

Biochemical and Functional Properties of Recombinant Human Sickle Hemoglobin Expressed in Yeast*

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Previous studies had indicated that recombinant and natural human sickle hemoglobin had similar chemical properties (Martin de Llano, J. J., Schneewind, O., Stetler, G., and Manning, J. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 918-922). In the present study, additional biochemical and physiological characterization of some primary through quaternary structural features of recombinant sickle hemoglobin are described. The molecular weight of the purified recombinant sickle hemoglobin was identical to natural sickle hemoglobin as determined by mass spectrometry, thus excluding extensive post-translational modification in the yeast system. Carboxypeptidases A and B together catalyzed the release of COOH-terminal amino acids at the same rate for recombinant and natural hemoglobin S, consistent with identity in their primary and secondary structures in this region of the molecule. The tryptic peptide maps of natural and recombinant hemoglobins were practically indistinguishable, indicating the same internal protein sequences for recombinant and natural hemoglobins. As a probe of the secondary structure of recombinant sickle Hb, the reactivity of the SH group of Cys-93(β) was investigated for the glutathione sickle hemoglobin adduct, which has significant anti-gelling and anti-sickling properties. The position of glutathione at Cys-93(β) was established by direct mass spectrometric analysis of enzyme digests; reduction of this derivative to the unmodified chains was also observed by mass spectrometry and by isoelectric focusing. The oxygen equilibrium curves of recombinant and natural sickle hemoglobin at high protein concentration were superimposable with identical Hill coefficients of 3.3. The response of recombinant sickle hemoglobin to chloride with respect to a lowered oxygen affinity was identical to that of natural sickle hemoglobin. The gelation properties of recombinant and natural sickle hemoglobins were identical at the high hemoglobin concentrations that occur in the red cell. Therefore, the yeast expression system synthesizes a completely functional recombinant sickle hemoglobin with the same biochemical and physiological

properties as natural sickle hemoglobin with respect to features characteristic of its primary through quaternary structures.

In sickle cell anemia, the abnormal hemoglobin S has the hydrophobic side chain of Val-6(β) substituted for the hydrophilic side chain of Glu-6(β) of hemoglobin A (1, 2). This substitution, which is on the exterior of the protein, leads to the aggregation of deoxygenated hemoglobin S tetramers, and distorts erythrocytes in the venous circulation. Although the initial point of aggregation is between Val-6(β) and the Phe-85(β)/Leu-88(β) region of adjacent HbS tetramers, there are other points of contact in the aggregate that serve to strengthen the overall stability of the polymer at low oxygen tensions (3-7). Although the identities of many contact sites in the aggregate are known, there is a lack of quantitative information on their relative contribution to the overall strength of the aggregate. Some of the known contact sites between tetramers are amenable to study with specific chemical modifying agents (8). However, such sites are usually hydrophilic in nature with enhanced reactivity because of their location in the protein. The introduction of recombinant DNA technology considerably broadens the scope of studies on the aggregation of sickle hemoglobin and opens the possibility of assessing the contribution of any interaction to the overall strength of the sickle hemoglobin polymer.

We have recently reported the expression of human sickle hemoglobin in the yeast *Saccharomyces cerevisiae* that carries a plasmid containing the human α - and β -globin cDNA (9); this system has several advantages over other systems that express hemoglobin such as correct NH₂-terminal processing and other features described under "Discussion." In those chemical properties that were evaluated, we found that the human sickle hemoglobin produced in yeast behaved like natural hemoglobin S (9). The present article examines the biochemical and functional properties of the recombinant HbS that are characteristic of the primary through quaternary features of hemoglobin. Such a thorough evaluation of the properties of recombinant HbS is considered a prerequisite to subsequent mutations on the hemoglobin molecule.

MATERIALS AND METHODS

Isolation of Natural Sickle Hemoglobin—It was considered important to document the source of the natural sickle hemoglobin samples that

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were used for comparison with the recombinant sickle hemoglobin. Venous blood samples from patients with sickle cell anemia were collected into vacutainer tubes that contained either heparin or EDTA as anticoagulant. The patients were clinically in a steady-state, had not received a blood transfusion within the prior 4 months, and were not chronically on any medication other than folic acid. Patients who donated blood samples for analysis of HbS function were young adults with homozygous sickle cell anemia documented by hemoglobin electrophoresis on alkaline cellulose or citrate agar; their hematocrits ranged from 24 to 29%, Hb from 80 to 97 g/liter. HbA₂ was not elevated. Natural sickle hemoglobin was prepared from red blood cells of such patients by procedures described previously (8). The new methods that were used for improved purification, as described below, were applied to both natural and recombinant sickle Hb for the studies described herein.

Preparation of Recombinant Sickle Hemoglobin—In our initial report on recombinant HbS in yeast (9) the cells were broken in an Omni-Mixer (Omni International, Waterbury, CT). In recent studies, we have found more efficient and more rapid breakage with a Bead Beater homogenizer (Biospec Products, Bartlesville, OK). With both methods it is important to bubble CO for several minutes into the suspension of the yeast cells in cold extraction buffer (9) prior to the homogenization in order to maintain the full function of the isolated hemoglobin (see below); all buffers used thereafter during purification were also bubbled with CO. In the initial fractionation of recombinant human sickle hemoglobin expressed in yeast, most of the non-heme yeast protein did not adhere to the conventional CM-52 cation exchange system; recombinant sickle hemoglobin eluted as a symmetrical component except for a small amount of heme-containing protein that eluted just prior to the main band (9). In the present article a batch procedure for the purification of larger amounts of the recombinant Hb and a new HPLC¹ purification procedure for the removal of minor contaminants are described.

Batch Purification of Recombinant Sickle Hemoglobin—The supernatant solutions (~300 ml), which were obtained from 3 batches of 70 g of yeast cells that were broken by the extraction procedure described previously (9), were mixed and dialyzed overnight against three changes of 12 liters each of 10 mM potassium phosphate, 0.5 mM EDTA, 0.5 mM EGTA, pH 5.85, that had been bubbled with CO. After centrifugation at 15,000 × g for 30 min at 4 °C, the supernatant containing the recombinant sickle Hb was mixed with 1.6 ml of carboxymethylcellulose (CM-52) resin equilibrated in the same buffer. The bright red slurry was stirred slowly for 1 h at 4 °C and then collected on a Millipore fritted glass filter, washed with 5–10 ml of buffer, and removed from the filter. Another 0.6 ml of the resin was added to the filtrate and the mixture was filtered again as described above. With this treatment more than 80% of the heme-containing protein was retained by the resin as determined by the absorbance in the 530–580 nm range for the initial and final filtrates. These resins (2.2 ml) were combined and added to the top of the bed of a CM-52 column (0.9 × 20 cm, final size) previously equilibrated in the same buffer. A linear gradient (150 ml of each buffer) between this buffer and 15 mM potassium phosphate, 0.5 mM EDTA, 0.5 mM EGTA, pH 8.0 (both bubbled with CO), was applied. The absorbance of the elute was monitored at 280 and 540 nm. Those fractions containing hemoglobin were pooled, bubbled with CO, and kept at –80 °C for further analysis.

