may be accounted for by changes in the precise contact and pseudocontact contributions to the shift. These mechanisms are powerful interactions, and electronic perturbations without massive conformational changes can readily lead to the observed shift differences.

The polypeptide conformational homology leads one to expect that heme methyls will be sensitive to the same pH* effects even though the root chemical shifts are different. The observed near identity in pH* profiles for the SMb hyperfine-shifted methyls is strong evidence that they are indeed the 5- and 8-methyl substituents.

The 5- and 8-methyls still appear in SMb outside the 0–10 ppm window, because they experience contact and pseudocontact shifts. Of these, it has been determined in heme prorteins in general that the contact mechanism is the main one for heme methyls (Shulman et al., 1971). These methyls in SMb can have large contact shift contributions because of delocalization of the electron spin of the iron into porphyrin molecular orbitals (La Mar & Walker-Jensen, 1979). On the saturated pyrrole of a chlorin, the methyls are more isolated from the unpaired spin and lose most of the major contact shift. In a model compound, iron octaethylchlorin (with eight ethyl

Table III: Comparison of Aromatic-Region Resonances for Cyanosulfmyoglobin and Cyanomyoglobin[a]

equine SMbFe^{III}CN				equine MbFe^{III}CN				whale MbFe^{III}Cn[b]				
L	δ_B	pK_a	Δ	L	δ_B	pK_a	Δ	L	δ_B	pK_a	Δ	assignment[b]
a	8.23	5.8	0.47	a	8.23	5.9	0.40	6	8.32	5.15	0.53	His-119 C2
b	8.11	5.8	0.74									
c	7.84	6.7	0.25	c	7.72	7.2	0.30	10	7.84	6.17	0.24	His-81 C4
d	7.74	7.0	0.88	d	7.70	6.9	0.99	2	7.82	6.35	1.14	His-81 C2
e	7.74	6.7	1.03	b	7.82	6.5	0.97	4	7.73	6.54	1.02	His-48 C2
f	7.71	6 (?)	<0.1	g	7.63	6 (?)	<0.1	12	7.71	5.82	0.13	His-24 C2
g	7.63	6.9	0.99	f	7.63	6.9	0.97	5	7.66	6.96	1.04	His-116 C2
h	7.63	6.2	1.05	e	7.67	6.2	0.96	7	7.71	5.56	1.18	His-113 C2
m	6.95	7.9	0.88	k	6.97	7.7	0.97	11	7.02	8.11	1.05	His-36 C2
								15	6.76	5.54	0.91	His-97 C2
i	7.17	6.9	0.33	h	7.16	6.9	0.33	13	7.24	6.92	0.35	His-116 C4
j	7.09	5.8	0.39	i	7.13	5.7	0.44	14	7.17	5.56	0.45	His-119 C4
n	6.90	6.9	0.31	m	6.87	6.8	0.34	17	6.94	6.38	0.36	His-48 C4[c]
o	6.90	5.6	0.13	l	6.91	5.6	0.14	19	6.95	5.58	0.05	His-36 C4 (?)[d]
p	6.75	6.4	0.42	o	6.81	6.3	0.44	18	6.85	5.68	0.42	His-113 C4
				p	6.75	6.0	0.46					
								22	6.46			His-24 C4
k	7.09	pH	invar[e]									
l	7.04	pH	invar	j	7.04	pH	invar					
				n	6.88	pH	invar	20	6.88	pH	invar	Trp H2
	8.67[f]				8.69[f]							
	8.54[f]				8.59[f]							
	8.38[f]				8.38[f]							
					8.22[f]							
					7.95[f]							
	7.57[f]											
	7.51[f]											
	7.37[f]				7.41[f]							
	7.32[f]											
	7.20[f]				7.21[f]							
	6.97[f]				6.95[f]			19	6.95[f]			
	6.81[f]				6.81[f]							
	6.63[f]				6.62[f]							

