Hb S mutated at αHis50 has improved solubility

Sickle Cell Hemoglobin with Mutation at αHis50 Has Improved Solubility*

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Background: Deoxy sickle cell hemoglobin (Hb S) tetramers polymerize in solution via lateral and axial contacts among neighbors.

Results: αHis50→Gln mutation can while αHis20→Gln mutation cannot improve the solubility of Hb S.

Conclusion: α2His50 interacts with the β1, CD corner while α2His20 has minimal interaction with β5, Glu22.

Significance: α2His50 contributes significantly to the polymerization of the Hb S tetramers.

SUMMARY

The unliganded tetrameric Hb S has axial and lateral contacts with neighbors and can polymerize in solution. Novel recombinants of Hb S with single amino acid substitutions at the putative axial [rHb (βE6V/αH20R), and rHb (βE6V/αH20Q)], lateral [rHb (βE6V/αH50Q)] or double amino acid substitutions at both the putative axial and lateral [rHb (βE6V/αH20R/αH50Q) and rHb (βE6V/αH20Q/αH50Q)] contact sites were expressed in Escherichia coli and purified for structural and functional studies. The 1H-NMR spectra of the CO and deoxy forms of these mutants indicate that substitutions at either αHis20 or αHis50 do not change the subunit interfaces or the heme pockets of the proteins. The double mutants show only slight structural alteration in the β-heme pockets. All mutants have similar cooperativity (n50), alkaline Bohr effect, and autoxidation rate as Hb S. The oxygen binding affinity (P50) of the single mutants is comparable to that of Hb S. The double mutants bind oxygen with slightly higher affinity than Hb S under the acidic conditions. In high salt, rHb (βE6V/αH20R) is the only mutant that has shorter delay time of polymerization and forms polymers more readily than Hb S with a Dextran-Csat value of 1.86 ± 0.20 g/dL. Hb S, rHb (βE6V/αH20Q), rHb (βE6V/αH50Q), rHb (βE6V/αH20R/αH50Q), and rHb (βE6V/αH20Q/αH50Q) have Dextran-Csat values of 2.95 ± 0.10, 3.04 ± 0.17, 11.78 ± 0.59, 7.11 ± 0.66 and 10.89 ± 0.83 g/dL, respectively. rHb (βE6V/αH20Q/αH50Q) is even more stable than Hb S under elevated temperature (60 °C).

Hb S is a naturally occurring mutant of human adult hemoglobin (Hb A) which has the normally occurring glutamate at the β6 position of Hb A replaced with a valine (1). Heterozygous individuals carrying both Hb S and Hb A genes are protected against Plasmodium infection (2). However, homozygous individuals carrying only the Hb S genes develop hemolytic anemia (3,4). The deoxygenated Hb S molecules form long fibers within the red blood cells, making them rigid and distorting them into the shape of a sickle. This leads to the blocking of capillary vessels and the destabilization of the red blood cell membranes and premature destruction of erythrocytes (5).

Electron microscopic (EM) studies proposed that the Hb S fibers contain 14 (6) or 16 (7) individual strands twisted in a rope-like...
fashion. These strands can be arranged into seven or eight double strands which are held together by inter-double strand, intra-double strand axial and intra-double strand lateral interactions (8,9). The crystal structure of deoxy-Hb S has been determined (8,10) and refined to 2.05 Å (11). In this communication, we adopt the designations of Padlan and Love (10,12) in denoting the subunits of the tetramers in the double strands. The β-subunits that provide acceptor pockets for the βVal6 are designated as βS1. The subunits that act as valine donors are assigned as βS2. The α-subunits are named according to the β-subunits that they form dimers with. According to the 2.05 Å structural model of Harrington et al. (11), each double strand is held together by the lateral interactions among βS1-subunits of one strands with the βS2- and α2-subunits of tetramers from the other strand (Fig 1A). The individual double strand is further stabilized by axial interactions between tetramers translated one unit cell along the vertical axis. The βS1-subunit of the lower tetramer makes contacts with the βS2- and the α2-subunits of the upper tetramer. In addition, α1- and α2-subunits interactions are also present (Fig. 1B). The model gives a static picture of interacting amino acid residues that stabilize the polymer.

Numerous natural or recombinant Hb S mutants with substitution(s) at the putative contact sites have been selected or generated for studying the mechanism and inhibition of Hb S polymerization. The hydrophobicity and the size of the side chain of the amino acid at βS6 or the βS24 position affects the solubility of the resulting mutants (13-15). Solubility can be viewed as the concentration of hemoglobin remains in solution after completion of polymerization. Mutations at the βS1 acceptor pocket can improve moderately the solubility of Hb S, but the mutants are inherently unstable (16-18). Himanen et al. replaced the βLys95 at the acceptor pocket with Ile and disrupted one of the lateral contact sites (19,20). The mutant is 2.6 times more soluble than Hb S under deoxy condition (19). Sivaram et al. removed one of the axial interactions by replacing the αLeu113 with histidine and improved the Hb S solubility by 1.8 times (21). Mutants that carry a replacement at one of the axial contact sites and a substitution at the acceptor pocket (αP114R/βT87K and αH20Q/βT87Q) have improved solubility but still less than twice of that of Hb S (22,23).

The αHis20 and the αHis50 residues are located on the surface of the Hb S tetramer. The imidazole nitrogen of α2His20 is putatively in close proximity to the carboxyl side chain of βS G122 (11). A βE22Q mutant presumably disrupted part of the axial interactions between Hb S tetramers and showed a moderate increase (1.28 folds) in solubility (24). The α2His50 is postulated in lateral hydrogen-bonding distance from the βS Asp79 and the βS Asn80 side chains (11). Surprisingly, Hb S mutants with substitutions at this position have not been generated to study its effect on Hb S polymerization.