Purification of Recombinant Sickle Hemoglobin by HPLC—After chromatography on CM-52, there were still some minor contaminants. For some purposes these minor impurities were tolerable but for the gelation and functional studies reported here, it was desirable to remove them. Further purification was achieved on a SynChropak CM 300 column (250 × 4.6 mm) (SynChrom, Inc.) on a Shimadzu LC-6A HPLC system at room temperature; a guard column (50 × 4.6 mm) was inserted between the injector and the column. The buffers used were similar to those previously described (10, 11), i.e. 30 mM bis-Tris, 1 mM EDTA, pH 6.4 (buffer A), and 30 mM bis-Tris, 150 mM sodium acetate, 1 mM EDTA, pH 6.4 (buffer B). Both buffers were filtered through 0.45-μm filter and degassed. The CO-hemoglobin sample was dialyzed against 30 mM bis-Tris, 30 mM sodium acetate, 1 mM EDTA, pH 6.4 (bubbled with CO), and concentrated if necessary; 200–400 μl were injected onto the column. The gradient used was from 20 to 70% buffer B over 10 min, 70 to 100% buffer B over 50 min, and then 15 min 100% buffer B, at a flow of 1 ml/min; the elution of the proteins was monitored at 280 nm or at 410 or 540 nm. The fractions comprising the major peak (Fig. 1) were pooled, bubbled with CO, and kept at –80 °C. Natural hemoglobin S was

also purified from hemolyzates of sickle cells by this procedure in order to compare its biochemical and functional properties with those of recombinant HbS.

Separation of Recombinant Globin Chains by Reverse Phase HPLC—Separation of the globin chains of natural and recombinant sickle hemoglobin was achieved by HPLC on a Vydac C-4 column (250 × 4.6 mm) at a flow of 1 ml/min with a gradient from 37.6 to 40.0% acetonitrile with 0.1% trifluoroacetic acid over a 6-min period and then to 44.8% during a 60-min period. In this system there was complete separation of the globin chains.

Carboxypeptidase Digestion of Recombinant and Natural HbS—Recombinant and natural hemoglobin S (0.5 mg of each), purified by the HPLC procedure described above, were subjected to digestion with 0.8% (w/w) carboxypeptidase A and 0.5% (w/w) carboxypeptidase B for 15 min at 37 °C in 70 μl of 75 mM Hepes, pH 8.0. The samples were then acidified to pH 3 with dilute HCl and centrifuged in an Amicon Centricron apparatus (10,000 molecular weight cut off). The filtrate was dried in a Savant Concentrator and the residue was dissolved in 0.1 ml of sodium citrate, pH 2.2. Analysis of amino acids released by the treatment with the carboxypeptidases was performed on a Beckman 6300 analyzer with a System Gold data handling system.

Tryptic Peptide Mapping of Recombinant and Natural HbS—Native HbS (3 mg) and recombinant HbS (r-HbS) (0.5 mg) were reduced and carboxymethylated as described by Crestfield *et al.* (12). After extensive dialysis against 0.1 M NH₄HCO₃, the samples were lyophilized. Trypsin (60 μl of 1 mg/ml in 0.1 M NH₄HCO₃) was added to natural HbS; 10 μl of the same trypsin solution was added to r-HbS to give a 2% (w/w) trypsin concentration for each sample. After incubation for 17.5 h at 37 °C, the samples were lyophilized to dryness, dissolved in H₂O and re-lyophilized. The residues were dissolved in 0.1% trifluoroacetic acid (about 1 ml) and 10% of the sample was loaded onto a C-18 Vydac HPLC column. The peptides were eluted with a linear gradient 0–50% B over 90 min and then 50–95% B over 30 min; A = 0.1% trifluoroacetic acid; B = 80% acetonitrile, 0.1% trifluoroacetic acid.

Isoelectric Focusing (IEF)—The pH 6–8 precast Hb Resolve system from Isolab was used. After application of the samples, the gel was subjected to a constant power of 10 watts (small gel) or 15 watts (large gel) for 40–60 min at 15 °C. After being fixed in 10% trichloroacetic acid for 20 min, the gel was washed extensively with several changes of H₂O, air-dried, and then stained with bromophenol blue for 5 min; destaining was performed in the ethanol/acetic mixture recommended by Isolab.

Preparation of Glutathione Derivative of Hemoglobins—Natural or recombinant HbS was incubated with 200 mM oxidized glutathione (GSSG) at pH 8.9 for 16 h at 25 °C. After dialysis against 10 mM potassium phosphate, pH 6.0, the treated natural hemoglobin was purified on a Toyo-Pearl CM 650 column (25 × 10 cm), which was eluted with a stepwise gradient of 10 mM potassium phosphate, pH 6.0, containing 0, 75, 100, or 150 mM sodium chloride.

Treatment of the Glutathione Derivative of Hemoglobins with Reducing Agents—Samples of glutathione-modified sickle hemoglobin were treated with a variety of reducing agents including 2-mercaptoethanol, dithiothreitol, or glutathione for varying times at different concentrations (1–50 mM) at room temperature. The effects of such treatments on glutathione-modified or unmodified hemoglobin samples were determined by the IEF system described above.

Analysis for the Glutathione Adduct by Amino Acid Analysis—In order to quantitate the number of glutathione moieties incorporated per Hb tetramer, the glutathione adduct prepared from natural HbS was treated with 0.1 M DTT in 10 mM potassium phosphate, pH 8.0, at room temperature for 60 min. The globin was then precipitated by addition of 10 volumes of ice-cold acetone containing 0.75% hydrochloric acid. The samples were permitted to stand for 15 min on ice and were then centrifuged in a microcentrifuge at 4 °C for 15 min; the supernatant was re-centrifuged twice. Aliquots were dried and subjected to amino acid analysis on a Beckman 6300 instrument with a System Gold attachment after hydrolysis in 6 N HCl for 20 h. Unhydrolyzed samples served as blanks.

