The Preparation and Some Physical Properties of Sulfhemoglobin*

(Received for publication, September 30, 1977)

ROBERT J. CARRICO,‡ JACK PEISACH, AND JAMES O. ALSEN

From the Departments of Molecular Pharmacology and Molecular Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461, and the Department of Physiological Chemistry, College of Medicine, Ohio State University, Columbus, Ohio 43210

Sulfhemoglobins were prepared from hemoglobin A and carboxypeptidase A-treated hemoglobin. The synthesis was achieved by oxidation of the ferric hemoglobins with H2O2 followed by reaction with excess hydrosulfide.

EPR spectra of these ferric sulfhemoglobins at high pH were well resolved from the resonances of contaminating ferric hemoglobins. Thus, quantitative EPR measurements provided a convenient method for estimating the purity of the sulfohemoglobin preparations. Results of such measurements were used to calculate the optical absorption spectrum of pure sulfhemoglobin. The intensity of the absorption band near 620 nm, an often used optical marker for sulfhemoglobin, is about 2 times greater than reported previously (Beutler, E. (1972) in Hematology (Williams, W. J., Beutler, E., Ersley, A. J., and Rundles, R. W., eds) p. 1381, McGraw-Hill Book Co., New York). Furthermore, the extinction coefficient and position of this band varied with oxygen tension. Under anaerobic conditions, it is at 619 nm (ε619 = 21.4) and at 1 atm it shifts to 623 nm (ε623 = 25.3).

Addition of CO to sulfhemoglobin shifts the absorption maximum from 619 to 613 nm and the extinction coefficient increases by 26%. The infrared stretching frequency of bound CO is 10 cm⁻¹ lower than that for CO bound to hemoglobin A. Acid-base titration of high spin ferric sulfhemoglobin to the low spin form has a pK of 7.8 compared to 8.1 for ferric hemoglobin titrated under the same conditions.

Sulf-Hb was first observed by Hoppe-Seyer (1) in 1863 as the green product of the reaction between HbO2 and H2S. In 1933, Kolin (3) showed that oxygen is essential for this reaction and that the product exhibiting a strong optical absorption band at 618 nm was distinctly different from the compound formed by the binding of sulfide directly to the iron of ferric Hb. Within a few years, Drabkin and Austin (4) calculated an optical absorption spectrum for sulf-Hb and their extinction coefficient for the strong band near 620 nm has been used as the clinical standard for measurement of sulfhemoglobin in blood (5). More recently, Berzofsky et al. (6) made careful measurements of the optical absorption spectrum of sulf-Mb and their results suggest that the original extinction coefficient for sulf-Hb is inaccurate by as much as a factor of two.

Various attempts have been made to prepare sulf-Hb as a pure species. Early work had shown that addition of reducing agents to reaction mixtures containing H2S and oxy-Hb improved the yield (7, 8). Nichol et al. (9) synthesized sulf-Hb in high yield by reaction of HbO2 with ascorbate and H2S in the presence of catalytic amounts of phenylhydroxylamine. Nichols (10) was able to improve the yield even further by reacting ferric Hb sequentially with H2O2, to form the ferryl compound (11) and then with H2S. Berzofsky et al. (6) optimized the conditions of the latter reaction scheme with Mb and synthesized sulf-Mb in 90% yield. They noted that optical spectral ratios used by previous workers to estimate the purity of sulf-Hb do not adequately define the purity of the protein.

As a continuation of studies on sulfheme proteins (6, 12–14), we have synthesized sulf-Hb in 90% yield by a procedure similar to that used for sulf-Mb (6). This report describes some of the physical and chemical properties of sulf-Hb and CPA sulf-Hb prepared from CPA-Hb. These preparations were used in subsequent studies of reversible ligand binding.

MATERIALS AND METHODS

Preparation of Hemoglobin A - Blood was drawn from laboratory personnel and treated with heparin to prevent clotting. Subsequent purification steps were performed at 0 to 5°C. The red blood cells were separated from the plasma by centrifugation and washed twice with globin cyanide. CPA-Hb, carboxypeptidase A-treated hemoglobin from which the 2 terminal amino acid residues of the β chains have been removed (2). CPA sulf-Hb, sulfhemoglobin prepared from CPA-Hb, K-P04 potassium phosphate buffer.

