A Role for the α113 (GH1) Amino Acid Residue in the Polymerization of Sickle Hemoglobin

EVALUATION OF ITS INHIBITORY STRENGTH AND INTERACTION LINKAGE WITH TWO FIBER CONTACT SITES (α16/23) LOCATED IN THE AB REGION OF THE α-CHAIN*

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A cluster of amino acid residues located in the AB-GH region of the α-chain are shown in intra-double strand axial interactions of the hemoglobin S (HbS) polymer. However, α-Leu-113 (GH1) located in the periphery is not implicated in any interactions by either crystal structure or models of the fiber, and its role in HbS polymerization has not been explored by solution experiments. We have constructed HbS Twin Peaks (βGlu-6→Val, α-Leu-113→His) to ascertain the hitherto unknown role of the α113 site in the polymerization process. The structural and functional behavior of HbS Twin Peaks was comparable with HbS. HbS Twin Peaks polymerized with a slower rate compared with HbS, and its polymer solubility (C_{sat}) was found to be about 1.8-fold higher than HbS. To further authenticate the participation of the α113 site in the polymerization process as well as to evaluate its relative inhibitory strength, we constructed HbS tetramers in which the α113 mutation was coupled individually with two established fiber contact sites (α16 and α23) located in the AB region of the α-chain: HbS(αLys-16→Gln, αLeu-113→His), HbS(αGlu-23→Gln, αLeu-113→His). The single mutants at α16/α23 sites were also engineered as controls. The C_{sat} values of the HbS point mutants involving sites α16 or α23 were higher than HbS but markedly lower as compared with HbS Twin Peaks. In contrast, C_{sat} values of both double mutants were comparable with or higher than that of HbS Twin Peaks. The demonstration of the inhibitory effect of α113 mutation alone or in combination with other sites, in quantitative terms, unequivocally establishes a role for this site in HbS gelation. These results have implications for development of a more accurate model of the fiber that could serve as a blueprint for therapeutic intervention.

Sickle cell anemia is a consequence of a point mutation (Glu-6→Val) at the sixth position in the β-chain of the hemoglobin molecule (1). The replacement of a charged residue with a hydrophobic one on the surface of the protein drastically reduces the solubility of the deoxygenated sickle hemoglobin (HbS)¹, leading to its polymerization into long helical fibers that are responsible for the clinical manifestations of sickle cell disease. Electron microscopy and crystallographic studies have suggested that both the deoxy HbS crystal and fiber are constructed from the same “Wisher-HbS” double strands (2–5). The model of the fiber structure derived from these analyses consists of seven Wisher-Love double strands (6–9).

The polymerization process is triggered by lateral interactions of the donor Val-β6 of a tetramer of one strand of the double strand with the acceptor pocket at the EF corner (elicited mainly by βPhe-85 and βLeu-88) of the β-chain of an adjacent molecule present in the second strand of the double strand. Subsequent intra-double strand and inter-double strand interactions involving several amino acid residues from both α- and β-chains contribute to the stabilization of the fiber structure. The polymerization-imparing or enhancing propensity of mutant hemoglobins, in a binary mixture of mutant hemoglobins and HbS, has facilitated the mapping of several contact residues of the HbS polymer (10–12). The list of contact sites has been expanded by subsequent studies involving chemical modifications of HbS (13, 14) and site-directed mutagenesis (15–19). However, the identities of all the fiber contacts that are predicted by model studies have not yet been tested in solution experiments. The fiber models themselves are not perfect, because they include several polymerization-insensitive sites, exclude polymerization-sensitive sites, and possibly underestimate or overestimate the number of contact residues (6–9). Thus, it is prudent to identify and authenticate all of the participating residues by solution polymerization studies. More importantly, knowledge of the inhibitory strength of each contact site and the combinatorial effects of two or more contact sites (interaction linkage) is imperative for designing effective antisickling agents or vectors for gene therapy that could bring about optimum inhibition of fiber formation needed for clinical amelioration of sickling. The relative strength of contact sites, as well as their “interaction linkage” relationships in terms of synergy and/or additivity, is largely unknown and is beginning to be addressed only now by solution polymerization studies (20).

We have chosen three sites, namely α16, α23, and α113, for further delineation of their contributions to the polymerization of HbS. Whereas sites αLys-16 and αGlu-23, located in the AB region, are established contact points in the HbS polymer, the participation of αLeu-113 (GH1) in the polymerization process is unknown. The α113 site is of interest because of its unique structural location. First, α113 is in sequence contiguity with a cluster of GH corner residues, α114, α115, and α116, which are pressure liquid chromatography; RP-HPLC, reverse phase HPLC; Fmoc, N-(9-fluorenlyl)methoxycarbonyl; FPLC, fast protein liquid chromatography.