Mass Spectrometry Analysis—Samples of tetrameric hemoglobin were subjected to mass spectrometric analysis on a matrix-assisted laser desorption time-of-flight mass spectrometer constructed at Rockefeller University and described elsewhere (13–15). The mass spectra were acquired by adding the individual spectra from 200 laser shots. Hemoglobin samples were prepared for laser desorption mass analysis as follows: the laser desorption matrix material (25–50 mM of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) or α-cyano-4-hydroxycinnamic acid) dissolved in formic acid/water/isopropyl alcohol, 1:3:2 (v/v/v). The hemoglobin sample in 10 mM potassium phosphate solution, pH 8, was then added to the matrix solution to give a final hemoglobin

¹ The abbreviations used are: HPLC, high pressure liquid chromatography; r, recombinant; IEF, isoelectric focusing; DTT, dithiothreitol; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

concentration of approximately 2 μM . A small aliquot (1 pmol) of this mixture was applied to the metal probe tip and dried with forced air at room temperature. The sample was then inserted into the mass spectrometer and analyzed. Horse heart myoglobin was used to calibrate the mass spectra.

Location of Glutathione Adduct by Mass Spectrometry of Enzyme Digests—Recombinant HbS either unmodified or modified with glutathione by synthesis (r-HbSSG) was digested with endoproteinase Glu-C (Boehringer Mannheim) in the absence or presence of a reducing agent, which was added before the digestion. In some studies, digestion with endoproteinase AspN was also performed. The resulting peptide mixtures were analyzed by matrix-assisted laser desorption mass spectrometry. For the endoproteinase GluC digestion, either 5 μl of 50 mM ammonium acetate, 4 M urea, pH 6.3 (non-reducing conditions), or 5 μl of 50 mM ammonium acetate, 4 M urea, 100 mM DTT, pH 6.3 (reducing conditions), were added to 5 μl of 50 mM ammonium acetate containing the hemoglobin samples (r-HbS, 20 μM ; or r-HbSSG, 30 μM). Endoproteinase GluC (1 μl of 0.1 mg/ml) in water was added to give an enzyme to substrate ratio of 1:70 (w/w) for r-HbS and 1:100 (w/w) for r-HbSSG. The incubations were carried out at 25 $^{\circ}\text{C}$ for various time intervals. An aliquot of the reaction mixture (1 μl) was added to 19 μl of the laser desorption matrix (4-hydroxy- α -cyanocinnamic acid) in formic acid/water/isopropyl alcohol, 1:3:2 (v/v/v), and analyzed as described above. For the digestion with endoproteinase AspN, 5 μl of 50 mM ammonium acetate, 4 M urea, pH 6.3, was added to 5 μl of 50 mM ammonium acetate containing the hemoglobin samples (r-HbS, 20 μM ; or r-HbSSG, 30 μM). Endoproteinase AspN (2.5 μl of 0.04 mg/ml) was added to give an enzyme to substrate ratio of 1:70 for r-HbS and 1:100 for r-HbSSG. The incubations were carried out at 37 $^{\circ}\text{C}$ for various time. Further analysis was done as described for the endoproteinase GluC digest.

Functional Studies—Recombinant or natural HbS, after HPLC purification on the CM-300 HPLC column as described above, was dialyzed against the appropriate buffer that had been bubbled with CO. The carbonmonoxy derivative of HbS was converted to the oxy derivative by two exposures to 200 watts of incandescent light in a round bottom flask in an atmosphere of pure O₂ as described previously (8). The conversion of CO-Hb to oxy-Hb was verified by spectral analysis on a Cary 2200. Each Hb sample was then concentrated using Amicon Centricon and Centriprep microseparation devices just prior to determination of its oxygen equilibrium properties on a modified Hem-O-Scan instrument. From the spectrum of the sample the amount of methHb was estimated to be <5%. Hill coefficients were calculated as described previously (8).

In order to measure the effect of chloride on the oxygen affinity of hemoglobin, solutions of NaCl in 50 mM bis-Tris acetate buffer, pH 7.5, were mixed with the hemoglobin sample (final Hb concentration: 0.4–0.5 mM) and the P₅₀ was measured at 37 $^{\circ}\text{C}$.

Measurement of Gelation Concentration—The method of Benesch *et al.* (16) which has a precision of ± 1 gm/dl, was employed. This procedure depends upon the sudden decrease in oxygen affinity when the sickle hemoglobin concentration reaches the critical value at which gelation begins; the junction of the biphasic lines is taken as the gelation concentration (c*). After removal of the CO as described above, the sample was concentrated to >300 mg/ml with an Amicon Microcon 10 device (10,000 molecular weight cut-off). The samples were in 0.1 M potassium phosphate buffer, pH 6.8; the P₅₀ was measured at 37 $^{\circ}\text{C}$ on a modified Hem O Scan instrument. Aliquots of concentrated samples were subjected to amino acid analysis as described above to calculate the hemoglobin concentration.

RESULTS

Purification of Recombinant Sickle Hemoglobin (r-HbS)—In a previous article (9) we reported the purification of recombinant HbS on a conventional cation exchange carboxymethylcellulose (CM-52) column. In that system, there was a minor hemoglobin fraction that eluted just before the main component. Heavily loaded IEF gels showed the presence of minor components. In order to perform the functional studies on the recombinant HbS described in this article, it was desirable to have a completely pure recombinant HbS sample. With this HPLC system (Fig. 1) it was possible to remove several minor components from the r-HbS sample obtained after chromatography on CM-52. The elution pattern is the same at 540, 410, and at 280 nm, indicating that these minor components were probably heme proteins. The small peaks eluting after the main

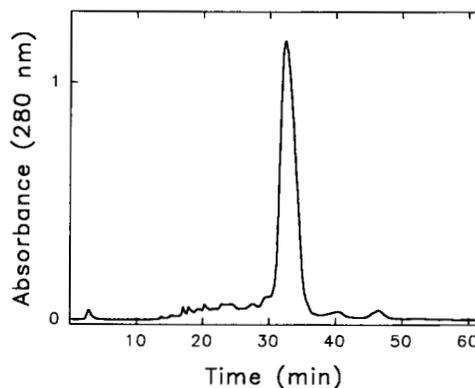


FIG. 1. Purification of recombinant human sickle hemoglobin by HPLC. The hemoglobin obtained from the yeast extract prepared as described previously by chromatography on CM-52 (9) was applied to a SynChropak CM300 column as described in the text.

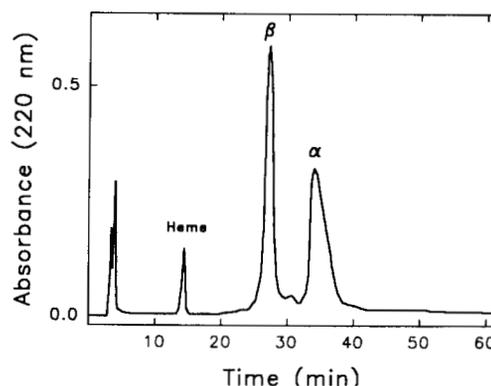


FIG. 2. Separation of α and β recombinant globin chains by HPLC. The natural and recombinant HbS was first purified on CM-52 (9) and then chromatographed on a Vydac C-4 column as described in the text. Both natural and recombinant HbS gave the same elution profile.

r-HbS component are probably oxidized at the heme as indicated by their spectra (data not shown). It is necessary to exclude these oxidized hemoglobins prior to determination of the gelation concentration. The same procedures were applied to the natural sickle hemoglobin purified from human red cells.

