

Participation and Strength of Interaction of Lysine 95(β) in the Polymerization of Hemoglobin S as Determined by Its Site-directed Substitution by Isoleucine*

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The role of Lys-95(β), which is on the exterior of the hemoglobin (HbS) tetramer, in the aggregation process has been addressed because there is a lack of agreement on its importance. The early studies on the aggregation of HbS in the presence of other mutant hemoglobins are consistent with the subsequent electron microscopic studies in demonstrating the participation of Lys-95(β) in gelation; the results of the crystal structure do not agree with these conclusions. Therefore, with the objective of clarifying its role we have carried out site-directed substitution of Lys-95(β) to an isoleucine residue. The mutation was introduced by polymerase chain reaction recombination methodology, and the absence of other mutations in the β -globin gene was established by sequencing the gene in its entirety. The recombinant mutant hemoglobin was expressed in yeast and characterized by peptide mapping and sequencing, which demonstrated that the only different tryptic peptide had the Ile substitution at position 95(β). The recombinant hemoglobin had the correct amino acid composition and molecular weight by mass spectrometric analysis. It was also pure as judged by isoelectric focusing. It was fully functional because it had an average Hill coefficient of 3.1 and responded normally to the allosteric regulators, chloride, 2,3-diphosphoglycerate, and inositol hexaphosphate. Of particular interest was the finding that this hemoglobin mutant aggregated at a concentration of about 40 g/dl, nearly twice that at which HbS itself aggregated (24 g/dl). Therefore, Lys-95(β) has a very important role in the aggregation process and is a good candidate site for the design of a therapeutic agent for sickle cell anemia.

The primary cause of sickle cell anemia is a single DNA base change encoding for the amino acid substitution Glu \rightarrow Val at position 6 of the β -chain of hemoglobin (1, 2). This replacement leads to a strong interaction between Val-6(β) and a hydrophobic pocket in the region of Phe-85(β)/Leu-88(β) of an adjacent tetramer in the deoxy conformation (3). Subsequent interactions, some hydrophobic and others hydrophilic in nature involving both lateral and axial intertetrameric contacts, lead to the aggregation of deoxy-Hb tetramers and eventual distortion and sickling of red cells in the venous circulation. The thermodynamics of the polymerization of sickle hemoglobin have been elucidated by Eaton and co-workers (4-7), as well as by other

investigators cited in Ref. 4.

Some of the interactions between HbS tetramers were identified by Bookchin and Nagel (8), who measured the sparing effect of other natural hemoglobin mutants with substitutions at various positions on the insolubility of deoxy-HbS. More recently, many of the contact sites and interactions have been identified by x-ray crystallography and electron microscopy of sickle hemoglobin (9-14). However, the strength of most of these interactions is not yet appreciated in quantitative terms. Using site-directed replacement, we are attempting to answer this question (15-17) for selected amino acids in sickle hemoglobin.

Other studies on sickle hemoglobin performed before the structure of the aggregate was known focused on the chemical modification of certain amino acid side chains of HbS in order to improve its solubility (18-21). These studies, as well as the results of Bookchin and Nagel described above, provided information on the participation of certain amino acids in the polymerization process. However, each approach had its limitations with respect to the sites that could be studied. For example, the chemical modification studies could address only those sites to which a given chemical reagent had some affinity, and this function could not generally be either predicted or directed. The experiments with the natural mutants could focus only on those sites for which natural mutants were available. With the advent of site-directed mutagenesis, especially in the yeast system (22, 23), the range of sites that can be evaluated is unlimited, because it is now possible to substitute any amino acid at any position on either the α - or the β -chain of the tetramer. The yeast expression system that we employ for HbS (15) has the human α - and β -globin genes on the same plasmid and produces a recombinant sickle hemoglobin that is identical by about a dozen biochemical and physiological criteria with the natural sickle hemoglobin purified from the red cells of sickle cell anemia patients (15-17). Most importantly, the gelling concentration of this recombinant sickle hemoglobin is the same as that of the hemoglobin S purified from human sickle red cells. These findings indicate that this system is well suited to produce sickle Hb double mutants to explore those areas of the hemoglobin S tetramer whose roles in the gelation process are not yet defined and to measure quantitatively the strength of such interactions at certain intertetrameric contact sites in the deoxy-HbS aggregate. In the initial study with this objective (17), a sickle hemoglobin double mutant was expressed in which Leu-88(β), part of the initial hydrophobic contact region with Val-6(β), was substituted by an Ala residue. Surprisingly, this conservative replacement led to a 30% decrease in gelation concentration of deoxy-HbS, a value that indicated the strength of this initial interaction. Adachi *et al.* (24) have also addressed this initial hydrophobic interaction by making substitutions at both the donor Val-6(β) and the part of the acceptor region involving Leu-88(β).