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¹ The abbreviations used are: HbS, sickle hemoglobin; HPLC, high performance liquid chromatography; RP-HPLC, reverse phase HPLC; Fmoc, N-(9-fluorenlyl)methoxycarbonyl; FPLC, fast protein liquid chromatography.
established or implicated intra-double strand contact sites of the HbS fiber (7, 11, 16). Second, the three-dimensional structure of the hemoglobin brings the AB region of the α-chain in close proximity to the GH corner. The involvement of several residues of the AB corner (α16, α20, α23) in HbS polymerization has been deduced from crystal structures and also been validated in solution experiments (21–24). However, α-Leu-113, which is located in the periphery of several physiologically relevant axial contacts in the AB-GH region, is not implicated in any contacts by crystal or model studies. Interestingly, a natural HB variant at this site, Hb Twin Peaks (α-Leu-113→His), is reported (25), but the functional properties of Hb Twin Peaks or its participation in deoxy HbS polymerization has not been examined. Here, we constructed HbS Twin Peaks (α-Leu-113→His, β-Glu-6→Val) to establish the role of the α113 site in HbS fiber generation. Furthermore, we have combined the α113 mutation at the GH corner with mutations of contact sites involving residues α16 and α23 of the AB region to see whether the inhibitory sites in the “contact-rich” AB-GH domain have additive or synergistic influence on the Val-6β dependent polymerization of HbS.

We have adopted a chemo-enzymatic strategy for the construction of α-globin mutants. The propensity of V8 protease to catalyze the ligation of complementary fragments, α1–30 and α31–141, to generate a full-length α-globin (α1–141) has been utilized for this purpose (26). Appropriate synthetic α1–30 segments were employed to incorporate desired mutations at sites α16 and α23. The Hb Twin Peaks mutation was introduced through the α31–141 segment of the marmoset (Callithrix argentata) α-chain, which contains a single amino acid substitution, α-Leu-113→His, with respect to the human α31–141 segment (27). HbS tetramers were assembled from β-chain and respective single and double mutant α-chains. The structural/conformational, functional, and polymerization behavior of mutants was studied with a view to examining the hitherto unknown role of the α113 (GH1) site in the HbS gelation process as well as to quantifying its inhibitory strength relative to selected AB region α-chain contact residues.

**MATERIALS AND METHODS**

CM-52 and DE-52 were purchased from Whatman. V8 protease was obtained from Pierce. The chemicals used in peptide synthesis were from Novabiochem. All other chemicals and reagents were of analytical purity and were procured from standard commercial sources. The hemoglobins from sickle cell patients and marmosets were purified form respective red cell hemolyzates by established procedures employing successive anion (DE-52) and cation (CM-52) exchange chromatography. The hydroxymercuribenzoate α- and β-chains were prepared as described previously (28). The chains were freed from heme by acid-acetone precipitation.

**Preparation of α1–30 and α31–141 Segments of Human or Marmoset α-Globin**—The complementary segments of α-globin needed for the semisynthesis of mutant chains were prepared by V8 protease digestion (26). The respective globins were dissolved in 10 mM ammonium acetate buffer (pH 4) at a concentration of 0.5 mg/ml and digested at 37 °C with V8 protease (1:200, w/v) for 3 h. The completion of digestion was ascertained by RPHPLC. The complementary segments, α1–30 and α31–141, from the respective digestion mixtures were isolated in pure form by gel permeation chromatography on a Sephadex G50 column. The column was equilibrated and run in 0.1% trifluoroacetic acid. The lyophilized sample of the digest was dissolved in the above solvent and loaded on to the column. The column was run at a flow rate of 45 ml/h, and the elution profile was monitored at 280 nm. The individual chromatographic profile of the α-globin digest (human or marmoset) showed only two peaks, α31–141 and α1–30, as expected from a single cleavage at the 30–31 peptide bond. The peak fractions from each digest were pooled separately and lyophilized.