HPLC Separation of Recombinant Globin Chains—Separation of the globin chains of natural and recombinant sickle hemoglobin was achieved by HPLC on a Vydac C-4 column as described under "Materials and Methods." In this system there is complete separation of the globin chains (Fig. 2). The elution position of the chains of recombinant sickle Hb coincided with those from natural HbS.

COOH-terminal Analysis of rHbS—Previously, we reported that the first 8 NH₂-terminal amino acids in the sequence of the α - and β -chains of the recombinant sickle Hb were identical to those of natural HbS (9). In order to ensure that the COOH terminus also had the correct sequence, the tetrameric recombinant HbS was subjected to treatment with a mixture of carboxypeptidase A and B. The results, which are shown in Table I, indicated that the COOH terminus of the α -chain was more prone to carboxypeptidase digestion than the COOH terminus of the β -chain. This behavior was found to be true for both natural and recombinant HbS. These experiments were performed on the native chains and the digest was deliberately limited so that any differences, if they existed, could be discerned. Thus, the amount of Arg released from both hemoglobins (3.1 and 3.5 nmol) was about one-half the theoretical value (6.8 and 7.8 nmol of Arg from recombinant and natural chains, respectively (Table I)). Since the amino acids and their rate of

TABLE I

Release of COOH-terminal residues of the α -chain of natural and recombinant sickle hemoglobin by carboxypeptidases A + B

Recombinant HbS tetramer (3.4 nmol) and natural HbS tetramer (3.9 nmol) were digested with a mixture of carboxypeptidases A + B as described in the text. Complete digestion of both α -chains per tetramer would have yielded 6.8 nmol of Arg from recombinant HbS and 7.8 nmol of Arg from natural HbS. The low values for His were taken as having been derived from the COOH terminus of the β -chain of Hb. A corresponding value for Tyr was subtracted from the total value for Tyr to represent the amount derived from the α -chain.

Amino acid	Recombinant HbS	Natural HbS
	<i>nmol</i>	
Arg	3.1	3.5
Tyr	0.6	0.6
Lys	0.7	0.5

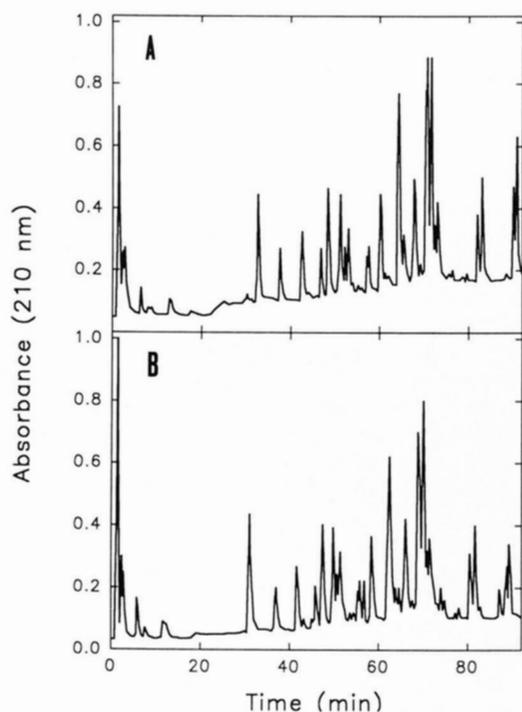


FIG. 3. Tryptic peptide maps of natural and recombinant HbS. The hemoglobins were isolated, reduced, and carboxymethylated, and digested with trypsin. Chromatography was performed on a Vydac C-18 column as described in the text. A, peptide map of recombinant HbS; B, peptide map of natural HbS.

release were the same for both recombinant and natural HbS, the COOH-terminal region of the α -chain of recombinant HbS has the same structural features as the corresponding region of natural HbS.

Tryptic Digestion of Natural and Recombinant HbS—In order to obtain information on the interior regions of the recombinant globin chains, the tetrameric proteins of natural and recombinant HbS were subjected to extensive digestion with trypsin and the digests were subjected to HPLC analysis. The pattern of the tryptic peptide maps in Fig. 3, A and B, for recombinant HbS and natural HbS, respectively, were practically identical. The number of peptides found in the digest was approximately correct, *i.e.* 29 different peptides and 1 free amino acid released from both α - and β -chains. These results are consistent with the conclusion that the recombinant sickle globin chains have the same internal sequence as natural HbS.

Preparation and Properties of Glutathione-modified Sickle Hemoglobin—In natural hemoglobin S, like hemoglobin A, the SH group of Cys-93(β) is highly reactive in the oxy conformation. However, in the deoxy conformation, the reactivity of Cys-

93(β) with various chemical modifiers is significantly reduced because it is shielded by the salt bridge between Asp-94(β) and His-146(β) (17). Hence, this reactivity is a measure of the secondary structure of hemoglobin in that region of the molecule. Glutathione (GSSG) is a modifier that undergoes a disulfide exchange reaction with some SH groups (18, 19). However, it is thought to react preferentially with Cys-93(β) of Hb, although this site of modification has never been fully documented or shown that it is exclusive. For that reason as well as the report that the glutathione adduct has a beneficial effect on sickle cell hemoglobin (20–23), studies on this derivative were undertaken for the recombinant and natural HbS. Such a modified HbS was prepared from natural and recombinant HbS by synthesis using oxidized glutathione (GSSG) as described under "Materials and Methods." The products of the natural HbS modified by glutathione, which were purified on Toyo-Pearl CM resin (Fig. 4), consisted of one major and two minor products. Component 1, which eluted early from the column, showed multiple bands on IEF (data not shown) and was not further characterized. Component 2 was a mixture as judged by its behavior on IEF (Fig. 4, *inset*). The major product (component 3) was homogeneous, as shown by its IEF pattern (Fig. 4, *inset*).

Effect of Reducing Agents on Glutathione-modified Sickle Hb—The IEF system described above was employed to evaluate the effects of several types of reducing agents on the modified sickle Hb tetramer prepared from both natural and from recombinant HbS. Reduced glutathione (GSH) was chosen as the reducing agent since its rate of reduction was rather slow (24). This property proved to be advantageous since the rates of reduction of natural and synthetic glutathione-modified Hb could be compared.