We report here the structural, functional, and polymerization properties of five recombinant Hb S mutants: rHb (βE6V/αH20R), rHb (βE6V/αH20Q), rHb (βE6V/αH50Q), rHb (βE6V/αH20R/αH50Q), and rHb (βE6V/αH20Q/αH50Q). We provide evidence that the putative axial interaction between αHis20 and βS Glu22, as suggested by an x-ray crystallographic study, has minimal contribution in stabilizing the Hb S polymers. The solubility of Hb S can be improved markedly by replacing the αHis50 with a glutamine. In particular, rHb (βE6V/αH50Q) and rHb (βE6V/αH20Q/αH50Q) are nearly 4 times more soluble than Hb S. Furthermore, rHb (βE6V/αH50Q) is more heat stable than Hb S. To our knowledge, rHb (βE6V/αH50Q) is the most soluble Hb S mutant reported.

MATERIALS AND METHODS

Materials

Blood samples were obtained from the local blood bank (a normal human donor) and National Institute of Health (an SS human donor) for the isolation of normal Hb A and Hb S, respectively. Restriction and related enzymes used in molecular biology work were purchased from New England Biolabs. The QuickChange site-directed mutagenesis kit was a product from Stratagene. Chromatographic materials used in hemoglobin purification were obtained from GE Healthcare. Reagent grade chemicals were purchased from Sigma and used without further purification.

Recombinant Proteins Expression and Purification
The expression vectors constructed in this study were derived from the pHE230 plasmid (22). The pH230 plasmid encodes the E. coli methionine aminopeptidase and synthetic human α- and β-globin genes under the control of separate tac promoters. The β-globin gene carries a βGlu6 to valine substitution for the expression of recombinant Hb S proteins. The pH230 was used as template in polymerase chain reactions to generate plasmids pH295 [rHb (βE6V/αH20Q)], pH2012 [rHb (βE6V/αH20R)], pH296 [rHb (βE6V/αH50Q)], pH297 [rHb (βE6V/αH20Q/ αH50Q)], and pH2022 [rHb (βE6V/αH20R/ αH50Q)]. The mutations on the plasmids were confirmed by DNA sequencing analysis of the entire α- and β-globin cDNAs.

The plasmids were transformed into JM109 cells for protein expression. Cells were grown in DM-4 medium (25) at 30 °C in a 20 liter fermenter (B. Braun Biotech International, model Biostat C). The induction and purification of recombinant hemoglobin (rHb) were carried out as previously described (26). Hb A and Hb S from whole blood were purified according to established methods in our laboratory (27,28). Briefly, red blood cells were lysed with water then loaded onto a Sephadex G-25 column equilibrated and eluted with 0.05 M Tris-HCl, pH 7.45, and 0.1 M NaCl. For the Hb S sample, it was further separated from other components by loading onto a Mono S column equilibrated with 10 mM phosphate, pH 6.8 and 0.5 mM EDTA. The column was developed with the 20 mM phosphate, pH 8.3 and 0.5 mM EDTA. Fractions corresponding to Hb S were collected. The Hb samples were then saturated with CO and stored frozen at -80 °C. The molecular weights of the Hb subunits were confirmed by mass spectrometry in an ion-trap instrument equipped with an electrospray ionization source. The amount of N-terminal methionine cleavage of the samples was estimated by Edman degradation (26). All rHb samples in this study had the correct molecular weights and less than 5% of unprocessed N-terminal methionine.

**Oxygen Binding Properties**

Oxygen dissociation curves were determined with a Hemox Analyzer (TCS Medical Products) according to Shen et al (26). The oxy-Hb samples at 0.1 mM Hb (in terms of heme) were prepared in 0.1 M sodium phosphate in the presence of catalase and superoxide dismutase to reduce the amount of met-Hb to less than 5% in all samples measured (29). Experiments were conducted at 29 °C as a function of pH. The experimental results were fit to the Adair equation using a nonlinear least-squares procedure. The partial pressure (in millimeters of Hg) at 50% oxygenation (P_{50}) and the Hill coefficient (n_{50}) were determined from each curve. The experiment values had an accuracy of ± 10% and standard deviations of ± 4% between runs.

**Dextran-Csat Assay and Polymerization Kinetic Measurements**

Dextran Csat assays were performed according to Tam et al. (28) for micro-sample handling. The polymerization progress curves were determined by the temperature-induced method of Adachi and Asakura (30) with modifications. Concentrated oxy-Hb sample (5-50 µl) was added to 3 ml of 1.8 M phosphate buffer, pH 7.4, and the solution was purged with nitrogen for 15 min. Sodium dithionite was then added to a final concentration of 10 mM and the mixture was transferred to a sealed cuvette. The concentration of deoxy-Hb in the sample was determined by light absorbance at 555 nm and calculated by using a millimolar extinction coefficient (mM^{-1}cm^{-1}) of 12.5. The cuvette was chilled in ice water and the temperature monitored closely with a thermocouple. Once the temperature in the cuvette dropped to 2 °C, it was placed in a Cary 50 UV-visible spectrophotometer (Varian) with a cuvette holder preheated and maintained at 30 °C. Immediately, light absorbance at 700 nm, which reflects the turbidity of the sample, was recorded at 15 sec intervals up to 2000 or 4000 sec depending on the polymerization speed of the sample. Routinely, the temperature of the sample increased from 2 to 30 °C in 325 sec. The delay time of polymerization (t_0) was determined from a A_{700} versus time plot according to Adachi and Asakura (30).