**Synthesis of α1–30 Analogos:** α16-Glu and α23-Glu—Peptides were synthesized by a standard solid phase N-(9-fluorenyl)methoxycarbonyl (Fmoc) strategy on a peptide synthesizer (model 90, Advanced Chemtech). For this, Wang resin pre-loaded with Fmoc-Glu was used as the starting material. The stepwise coupling of Fmoc amino acids was performed with N,N′-disopropylcarbodiimide/1-hydroxybenzotriazole activation procedure. The coupling of each step was monitored by a Kaiser test (29), and wherever necessary, a double coupling was used to increase the yield. On completion of the synthesis, the peptide was cleaved from the resin and deprotected with an appropriate volume of a mixture containing trifluoroacetic acid, ethanedithiol, phenol, thioanisole, and water (80:5:5:5, v/v). The resin was removed by filtration, and the crude cleaved peptides were precipitated using cold diethyl ether. The peptides were purified by RPHPLC, and their chemical purity was established or implicated. The experimental masses of the peptides were in agreement with their theoretical masses: α-Lys-16→Glu (observed mass of 3040.73 Da (theoretical mass, 3040.73)); α-Glu-23→Gln (observed mass of 3040.50 (theoretical mass, 3039.39).

**Construction of Mutant α-Globins**—V8 protease-mediated semisynthesis of α-globin was carried out at 4 °C in 50 mM ammonium acetate buffer (pH 6) containing 30% 1-propanol. For this, the lyophilized samples of natural or synthetic analogs of α1–30 and human or marmoset α31–141 were individually prepared in water. Suitable volumes of the complementary fragments were mixed to obtain a 1:1 molar ratio and lyophilized. The lyophilized material (150 mg) was dissolved in 6 ml of 84 mM ammonium acetate buffer (pH 6). To this solution, 3 ml of 1-propanol was added. The mixture was cooled on ice, subsequent to which 1 ml of V8 protease solution (1.5 mg/ml in water) was added. The ligation reaction mixture was incubated at 4 °C for 24 h. The reaction was stopped by addition of 2 ml of 5% trifluoroacetic acid and lyophilized. The semisynthetic α-globin was isolated from the reaction mixture by CM52-urea chromatography, extensively dialyzed against 0.1% trifluoroacetic acid, and lyophilized (26). The semisynthetic yield of the protein varied between 35 and 45%. The identity of the α-globin construct was checked by mass spectrometry and tryptic peptide mapping.

**Reconstitution of α-Globin and the β-Chain into HbS Tetrads**—The semisynthetic α-globin was reconstituted with heme and the β-chain into tetrameric hemoglobin through the “Alloplex pathway” as described previously (26). The reconstituted tetramers were purified by CM-52 chromatography. The heme stoichiometry in purified tetramers was ascertained by 280:540 nm absorbance ratios. The A280/A40 Ratio for native HbS was 2.54. For reconstituted tetramers, this ratio varied between 2.49 and 2.55. The tetramers were checked for the correct stoichiometry of chains by RPHPLC. The α- and β-chains from each Hb were isolated and subjected to electrospray mass spectrometry and tryptic peptide mapping to ensure that the reconstitution procedure did not alter the chemical integrity of the chains.

**Spectroscopic Studies**—The spectra were recorded on a Lambda Bio20 spectrophotometer (PerkinElmer Life Sciences). The UV region derivative spectra were recorded in the first derivative mode of the spectrophotometer. The hemoglobin concentration used for the spectral measurements was ~50 µM on hemoglobin basis. Circular dichroism spectra were recorded on a J710 spectropolarimeter (Jasco) fitted with a Peltier-type constant temperature cell holder (PTC-348W). The calibration of the equipment was done with (+)10-camphorsulfonic acid.

**Analytical Procedures**—The synthetic peptides were purified by RPHPLC on an aquapore RP300 column (250 × 7 mm) using a 4–72% linear gradient of solvent B (acetonitrile containing 0.1% trifluoroacetic acid) in 130 min at a flow rate of 2 ml/min. Globin chains from respective hemoglobins were separated on a similar column of a smaller dimension (250 × 4.6 mm) under identical conditions but at a flow rate of 0.7 ml/min.

Analytical anion-exchange chromatography of HbS constructs was performed by FPLC (AKTA, Amersham Pharmacia Biotech) on a Mono Q HR5/5 column. The respective protein samples were prepared in Tris acetate buffer (50 mM, pH 8.5) and loaded on the column that was pre-equilibrated with the same buffer. The samples were chromatographed using a linear pH gradient of 50 mM Tris acetate buffer, pH 8.5 to 7.0 over 20 min with a 1 ml/min flow rate. The elution profile was monitored at 540 nm.

**Electrospray mass spectrometric analysis was carried out on a VG Platform (Fisons) mass spectrometer.** The instrument was usually calibrated with standard myoglobin solution. Appropriate amounts of globin chains isolated from each HbS sample by RPHPLC were taken in 50% acetonitrile containing 1% formic acid and analyzed under the positive ion mode. The spectra of globins produced a series of protonated species typically ranging from 13 to 22 positive charges. The average molecular mass of each globin from the respective spectra was obtained.
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by using the software provided by the manufacturer.