As shown in Fig. 5, the recombinant HbS (*lane b*) had the same mobility as the major Hb band in the hemolyzate of a patient with sickle cell anemia (*lane a*, lower band; the upper band in *lane a* is fetal Hb). Unmodified HbS did not undergo a change in electrophoretic mobility upon treatment with reduced GSH. The synthetic mixed disulfide of recombinant sickle Hb and glutathione (*lane c*) migrated to the same position as the glutathione adduct prepared from natural HbS (*lane h*). The glutathione adducts of both hemoglobins were reduced at similar rates as shown by comparison of *lane d* with *lane i*

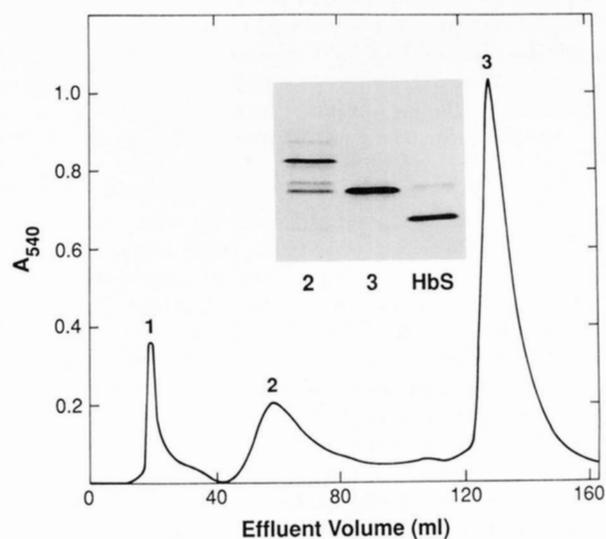


FIG. 4. Purification of synthetic glutathione-modified hemoglobin S. Natural hemoglobin S was treated with oxidized glutathione and then purified on Toyo-Pearl CM650 resin as described in the text. The isoelectric focusing pattern of each purified component, which is shown in the *inset*, indicates that all of the HbS was modified. The major component 3 was used for subsequent studies.

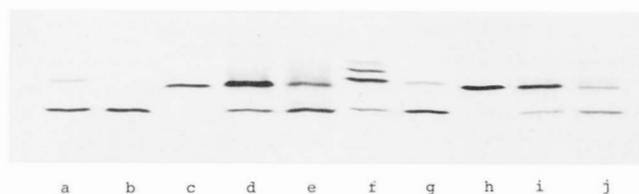


FIG. 5. Reduction of the glutathione adduct of natural and recombinant hemoglobin S. The purified glutathione adducts were treated with 33 mM reduced glutathione and subjected to isoelectric focusing, as described in the text. Lane a, natural HbS (lower band) with HbF (upper band); lane b, recombinant HbS; lane c, glutathione adduct of recombinant HbS; lane d, sample in lane c reduced with GSH for 45 min; lane e, sample in lane c reduced with GSH for 120 min; lane f, standards from top to bottom: hemoglobins A, F, and S, lane g, natural HbS; lane h, glutathione adduct of natural HbS; lane i, sample in lane h reduced with GSH for 45 min; lane j, sample in lane h reduced with GSH for 120 min.

TABLE II
Amino acid analysis of the glutathione modifier released from hemoglobin by reduction

Natural sickle hemoglobin was modified by synthesis with oxidized glutathione as described in the text. The modifier was released from the hemoglobin by reduction as described in the text and then separated from the protein by precipitation. After acid hydrolysis, the amounts of free amino acids (in the range of 1–2 nmol) were determined as described under "Methods and Materials."

Amino acid	Amount released/ Hb tetramer
	<i>mol/mol</i>
Glu	2.2
Gly	1.9
Cys	1.4

and lane e with lane j. Treatment of modified HbS with GSH for longer periods led to progressively more formation of unmodified HbS until eventually all of it was deblocked. Other agents such as dithiothreitol and β -mercaptoethanol were also effective in reducing the modified sickle hemoglobin to unmodified HbS (data not shown).

Amino Acid Analysis of Glutathione Hemoglobin S Adducts—The synthetic glutathione adduct with hemoglobin S was also analyzed by amino acid analysis after cleavage from the protein by reduction in order to verify the presence of glutathione and to quantitate how much had reacted with hemoglobin S. The sample was treated with 0.1 M DTT and the material released by reduction was separated from Hb by precipitation of globin in acetone: HCl as described under "Materials and Methods"; part of this sample was hydrolyzed in 6 N HCl and the remainder was not; the latter portion served as a control. Both samples were subjected to amino acid analysis. The results, which are shown in Table II, indicated that glutathione was released from hemoglobin by the reduction treatment. The nearly stoichiometric amounts of glutamic acid, glycine, and cysteine found after subtraction of the small amounts of amino acids in the blank were direct evidence for the identification of the adduct as glutathione attached to hemoglobin. The results were consistent with the incorporation of glutathione at two of the six SH groups per Hb tetramer. The identification of the site of attachment was achieved by direct mass spectrometry of enzyme digests of the protein, as described next.

Mass Spectrometric Analyses of Recombinant Hemoglobins Prior to and after Modification with Oxidized Glutathione

—Matrix-assisted laser desorption mass spectrometric analysis of the purified r-HbS yielded molecular masses of, respectively, 15,126 and 15,837 Da for the α - and β -chains (Fig. 6A). These experimentally determined mass values agree, within the error of the measurement (± 2 Da), with the values of 15,126 and 15,838 Da calculated for the α - and β -chains, respectively, of HbS.

The purified synthetic adduct of glutathione with r-HbS was also analyzed by mass spectrometry (Fig. 6B). The mass spectrum was dominated by peaks corresponding to the unmodified α -chain (molecular mass 15,126 Da) and a modified β -chain (molecular mass 16,142 Da). The mass of the modified β -chain is consistent with the addition of a single glutathione moiety to each β -chain of r-HbS (measured mass increase 305 Da; calculated mass increase 305 Da). No peak corresponding to the addition of more than one glutathione moiety per chain was observed. These results indicated that the modification occurred exclusively on the β -chain and that only one of its two free SH groups is modified.

Mass Spectrometric Analyses of Glutathione-Hemoglobin Adducts Prior to and after Partial Reduction with Glutathione—Matrix-assisted laser desorption mass spectrometric analysis of the purified synthetic adduct of glutathione with natural hemoglobin (HbS) yielded molecular masses of, respectively, 15,126 Da for the unmodified α -chain and 16,139 Da for modified β -chain (Fig. 7A), in agreement with the modification observed with r-HbS by IEF analysis. When the glutathione-hemoglobin adduct was subjected to reduction by 30 mM GSH (as described above for the IEF experiments), mass spectrometric analysis revealed that approximately half of the modified β -chains (molecular mass 16,144 Da) was converted to unmodified β -chain (molecular mass 15,838 Da) (Fig. 7B). The measured mass loss of 306 Da is close to that predicted for the reductive release of a single glutathione moiety (305 Da). No effect was observed on the molecular mass of the α -chain upon reduction.