‡ Present address, Miles Laboratories, Inc., Elkhart, Ind. 46514.

The abbreviations used are: Hb, hemoglobin A and Mb, myoglobin and these are preceded by "sulf" for the corresponding sulfhemoglobin compounds or followed by a symbol to indicate a bound ligand (or both), e.g. HbO2 for oxyhemoglobin or ferric HbCN for ferric hemo...
Preparation and Properties of Sulfhemoglobin

3 volumes of 0.15 M NaCl. Lysis was achieved by addition of 10 volumes of water, and stroma were removed by centrifugation at 30,000 × g for 30 min. The supernatant was dialyzed against I = 0.01 tris(hydroxymethyl)aminomethane buffer, pH 7.2. The conditions used for formation of this derivative, the prosthetic group of sulf-Hb, is converted to hemin (12). Therefore, the extinction coefficient for the pyridine-hemochromogen of protohemin was employed (21).

**Sulphhemoglobin—Concentrations of mixtures of heme and sulfheme proteins were measured by the pyridine-hemochromogen method.** To determine absolute purity (see below).

**Oxidation of ferrous sulf-Hb with K,Fe(CN)₅⁻ was accomplished by the procedure given above for ferrous Hb.**

**Measurement of Sulphhemoglobin**

Ferrous Hb was the major product formed by the reaction of ferrous sulf-Hb with K,Fe(CN)₅⁻. Under the conditions used for formation of this derivative, the prosthetic group of sulf-Hb is converted to hemin (12). Therefore, the extinction coefficient for the pyridine-hemochromogen of protohemin was employed (21).

**pH Titrations of Peric Hemoglobin and Peric Sulfhemoglobin**

Ferrous Hb was equilibrated with 0.1 M K-P₅₀₀, pH 8.0, and then was stored in 0.2 M NaCl. Lysis was achieved by addition of several microliters of 1 M NaOH accompanied by vigorous stirring. Occasionally, traces of precipitated protein appeared and were removed by centrifugation before optical spectra were recorded. The precipitate constituted a very small fraction of the total protein (<1%), as shown by pyridine-hemochromogen measurements at the beginning and end of the titrations. The titration data were analyzed by a computer-facilitated least squares fitting algorithm described previously (22).

**Optical Absorption Spectroscopy**

The spectra were measured with a Cary 14 spectrophotometer equipped with a 14R instrument using cells with a 1-cm light path. A cold ethanol/water solution was circulated through a jacketed cuvette holder for measurement of spectra at 0°C. For calculation of absorption spectra of pure sulf-Hb, the optical densities were digitized manually at 5-nm intervals (at 2-nm intervals near the peaks) and the contributions from contaminating heme proteins were subtracted.

**Anoecic protein solutions used for optical absorption measurements were prepared at 0°C in 500-ml spherical tonometers.** Optical measurements at O₂ pressures greater than 1 atm were conducted in a pressure vessel (14 cm diameter, 14 cm height). The solution contained 0.8 g of air/mg protein.

**EPR Spectroscopy**

EPR spectra were taken with an X-band spectrometer operating at microwave frequencies between 9000 and 9000 MHz (23). Protein solutions (0.70 ml) containing 50 to 100 µM heme or heme derivative were pipetted into an EPR cavity described previously (6), frozen in liquid N₂, and cooled to 1.5 K under pumped helium. Magnetic field calibrations were measured with a digital Hall probe which was standardized against the NMR frequency of H₂O. The magnetic field strength and the EPR spectrum were recorded simultaneously on a two-channel strip chart recorder and the microwave frequency was determined with a wavemeter. Quantitation of EPR spectra was accomplished by the method employed previously using a high spin ferric Mb standard (6).