Oxygen Affinity Measurements—The oxygen affinity of hemoglobins was measured by a Hemox-Analyzer (TCS Medical Products, New Hope, PA) at 29 °C in 0.1 M sodium phosphate buffer, pH 7.4. The hemoglobin concentration was ~0.1 mM based on heme. The \( P_50 \) value (partial oxygen pressure at 50% saturation) and the Hill coefficients \( (n_{Hill}) \), a measure of cooperativity, were determined from each dissociation curve.

Measurement of Gelation Concentration, \( C_{sat} \)—The gelation concentration of HbS constructs was determined by the dextran-\( C_{sat} \) method of Bookchin et al. (30). This method allows measurement of \( C_{sat} \) under near-physiological conditions and at a much lower concentration of Hbs (about 5-fold or less) than that required in standard \( C_{sat} \) assays but essentially provides the same information. Briefly, a suitable aliquot of a concentrated solution of hemoglobin in potassium phosphate buffer (0.05 M, pH 7.5) was taken in a 1.5-ml microcentrifuge tube. A concentrated dextran (70 KDa) solution prepared in the same buffer was added to the aliquot and mixed well. This mixture was overlayed with 0.5 ml of mineral oil, chilled on an ice bath, and deoxygenated with an anaerobically prepared dithionite solution through an airtight Hamilton syringe. The final concentrations of dextran and dithionite in the mixture were 120 mg/ml and 0.05 M, respectively. The above deoxygenated sample was allowed to polymerize at 37 °C for 30 min, after which the gel under the oil layer was disrupted with the plunger of a Hamilton syringe. The tube was centrifuged at room temperature at 14,000 rpm for 30 min. The above process of gel disruption and centrifugation was repeated twice, subsequent to which the oil layer was aspired, and suitable aliquots from the supernatant were taken for estimation of \( C_{sat} \) by Drabkin’s reagent.

Kinetics of Polymerization—The delay time kinetics of deoxyhemoglobin were studied in 1.8 M phosphate buffer (pH 7.25) as described by Adachi and Asakura (31, 32) using a Cary 400 spectrophotometer equipped with a Peltier temperature controller. The polymerization of deoxyhemoglobin samples was initiated by a temperature jump from 3 to 30 °C within 10 s, and the progress of the reaction was followed by monitoring turbidity changes at 700 nm. The delay time was calculated from the kinetic traces.

RESULTS

Assembly and Chemical Characterization of HbS Twin Peaks (αLeu-113→His, βGlu-6→Val)—The construction of the \( \alpha \)-chain of Hb Twin Peaks (αLeu-113→His) through the \( \alpha \)-globin semisynthetic strategy involves the insertion of human \( \alpha1–30 \) with an \( \alpha31–141 \) segment containing the Twin Peaks mutation. The two peaks isolated from G-50 chromatography of the V8 protease digest of the marmoset α-globin were subjected to electrospray mass spectrometry. The reported sequence of marmoset \( \alpha \)-globin (27) contains amino acid substitutions at four sites compared with the human (TSS, A19S, E23D, and L113H). The experimental masses obtained for the two peaks, 3029.23 and 12126.05 Da, respectively, were in agreement with the calculated masses of the complementary fragments of marmoset \( \alpha \)-globin (30, 3028.32 Da; \( \alpha31–141, 12127.99 \) Da).

The semisynthetic Twin Peaks \( \alpha \)-globin was obtained by ligation of human \( \alpha1–30 \) and marmoset \( \alpha31–141 \) fragments through the V8 protease-catalyzed reaction as described under “Materials and Methods.” The purified material was reconstituted with the \( \beta \)-chain and heme to obtain the tetramer. HbS Twin Peaks was isolated in pure form CM-52 chromatography. The purity of the protein was further established by FPLC. Under identical chromatographic conditions, HbS Twin Peaks eluted slightly earlier than the native HbS from the Mono Q anion-exchange column (Fig. 1). This elution behavior of HbS Twin Peaks was consistent with the replacement of Leu by His at the \( a1.13 \) site in HbS.