Determination of the Site of Glutathione Modification of Recombinant HbS—Mass spectrometric analysis of recombinant HbSSG (Fig. 6) and natural HbSSG (Fig. 7) indicated that only one of the two cysteines (Cys-93(β) or Cys-112(β)) in the β -chain was modified. To determine the site of modification of r-HbS with glutathione, r-HbSSG was partially digested with endoproteinase GluC and the resulting mixture of peptide products subjected to mass spectrometric analysis (Fig. 8A). For purposes of comparison, unmodified r-HbS was subjected to the same analysis (Fig. 8B). An ion peak unique to the digestion products of r-HbSSG was observed at m/z 6437 (Fig. 8A), arising from the protonated peptide 44–101(β) modified by the addition of a glutathione entity (calculated $m/z = 6131 + 305 + 1 = 6437$). No significant ion signal was observed for the unmodified peptide 44–101(β). These results are consistent with the quantitative presence of a single glutathione adduct at Cys-93(β). The mass spectrum of the digestion products of r-HbSSG (Fig. 8A) also exhibited a peptide fragment ion with m/z 4931, corresponding to the protonated unmodified peptide 102–146(β) (calculated $m/z = 4932$), as well as a lower intensity ion at m/z 5236, corresponding to peptide 102–146(β) partially modified by glutathione at Cys-112 (calculated $m/z = 5236$). Together, these data indicate that the primary site of modification is Cys-93(β). The small degree of modification occurring at Cys-112 may arise by disulfide interchange between Cys-93 and Cys-112.

r-HbSSG and r-HbS were also subjected to digestion with endoproteinase GluC in the presence of the reducing agent dithiothreitol. The mass spectra of the resulting peptide fragments are shown in Fig. 8, C and D. As expected, the spectra revealed no evidence for modification by glutathione. The absence of any peaks in the area of the modified peptides indicates that there was no interference with different peptides of similar mass and, therefore, confirms the previous assignment of the modified peptides.

Oxygen Binding Studies—The oxygen equilibrium curves of natural and recombinant HbS at high protein concentrations of 17–18 g/dl (just below the gelation concentration of nearly 24

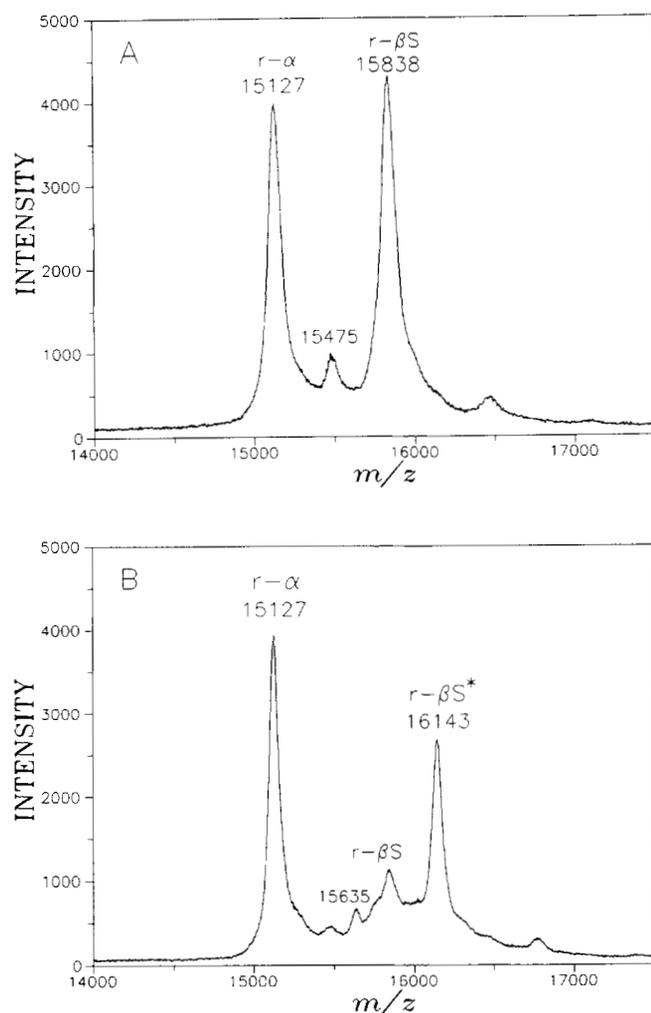


FIG. 6. Mass spectrometric analysis of recombinant sickle hemoglobin. A, recombinant sickle hemoglobin (r-HbS) showing peaks corresponding to the protonated α - and β -chains (designated α and βS). The matrix used to obtain the spectrum was α -cyano-4-hydroxycinnamic acid. The small peak at m/z 15,475 likely arises from a doubly protonated heterodimer of the α - and β -chains. The origin of the weak peak at m/z 16,471 has not yet been elucidated. B, recombinant sickle hemoglobin after reaction with oxidized glutathione (r-HbSSG) showing peaks corresponding to the protonated unmodified α -chain and a protonated modified β -chain (designated r- βS^*). A small peak is observed at a mass corresponding to the unmodified β -chain (designated r- βS). The matrix used to obtain the spectrum was α -cyano-4-hydroxycinnamic acid. The small peak at m/z 15,635 likely arises from a doubly protonated heterodimer of the α -chain and the modified β -chain. The origin of the weak peak at m/z 16,768 has not yet been determined.

g/dl; Fig. 10) are shown in Fig. 9. These conditions were chosen because a hallmark of the physiological properties of sickle Hb is its propensity to undergo a significant lowering of its oxygen affinity at high Hb concentrations, *i.e.* approaching those in the red cell. The results in Fig. 9 indicate that the curves for both recombinant and natural HbS are superimposable. The P_{50} value for each Hb was 42 mm Hg. The Hill coefficient for each was identical, $n = 3.3$, which shows a very high degree of cooperativity (Fig. 9, inset).