**Measurement of Infrared Spectra**

A Digibal infrared interferometer with 2 cm⁻¹ resolution was employed. Sample cells equipped with CaF₂ windows provided a 0.05-cm light path. A digital computer program calculated a Fourier transform from the interferogram data. The interferograms were the sum of 1612 interferograms collected at a rate of 2 scans/sec. Positions of absorption maxima were determined to within ±0.5 cm⁻¹. Sulf-Hb CO was prepared and processed in a darkened room to minimize degradation of the prosthetic group by light (10).

**RESULTS**

**Factors Affecting Yield and Stability of Sulfhemoglobin**

As sulf-Hb is synthesized from ferryl Hb, the optimal conditions for formation and stability of the latter compound were determined. Reaction of ferric Hb with H₂O₂ was conducted at pH 7.0 and 0°C because human cells contain a 1-cm light path. A variation of the analogous compound, ferryl Mb, is developed. A concentration of 0.1 M K-P₅₀₀, pH 8.0, with a 5-min reaction time before addition of catalase gave maximal yield of the ferryl compound as determined by the intensity of the absorption maximum at 545 nm. In order to maximize the yields of sulf-Hb, the level of (NH₄)₂S and also the pH of reaction were varied. A 20-fold excess of (NH₄)₂S per heme added at pH 5.0 gave the best yield.

Ferrous sulf-Hb was the major product formed by the procedure described above (see results of EPR measurements).

**J. B. Wittenberg, B. A. Wittenberg, and L. Kampa, personal communication.**
The ferrous protein was stable for at least several months when stored in liquid nitrogen. A sample stored for 4 months was thawed and mixed with dithionite under anaerobic conditions. Changes in the optical absorption spectrum indicated that about 6% of the sulf-Hb was in the ferrous state. Spectra of the hydroxide derivatives were hydroxy form of the protein is derived from ferrous sulf-Hb preparation was 85% pure assuming that the ferric sulf-Hb hydroxide accounted for as much as 85% of total paramagnetic material. This means that the original sulfhemoglobin, ferric CPA sulfhemoglobin, and ferric hemoglobin sulfide and the minority having the same EPR as ferric Hb sulfide (6) (Table I).

Sulfides of ferric sulf-Hb and ferric Hb were detectable by EPR in a fresh preparation of ferrous sulf-Hb. Quantitation of the EPR showed that these components represented no more than 5% of the protein in the sample. The remainder of the sample was in the ferrous oxidation state and was thus EPR-silent. The same results were obtained with CPA sulf-Hb.

Ferrous sulf-Hb was oxidized with K₂Fe(CN)₆ and examined by EPR in a K-P, buffer, pH 5.4. The major component was a high spin species with absorption derivative features near g = 6 and g = 2. Much weaker resonances attributed to the low spin forms of ferric Hb and ferric sulf-Hb were also detected. Results of EPR measurements for ferrous and ferric CPA sulf-Hb were essentially the same as those for the corresponding forms of sulf-Hb.

Adjustment of a ferric sulf-Hb solution to pH 10 by addition of glycine buffer (final concentration, 0.4 M) alters the EPR radically. The resonances of the high spin ferric iron seen at pH 5.4 were replaced by those of a low spin ferric species. The EPR of this new material is quite similar to that observed for ferric sulf-Hb at high pH (6) and is thus ascribed to ferric sulf-Hb hydroxide (Table I). In addition, the EPR of the ferric sulf-Hb sulfide minority species described above was observed along with a minority of ferric Hb hydroxide which is derived from residual unreacted hemoglobin in the sample.

Quantitation of the EPR from each of the three low spin ferric species observed at high pH showed that the concentration of ferric sulf-Hb hydroxide accounted for as much as 85% of total paramagnetic material. This means that the original ferrous sulf-Hb preparation was 95% pure assuming that the hydroxy form of the protein is derived from ferrous sulfhemoglobin which was present before oxidation and adjustment of pH.

As has been demonstrated by Nichol et al. (9), ferric sulf-Hb binds reversibly to many of the same ligands as does ferric Hb. The addition of cyanide to ferric sulf-Hb converts to it a low spin cyanide derivative. The EPR parameters are given in Table I. The EPR of CPA sulf-Hb cyanide, hydroxide, and sulfide, are virtually the same as the corresponding derivatives of sulf-Hb. Therefore, the purity of CPA sulf-Hb preparations could also be determined by means of EPR measurements at alkaline pH. Here, the purity was determined as 85%.

