Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 918–922, February 1993
Medical Sciences

Recombinant human sickle hemoglobin expressed in yeast

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Communicated by Bruce Merrifield, October 28, 1992

ABSTRACT **Sickle hemoglobin has been expressed in the yeast *Saccharomyces cerevisiae* after site-directed mutagenesis of a plasmid containing normal human α- and β-globin genes. Cassette mutagenesis of this plasmid was achieved by inserting a DNA fragment containing the β-globin gene in the replicative form of M13mp18 to make a point mutation and then reconstituting the original plasmid containing the mutated β-globin gene. Pure recombinant hemoglobin S was shown to be identical to natural sickle hemoglobin in its ultraviolet and visible absorption bands and by gel electrophoresis, isoelectric focusing, amino acid analysis, mass spectrometry, partial N-terminal sequencing, and functional properties (P_{50}, cooperativity, and response to 2,3-bisphosphoglycerate). In yeast and in mammalian cells, cotranslational processing yields the same N-terminal valine residues of hemoglobin α- and β-chains, but in bacterial expression systems the N terminus is extended by an additional amino acid because the initiator methionine residue is retained. Since the N-terminal valine residues of both chains of hemoglobin S participate in important physiological functions, such as oxygen affinity, interaction with anions, and the Bohr coefficient, the yeast expression system is preferable to the bacterial system for recombinant DNA studies. Hence, mutagenesis employing this expression system should permit definitive assignments of the role of any amino acid side chain in hemoglobin S aggregation and could suggest additional approaches to therapeutic intervention. The engineering of this system for the synthesis of sickle hemoglobin and its purification to homogeneity in a single column procedure are described.**

The genetic mutation underlying sickle cell anemia leads to the replacement of Glu-6 on the β-chain with a valine residue (1, 2). The substitution of a hydrophobic side chain for a hydrophilic one on the exterior of the protein (3) promotes the aggregation of deoxygenated hemoglobin (Hb) tetramers (4, 5) that eventually distort the erythrocyte into a sickled form in the venous circulation (6). One approach toward treatment of sickle cell anemia focuses on the HbS molecule itself by reacting it with chemical modifiers that directly alter the functional properties of the protein and indirectly reduce aggregation *in vitro* (7, 8). The number and nature of such susceptible sites has been limited to those hydrophilic side chains with enhanced reactivity because of their location in the protein (8, 9). The availability of recombinant DNA technology now permits studies at any site on the HbS tetramer, such as hydrophobic amino acid side chains heretofore not possible to modify by other methods. For sickle cell HbS, the ability to alter hydrophobic sites is especially important since the initial and some of the subsequent stages of aggregation involve hydrophobic interactions. Although the identity of some of these contact sites in the aggregate is known (3–5, 10), there is a lack of information on other contact sites and their contribution to the overall strength of the interactions in the HbS aggregate. In this communication, we describe the expression of recombinant (r) human HbS in a yeast system as the initial study for site-specific mutagenesis at any site in the tetramer to provide additional information on the details of the aggregation process.

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METHODS AND MATERIALS

Reagents and Enzymes. The restriction endonucleases, T4 DNA ligase, and alkaline phosphatase were purchased from Boehringer Mannheim and T4 polynucleotide kinase was from New England Biolabs. The DNA sequencing kit and T7 DNA polymerase (Sequenase Version 2.0) were purchased from United States Biochemical. The oligonucleotides were synthesized by Operon Technologies (Alameda, CA). To create the sickle mutation in the β-globin gene, the oligonucleotide 5′-CAGACTTCTCCAACAGGAGTCAG was used; the underlined base was used to cause the mutation. All the other reagents were of the highest purity available. Sequencing of the β-globin gene employed two different primers.

***Escherichia coli* Strains.** *E. coli* XL1-Blue was used in most of the experiments. *E. coli* BW313 was used in the site-directed mutagenesis step, as described by Kunkel (11). *E. coli* cells were grown in LB medium, adding antibiotics when required, and incubated at 37°C.