The purified HbS Twin Peaks was analyzed by reverse-phase HPLC to establish the stoichiometry and chemical integrity of the globin chains. The \( \alpha \)- and \( \beta \)-chains of HbS Twin Peaks were separated on a C8 column (RP300) using an acetonitrile-trifluoroacetic acid-water solvent system and compared with native HbS. The chromatographic profile showed identical retention times for \( \beta \)-chains from both samples and indicated correct stoichiometry of the \( \alpha \)- and \( \beta \)-chains in HbS Twin Peaks (Fig. 1, inset). Interestingly, the order of elution of chains of HbS Twin Peaks was reversed as compared with natural HbS. The \( \alpha \)-chain of HbS Twin Peaks eluted earlier than the \( \beta \)-chain, suggesting that Leu to His substitution exerted considerable influence on the chromatographic behavior of the \( \alpha \)-chain. To rule out the possibility that the above elution behavior was a consequence of chemical modifications during semisynthesis or tetramer assembly, \( \alpha \)- and \( \beta \)-chains of HbS Twin Peaks were isolated by reverse-phase HPLC and subjected to electrospray mass spectrometry. The molecular mass of the isolated \( \alpha \)-chain (15150.03 Da) obtained by electrospray mass spectrometry agreed very well with the calculated value of 15150.36 Da for the \( \alpha \)-chain of HbS Twin Peaks. Likewise, the experimental mass of the \( \beta \)-chain (15837.99 Da) was in accord with the calculated mass of the \( \beta \)-chain (15837.25 Da). Taken together, the results unambiguously established the chemical integrity of HbS Twin Peaks.

Structural Characterization of HbS Twin Peaks—The CD spectrum (Fig. 2) in the soret region for HbS Twin Peaks was similar to that of the HbS, suggesting that interactions of heme with the relevant aromatic residues in the mutant protein are maintained and that the Leu-113→His mutation does not have a deleterious effect on the folding of the heme pocket.

UV spectroscopy was employed to further probe the quaternary structural status of HbS Twin Peaks. The ligand-dependent fine spectral changes around 290 nm are considered as diagnostic of the quaternary structure of the Hb molecule (33–35). The first-derivative UV spectra of oxy (liganded) and deoxy (unliganded) forms of HbS Twin peaks and native HbS were compared to assess the presence of gross quaternary structural changes, if any, in HbS Twin Peaks (Fig. 3). Both HbS and HbS Twin Peaks displayed fine structural characteristics with a peak at 289 nm and a double minimum at 285 and 293 nm, respectively. In both Hbs, the magnitude of the double minimum was reduced to about half upon deoxygenation. These results suggest that quaternary structural features of native
HbS are preserved in HbS Twin Peaks.

**Oxygen Affinity of HbS Twin Peaks**—The functional aspect of HbS Twin Peaks was assessed by measuring the oxygen affinity at pH 7.4 in 0.1 M sodium phosphate buffer at 29 °C using a hemoglobinometer. The hemoglobin concentration was about 50 mg/ml. Solid line, HbS; broken line, HbS Twin Peaks. MRE, mean residue ellipticity.

**Polymer Solubility of HbS Twin Peaks**—The gelation concentration of high concentrations of dextran developed by Bookchin et al. (30) and as described under “Materials and Methods.” The hemoglobin concentration in the supernatant (C$_{sat}$) was determined by Drabkin’s method. Ø, HbS; △, HbS Twin Peaks.

The functional aspect of HbS Twin Peaks was assessed by measuring the oxygen affinity at pH 7.4 in 0.1 M sodium phosphate buffer at 29 °C using a hemoglobinometer. The hemoglobin concentration was 0.64 mg/ml. Solid line, HbS; broken line, HbS Twin Peaks. MRE, mean residue ellipticity.

![Soret region CD spectra of HbS Twin Peaks. The spectra were recorded at 10 °C in 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 M chloride. Solid lines and broken lines represent the spectra of deoxy and oxy forms of the hemoglobin, respectively. 1, native HbS; 2, HbS Twin Peaks.](Image 101x347 to 246x539)

![First derivative UV spectra of liganded and unliganded HbS Twin Peaks. The spectra of oxy and deoxy forms of the protein were recorded at 25 °C in the first derivative mode of the spectrophotometer. The hemoglobin concentration used was about 50 μM (on heme basis) in 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 M chloride. Solid lines and broken lines represent the spectra of deoxy and oxy forms of the hemoglobin, respectively. 1, native HbS; 2, HbS Twin Peaks.](Image 103x596 to 244x729)