**Autoxidation and Thermal Stability of rHb S Mutants**

Autoxidation rate (k_{ox}) of the samples was determined as described (31). The concentration of oxy-Hb at each time point was calculated according to the equation [Oxy-Hb] = 0.2174A_{560} − 0.0573A_{577} − 0.1616A_{630} and expressed as percent of the original sample. The logarithm
(base 10) of the percent oxy-Hb was plotted against time and the slope of the plot gives the autoxidation rate. Thermal stability measurements were performed according to the outline of Adachi et al. (16). Briefly, oxygenated samples (50 μL, ~20 mg/mL) in 0.1 M sodium phosphate, pH 7.0, and 1 mM EDTA were aliquoted in quadruplicates into thin-walled polypropylene tubes and inserted into a GeneAmp PCR System 2700 (Applied Biosystems). The instrument was programmed to run at 60 °C for 10 min then cooled to 25 °C in 1 min. The precipitates in the samples were removed by centrifugation and the absorbance of the supernatants at 577 nm readings. The background readings were treated as background and subtracted from the amount of precipitated proteins was calculated accordingly and expressed as percentage.

**Structural Study with ^1^H NMR Spectroscopy**

Proton NMR spectra of Hb A, Hb S and its five mutants were obtained at 29 °C on Bruker Avance DRX-300 or DRX-600 spectrometers. Hb samples in carbonmonoxy form were concentrated to approximately 5% (3.1 mM in terms of heme) and exchanged into 0.1 M sodium phosphate at pH 7.0 in 95% water and 5% deuterium oxide (D₂O). Hb S and rHb mutants carrying substitution at the α50 position [rHb (βE6V/αH50Q), rHb (βE6V/αH20Q/αH50Q) and rHb (βE6V/αH20R/αH50Q)] were diluted to 2% and 4% solutions, respectively, before converting into the deoxy form for the NMR measurements. A jump-and-return pulse sequence was used to suppress the water signal (32).

**RESULTS**

**Oxygen-Binding and Cooperativity Properties of rHbs**

The O₂-binding properties of Hb A, Hb S and Hb S recombinant mutants with additional single [rHb (βE6V/αH20Q), rHb (βE6V/αH20R), rHb (βE6V/αH50Q)] or double [rHb (βE6V/αH20Q/αH50Q) and rHb (βE6V/αH20R/αH50Q)] substitutions are summarized in Table 1. The α50 and the αHis50 are located on the surface of the tetramer (33). As expected, substitutions at these positions do not change significantly the O₂-binding properties of the macromolecules. The O₂-binding curves of the Hb S single mutants are essentially indistinguishable from that of Hb A or Hb S, while the Hb S double mutants have slightly higher O₂-binding affinity at low pH. The P₅₀ values determined for the double mutants at acidic pH are approximately 20% lower than those of Hb S. All mutants in this study have Hill coefficients (n₅₀) of 2.5 or higher (Table 1). These results are indicative that these rHbs are cooperative in binding O₂.

Hemoglobin releases hydrogen ions upon oxygenation, and this alkaline Bohr effect can be expressed mathematically as ΔH⁺ = -Δlog P₅₀/ΔpH (34). The number of protons released per heme for Hb A, Hb S, and the recombinant mutants are listed in Table 1. The rHbs release 0.43 - 0.48 proton per heme, while Hb A and Hb S release 0.46 and 0.48 proton per heme, respectively. Hence, the mutants we generated are effective oxygen carriers.

**Dextrans-Csat and Polymerization Kineic Measurements**

The solubility of Hb S and its recombinant mutants in the presence of Dextrans and low ionic strength buffer were determined according to the micro-assay of Tam et al. (28) and are listed in Table 2. Harrington et al. (11) estimated that in the double stranded form of Hb S fibers, the imidazole nitrogen of the α2His20 is 3.55 Å from the side chain oxygen of β1Glu22 and suggested they could form axial interaction. By eliminating the positive charge from the αHis20, the rHb (βE6V/αH20Q) mutant has a Csat value of 3.04 ± 0.17 g/dL, which is close to that of Hb S (2.95 ± 0.10 g/dL). By elongating the side chain, the rHb (βE6V/αH20R) mutant apparently has improved interaction with neighboring tetramers, and the Csat value decreased to 1.86 ± 0.20 g/dL. These results imply that the interaction between the α50 and the βGlu22 of Hb S is minimal under Dextran-CSAT assay condition at pH 7.5. In contrast, eradicating the positive charge from the α50 position improves the solubility of the mutated rHb S. The rHb (βE6V/αH50Q) mutant has a Csat value of 11.78 ± 0.59 g/dL, which is 4 times higher than Hb S. This substitution can also improve the solubility of the rHb S mutated at the α50 position. The rHb (βE6V/αH20R/αH50Q)
mutant has a $C_{\text{sat}}$ value of $7.11 \pm 0.66$ g/dL, which is 3.8 and 2.4 folds higher than that of the 
$rHb$ ($\beta E6V/\alpha H20R$) mutant and $Hb$ $S$, respectively. The 
rHb ($\beta E6V/\alpha H20Q/\alpha H50Q$) mutant has a $C_{\text{sat}}$ value of $10.89 \pm 0.83$ g/dL, 
which is approximately 3.6 folds higher than that of the 
rHb ($\beta E6V/\alpha H20Q$) mutant and $Hb$ $S$. Therefore, the solubility of $Hb$ $S$ can be improved 
by disrupting the lateral contacts between the 
$\alpha_2$CD and the $\beta^3_{1}EF$ corners.