***Saccharomyces cerevisiae* Strain and Culture Conditions.** The strain used was GSY112 (*cir°* *Matα*, *pep4::HIS3*, *prb1-Δ1.6R*, *his3-Δ200*, *ura3-52*, *leu2::hisG*, *can1*). The yeast was grown in rich medium (YPD). To select those cells containing the pGS389 plasmid (see below), agar plates of complete minimal medium either without uracil or without uracil and L-leucine were used. To maintain the presence of this plasmid in the yeast, the incubations were in complete minimal liquid medium without uracil or L-leucine at 30°C. Liquid cultures were shaken at 300 rpm in Fernbach flasks.

Plasmids. pGS389 (15.5 kbp), which is an *E. coli*/yeast shuttle plasmid that contains the human α- and β-globin cDNAs, was used (12). The expression cassette containing both globin genes and their promoters can be excised as a *Not* I fragment. pGS189 (5.3 kbp) is a derivative of pSK(+) plasmid (Stratagene) and contains the α/β-globin expression cassette named above. This plasmid contains an ampicillin-selectable marker and can replicate only in *E. coli*. The digestion of the plasmid with *Sph* I yields a DNA fragment of 1.2 kbp containing the β-globin cDNA.

Site-Directed Mutagenesis. The plasmid pGS189 was digested with *Sph* I into two DNA fragments of 1.2 and 4.1 kb. The 1.2-kb fragment containing the β-globin cDNA was inserted into the replicative form of M13mp18 previously digested with the same restriction enzyme, so that the phage contains the sense DNA strand of the β-globin gene.

E. coli BW313 was transfected with that recombinant phage, and the oligonucleotide described above was used to create the mutation $\beta^{6Glu \rightarrow Val}$ by the procedure described by Kunkel (11). The presence of this mutation was confirmed by sequencing the single-stranded DNA of the mutant phage. The replicative form of the mutant phage was digested with

Abbreviations: Hb, hemoglobin; r, recombinant.

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Sph I and the fragment containing the mutated β-globin gene was recombined with the large *Sph* I fragment of pGS189 creating pGS189^s. The correct orientation of the recombinant fragments was confirmed by DNA sequencing. The plasmid pGS189^s was treated with *Not* I to release the cassette with the sickle globin gene, which was subsequently inserted into pGS389 digested with the same restriction enzyme. The correct orientation of the DNA fragments was again confirmed by DNA sequencing, and the recombinant plasmid was transformed into yeast GSY112 *cir*^{o} strain by using the lithium acetate method (13). Transformed cells were selected on complete minimal medium without uracil. To increase the copy number of plasmids per cell, the selected clones were restreaked up to four times on agar plates of complete minimal medium lacking uracil and L-leucine (14).

Growth of Yeast and Induction of HbS. The procedure to culture the yeast strain GSY112 [pGS389^s] and to induce the synthesis of rHbS is based on that described for rHbA (12). Typically, an inoculum of yeast was grown in 200 ml of complete minimal medium without uracil or L-leucine; after 24 h another 800 ml of the same medium was added to the inoculum and the incubation continued for another 20 h. This culture was diluted with 9 liters of YP medium and 2% ethanol as the carbon source was added. After each increase of 3.0 in A_{600}, an additional 1% ethanol was added. When the A_{600} reached 10, Hb expression was induced by the addition of 2% galactose (20% galactose stock, sterile filtered). After 24 h, the culture was harvested by centrifugation at 7000 × *g* for 10 min at 4°C. The cells were washed with the extraction buffer (20 mM sodium phosphate/20 mM NaCl/10 mM benzamidine/0.5 mM EDTA/0.5 mM EGTA/0.1% Triton X-100, pH 8.2) and collected under the same conditions. If not used immediately, the cell pellets were stored frozen at −80°C.