**Polymer solubility of HbS Twin Peaks.** The measurements were performed in the presence of dextran in 50 mM potassium phosphate buffer (pH 7.5) and at 37 °C as described under “Materials and Methods.” The hemoglobin concentration in the supernatant (C$_{sat}$) was determined by Drabkin’s method. Ø, HbS; △, HbS Twin Peaks.
yield of the semisynthetic α-chain strategy was carried out in 1.8 M potassium phosphate buffer, pH 7.25. The polymerization of the deoxy form of the protein was initiated by a temperature jump from 4 to 30 °C within 10 s, and the progress of the reaction was continuously monitored at 700 nm. α-α T––x, HbA Twin Peaks (0.5 mg/ml); x––x, HbS Twin Peaks (0.78 mg/ml); α––α, HbS (0.5 mg/ml); x––x, HbA Twin Peaks (1 mg/ml). α-α T––x, HbA (1 mg/ml); x––x, HbA Twin Peaks (1 mg/ml). b, x––x, HbS (0.78 mg/ml); α––α, HbS Twin Peaks (0.78 mg/ml).

Fig. 5. Kinetics of polymerization of HbS Twin Peaks. Polymerization of hemoglobin was carried out in 1.8 M potassium phosphate buffer, pH 7.25. The polymerization of the deoxy form of the protein was initiated by a temperature jump from 4 to 30 °C within 10 s, and the progress of the reaction was continuously monitored at 700 nm. α-α T––x, HbA Twin Peaks (0.5 mg/ml); x––x, HbS Twin Peaks (0.5 mg/ml); α––α, HbS (0.5 mg/ml); x––x, HbA Twin Peaks (1 mg/ml). b, x––x, HbS (0.78 mg/ml); α––α, HbS Twin Peaks (0.78 mg/ml).

The globin chains were isolated by RPHPLC of respective tetramers and subjected to electrospray mass spectrometry analysis. The values in daltons represent the average mass based on natural isotopic abundance. The theoretical mass (calculated) of each chain is indicated in parentheses.

Table I

| Sample            | α-Chain | β-Chain |
|-------------------|---------|---------|
| HbS (native)      | 15127.66 (15126.38) | 15839.51 (15837.25) |
| HbS(αK16Q)        | 15128.25 (15126.33) | 15839.99 (15837.25) |
| HbS(αK16Q, L113H) | 15151.72 (15150.31) | 15837.99 (15837.25) |
| HbS(αE23Q)        | 15124.82 (15125.39) | 15838.99 (15837.25) |
| HbS(αE23Q, L113H) | 15150.70 (15149.37) | 15838.89 (15837.25) |

Table II

| Sample            | P50 | a | b | Csat | Csat/Csat (HbS) |
|-------------------|-----|---|---|------|-----------------|
| HbS (native, control) | 8.5 | 2.5 | 29.6 | 1.00 |
| HbS(αL113H) | 8.0 | 2.4 | 53.4 | 1.80 |
| HbS(αK16Q) | 7.5 | 2.5 | 38.7 | 1.30 |
| HbS(αE23Q) | 7.0 | 2.4 | 44.1 | 1.49 |
| HbS(αK16Q, L113H) | 7.0 | 2.4 | 60.1 | 2.03 |
| HbS(αE23Q, L113H) | 7.5 | 2.5 | 55.4 | 1.87 |

* Oxygen equilibrium curves were measured using Hemox Analyzer at pH 7.4 in 0.1 M sodium phosphate buffer at 29 °C. The concentration of hemoglobin for each sample was approximately 0.1 mM on heme basis. P50 values were reproducible within ±0.5 units.

± All Csat measurements were performed in the presence of dextran as described in the text. Each value represents an average of at least two or more replicates. Variation within the replicates was always less than 3%.

HbS sample were in good agreement with their respective theoretical masses (Table I).

Functional Characterization—The oxygen affinities of all of the HbS mutants (Table II) were in the normal range (P50 = 7.0–7.5), albeit slightly higher than that of native HbS (P50 = 8.5). Hill coefficients of all of the mutants were also normal, indicating preservation of HbS-like quaternary structure. HbS(αL163→Glu) has been studied previously and found to exhibit the usual functional properties (21). The normal oxygen binding behavior of mutants, HbS(αK16Q) or HbS(αK16Q, L113H), suggests that abrogation of the positive charge or reversal of the charge polarity at the α16 site does not appreciably affect the oxygen binding behavior of the Hb.