The polymerization kinetic of the $rHb$ $S$ mutants was determined in 1.8 M phosphate buffer, 
$\text{pH}$ 7.4, at 30 ºC. The increase in turbidity of the 
sample, as measured by light scattering at 700 nm, 
reflects sample polymerization. The $A_{700}$ values at 
various time points for each protein concentration 
can be plotted as a function of time to generate a 
sigmoidal curve. Some representative results for 
$Hb$ $S$ and its five mutants are presented in Figure 2. 
The intercept of the slope at the $x$-axis gave the 
delay time of polymerization ($t_d$). The delay time 
between the beginning of the experiment and the 
onset of polymerization depends on the initial 
$\text{hemoglobin}$ concentration and increases as the 
protein concentration decreases. The logarithmic 
plots of the reciprocal of delay time ($t_d^{-1}$) versus 
concentration of proteins are presented in Figure 
3 and summarize the data for multiple mutants at 
various concentrations for direct comparison. The 
plots for $rHb$ ($\beta E6V/\alpha H20Q$), $rHb$ ($\beta E6V/\alpha H50Q$), $rHb$ ($\beta E6V/\alpha H20Q/\alpha H50Q$) and 
rHb ($\beta E6V/\alpha H20R/\alpha H50Q$) fall to the right of 
that representing $Hb$ $S$. The results indicate that 
these mutants have longer polymerization delay 
time than $Hb$ $S$. The plot for $rHb$ ($\beta E6V/\alpha H20R$) 
is shifted to the left of the plot for $Hb$ $S$. The result 
suggests that $rHb$ ($\beta E6V/\alpha H20R$) forms polymers 
faster than $Hb$ $S$. Among these six plots in Figure 
3, only the line representing $rHb$ ($\beta E6V/\alpha H20R$) 
has a slope (1.91) significantly steeper than others 
(1.46 – 1.79). The slope of the line (m) is related 
to the size of the nucleus in forming aggregates 
(35,36). Hence, the nuclei formed in the $rHb$ 
($\beta E6V/\alpha H20R$) solution are larger than for $Hb$ $S$ 
and other mutants in this study.

**Autoxidation and Thermal Stability**

It has been suggested that Hb mutants 
have the potential to be used in gene therapy for 
sickle cell anemia (22). It is imperative to 
determine the autoxidation rate ($k_{\text{ox}}$) and stability 
of the putative candidates. The successful 
candidate should have $k_{\text{ox}}$ and stability similar to 
that of $Hb$ $S$, if not $Hb$ $A$. The $k_{\text{ox}}$ of $Hb$ $A$, $Hb$ $S$, 
and its mutants were determined and are listed in 
Table 2. $Hb$ $S$ has a $k_{\text{ox}}$ of 0.0029 $\pm$ 0.0002 h$^{-1}$, 
which is 3.4 times higher than that of $Hb$ $A$ 
(0.00086 $\pm$ 0.0002 h$^{-1}$). The $Hb$ $S$ mutants in this 
study all have substitution(s) on the protein 
surface and away from the $\text{heme}$ pocket (33). 
Consequently, these mutants have $k_{\text{ox}}$ ranging from 
0.0023 to 0.0031 h$^{-1}$, very similar to that of $Hb$ $S$.

Reportedly, oxy-$Hb$ $S$ was found 
mechanically (18) and thermally (16,18) less 
stable than oxy-$Hb$ $A$. We examined the thermal 
stability of the oxy-form of $Hb$ $A$, $Hb$ $S$, and its 
mutants in a PCR thermal cycler, which has a 
more precise temperature control than previous 
studies. $Hb$ $S$ has $15.7 \pm 0.2 \%$ of denatured 
protein after heating at 60 ºC for 10 min. $Hb$ $A$ has 
only $5.8 \pm 0.1 \%$ denatured protein under the same 
experimental condition. The results for the $Hb$ $S$ 
mutants are listed in Table 2. With the exception 
of $rHb$ ($\beta E6V/\alpha H50Q$), all mutants have thermal 
stability equal to or better than $Hb$ $S$. In particular, 
rHb ($\beta E6V/\alpha H20Q/\alpha H50Q$) has only $12.0 \pm 0.3$ 
% denatured protein under the same treatment, 
significantly better than $Hb$ $S$. Apparently, $Hb$ $S$ 
mutants with substitution(s) on the protein surface 
are thermally more stable than $Hb$ $S$ with 
additional mutation in the acceptor pocket (16,18).

**Structural Properties Investigated with $^1$H- 
NMR**

The possible conformational changes of the 
rHb $S$ mutants were investigated with $^1$H-NMR. 
$\alpha$His122 and $\alpha$His103 are located at the $\alpha_1\beta_1$ 
interface and the NH$_{\text{e1}}$ of their side chains give 
signals at 12.9 and 12.1 ppm, respectively (37-39). 
The exchangeable proton resonance spectra of the 
CO-form of the samples show clearly that these 
two amino acid residues have not been disturbed 
in the five mutants studied (Figure 4A). 
$\beta$Trp37 is located at the $\alpha_1\beta_2$ interface and 
its NH$_{\text{e1}}$ atom gives a resonance peak at 10.6 ppm 
(39,40). As demonstrated in Figure 4A, the 
resonances between 9.5 and 11.0 ppm are similar 
among $Hb$ $A$, $Hb$ $S$, and the five recombinant 
mutants. Therefore, substitutions at $\alpha$His20 
and $\alpha$His50 do not change the quaternary structure of 
the liganded $Hb$ $A$ at the $\alpha_1\beta_2$ interface.
Resonances from 0 to -3 ppm belong to the non-exchangeable ring current-shifted protons and they provide insight about the tertiary structure of the heme pockets. The γ₂-CH₃ groups of αVal62 and βVal67 of the distal heme pocket give resonances at -1.75 and -1.82 ppm relative to DSS, respectively (41,42). The signals at -1.75 ppm are similar among all six proteins studied. However, the signal at -1.82 ppm is shifted slightly upfield for the rHb (βE6V/αH20R/αH50Q) and the rHb (βE6V/αH20Q/αH50Q) mutants, even though the mutations are on the α-subunit (Figure 4B). Moreover, the resonances between -0.7 and -1.1 ppm for the double mutants differ significantly from the other five proteins. But, we have not yet assigned the resonances for these signals and cannot comment on their importance.