[a] Data were obtained at 25 °C for the ferric cyano complexes of equine skeletal muscle myoglobin and sulfmyoglobin. Four entries are given for each form. The column labeled L is the peak label as given on the spectra of Figure 6, δ_B is the chemical shift at alkaline pH, pK_a is the computed best fit acid constant from the titration experiments, and Δ is the total change in chemical shift upon going from alkaline to acidic conditions. Resonances f and g, respectively, for sulf and normal myoglobin showed only slight pH effects such that the computed values of pK are uncertain. [b] Data for spermwhale ferric cyanomyoglobin were taken from Carver & Bradbury (1984), as were the listed assignments. The table is intended to imply that the resonances sharing a common row are analogous spin systems in the three forms. Analogies were assigned by considering respective values for δ_B, pK_a, and Δ. [c] Ascribing observed resonances n and m to His-48 C4 protons with pK_a's of 6.9 and 6.8, respectively, does not seem fully consistent with the assignment of resonances e and b to His-48 C2 protons because of the lower respective pK_a's of 6.7 and 6.5, respectively. This may reflect an extreme case of experimental error in fitting the pK_a for the C4 protons (the C2 protons with a larger pH jump are felt to provide the more reliable experimental data). [d] Literature assignment for the His-36 C4 does not seem right because of the discrepancy with the pK_a for the assigned His C2 at 8.1. [e] No chemical shift change with changes in pH*. [f] Unassigned aromatics.

substituents and one saturated pyrrole also bearing two bonded protons), the methylene protons of the saturated ring substituents are found upfield of tetramethylsilane, while the methylene protons of unsaturated pyrrole substituents are hyperfine-shifted far downfield (Stolzenberg et al., 1981). We are interpreting the loss of a hyperfine-shifted 1-methyl resonance downfield of 10 ppm, from the SMb spectrum as compared to normal Mb, as being due to the fact that it now resides on a saturated pyrrole and, hence, has lost its previous contact shift contributions.

The argument has flaws, the most glaring of which is the fact that the 3-methyl has not been resolved in either our Mb or SMb spectra. The possibility does exist that ring B is the site of saturation and that the 1-methyl has moved upfield because of a rearrangement of the unpaired spin density, or a new major pseudocontact contribution.

Assignment of the saturation site as ring A is supported in part by the assignment of His C2 protons in Table III. His-36 is sufficiently close to the buried pyrrole ring B that we expect a local conformational perturbation due to B modification would have had some impact on the titration behavior of His-36. But, the titration behavior of the His-36 C2 proton retains its distinctive base chemical shift (ca. 7.0 ppm) and unusual pK_a (near 8) in SMbFe^{III}CN. Ring A is not near to any His residues, except for the proximal and distal His residues, but these have been unobservable in a ferric cyanomyoglobin.

Modification of ring A is consistent with the known three-dimensional structure of myoglobins. Crystallography has shown that there is a hole in the interior of Mb surrounded by a cluster of hydrophobic residues on the proximal histidine side of the heme, directly over the 1,2-substituent positions of the heme (Takano, 1977; see especially Figure 8). This cavity binds xenon (Schoenborn et al., 1965; Tilton & Kuntz, 1982) and cyclopropane (Shulman et al., 1969). The sulfur modification of myoglobin is a unique reaction that depends upon some unique aspect of the protein–heme complex. The reaction does not occur on isolated hemes or on general heme proteins. The cavity above ring A may be the key structural element that provides a sheltered microenvironment directly adjacent to the heme in which the unusual chemistry may take place. It has been suggested that the reaction proceeds through a sulfur radical (Nichol et al., 1968; Berzofsky et al., 1972b). The cavity may provide a locale for such a radical where the most susceptible site toward radical attack is the pyrrolic π-electron system.

This evidence for partial identification of the site of sulfur addition does not address the question of the actual structure

of the sulfur adduct. The proposed episulfide structure is the best current hypothesis to explain the available data, but we conclude on a cautious note that this hypothesis requires further confirmation. Localization of the addition site to the A ring, or with less probability the B ring, raises the issue of whether the 2- or 4-vinylic substituents may participate in either the final structure or the mechanism of formation. Michel (1938a,b) has reported that hemoglobin reconstituted with hematoheme still could be treated to form sulfglobin. Hematoheme contains 2- and 4-(hydroxyethyl) groups instead of vinyls. At face value, this seems to argue that the vinyl substituents are not obligatory for sulfheme formation. However, hematoheme preparations are often contaminated with appreciable quantities of heme with one or two vinyl substituents due to incomplete hydrolysis in the hematoheme formation plus partial dehydration in subsequent workup. The procedures used at that time for sulfglobin formation are now known to produce low yields with respect to the fraction of globin converted (Berzofsky et al., 1971a), and it is possible that the observed sulfheme reaction was due to contaminating vinyl hemes. The possibility of vinyl-group participation should be considered a viable hypothesis.

Added in Proof

Recent evidence from resonance Raman studies (Andersson et al., 1984, 1985) has been interpreted to "suggest the presence of at least one, and probably both, vinyl substituents on the iron chlorin moiety of SMb". The first part of the suggestion is clearly consistent with the evidence presented in this paper, while the second is not necessarily inconsistent.