Polymer Solubility of the Mutants—The Csat values of all HbS constructs were obtained in the presence of dextran under similar conditions as those used for HbS Twin Peaks. The Csat values of the single as well as double mutants were significantly higher than the Csat of native HbS, albeit to different extents (Table II). The innate Csat of HbS (30 mg/ml) increased by nearly 30% (39 mg/ml) and 49% (44 mg/ml) with point mutations of α16 and α23 sites, respectively. This increase in Csat for the above mutants was markedly less than that observed for HbS Twin Peaks; Csat of HbS Twin Peaks (53 mg/ml) was about 80% higher relative to native HbS. The Csat of the double mutant comprising sites α16 and α113 (60 mg/ml) was twice that of HbS and significantly higher than that of the mutation of the α113 site alone. In contrast, the double mutant consisting of α23 and α113 sites produced a Csat (55 mg/ml) that was very similar to that of HbS Twin Peaks.

Discussion

A 14-strand model of the HbS fiber composed of seven Wishner-Love double strands and stabilized by intra- and inter-double strand interactions is now well accepted, although am-

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The $C_{\text{sat}}$ values for the listed sites were determined in the presence of dextran under similar conditions except for minor differences related to the estimation of supernatant Hb concentration subsequent to gelation. Whereas $C_{\text{sat}}$ values of HbSs containing mutations at sites α16, α23, α113, α16/113, and α23/113 (present study) were obtained by measuring the Hb concentration in the supernatant by Drabkin’s procedure, concentration estimates in the case of other sites ($C_{\text{sat}}$ values represented in parentheses) were made by quantitative amino acid analysis. The apparent difference between $C_{\text{sat}}$ values of HbS (Val-6β) obtained in the present report (30 mg/ml) and the previous studies (34 mg/ml) is presumably related to the method of Hb estimation. Nonetheless, this small difference may not have a bearing on the comparative analyses of $C_{\text{sat}}$ data.

### Table III

| Site   | Mutation(s) | Dextran $C_{\text{sat}}$ | Reference |
|--------|-------------|--------------------------|-----------|
| β6    | Glu→Val (HbS) | 30(34)                  | Present study/18, 19, 41 |
| α16   | Lys→Gln     | 39                       | Present study |
| α23   | Glu→Gln     | 44                       | Present study |
| α113  | Leu→His     | 53                       | Present study |
| α85   | Asp→Lys     | (53)                     | 15         |
| β88   | Leu→Ala     | (67)                     | 41         |
| β95   | Lys→Ile     | (90)                     | 41         |
| β121  | Glu→Arg     | (24)                     | 19         |
| α16/113 | Lys→Gln/Leu→His | 60                      | Present study |
| α23/113 | Glu→Gln/Leu→His | 55                      | Present study |
| β88/95 | Leu→Ala/Ala→Ile | (91)                  | 41         |
| β75/112 | Asp→Tyr/Glu→Arg | (12)                   | 19         |
| α6/β75/112 | Asp→Ala/Asp→Tyr/Glu→Arg | (7)                  | 19         |

bignities persist regarding the arrangement of the crystal double strands within the fiber (9). Whereas intra-double strand contacts of the fiber are similar to those found in the single crystals of HbS, inter-double strand contacts are somewhat different. This is due to the fact that double strands in the crystal are straight, whereas packing requirements entail twisted strands. The map of contact sites displayed by different fiber models varies considerably depending on the distance of separation used between Wishner-Love double strands to achieve a reasonable packing (6–8). Most of the interactions within the double strand consist of residues from the β-chain, and those involved between the double strands emanate predominantly from the α-chains. In this connection, it is noteworthy that the discrepancies between the molecular modeling and solution experiments largely relate to the contact sites involving α-chain residues. Therefore, further delineation of interactions in the HbS fiber, particularly of those surface residues of the α-chain that are not implicated in contacts by fiber models or solution studies, appears to be necessary, because it is likely to provide information on the inter-double strand contacts specific to the fiber. The major aim of the present study was to explore the role of one such residue, α-Leu-113 (GH1), by solution polymerization experiments.

We chose to replace the Leu residue at the α113 site by His because this point mutation is present in the α31–141 segment of the marmoset α-chain and was compatible with our semi-synthetic strategy. Because this mutation (α-Leu-113→His) is also naturally present in Hb Twin Peaks (25), it would be expected to have no effect on hemoglobin stability and function. Indeed, the environment of heme and the αβd interface in Hb Twin Peaks was found to be similar to that in HbS, indicating that HbS Twin Peaks assumes an HbS-like quaternary structure. The $P_{\text{maj}}$ and Hill coefficient of HbS Twin Peaks were also similar to those of HbS. Thus, the inhibition of polymerization seen with HbS Twin Peaks, both in high phosphate buffer and in the presence of dextran, reflects the true inhibitory potential of the α113 residue.