The ¹H-NMR spectra for the deoxy form of Hb A, Hb S, rHb (βE6V/αH50Q), rHb (βE6V/αH20R/αH50Q) and rHb (βE6V/αH20Q/αH50Q) are presented in Figure 5. The deoxy form of Hb S mutants with single substitution at αHis20 are at least 3-fold less soluble than mutants carrying substitution at the αHis50 position, and they were excluded from the NMR experiments. The hyperfine-shifted proton resonances of the N₃H exchangeable proton of αHis87 and βHis92 in the proximal heme pocket appear at 63 and 76 ppm from DSS, respectively (43,44). The signals for the mutants, Hb S and Hb A appear at the same positions (Figure 5A).

Figure 5B presents the spectral region of the deoxy form of the proteins between 11-25 ppm downfield from DSS. The porphyrin rings of the α- and β-subunits resonate at 17.0 and 22.6 ppm, respectively (45). The signals at 14.1 and 11.2 ppm have been assigned to the H-bonds between αTyr42-βAsp99 and βTrp37-αAsp94, respectively. These residues are located in the α₁β₂ interface and the resonances are important T-state markers of the deoxy Hb A (39,46,47). We did not detect any difference in chemical shift for these signals among the five proteins. The signals at 12.9 and 12.1 ppm belong to αHis122 and αHis103, as in the spectra for the liganded form of the molecules. Again, we cannot detect any shift in the signals for the mutants.

In summary, the β-heme pockets of the liganded form of rHb (βE6V/αH20R/αH50Q) and rHb (βE6V/αH20Q/αH50Q) changed slightly when compared to that of Hb A or Hb S. Besides that, the tertiary and quaternary structures of the four mutants are similar to that of Hb A or Hb S.

Discussion

The oxy-forms of Hb A and Hb S have similar solubility. Upon deoxygenation, the solubility of Hb A was decreased by half while that of Hb S diminished by 100 times (48). The primary sequences of Hb S and Hb A differ in a single amino acid. Hb S has a hydrophobic valine instead of a hydrophilic glutamic acid at the β6 position. Furthermore, mutants with isoleucine (49) and lysine (13,50) at the β6 position have lower and higher solubility, respectively, than Hb S. Therefore, lowering solubility and forming polymers can be attributed to this single amino acid substitution, a direct result of introducing undesirable interactions among surface residues of neighboring tetramers.

We have generated five Hb S mutants for this study. Three of them carry an additional substitution at the α20 or the α50 position [rHb (βE6V/αH20R), Hb (βE6V/αH20Q) and rHb (βE6V/αH50Q)] while the other two carry mutations at both α20 and α50 positions [rHb (βE6V/αH20R/αH50Q) and rHb (βE6V/αH20Q/αH50Q)]. These Hb S mutants have substituted amino acid(s) on the surface of the tetrameric protein and away from the heme pockets, the α₁β₁ and the α₁β₂ interfaces. Hence, these mutants are structurally (Figures 4 and 5) and functionally (Figure 2 and Tables 1 and 2) similar to Hb S. Their autoxidation rate and thermal stability are similar to, and in some case [rHb (βE6V/αH20Q/αH50Q)] better than, Hb S.

EM studies (6,51) indicate the double strands within the Hb S fibers have a slight helical twist. The results give an overall conformation of the fiber but cannot pinpoint the interacting residues with certainty. Data from X-ray crystallography have higher resolution but the double strands found in the Hb S crystals are straight and do not have the helical twist (10-12).

Harrington et al. (11) refined the crystal structure of deoxy Hb S to 2.05 Å, then employed the program CONTACT to identify inter- and intra-strand interacting residues. With this approach, 34 residues from the β₁/β₂ subunits and
9 residues from the \( \alpha_1/\alpha_2 \) subunits are suggested to be within interacting distances either laterally or axially among Hb S tetramers. These putative interacting residues are listed in Table 3.