In the present study, the particular site that we substitute is

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Lys-95(β), which is on the exterior of the hemoglobin tetramer, yet near the Phe-85/Leu-88 hydrophobic pocket. The role of this amino acid side chain in the aggregation process has been implicated in some studies but not in others so that its role at present is uncertain. When the natural Hb mutants with Lys-95(β) replaced either by asparagine (Hb Detroit) or by glutamic acid (HbN Baltimore), neutral polar and charged polar side chains, respectively, were mixed with HbS, the gelling concentration of the latter increased from about 29 g/dl for HbS to 31.6 and 37.0 g/dl in the presence of the two mutant hemoglobins, respectively (8). However, the crystal structure of sickle hemoglobin (9, 10) did not show the participation of Lys-95(β) in the aggregate, although it did not specifically exclude this site. The findings of Edelstein and co-workers (13, 14) and of Josephs and co-workers (11), who used electron microscopic studies of the sickle HbS fiber, show involvement of Lys-95(β) as a contact site. Dickerson and Geis (25) have pointed out this distinct difference between the crystal and fiber structures of HbS. Hence, our objective in this communication is to answer the question of its involvement in the aggregation process and to establish its role quantitatively.

MATERIALS AND METHODS

Reagents—The restriction endonucleases, T4 polynucleotide kinase, alkaline phosphatase, and DNA ligase were from Boehringer Mannheim. The DNA sequencing kit and the T7 DNA Polymerase (Sequenase version 2.0) were obtained from U. S. Biochemical Corp. The GeneAmp PCR¹ reagent kit and AmpliTaq DNA polymerase were purchased from Perkin-Elmer. The oligonucleotides were synthesized by Operon Technologies (Alameda, CA). Universal pUC/M13 Forward and M13 Reverse were obtained from Promega and U. S. Biochemical Corp., respectively. The Prep-A-Gene DNA purification kit was from Bio-Rad. All the other reagents were of analytical purity.

Yeast Expression System—The double mutant Hb was expressed in yeast (*Saccharomyces cerevisiae* GSY112 *cir*^o) as described previously (15–17) after making the desired mutation in the α - and β -globin gene-containing plasmid by site-directed mutagenesis. pGS389 is a yeast/*Escherichia coli* shuttle plasmid containing the human α - and β -globin cDNAs and the 2- μ m yeast element (22). It can replicate in *E. coli*, where the selection is facilitated by the use of its ampicillin resistance gene. The transcription of the globin genes is induced by growing the cells in a medium containing galactose. pGS189 is a derivative of Bluescript II SK(+) plasmid containing the human α - and β -globin cDNAs and replicates only in *E. coli* (15–17).

To prepare the E6V/K95I(β) double mutant, an *Xho*I fragment of pGS189 containing the sickle β -globin cDNA was first inserted in the *Xho*I site of Bluescript SK(+) (12). This plasmid was used as a template in the PCR reactions. Two overlapping PCR products were synthesized by a PTC-100-60 instrument (MJ Research Inc., Watertown, MA) as described earlier (12) using separately the 5'-ATCCACGTGCAGGAT-GTCACAGTGCAG and pUC/M13 Forward and the 5'-CTGCACGTG-GACATCCTGCACGTGGAT and M13 Reverse primers. The underlined bases were those used to bring about the desired mutation. These products were recombined in a separate PCR reaction by using the pUC/M13 Forward and M13 Reverse primers. The final amplified DNA was digested by *Xho*I, and the 1280-base pair fragment was purified from agarose gel by a Prep-A-Gene DNA purification kit. This fragment was subcloned to the 4130-base pair *Xho*I-fragment of pGS189. The correct insertional direction was confirmed by restriction mapping, and the sequence was verified by the conventional dideoxy method. Finally, the α - and β -globin gene cassette was isolated as a *Not*I fragment after digesting the newly synthesized pGS189 derivative with *Not*I and *Bgl*II and inserted into pGS389 previously digested with *Not*I. The yeast cells were transformed by this plasmid using a lithium acetate method referred to in Refs. 15–17, and the transformants were selected on a complete minimal medium without uracil (15).