Isolation of rHbS. Yeast cells [70 g (wet weight)] were resuspended in 100 ml of the extraction buffer and 200 g of acid-washed glass beads (425–600 μm, Sigma) was added in the 200-ml stainless steel mixing chamber of the Omni-Mixer homogenizer with a titanium blade (Omni International, Waterbury, CT). After several trials, it was found to be advantageous to bubble the mixture with CO gas just before the homogenization. OxyHb was thus converted to CO-Hb and oxidation to metHb was thereby minimized. The vessel was placed in ice water and the homogenizer was operated at 5000 rpm for four 5-min periods with 3-min intervals; with this procedure, the temperature of the homogenate did not rise above 10°C. The suspension, which had a grey hue, was decanted. The beads were washed with 25 ml of the extraction buffer and both suspensions were mixed and centrifuged at 4°C and 15,000 × *g* for 20 min. The supernatant (≈100 ml), at pH 6.3–6.5, was reddish and cloudy. It was concentrated to 20 ml by using both an ultrafiltration chamber (Amicon) and Centriprep tubes (Amicon) (membrane molecular mass cut-off of both, 10 kDa). The sample was dialyzed overnight against three 1-liter volumes of 10 mM potassium phosphate/0.5 mM EDTA/0.5 mM EGTA, pH 5.85, bubbled with CO. After centrifugation at 15,000 × *g* for 30 min at 4°C, the red supernatant (≈20 ml) was applied to a carboxymethyl-cellulose (CM-52) column (0.9 × 20 cm), previously equilibrated in the buffer used in the dialysis step; 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 CO bubbled) was applied. The absorbance of the elute was monitored at 280 nm and at 540 nm. Those fractions corresponding to peaks containing protein absorbing at 540 nm were bubbled with CO and kept at −20°C for further analysis.

Analytical Procedures. Absorption spectra were recorded on a Cary 2200 instrument. Amino acid analysis of 24-h acid hydrolyzates of globin chains was performed on a Beckman 6300 instrument with a System Gold data handling system. Protein sequencing was performed on an Applied Biosystems gas-phase system. Isoelectric focusing was done with the pH 6–8 Resolve-Hb kit (Isolab) on a Pharmacia Multiphor II system at 15°C. The oxygen dissociation curves were recorded on a modified Hem-O-Scan instrument.

RESULTS

Growth of Yeast Harboring Plasmids for HbA and HbS. To determine whether it was deleterious for yeast to synthesize HbS, the growth rates and yields of normal Hb and HbS were compared. In general, yeast synthesized about equivalent amounts of HbA and HbS at about the same rate (1–2 mg of purified Hb per liter of liquid culture, depending on the amount of cell breakage). Hence, the presence of HbS was not harmful to the yeast (i.e., in the amounts produced), and there was no adverse effect on the metabolism or the morphology of the yeast.

Purification of HbS. Breakage of the yeast cells with an Omni-Mix homogenizer in the presence of glass beads afforded a convenient way to obtain a cell extract. Chromatography of the extract on carboxymethyl-cellulose (CM-52) gave the pattern shown in Fig. 1. The column fractions were monitored at 280 nm and at 540 nm; the former wavelength provided a profile of all proteins, nucleic acids, and other ultraviolet-absorbing material. The 540-nm wavelength detects only the presence of heme-containing proteins. Most of the material in the extract did not adhere to the resin and was eluted close to the void volume of the column. After application of the gradient, several more nonheme proteins preceded a peak of Hb, which eluted late in the gradient. The elution position of this heme protein corresponds to that of natural oxyHbS purified from human sickle erythrocytes. The fractions comprising the main peak were pooled and bubbled with CO.

Molecular Weight of rHbS Subunits. The rHbS isolated on CM-52 (Fig. 1) behaved predominantly as a single band upon electrophoresis (SDS/PAGE) (Fig. 2, lane c). Natural HbA and HbS were included for comparison (Fig. 2, lanes a and b, respectively). The degree of purification by the single column chromatography on CM-52 (Fig. 1) can be appreciated by a comparison of lanes a and d; the latter sample indicates the large number of proteins in the yeast cell extract before fractionation on the CM-52 column. The molecular weight of the rHbS subunits was in the correct range of ≈16,000 by comparison with standards.

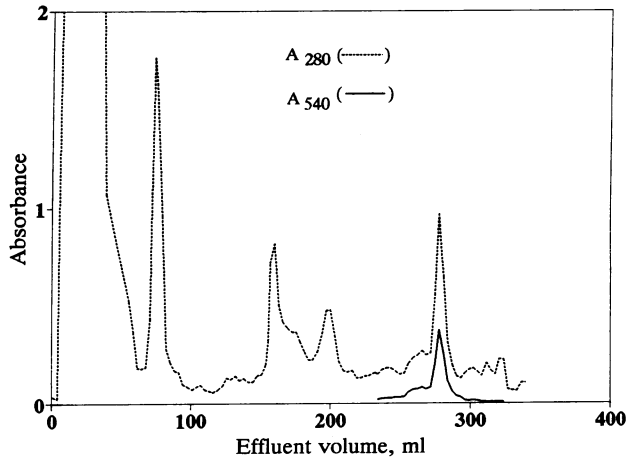

FIG. 1. Purification of rHbS. A column of carboxymethyl-cellulose (CM-52) was used as described in the text. The amount of protein in the yeast extract applied was about 850 mg and the yield of purified Hb was 2.7 mg.