References

Andersson, L. A., et al. (1984) *J. Biol. Chem. 259*, 15340.

Andersson, L. A., Loehr, T. M., Chang, C. K., & Mauk, A. G. (1985) *J. Am. Chem. Soc. 107*, 182–191.

Berzofsky, J. A., Peisach, J., & Blumberg, W. E. (1971a) *J. Biol. Chem. 246*, 3367–3377.

Berzofsky, J. A., Peisach, J., & Blumberg, W. E. (1971b) *J. Biol. Chem. 246*, 7366–7372.

Berzofsky, J. A., Peisach, J., & Alben, J. O. (1972a) *J. Biol. Chem. 247*, 3774–3782.

Berzofsky, J. A., Peisach, J., & Horecker, B. L. (1972b) *J. Biol. Chem. 247*, 3783–3791.

Bothelho, L. H., & Gurd, F. R. N. (1978a) *Biochemistry 17*, 5188–5196.

Bothelho, L. H., & Gurd, F. R. N. (1978b) *Biochemistry 17*, 5197–5205.

Bradbury, J. H., & Carver, J. A. (1984) *Biochemistry 23*, 4905–4913.

Brittain, T., & Greenwood, C. (1982) *Biochem. J. 201*, 153–159.

Brittain, T., Greenwod, C., & Barber, D. (1982) *Biochim. Biophys. Acta 705*, 26–32.

Buckley, J. A. (1982) *Bull. Environ. Contam. Toxicol. 29*, 637–644.

Carrico, R. J., Peisach, J., & Alben, J. O. (1978) *J. Biol. Chem. 253*, 2386–2391.

Carver, J. A., & Bradbury, J. H. (1984) *Biochemistry 23*, 4890–4905.

Drabkin, D. L., & Austin, J. H. (1935) *J. Biol. Chem. 112*, 51–65.

LaMar, G. N., & Walker-Jensen, F. A. (1979) *Porphyrins 4B*, 61–157.

Lambert, M., Sonnet, J., Mahieu, P., & Hassoun, A. (1982) *J. Toxicol. 19*, 45–50.

Mayer, A., Ogawa, S., Shulman, R. G., Yamane, T., Cavaleiro, J. A. S., Gonsalves, A. M. R., Kenner, G. W., & Smith, K. M. (1974) *J. Mol. Biol. 86*, 749–756.

Michel, H. O. (1938a) *J. Biol. Chem. 123*, lxxxv.

Michel, H. O. (1938b) *J. Biol. Chem. 126*, 323.

Mills, C. F., Bremner, I., Young, B. W., & Davies, N. T. (1982) in *Trace Elements in the Metabolism of Man and Animals* (Gawthorne, J. M., McHowell, J., & White, C. L., Eds.) pp 549–552, Springer, Berlin.

Morell, D. B., Chang, Y., & Clezy, P. S. (1967) *Biochim. Biophys. Acta 136*, 121–130.

Nichol, A. W., Hendry, I., Morell, D. B., & Clezy, P. S. (198) *Biochim. Biophys. Acta 156*, 97–108.

Nicholls, P. (1961) *Biochem. J. 81*, 374.

Ohms, J. P., Hagenmaier, H., Hayes, M. B., & Cohen, J. S. (1979) *Biochemistry 18*, 1599–1602.

Rifkind, J. M. (1981) *Met. Ions Biol. Syst. 12*, 191–232.

Rifkind, J. M. (1983) *Met. Ions Biol. Syst. 15*, 275–317.

Schoenborn, B. P., Watson, H. C., & Kendrew, J. C. (1965) *Nature (London) 207*, 28–30.

Sheard, B., Yamane, T., & Shulman, R. G. (1970) *J. Mol. Biol. 53*, 35–48.

Shulman, R. G., Peisach, J., & Wyluda, B. J. (1969) *J. Mol. Biol. 48*, 517–523.

Stolzenberg, A. M., Strauss, S. H., & Holm, R. H. (1981) *J. Am. Chem. Soc. 103*, 4763–4778.

Takano, T. (1977) *J. Mol. Biol. 110*, 537–568.

Tilton, R. F., Jr., & Kuntz, I. D., Jr. (1982) *Biochemistry 21*, 6850–6857.

Tomoda, A., Kakizuka, A., & Yoneyama, Y. (1983) *Biomed. Biochim. Acta 42*, 173–174.

Zwart, A., Buursma, A., Van Kampen, E. J., & Zijlstra, W. G. (1984) *Clin. Chem. 30*, 373–379.