Growth of Yeast and Purification of Recombinant Hb—The yeast was grown in YP medium (22) for 4 days using ethanol as the carbon source. The promoter controlling the transcription of the globin genes was

induced for 20 h by the addition of galactose; collection and breakage of the cells has been described earlier (15–17, 26). The purification of the double mutant Hb was accomplished by chromatography on CM-Cellulose 52 and Synchropak CM-300 HPLC columns (16). The gradient for CM-300 was optimized to fit the elution characteristics of the newly synthesized double mutant.

Mass Spectrometry Analysis—The hemoglobin K95I sample was subjected to mass spectrometric analysis on a matrix-assisted laser desorption time-of-flight mass spectrometer constructed at Rockefeller University and described elsewhere (27, 28). The mass spectra were acquired by adding the individual spectra of 200 laser shots. Hemoglobin samples were prepared for laser desorption mass analysis as follows: the laser desorption matrix material (4-hydroxy- α -cyano-cinnamic acid) was dissolved in 50 mM formic acid/water/isopropanol (1:6:4) (v/v/v). A 10 mM bis-Tris acetate solution, pH 7.5, containing the hemoglobin sample was then added to the matrix solution to give a final hemoglobin concentration of approximately 2 μ M. A small aliquot (0.5 μ l) of this mixture was applied to the metal probe tip and dried at room temperature with forced air. The sample was then inserted into the mass spectrometer and analyzed. Bovine cytochrome *c* was used for internal calibration.

Analytical Methods—Isoelectric focusing, amino acid analysis, and other procedures were performed as described earlier (15–17, 26). A Vydac C-4 reversed phase column was used for the separation of the globin chains. The hemoglobin samples were injected onto the column previously equilibrated with 38% acetonitrile in 0.1% trifluoroacetic acid. The isolated β -globin chains were digested with trypsin, and the resulting peptides were separated on a Vydac C-18 column with a linear gradient of acetonitrile from 12 to 80% (15, 16). The amino acid sequence of the mutant tryptic peptide was determined on an Applied Bioscience instrument. For determination of the concentration of DPG and IHP, inorganic and total phosphate were determined by the spectrophotometric method of Lowry *et al.* (29). Hydrolysis was accomplished by using 11 N sulfuric acid, 4.7% perchloric acid.

Functional Studies—The oxygen dissociation curves were determined at 37 °C on a modified Hem O Scan instrument (Aminco) as described previously (15–17, 26). Before the measurements, the Hb samples were dialyzed and converted to the oxy form (30, 31). When evaluating the effects of allosteric modulators, the samples were in 50 mM bis-Tris buffer, pH 7.4. Prior to determination of the concentration of hemoglobin at the onset of gelation (*C*^{*}) as described by Benesch *et al.* (32), the samples in 100 mM potassium phosphate buffer, pH 6.8, were concentrated using CentriPrep, Centricon, and MicroCon ultrafiltration devices (Amicon; molecular weight cut-off of 10,000). The final protein concentrations were verified by amino acid analysis on a Beckman 6300 analyzer.

RESULTS

Expression and Purification of the Double Mutant—The hemoglobin double mutant, Hb E6V/K95I(β), was expressed in yeast from the plasmid pGS389 containing the human α - and β -globin cDNAs with the desired substitutions introduced by the two mutagenic oligonucleotides described under "Materials and Methods." The final product synthesized by PCR was rather large (about 1,300 base pairs), and the sequence of the entire β -gene was checked by the dideoxy method. No mutations other than the two desired (*i.e.* encoding the amino acid substitutions Glu-6(β) \rightarrow Val and Lys-95(β) \rightarrow Ile) were detected. The expression level of the recombinant hemoglobin, which was 5–10 mg/liter, was not markedly influenced by increasing the galactose concentration from 2 to 3% during the induction period. In addition to the K95I(β) recombinant Hb, only small amounts of minor hemoglobins were detected during the 20-h induction.