Functional Binding of Chloride to rHbS—It has been known for many years that chloride binds to hemoglobin to cause a lowering of its oxygen affinity (25). There are about five established oxygen-linked chloride binding sites. The results shown in Table III indicate that chloride is just as effective in lowering the oxygen affinity of recombinant HbS as it is with natural HbS; these determinations were performed at lower Hb con-

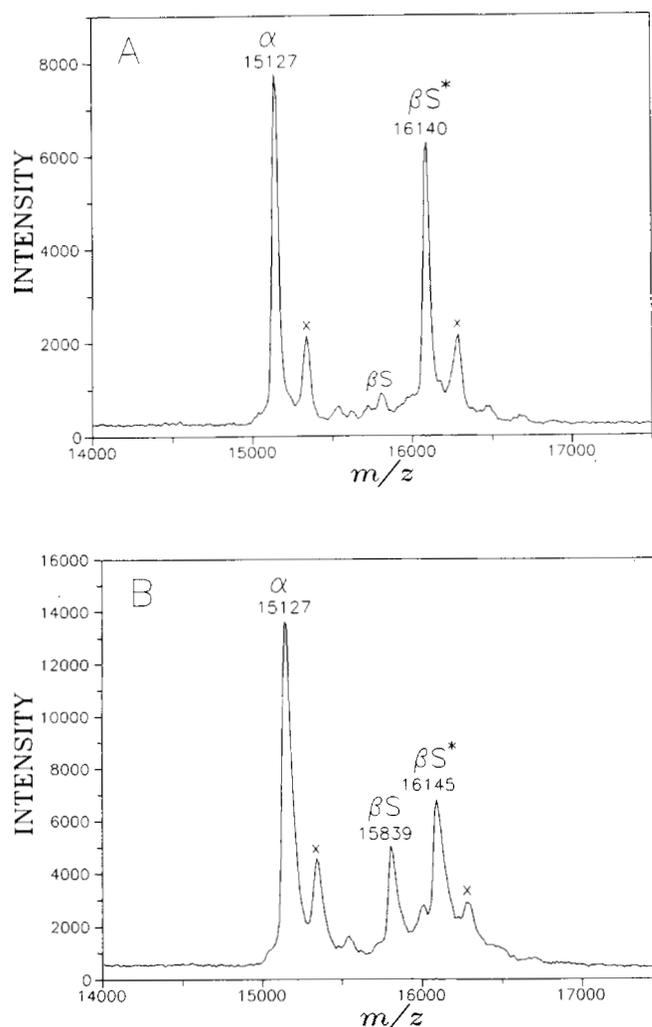


FIG. 7. Matrix-assisted laser desorption mass spectrum of un-reduced and reduced hemoglobin-glutathione adducts. A, synthetic glutathione-hemoglobin adduct showing peaks corresponding to the protonated unmodified α -chain and a protonated modified β -chain (designated βS^*). Matrix, sinapinic acid. The peaks labeled X are artifacts arising from the adduction of dehydrated sinapinic acid with the protein chains (Ref. 13). The origin of the other small peaks in the spectrum is currently under investigation. B, synthetic glutathione-hemoglobin adduct after treatment with reduced glutathione. Matrix, sinapinic acid. For a discussion of the peaks labeled X and the other minor peaks, see A above.

centrations than those in Figs. 9 and 10, hence the lower P_{50} values. Thus, at low concentrations of chloride, *i.e.* 10 and 20 mM, there was little response of either natural or recombinant Hb to chloride. However, at higher concentrations of NaCl the oxygen affinity of each hemoglobin was lowered to about the same extent by the same concentration of chloride. Hence, in its functional response to the allosteric regulator chloride, the recombinant HbS behaved like natural HbS.

Gelation of Recombinant HbS—One of the unique features of HbS that distinguishes it from HbA is its aggregation at high hemoglobin concentrations such as those present in the intact erythrocyte. Indeed, this is the underlying cause of sickle cell anemia. In the procedure of Benesch *et al.* (16), which was used to measure the gelation concentration of HbS, the P_{50} was determined at a number of hemoglobin concentrations. This procedure takes advantage of the sudden and rapid decrease in the oxygen affinity (increase in P_{50}) that occurs at the onset of gelation. Thus, an oxygen equilibrium curve is determined at every Hb concentration tested. Full cooperativity was found for

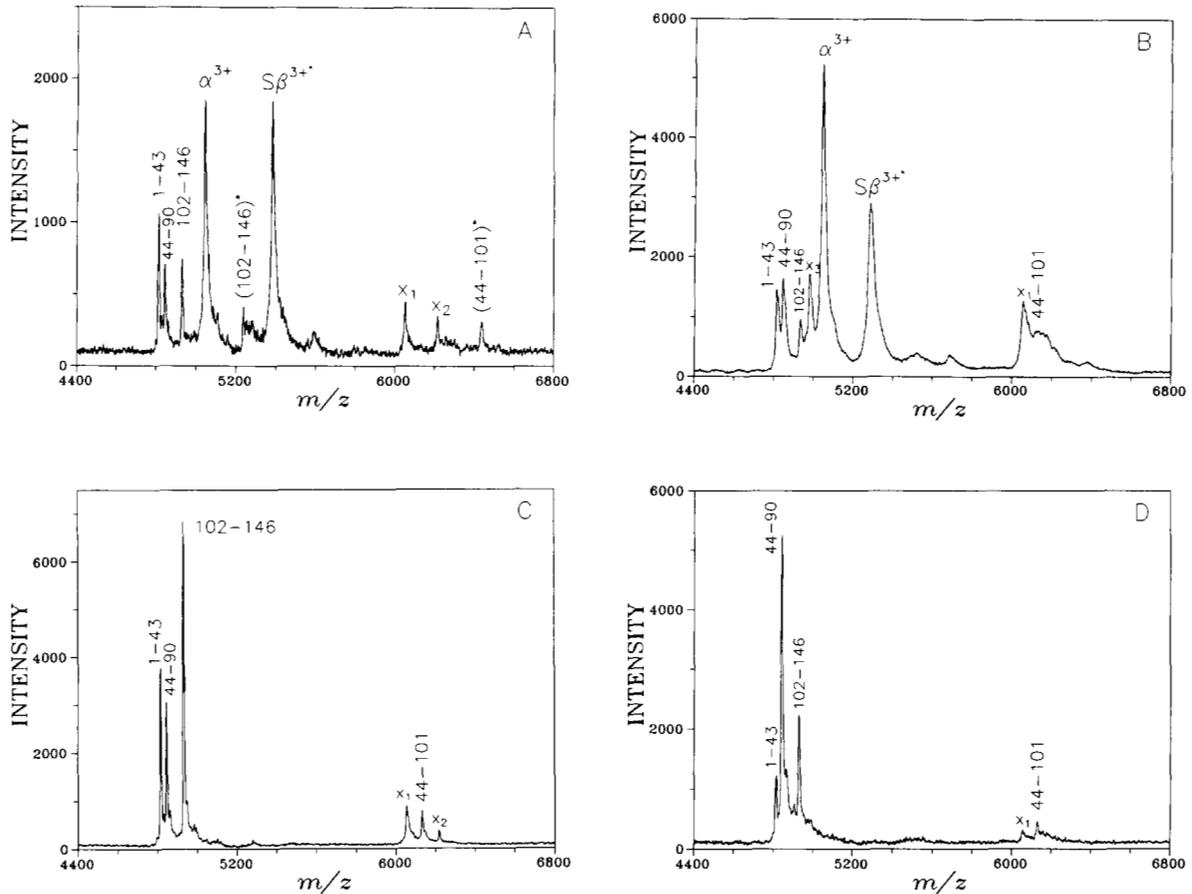


FIG. 8. Matrix-assisted laser desorption mass spectrum of the peptide fragments produced by the digestion with endoproteinase GluC of: **A**, recombinant glutathione-modified sickle hemoglobin (r-HbSSG); **B**, recombinant sickle hemoglobin (r-HbS); **C**, r-HbSSG in the presence of reducing agent (DTT); and **D**, r-HbS in the presence of reducing agent (DTT).