The participation of the α113 site in the polymerization of HbS was further corroborated by the polymer solubility data of double mutants involving sites α113 and α23 or α16. The dextran $C_{\text{sat}}$ of point mutants at α16 or α23 (39 and 44 mg/ml, respectively) was much lower than the $C_{\text{sat}}$ of HbS Twin Peaks (α113; 53 mg/ml), suggesting that the above AB region α-chain residues are relatively weak fiber contacts as compared with the α113 site. In contrast, the $C_{\text{sat}}$ of the double mutants comprising sites α16 and α113 (60 mg/ml) or α23 and α113 (55 mg/ml) was higher than or comparable with that of HbS Twin Peaks. Thus, the inhibitory effect of α113 was additive with α16 but not with α23. This is in accord with the crystal structure of deoxy HbS, which shows axial interactions of αLys-16 with αPro-114 and α Ala-115 residues located at the GH corner (37). The interactions of Lys-16 are likely to be perturbed by Gln mutation. Indirect effects of Lys-16 replacements on the α113 mutation are also conceivable in view of the sequence proximity of α113 to the above GH corner sites. In contrast, interactions of the αGlu-23 site do not involve the GH corner residues.

The evaluation of the individual strength of contact sites in quantitative terms has been a current subject of intense focus. In recent studies, site-directed mutagenesis has been employed to generate perturbation in both α- and β-chain contact points with sites in question mutated individually or in combination. The α-chain contacts have also been probed through construction of interspecies hybrids and semisynthetic chimeric hemoglobin (38–40). Nonetheless, these interspecies hybrids contain a large number of mutations, at contact sites or otherwise, compared with the human α-chain, that interfere with the unambiguous assignment of the role of each residue vis à vis the synergistic and additive nature of their interactions. Given the large number of participating residues and the complexity of the polymerization process, precise delineation of the individual or combinatorial strength of sites could be ideally achieved by systematic variation of surface residues through DNA-based site-directed mutagenesis (16, 18, 19, 41). The semisynthetic method, although restricted in scope, could also be effectively applied in appropriate situations as demonstrated in the present study.

We are now in a position to compare the individual strength of α113, α16, and α23 sites with other sites and also the interaction linkage of some fiber contacts in quantitative terms (Table III), because the $C_{\text{sat}}$ values of respective HbS mutants in all these cases have been determined in the presence of dextran under similar conditions, as described by Boekchin et al. (30). The inhibitory strength of sites varies as follows: $β95 > β88 > α113 > α85 > α23 > α16$. The inhibitory strength of α113 is similar to that of α85 but significantly lower than that of $β95$ or $β88$. The other two sites, α16 and α23, are rather weak. Among the above sites, $β95$ and α85 residues are implicated in inter-double strand contacts, whereas $β88$ is involved in crucial primary interactions with Val-6β. We envision the
α113 site as a potential inter-double strand contact point of the fiber, because it is not implicated in interactions by crystal and model studies. The remaining two sites (α16 and α23) with lowest inhibitory propensity are intra-double strand contacts. Against this scenario, it appears that the inhibitory strength of fiber-specific inter-double strand contact sites is likely to be greater than those involved in intra-double strand contacts. It is instructive to note that multiple mutations of the inter-double strand contacts in the pig α-chain relative to human were posited as prime sites that contributed to the total abrogation of polymerization in interspecies hybrid HbS composed of pig α-chain and human β-chain (38). The influence of simultaneous perturbation of two or more contact sites has been analyzed in quantitative terms only for a few combinations of sites. Whereas a non-additive inhibitory effect was seen for β88/β95 residues, the potentiation of polymerization by αβ75/β121 appeared to be additive. Our data show that the inhibitory effect of the α113 site was non-additive with α23 but additive with α16. So far, a synergistic influence between two contact sites has not been demonstrated in any definitive fashion. The knowledge of the individual strength of all of the fiber contact sites in quantitative terms appears to be an immediate need, because it would help discriminate weak and strong sites, thereby restricting the number of combinations that could be assessed as potential therapeutic targets.

In conclusion, we unequivocally established a role for the α113 amino acid residue in the polymerization of HbS. We not only demonstrated the participation of this site in the gelation process but also quantified its strength of interaction. The α113 amino acid residue is not postulated in any interactions in the crystal structure of HbS and is also excluded by the models of the fiber. These considerations lead us to believe that the α113 site might be involved in fiber-specific inter-double strand contacts. Further solution copolymerization studies of HbS Twin Peaks coupled with electron micrographic analysis of the HbS Twin Peaks fiber should help delineate the stereochemistry of the α113 residue in the polymer and facilitate efforts to develop an accurate model of the HbS fiber.

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