After purification on a CM-Cellulose 52 column as described earlier (15–17), the double mutant eluted as a single peak when rechromatographed analytically on the SynchroPak CM-300 HPLC column. Its purity was verified by isoelectric focusing as described previously (15–17), and its migration was consistent with the charge differences at the two mutation sites (*i.e.* the removal of one negative charge in sickle Hb (Glu \rightarrow Val) compensated for by the removal of one positive charge in the double mutant (Lys \rightarrow Ile)) (Fig. 1). Thus, the double mutant is near

¹ Abbreviations: PCR, polymerase chain reaction; DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; HPLC, high performance liquid chromatography; K95I(β), another name for the double HbS mutant E6V/K95I(β).

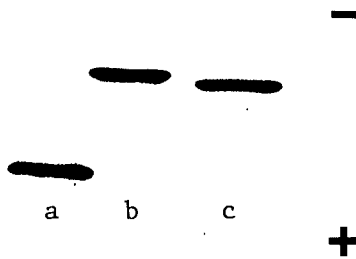


FIG. 1. Isoelectric focusing of the purified K95I(β) Hb. A gel from Isolab (pH 6–8) was electrophoresed at 10 W for 45 min. Lane a, natural HbS; lane b, natural HbA; lane c, K95I(β) Hb.

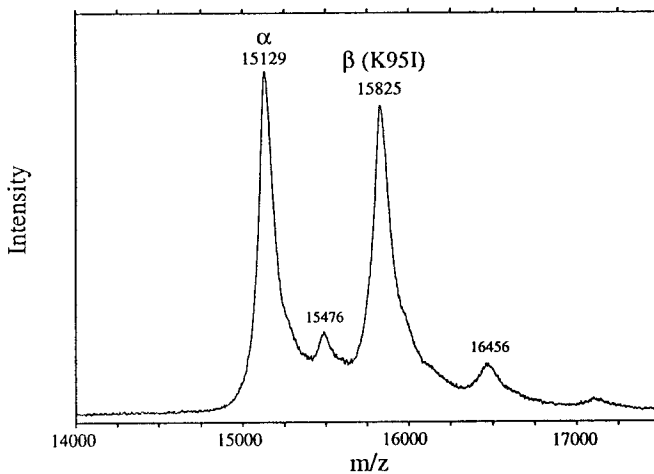


FIG. 2. Mass spectrometric analysis of the double mutant sickle hemoglobin with Lys-95(β) substituted by Ile. Matrix-assisted laser desorption mass spectrum of HbS K95I(β) is shown. Peaks corresponding to the protonated α - and β -chains are designated α and β (K95I), respectively. The small peak at m/z 15,476 likely arises from a doubly protonated heterodimer of the α - and β -chains. The origin of the weak peak at m/z 16,456 has not been elucidated.

HbA upon isoelectric focusing. Both mutations are on the exterior of the protein, so that the full effect of these pK_a changes is reflected in its electrophoretic behavior.

Mass Spectrometry—The molecular mass of the purified E6V/K95I(β) double Hb mutant was determined by matrix-assisted laser desorption mass spectrometry (27, 28, 33). In this procedure hemoglobin is dissociated into its constituent α - and β -subunits and their individual molecular masses are measured by a time-of-flight method. A molecular mass of 15,823.9 mass units was obtained for the β -subunit (Fig. 2). This value agrees well with the calculated molecular mass of 15,823.3 mass units. The difference of 14.4 Da from the mass of sickle Hb (15,838.3 mass units) is within experimental error of the expected difference of 15 Da between a Lys (146.2 mass units) and an Ile (131.2 mass units) residue. The molecular mass obtained for the α -chain (15,128.4 mass units) is consistent with the calculated value (15,126.4 mass units) within the error of the measurement.