According to the model of Harrington et al. (11), the axial contacts can be grouped into: (i) the GH corner (\( \alpha_1 \text{Pro114 and } \alpha_1 \text{Ala115} \)) of the \( \alpha_1 \) subunit of the lower tetramer interacts with the GH corner (\( \beta_2 \text{Glu116} \) and A helix (\( \beta_2 \text{Lys16} \)) of the \( \alpha_2 \) subunit of the upper tetramer; (ii) the A helix (\( \beta_2 \text{Gly16}, \beta_2 \text{Lys17} \) and \( \beta_2 \text{Val18} \)) of the \( \beta_2 \) subunit of the lower tetramer interacts with the G helix (\( \beta_1 \text{His119} \) and \( \beta_1 \text{Glu22} \)) of the \( \alpha_2 \) subunit of the upper tetramer; and (iii) the G helix (\( \beta_1 \text{His117} \) and the GH corner (\( \beta_1 \text{Phe118} \) and \( \beta_2 \text{Lys120} \)) of the \( \beta_2 \) subunit of the upper tetramer; (iii) the G helix (\( \beta_1 \text{His117} \) and the GH corner (\( \beta_1 \text{Phe118} \) and \( \beta_2 \text{Lys120} \)) of the \( \beta_2 \) subunit of the upper tetramer interacts with the GH corner (\( \alpha_2 \text{Pro114 and } \alpha_2 \text{Ala115} \)) of the \( \alpha_2 \) subunit of the upper tetramer; and (iv) the B helix (\( \beta_1 \text{Glu22} \)) of the \( \beta_1 \) subunit of the lower tetramer interacts with the B helix (\( \alpha_2 \text{His20} \)) of the \( \alpha_2 \) subunit of the upper tetramer. These interacting residues are presented as a diagram in Fig. 1B. Since the coordinates in the PDB file 2HBS cannot pinpoint the relative positions of the interacting residues, we summarized the interacting residues as Wishner et al. (8).

The lateral interactions contributing to the Hb S polymerization are mainly between \( \beta_1 \) of one strand with the \( \beta_2 \) of the neighboring strand. Seven residues from the A helix, including the substituted \( \beta_1 \text{Val6} \), plus two residues from the H helix of \( \beta_2 \) interact with 13 residues from the E and F helices, and the EF and FG corners of the neighboring \( \beta_2 \) subunit (Table 3 and Fig. 1A). The interactions between the \( \beta_1 \text{EF corner} \) (\( \beta_1 \text{Asp79} \) and \( \beta_2 \text{Asn80} \)) and the \( \alpha_2 \) CD corner (\( \alpha_2 \text{Ser49} \) and \( \alpha_2 \text{His50} \)) constitute the only lateral contact between \( \beta_2 \) and \( \alpha \) subunits. In particular, the side chains of \( \beta_1 \text{Asp79} \) and \( \alpha_2 \text{His50} \) are reportedly separated by only 2.83 Å (11). However, according to the deposited coordinates (PDB 2HBS) of the two Hb S tetramers in the unit cell, the imidazole nitrogen of \( \alpha_2 \text{His50} \) is 6 Å from the side chain oxygen of \( \beta_1 \text{Asp79} \). The distance between the side chains of \( \alpha_2 \text{His50} \) and \( \beta_1 \text{Asn80} \) is shorter (3.86 Å), but still too far for hydrogen bonding. Therefore, these results generated from docking calculations should be confirmed with biochemical and mutagenesis studies.

Careful scrutiny of the docking results reveals that only five (\( \alpha_1 \text{Lys16, } \alpha_1 \text{His20, } \alpha_1 \text{Pro114, } \alpha_2 \text{Ala115 and } \alpha_2 \text{Glu116} \)) and two (\( \alpha_2 \text{Ser49 and } \alpha_2 \text{His50} \)) residues on the \( \alpha_1 \) subunits contribute to either axial or lateral interactions, respectively. Russu and Ho (52) have suggested the presence of histidyl residues at contact areas between Hb S molecules in pre-gelation aggregates. Therefore, \( \alpha_1 \text{His20 and } \alpha_1 \text{His50} \), the only two histidines among the list, were selected for mutation and its effect on Hb S solubility was studied.

Presumably, \( \alpha_1 \text{His20 and } \beta_2 \text{Glu22} \) form an interacting pair in the Hb S fibers. Nagel et al. (53) compared the \( \beta_2 \) and \( \gamma \) sequences of the hemoglobin subunit, and determined the minimum gelling concentration of a mixture of Hb S and Hb G Coushatta (\( \beta_2 \text{Glu22} \) to Ala). They suggested \( \beta_2 \text{Glu22} \) possibly partially stabilized the deoxy Hb S polymers. Acharya & Seetharam (54) modified the \( \beta_2 \text{Glu22} \) and the \( \beta_2 \text{Glu43} \) with glucosamine and they observed an increase in the solubility of the modified deoxy Hb S by about 55%. However, addition of glucosamine not only eliminated the carboxyl side chain of \( \beta_2 \text{Glu22} \) but appended also a bulky carbohydrate derivative that might interfere with subunit-subunit interactions. Therefore, whether disrupting the putative \( \alpha_1 \text{His20 and } \beta_2 \text{Glu22} \) interaction can improve Hb S solubility is debatable.

Hb S carrying mutations at the \( \alpha_2 \) position have been studied (23, 24, 55). Rhonda et al. (55) measured the solubility of an equal mixture of Hb S and Hb Le Lamentin (Hb S with \( \alpha_1 \text{His20} \) to Gln mutation) and claimed a 1.6 fold increase in Csat value. Srinivasulu et al. (23) measured the solubility of Hb Le Lamentin with an oxygen affinity method and reported only a 26% increase in Csat. Banerjee et al. (24) used the Dextran-Csat method of Bookchin et al. (56) and reported a 28% increase in Csat value for the same mutant.

We used the Dextran-Csat method of Tam et al. (28) developed for micro-sample handling in this study. Our Hb Le Lamentin [rHb (\( \beta_2 \text{E6V/} \alpha_1 \text{H20Q} \))] has a Csat value similar to that of Hb S without any pronounced improvement (Table 2), even though the polymers formed slower than for Hb S (Fig. 3). The 3.55 Å distance (11)
between the N\textsuperscript{2} atom of the \(\alpha_2\)His20 and the O\textsuperscript{2} atom of the \(\beta_1\)Glu22 is possibly too long for optimal hydrogen bond formation. However, the structure of Hb in solution is flexible and its backbone and side chains can exhibit various types of motions (see (57) for review). Hence, it is conceivable that a subpopulation of the Hb possibly assumes a conformation with a shorter distance between \(\alpha_2\)His20 and \(\beta_1\)Glu22 for hydrogen bonding. The conservative substitution of Gln for His at the \(\alpha_2\) position disrupts this interaction and abolished the contribution of \(\alpha_2\)His20 and \(\beta_1\)Glu22 to the polymerization process, resulting in a lengthening in the delay time of polymerization.