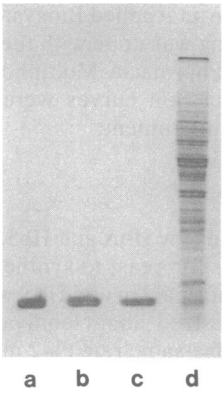

FIG. 2. SDS/PAGE of HbS purified from yeast extract. Purified natural HbA and HbS were applied to lanes a and b, respectively. The rHbS purified as described in Fig. 1 is shown in lane c. A sample of the yeast extract was applied to lane d.

A more precise determination of the molecular weight of the rHbS was obtained by mass spectrometry. In this analysis the Hb subunits were separated so that it was possible to determine their individual molecular weights. Molecular weights of 15,126.6 ± 1 and 15,839.5 ± 1 were found for the α- and β-chains, respectively; these values agree well with the calculated molecular weights of 15,126 and 15,837 for the natural α- and β^s-chains, respectively.

Spectral Properties. The absorption maxima and extinction coefficients of the rHbS in the UV-visible range were nearly the same as the corresponding values for natural HbS. The ratios of these absorbances, which are shown in Table 1, indicate that these proteins are indistinguishable with respect to these spectral properties.

Isoelectric Focusing. The sample of rHbS, purified as described in Fig. 1, was analyzed by isoelectric focusing (Fig. 3). The results indicate that the rHbS (lane c) migrated in exactly the same position as natural HbS (lane b). A sample of natural human HbA is shown in lane a to indicate the high resolving ability of this system and the absence of the latter in the rHbS.

HPLC Analysis. Separation of the subunits of natural HbS and rHbS was achieved by HPLC on a Vydac C_4 column (data not shown). In this system there was complete separation of the globin chains from one another. Moreover, the elution position of the chains of rHbS coincided exactly with those of the chains of natural HbS. These purified recombinant and natural globin chains were used for further characterization by amino acid analysis and N-terminal sequencing as described next.

Amino Acid Analysis. The α- and β-chains isolated by HPLC on Vydac C_4 as described above were subjected to amino acid analysis after acid hydrolysis (Table 2). The results indicate that the recombinant globin chains are pure since their amino acid compositions are in good agreement with analyses of the globin chains from natural HbS isolated by the same HPLC procedure, as well as the published values (15).

N-Terminal Sequencing. The α- and β-chains separated by HPLC on a Vydac C_4 column as described above were subjected to N-terminal analysis by Edman degradation. The results (Table 3) for the first 8 amino acid residues of each chain clearly indicate that the sequences, which were obtained in high yield, are correct for the N-terminal segment of each α- and β-subunit. Furthermore, the presence of a valine at position 6 of the β-chain confirms that the mutagenesis was at the correct position.

Table 1. Spectral properties of natural HbS and rHbS

Spectral ratio	rHbS	Natural HbS	Published values
568/555	1.22	1.25	1.26
539/555	1.24	1.25	1.27
539/568	1.02	1.00	1.00
420/539	13.71	13.07	13.37
420/568	13.97	13.07	13.42
280/539	2.69	2.53	—
420/280	5.1	5.16	—

Values are for CO-Hb. Published values are from ref. 15.

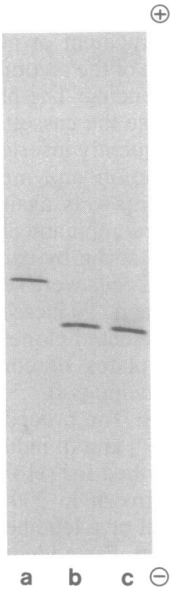

FIG. 3. Isoelectric focusing of recombinant and natural Hb. A gel (pH 6–8) (Isolab) was electrophoresed at 10 W for 45 min. Lanes: a, natural HbA; b, natural HbS; c, rHbS.