Optical Absorption Spectra of Ferrous Sulfhemoglobin — The optical absorption spectrum of freshly prepared sulf-Hb equilibrated with atmospheric oxygen has an intense band near 621 nm and weaker bands at 544 and 575 nm (Fig. 1). The positions and intensities of these bands vary reversibly with O₂ concentration. As the O₂ pressure was increased to 1 atm, the 621 nm band increased in strength and shifted to 623 nm. The protein appeared to be saturated at 1 atm of oxygen because further changes in the spectrum were not detectable at pressures up to 130 atm.

At very low O₂ pressure (less than 0.02 mm of O₂), the absorption band shifts to 619 nm and decreases in strength. The bands at 544 and 575 nm also diminished, while the absorption increased in the region of 550 to 565 nm. The spectral changes in the 540 to 575 nm region are attributed in part to deoxygenation of ferrous-Hb which is present as a contaminant in sulf-Hb preparations. This conclusion is supported by the observation of ferric HbOH, detected by EPR, in alkaline preparations of oxidized sulf-Hb.

The optical contributions of oxy- and deoxy-Hb (about a 10% impurity) were stripped from the optical absorption spectra of oxy- and deoxy-sulf-Hb, respectively, utilizing EPR data to estimate the relative contributions of each species. Only the contributions of Hb were stripped from the spectra since the sulfides of ferric Hb and ferric sulf-Hb represented a smaller proportion of the contaminants in the ferrous sulf-Hb preparation and it was therefore assumed that they did not contribute substantially to the optical spectra. Fig. 2 shows spectra calculated in this way for pure oxy- and deoxy-sulf-Hb. The three absorption bands between 500 and 600 nm, in addition to the intense band near 620 nm, are readily apparent in both spectra. Optical absorption data for the Soret band and the intense absorption band near 620 nm are listed in Table II. The millimolar extinction coefficient of oxy-sulf-Hb at 625 nm

---

**Table I**

Magnetic parameters of some low spin derivatives of ferric sulfhemoglobin, ferric CPA sulfhemoglobin, and ferric hemoglobin.

The proteins were oxidized to the ferric form with K₂Fe(CN)₆ as described under “Materials and Methods.” Cyanide and sulfide concentrations were 5 mM. Spectra of the hydroxide derivatives were measured with solutions adjusted to pH 10 with glycylglycine buffer.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Ferric Sulf-Hb</th>
<th>Ferric CPA Sulf-Hb</th>
<th>Ferric Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g₁</td>
<td>g₂</td>
<td>g₃</td>
</tr>
<tr>
<td>OH⁻</td>
<td>2.44</td>
<td>2.21</td>
<td>1.88</td>
</tr>
<tr>
<td>CN⁻</td>
<td>2.74</td>
<td>2.40</td>
<td>1.65</td>
</tr>
<tr>
<td>SH</td>
<td>2.36</td>
<td>2.25</td>
<td>1.93</td>
</tr>
</tbody>
</table>

* — not measured.
Preparation and Properties of Sulfhemoglobin

**FIG. 2.** Computed optical absorption spectra for oxy- and deoxy-sulfhemoglobin. Optical absorption spectra of a sulfhemoglobin preparation in 0.1 M K-P$_2$, pH 8.2, at 0°C were measured under anaerobic conditions and at 1 atm of O$_2$. Results of EPR measurements were used to calculate the relative amounts of Hb and sulf-Hb in the preparation. The optical spectra of pure oxy- and deoxy-sulf-Hb were obtained by manually subtracting the contributions of oxy- and deoxy-Hb, respectively.