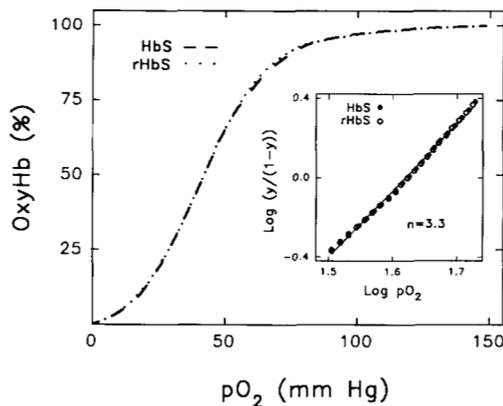


FIG. 9. Oxygen binding curves of natural and recombinant sickle hemoglobins. The curves were obtained on concentrated Hb samples, 17.2 and 18.4 g/dl for natural and recombinant HbS, respectively, in 0.1 M potassium phosphate, pH 6.8, at 37 °C.

both recombinant HbS ($n = 3.2$ (average of seven measurements: precision ± 0.15)) and for natural HbS ($n = 3.4$ (average of nine measurements: precision: ± 0.19)). As shown in Fig. 10, the gelation concentration (23.7 g/dl) obtained for both recombinant and natural HbS purified by the same procedures described in this study are the same. Hence, the data are plotted with common lines to illustrate this point. Thus, the recombinant HbS expressed in yeast retains the ability to aggregate in a manner identical to that of natural HbS; this result indicates that the quaternary structures of recombinant and natural

TABLE III

Effect of NaCl on the oxygen affinity of natural and recombinant sickle hemoglobins

P_{50} values are ± 1 mm Hg. The Hb concentration was 0.4–0.5 mM (2.5–3 gm/dl). Hill coefficient values averaged 2.8 ± 0.03 (three measurements) for recombinant HbS and 2.9 ± 0.06 (three measurements) for natural HbS.

NaCl	Recombinant HbS, P_{50} mm Hg	Natural HbS
<i>mM</i>		
0	10	10
10	11	10
20	10	10
100	15	12
200	16	17
500	21	23
1000	25	28

HbS are the same, at least as ascertained by the tests used in this study.

DISCUSSION

The yeast expression system for recombinant hemoglobin has many features that make it conducive to study any contact site in aggregating tetramers of human sickle hemoglobin. For example, the NH_2 -terminal processing of the human hemoglobin expressed in yeast is the same as that for human sickle hemoglobin in human red cells (26). This is an important consideration for studies on hemoglobin, in general, because the NH_2 -terminal residues are critical for some of its functions (17). The correct NH_2 -terminal residues are also important for sickle hemoglobin because several anti-sickling agents are directed at

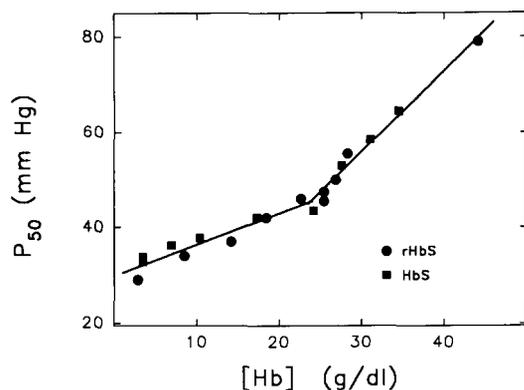


FIG. 10. Gelation of natural and recombinant sickle hemoglobin. The procedure of Benesch *et al.* (16) was used on Hb samples in 0.1 M potassium phosphate, pH 6.8, at 37 °C. The point of intersection of the biphasic lines, which are common for recombinant and natural HbS, represents the concentration at which HbS starts to aggregate. Hill coefficient values averaged 3.2 ± 0.15 for recombinant HbS and 3.4 ± 0.19 for natural HbS.

this site (8). Another advantage of the yeast expression system for human sickle Hb is that the problems encountered with fusion proteins are avoided. Furthermore, the endogenous heme of yeast is used for synthesis of the recombinant hemoglobin, obviating the need for reconstituting with heme and the subsequent reduction to the ferrous state. More extensive comparisons of the yeast expression system with other systems that also synthesize hemoglobin are discussed in Refs. 9, 26, and 27. We have seen no evidence of improper folding of recombinant hemoglobin as shown by the results in this and the previous studies (9).

The yeast system should facilitate the synthesis of double mutants of hemoglobin having changes at any position in the α - or β -chain. However, before initiating such studies it was considered important to establish that the recombinant human sickle hemoglobin had the same properties as sickle hemoglobin isolated from human red cells. In the present study, this claim is supported by several lines of evidence, including the internal protein sequence, the properties of the COOH-terminal amino acids of the α - and β -chains, and their functional properties with respect to their response to chloride anion. Of particular importance was the finding that the aggregation properties of sickle hemoglobin expressed in yeast were superimposable with the hemoglobin isolated from the erythrocytes of sickle cell anemia patients. Thus, the yeast expression system makes a fully functional hemoglobin tetramer with respect to those features of the primary through quaternary structures that we have studied in this article.

The properties of the adduct of glutathione and hemoglobin

have been described previously (18–23). We used this derivative as a probe of the important region around Cys-93(β). Studies on the preparation of this derivative, its purification, and its location were established by the mass spectrometry studies described in this article. This particular derivative has several anti-sickling properties such as an increased oxygen affinity and a decreased capacity to aggregate that make it appealing for further study. Both of these properties act in concert to prevent erythrocyte sickling. If a mild enough treatment could be found to convert a fraction of red cell glutathione to the oxidized form, which could subsequently attach to hemoglobin *in vivo*, it might represent an effective modality for the treatment of sickle cell anemia.

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