Functional Studies. After purification of rHbS on the CM-52 column, as described in Fig. 1, and prior to determination of the oxygen equilibrium curve, the sample was treated with sodium dithionite (0.15 M) to remove traces of metHb. The Hb solution was layered on a Sephadex G-25 column (1 × 25 cm) equilibrated in 50 mM Bistris acetate (pH 7.5) containing sodium dithionite; the column was eluted with the same buffer without sodium dithionite. The Hb fractions, which were separated from the reducing agent, were collected and the CO derivative of HbS was converted to the oxy derivative by two exposures to a 200-W incandescent light in a round bottom flask in an atmosphere of pure O_2, as described (16). The Hb sample, in 50 mM Bistris acetate (pH 7.5), was then concentrated to ≈0.5 mM (tetramer) by using a Centriprep and Centricon apparatus just prior to determination of its oxygen equilibrium properties on a modified Hem-O-Scan instrument.

Table 2. Amino acid analysis of recombinant α and β^s-globins

	α-chain		β^s-chain	
Amino acid	Theory	Found	Theory	Found
Asx	12	12.5	13	13.5
Thr	9	8.8	7	7.2
Ser	**11**	**10.4**	**5**	**5.5**
Glx	**5**	**5.7**	**10**	**10.6**
Pro	7	6.7	7	6.1
Gly	**7**	**7.2**	**13**	**12.5**
Ala	**21**	**21.1**	**15**	**15.1**
Cys	1	1.0	2	2.1
Val	**13**	**11.8**	**19**	**16.7**
Met	2	1.6	1	1.1
Ile	0	0	0	0
Leu	18	18.8	18	18.4
Tyr	3	2.4	3	3.1
Phe	7	6.7	8	7.9
His	10	10.0	9	9.0
Lys	11	12.1	11	11.9
Arg	3	3.2	3	3.3

Five amino acids shown in boldface type are those for which there are significant differences between the α- and β-chains (15).

Table 3. Partial N-terminal sequences of α- and β-chains of rHbS

Chain	Sequence
α	H_2N-Val - Leu - Ser - Pro - Ala - Asp - Lys - Thr- (215) (110) (59) (141) (148) (122) (129) (76)
β	H_2N-Val - His - Leu - Thr - Pro - Val - Glu - Lys- (330) (135) (228) (102) (122) (172) (92) (106)

Sequence was initiated with 200–300 pmol of each subunit purified by HPLC. The numbers in parentheses below each amino acid are the pmol of phenylthiohydantoin derivative amino acid recovered at each cycle of Edman degradation. The valine residue, which is 6 residues from the N terminus, is the one formed by the mutagenesis.

Measurement of the oxygen equilibrium curve for rHbS in the presence of 0.1 M KCl indicated that it had a P_{50} value of ≈15 mmHg (1 mmHg = 133 Pa) (Fig. 4), close to the value that we found for natural HbS. These determinations were done below the gelling concentration of HbS. The cooperativity of rHbS (Hill coefficient, $n = 2.4$; Fig. 4 *Inset*) was the same as that for natural HbS. To determine whether the rHbS was responsive to allosteric regulators, the effect of 2,3-bisphosphoglycerate was evaluated. Addition of 2,3-bisphosphoglycerate reduced the oxygen affinity of rHbS and natural HbS to the same extent, i.e., with a right shift of 10 mmHg in the oxygen dissociation curve.

DISCUSSION

The synthetic human α- and β-globin genes for normal Hb (17) and for a few other mutant Hbs (18–22, 27) have been expressed in *E. coli*. However, the Hb produced in this system is not processed at its N terminus in the same way that human Hb is processed in human reticulocytes. In the bacterial system, the initiator methionine residue is usually retained; i.e., it is not removed by bacterial processing enzymes to expose the second valine residue as the new N terminus of mature human HbS chains. Depending upon the intended use of these rHbs, the presence of this N-terminal methionine residue may not be detrimental. However, for HbS where modifications of the N-terminal valine of either the α- or β-chains (9, 16) have dramatic effects on its oxygen equilibrium and gelation properties, this will present serious problems in the interpretation of any data obtained in mutagenesis studies using such a system. In the present communication, the high yield in N-terminal sequencing of only valine at the N terminus of each mature α- and β-globin subunit is consistent with the complete removal of the initiator methionine residue by yeast processing enzymes. Another advantage of the yeast expression system is that the α- and β-globin genes are on the same plasmid (12) and hence soluble Hb is produced already combined with the endogenous heme of yeast. In the bacterial system, expression of a stable α-globin chain has been difficult because it is apparently degraded (20). Therefore, in most cases (19, 20) native α-chains are used to reconstitute hybrid tetramers. In the *E. coli* system, the β-globin is produced as a fusion protein that forms insoluble inclusion bodies and stringent conditions are required for their solubilization. After enzymic cleavage of the fusion β-globin and reconstitution with exogenous heme, the individual chains are then combined and the heme is reduced to the ferrous state. Such problems are not encountered in the yeast expression system designed by Wagenbach *et al.* (12) and reengineered as described in this communication.