**TABLE II**

Optical maxima and extinction coefficients for deoxy-, oxy-, and carboxysulfhemoglobin

<table>
<thead>
<tr>
<th>Species</th>
<th>Absorption maxima</th>
<th>Extinction coefficient ($\epsilon_{max}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxy</td>
<td>619</td>
<td>21.4</td>
</tr>
<tr>
<td>Oxy</td>
<td>623</td>
<td>25.3</td>
</tr>
<tr>
<td>Carboxy</td>
<td>618</td>
<td>27.9</td>
</tr>
</tbody>
</table>

The optical maximum for deoxy-sulf-Hb at 619 nm is 25.3% or 28% greater than that of the deoxygenated protein at 619 nm.

Equilibration of deoxy-sulf-Hb with CO at atmospheric pressure reversibly shifts the major absorption in the visible from 619 to 613 nm with an increase of 26% in the extinction coefficient (Table II).

**Optical Absorption Spectra of Ferric Sulfhemoglobin** — EPR data discussed above indicate that ferric sulf-Hb at pH 5.4 is primarily high spin. Fig. 3 shows the optical absorption spectrum of a preparation of ferric sulf-Hb at this pH. The spectrum has an absorption band in the near infrared region ($\lambda_{max} = 717$ nm), and a stronger one at 596 nm in the visible (see Fig. 3). At elevated pH, the EPR spectrum of ferric sulf-Hb is converted to that of the low spin hydroxide derivative. Under similar conditions of pH, the optical absorption band at 717 nm diminishes and a new one appears at 667 nm.

**Acid-Base Titrations of Ferric Sulfhemoglobin** — In the absence of competing ions, the heme iron of ferric Hb binds water (24). Protons dissociate reversibly from this water according to the reaction:

$$\text{Heme-H}_2\text{O} \rightleftharpoons \text{Heme} + \text{OH}^- + \text{H}^+$$

The extent of this reaction as a function of pH has been measured optically (24). Similar acid-base titrations can be performed with ferric sulf-Hb by measurement of changes in the absorbance at 717 nm. Good isosbestic points were obtained in the region of the optical spectral measurements (640 to 800 nm). A plot of optical absorbance at 717 nm against pH is presented in Fig. 4. A least squares fit of these data gave a pK of 7.8 for the proton dissociation. Similar titrations were conducted with ferric Hb utilizing changes in absorbance at 576 nm, and a pK of 8.1 was obtained. This value is the same as that measured by Antonini et al. (24).

**Infrared Measurements** — The CO stretching frequency of gaseous CO occurs in the infrared region at 2143 cm$^{-1}$. Binding of CO to hemoglobin A in 0.1 M K-P$_2$ buffer, pH 8.2, shifted this frequency to 1951.6 cm$^{-1}$, which agrees well with results of previous measurements by Caughey et al. (25). The stretching frequency which we determined for CPA-HbCO is the same as that for Hb. A preparation of sulf-HbCO showed two infrared absorption bands, one at 1951.6 cm$^{-1}$ arising from
Drabkin and Austin (4) made the first quantitative spectrophotometric measurements on sulf-Hb and calculated a millimolar extinction coefficient of 10.6 for the absorption maximum near 620 nm, assuming 100% conversion of Hb to sulf-Hb. Our values for the extinction coefficient are approximately 2 times greater indicating that they had about 50% conversion to sulf-Hb. Nijveld (27) used the Drabkin and Austin (4) extinction coefficient to calculate the stoichiometry of oxidation-reduction titrations of sulf-Hb with $K_{Fe(III)}$. He reported that only half an equivalent of $K_{Fe(III)}$ will oxidize the prosthetic group. Recalculations of his results using our extinction coefficient show that about 1 eq of sulfhemoglobin is oxidized per eq of $K_{Fe(III)}$. Thus, sulf-Hb, with the optical spectrum shown in Fig. 2, appears to be in the ferrous oxidation state. This conclusion is supported by our observations that sulf-Hb does not have an EPR spectrum, and furthermore, that the optical spectrum does not change significantly on addition of dithionite. In addition, titrations conducted with sulf-Mb and $K_{Fe(III)}$ demonstrated that 1 oxidizing eq is required for conversion of the protein from the ferrous to the ferric form (14).