To further explore the possible interaction between \(\alpha_2\)His20 and \(\beta_5\)Glu22, we generated the rHb \((\beta_1E6V/\alpha_2H20R)\) mutant. rHb \((\beta_1E6V/\alpha_2H20R)\) has a Csat value of 1.86 ± 0.20 g/dL (Table 2), which is only 2/3 of that of Hb S. It has also a shorter delay time of polymerization and the size of nucleus in forming aggregates is larger than for Hb S (Figure 3). Hence, the interactions between neighboring Hb S tetramers are intensified by introducing a longer side chain carrying a positive charge at the \(\alpha_2\) position. Adachi et al. have investigated the polymerization of Hb S in the presence of Hb A\textsubscript{2} variants carrying Val, Glu and Thr at positions 6, 22 and 87 of the \(\delta\)-subunit (58). They concluded also that it is not necessary to change Glu to Ala at the \(\beta_2\) position in preparing anti-sickling hemoglobin. Therefore, we conclude that the interaction between \(\alpha_2\)His20 and \(\beta_5\)Glu22 contributes minimally to the polymerization of Hb S.

The lateral interactions among residues on the \(\beta_2\) subunit with the neighboring \(\beta_5\) EF pocket have been studied extensively. For instance, mutants with an aromatic residue (phenylalanine or tryptophan) at the \(\beta_6\) position are more soluble than Hb S (59). Probably, it is more difficult to insert a bulky side chain into the EF pocket. The observation that the rHb \((\beta_1E6V/\beta_2L88F)\) mutant, with an aromatic residue in the EF pocket, has also higher solubility substantiates this speculation (16). Other residues on or near the acceptor site have also been mutated to disrupt the lateral interactions in various studies. In general, the mutants have moderately improved solubility (16,18,19,22,60,61) but some of them reportedly suffered from thermal instability (16,18,61).

According to the model proposed by Harrington et al., (11) \(\beta_5\)Asp79 interacts with both \(\alpha_2\)Ser49 and \(\alpha_2\)His50, while \(\beta_5\)Asn80 interacts only with \(\alpha_2\)His50. Nagel et al (53) have investigated the solubility of Hb S in the presence of an Hb A\textsubscript{2} mutant containing a \(\beta_1\)Asn80\(\rightarrow\)Lys mutation and observed a 19% increase in solubility. McCune et al. (62) generated an Hb S mutant with addition \(\beta_1\)Glu22\(\rightarrow\)Ala and \(\beta_5\)Asn80\(\rightarrow\)Lys substitutions. This mutant is better than Hb A, but less effective than Hb F in inhibiting the polymerization of Hb S. Srinivasulu et al. (63) substituted the \(\alpha_1\)Ser49 on Hb S with Arg and observed a 60% increase in solubility. However, the contribution of \(\alpha_2\)His50 in stabilizing the Hb S polymer has never been tested.

To address this issue, we generated the rHb \((\beta_1E6V/\alpha_2H50Q)\) mutant which has a Csat value 4 times higher than that of Hb S (Table 2). The results show clearly that removing the positive charge on \(\alpha_1\)His50 interrupts its interaction with the neighboring \(\beta_5\)Asp79 and/or \(\beta_5\)Asn80. Furthermore, this substitution at the \(\alpha_1\)His50 position improves also the solubility of rHb \((\beta_1E6V/\alpha_2H20R)\) and rHb \((\beta_1E6V/\alpha_2H20Q)\). The resulting rHb \((\beta_1E6V/\alpha_2H20Q/\alpha_1H50Q)\) mutant has a Dextran-Csat value of 10.89 ± 0.80 g/dL, which is 3.7 times higher than Hb S. It has similar autoxidation rate and better thermal stability than Hb S (Table 2). The rHb \((\beta_1E6V/\alpha_2H20R/\alpha_1H50Q)\) mutant has a Dextran-Csat value of 7.11 ± 0.66 g/dL (Table 2), which is 3.8 times better than the rHb \((\beta_1E6V/\alpha_2H20R)\) mutant. Both mutants have longer delay time of polymerization compared to that of Hb S (Figure 3). Furthermore, the results also suggest the effects of mutation at positions \(\alpha_2\) and \(\alpha_1\) are independent of each other.

In summary, the results in this report show that: (i) the interaction between \(\alpha_2\)His20 and \(\beta_5\)Glu22 is minimal in stabilizing the Hb S polymer; and (ii) the Hb S polymer can be destabilized by replacing the histidyl residue at the \(\alpha_50\) position with a Gln. The resulting protein is functionally and structurally similar to Hb S. The mutant carrying the \(\alpha_1\)His50\(\rightarrow\)Gln substitution can be a candidate for the gene therapy of sickle cell disease.
Conflict of interest—the authors declare that they have no conflicts of interest with the contents of this article.