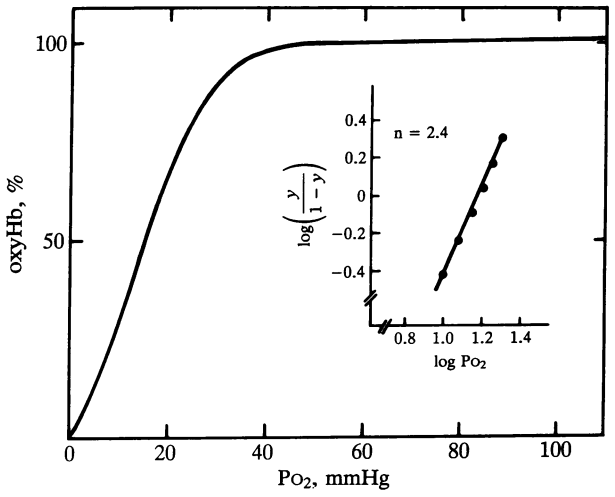

FIG. 4. Oxygen dissociation curve and Hill plot of rHbS. The oxygen equilibrium curve was obtained at 37°C on rHbS (0.5 mM in tetramer) in 50 mM Bistris, pH 7.5/0.1 M KCl. (*Inset*) Hill plot where the logarithm of the fractional oxygen saturation (y) is plotted against the logarithm of the partial pressure of oxygen (P_{O_2}).

Bookchin *et al.* (10) first elucidated some of the important contact sites in the HbS aggregate by determining the effect of naturally mutant Hbs having substitutions at sites other than Val-6(β) on the extent of aggregation of HbS. Benesch *et al.* (23) demonstrated that the aggregation even involved contact sites on the α-chain. The crystal structures of deoxyHbS reported by Wishner *et al.* (3) and by Magdoff-Fairchild and Chiu (24) as well as the detailed electron micrographs of Edelstein and Crepeau (4) and of Wellems and Josephs (5) give an indication of the complexity of aggregated deoxyHbS tetramers and the diverse number of contact sites involved. The studies of Noguchi and Schechter (25) and those of Hofrichter *et al.* (26) have revealed the thermodynamics of the aggregation process. All of these studies have suggested potential points of intervention in the aggregation process. However, the importance of most of these contact sites has not been experimentally tested either because they are not susceptible to chemical modification or because a natural mutant has not been reported. Therefore, there is a need for an experimental approach to obtain information on these other contact sites in the aggregate. Systematic site-directed mutagenesis studies using the yeast expression system described in this communication should be a procedure to provide that information because a rHbS with the structure and functional properties identical with natural HbS can be readily obtained in reasonable yield and with a relatively simple purification procedure. Therefore, it should be possible to prepare double mutants of Hb—i.e., a HbS combined with a mutation at any other site in the tetramer.

Note Added in Proof. Preliminary data indicate that the polymerization of deoxy rHbS in high-ionic-strength phosphate buffer resembles that of natural deoxy HbS.

We are grateful to Adelaide Acquaviva and to Laura Manning for their skillful assistance with the typescript. The advice of Dr. Thelma Chen and of Dr. Zvi Bohak in the initial phases of these studies is gratefully acknowledged. We are indebted to Dr. Klaus Schneider and Dr. Brian Chait for determining the molecular weight of the subunits of the sickle rHb. This work was supported in part by National Institutes of Health Grant HL18819 and by Biomedical Research Support Grant BRSG-507-RR-07065 from the Division of Research Resources, National Institutes of Health, to Rockefeller University.

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