Like hemoglobin, ferric sulf-Hb can be titrated to a low spin form on addition of alkali. The pH values for these titrations were 7.8 and 8.1 for sulf-Hb and Hb, respectively, which means that the equilibrium constants for these reactions differ by a factor of two. Assuming that the protein structure in the vicinity of the prosthetic group was not altered on formation of the sulfhemoglobin protein, the titration data indicate that the O—H bond of the water presumably bound at the iron of the sulfhemoglobin is weaker than that bound to ferric Hb. Additional differences in bond strengths of ligands bound to the prosthetic groups of Hb and sulf-Hb are seen with CO bound to the ferrous proteins if one assumes that the local protein environment of the prosthetic group is not altered. The stretching frequency of CO bound to Hb is 1951.6 cm$^{-1}$ which is 10 cm$^{-1}$ lower than 1961.4 cm$^{-1}$ for sulf-Hb CO. This difference in bond energies appears to reflect differences in the electronic structures of the prosthetic groups. The implications of these differences in terms of $O_2$ and CO-binding affinities will be discussed in the following paper of this series.\(^2\)

REFERENCES


---

**Preparation and Properties of Sulfhemoglobin**

Fig. 5. An infrared spectroscopic comparison of the CO stretching frequency of carbon monoxide-ligated hemoglobin A (Hb A), sulfhemoglobin (Sulf-Hb), and sulfhemoglobin prepared from carboxypeptidase A-treated hemoglobin A (CPA Sulf-Hb). As can be seen, the hemoglobin A contamination is greater in the sulfhemoglobin protein prepared from carboxypeptidase-treated hemoglobin.

HbCO in the preparation and one at 1961.4 cm$^{-1}$ arising from sulf-HbCO (Fig. 5). Carbon monoxide bound to CPA sulf-Hb has the same stretching frequency as carbon monoxide bound to sulf-Hb.

**DISCUSSION**

The procedure described by Berzofsky et al. (6) for synthesis of 90% pure sulf-Mb gave only 40 to 60% yields of the sulfhemoglobin when applied to Hb. We varied the reaction conditions and found that longer reaction time with $H_2O_2$ and buffering the $(NH_4)_2S$ solution at pH 5.0 increased the yields to as high as 85%.

Earlier workers estimated the purity of their sulf-Hb preparations from the optical absorption ratio $A_{590 nm}/A_{630 nm}$ (9, 26). This ratio does not provide a satisfactory estimate of purity because the intensity of the absorption band near 620 nm, as well as the one at 580 nm, varies markedly with oxygen pressure. For example, at 1 atm of $O_2$, the ratio can be as high as 3.5 for pure sulf-Hb while at low $O_2$ pressure it is 2.6 (Fig. 2).

A better estimation of the purity of sulf-Hb preparations is provided by EPR measurements. Since the EPR of ferric sulf-HbOH is well resolved from the resonances of impurities in sulf-Hb preparations, integration of the spectra provides a reliable estimate of the relative amounts of each compound in a preparation. Results of quantitative EPR measurements were used to calculate optical spectra for pure oxy- and deoxy-sulf-Hb (Fig. 2). The millimolar extinction coefficient for pure deoxy-sulf-Hb at 619 nm is 21.4. At high $O_2$ pressure, the maximum shifts to 623 nm and increases in intensity by 18%. These extinction coefficients are in the same range as that reported for pure sulf-Mb (6). However, the intensity of the absorption band of sulf-Mb ($\varepsilon_{580} = 24$) remains nearly constant and shifts 7.3 nm to the red during oxygenation of the protein (14). The changes in the optical absorption spectrum of sulf-Hb when the $O_2$ concentration is varied are the first reported evidence that the protein binds this ligand. More detailed results of $O_2$ binding measurements are reported separately.\(^3\)
Preparation and Properties of Sulfhemoglobin

The preparation and some physical properties of sulfhemoglobin.
R J Carrico, J Peisach and J O Alben


Access the most updated version of this article at [http://www.jbc.org/content/253/7/2386.citation](http://www.jbc.org/content/253/7/2386.citation)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/253/7/2386.citation.full.html#ref-list-1) to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/253/7/2386.citation.full.html#ref-list-1](http://www.jbc.org/content/253/7/2386.citation.full.html#ref-list-1)