Author contributions—MFT and CH designed the study and wrote the paper. TCST purified all the samples in this study, performed and analyzed the experiments in Table 2. VS collected and analyzed all NMR spectra. NTC performed and analyzed the experiments shown in Table 1. MZ performed and analyzed the experiments shown in Figures 2 and 3. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES

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2The abbreviations used are: Hb A, human adult hemoglobin; sickle cell hemoglobin, Hb A with βGlu6→Val (βE6V) mutation; rHb, recombinant hemoglobin; rHb (βE6V/αH20R), recombinant sickle cell hemoglobin with additional αHis20→Arg mutation; rHb (βE6V/αH20Q), recombinant sickle cell hemoglobin with additional αHis20→Gln mutation; rHb (βE6V/αH50Q), recombinant sickle cell hemoglobin with additional αHis50→Gln mutation; rHb (βE6V/αH20R/αH50Q), recombinant sickle cell hemoglobin with additional αHis20→Arg and αHis50→Gln mutations; rHb (βE6V/αH20Q/αH50Q), recombinant sickle cell hemoglobin with additional αHis20→Gln and αHis50→Gln mutations; met-Hb, methemoglobin; HbCO, carbonmonoxy hemoglobin; deoxy-Hb, deoxy hemoglobin; P50, partial O2 pressure at 50% saturation; n50, Hill coefficient at 50% O2 saturation; Csat, solubility; td, delay time of polymerization; kauto, autoxidation rate; NMR, nuclear magnetic resonance.

FIGURE LEGENDS

Figure 1. Putative amino acid residues that participate in (A) lateral and (B) axial contacts in double strand Hb S polymers. The lateral contacts are drawn according to coordinates in the PDB 2HBS. Residues from α2, βS1 and βS2 subunits are colored in cyan, blue and coral, respectively. The axial contacts are presented as in (8). The yellow double arrows depict the interacting subunits.

Figure 2. Polymerization of Hb S (A) and its recombinant mutants. Polymer formation was assessed by light scattering at 700 nm in 1.8 M phosphate buffer at pH 7.4. Data are presented for each protein at two different concentrations. The scales on the x- and y-axis vary among the panels.

Figure 3. Kinetics of polymerization of Hb S and its recombinant mutants in concentrated phosphate buffer. The log values of the reciprocal of the delay times of polymerization are plotted against the log of hemoglobin concentrations. The slopes (m) of the plots are indicated.

Figure 4. 1H-NMR spectra of the CO form of Hb A, Hb S and rHbs. Spectra of (A) exchangeable proton resonances and (B) ring current-shifted proton resonances were acquired at 600 MHz in 95% H2O, 5% D2O, and 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C.

Figure 5. 1H-NMR spectra of the deoxy form of Hb A, Hb S and rHbs. Spectra of (A) the hyperfine-shifted NδH proton resonances of the proximal histidyl residues and (B) the hyperfine-shifted and exchangeable proton resonances were acquired at 300 MHz in 95% H2O, 5% D2O, and 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C.
Table 1: Oxygen binding properties and Bohr Effect of Hb S and recombinant mutants

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<th>pH</th>
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Experiments were conducted in 0.1 M sodium phosphate buffer at 29º C in the presence of a methemoglobin reductase system (29). Values for Bohr Effect were estimated from the pH range in parentheses. Experiment results have standard deviation less than ± 4% between runs. *Data from Tam et al. (31).
Table 2: Dextran-Csat value, autoxidation rate and thermal stability of Hb S and recombinant mutants

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<th>Dextran-Csat (g/dL)</th>
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<th>Thermal Stabilityᵃ</th>
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<td>Hb S</td>
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ᵃ Percent denatured proteins after heating at 60 ºC for 10 min.
N.D., not determined.
Table 3: Putative amino acid residues that participate in lateral and axial contacts in double strand Hb S polymers (11). Interacting residues are highlighted with the same color or connected with double-headed arrows.

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Figure 1. Putative amino acid residues that participate in (A) lateral and (B) axial contacts in double strand Hb S polymers. The lateral contacts are drawn according to coordinates in the PDB 2HBS. Residues from $\alpha_2$, $\beta^S_1$ and $\beta^S_2$ subunits are colored in cyan, blue and coral, respectively. The axial contacts are presented as in (8). The yellow double arrows depict the interacting subunits.
Figure 2. Polymerization of Hb S (A) and its recombinant mutants. Polymer formation was assessed by light scattering at 700 nm in 1.8 M phosphate buffer at pH 7.4. Data are presented for each protein at two different concentrations. The scales on the x- and y-axis vary among the panels.
Figure 3. Kinetics of polymerization of Hb S and its recombinant mutants in concentrated phosphate buffer. The log values of the reciprocal of the delay times of polymerization are plotted against the log of hemoglobin concentrations. The slopes (m) of the plots are indicated.
Figure 4. $^1$H-NMR spectra of the CO form of Hb A, Hb S and rHbs. Spectra of (A) The exchangeable proton resonances and (B) ring current-shifted proton resonances were acquired at 600 MHz in 95% H$_2$O, 5% D$_2$O, and 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C.
Figure 5. $^1$H-NMR spectra of the deoxy form of Hb A, Hb S and rHbs. Spectra of (A) the hyperfine-shifted $^1$H proton resonances of the proximal histidyl residues and (B) the hyperfine-shifted and exchangeable proton resonances were acquired at 300 MHz in 95% $^2$H$_2$O, 5% $^2$D$_2$O, and 0.1 M sodium phosphate buffer at pH 7.0 and 29 °C.
Sickle Cell Hemoglobin with Mutation at αHis50 Has Improved Solubility
Ming F. Tam, Tsuey Chyi S. Tam, Virgil Simplaceanu, Nancy T. Ho, Ming Zhou and Chien Ho

J. Biol. Chem. published online July 16, 2015

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