HPLC Analysis of Globin Chains and Amino Acid Analysis—For further characterization, the α - and β -chains were first separated by HPLC. The double mutant β -chain eluted after the α -chain (Fig. 3) with an elution time of 56.2 min compared with an elution time of 36.6 min for a normal β -chain and 38.7 min for a sickle β -chain. Thus, the double mutant β -chain showed a dramatic difference in its elution behavior compared to both sickle Hb and HbA. Indeed, the order of elution of α - and β -globin chains was the reverse of that usually observed (Fig. 3). Subtle changes in the amino acid composition of polypep-

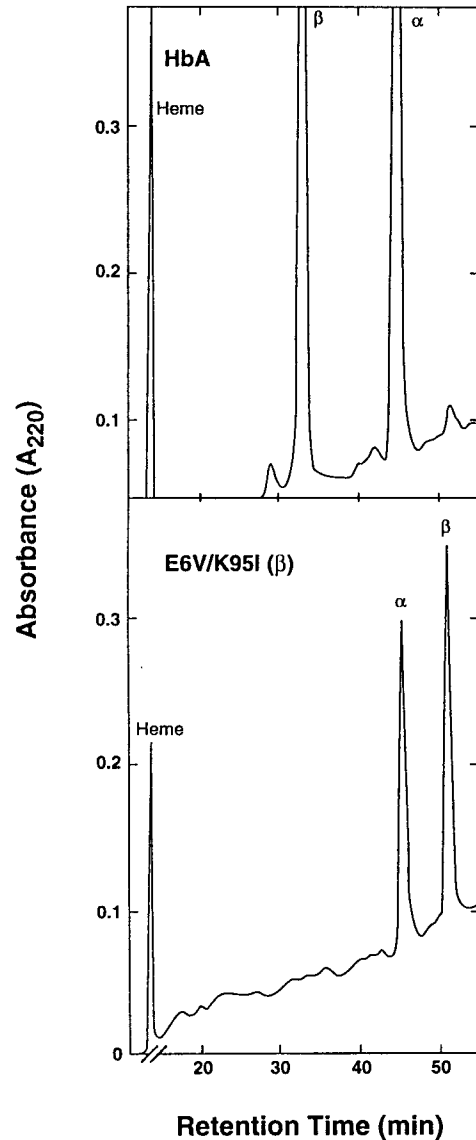


FIG. 3. Separation of the α - and β -globin chains of K95I(β) Hb by HPLC. The purified double mutant was injected in a Vydac C-4 column previously equilibrated with 38% acetonitrile in 0.1% trifluoroacetic acid.

tides can lead to dramatic changes in their behavior on reverse phase HPLC columns, which is not always strictly according to the hydrophobicity of the amino acids. The influence of the Ile residue was probably accentuated due to its location on the outer surface of the β -subunit.

The α - and β -chains were identified by the amounts of Ser, Glu, Gly, Ala, and Val, which are the amino acids that show significant differences between the two chains. The amino acid composition of the isolated β -chain showed the theoretical value of 1 mol of Ile/mol of Hb chain. The values for the other amino acids were in reasonable accord with the known composition (Table I). The choice of Ile as the substitution also facilitated the identification of the mutation site, because natural HbS does not contain Ile. No Ile was found in the α -chain, which also had the expected composition.

Peptide Mapping and Sequencing of the Mutant Peptide—The double mutant β -chain isolated as described in Fig. 3 was subjected to tryptic digestion, and the peptides were analyzed on a Vydac C-18 column. The mutant peptide eluted in a unique position (Fig. 4, bottom) compared with the peptide map shown of this region of HbA (Fig. 4, top). Amino acid analysis of

TABLE I

Amino acid analysis of the α - and β -chains of K95I(β) Hb

The α - and β -chains were isolated as described in the legend to Fig. 2. The values have been normalized to the values found for Pro, which was set at the theoretical value of 7.0. The values for Thr and Ser are uncorrected for their 5–7% destruction during acid hydrolysis. Under the conditions of acid hydrolysis employed, Cys, Met, and Trp are destroyed. For the amino acid values that are underlined, there is a significant difference between their amounts in the α - and β -chains.

| Amino acid | Found | | Theory | |
|------------|-----------------|----------------|-----------------|----------------|
| | α -Chain | β -Chain | α -Chain | β -Chain |
| Asx | 10.1 | 10.8 | 12 | 13 |
| Thr | 6.5 | 5.4 | 9 | 7 |
| Ser | <u>8.5</u> | <u>4.7</u> | <u>11</u> | <u>5</u> |
| Glx | <u>5.2</u> | <u>9.9</u> | <u>5</u> | <u>10</u> |
| Pro | 7 | 7 | 7 | 7 |
| Gly | <u>7.2</u> | <u>12.7</u> | <u>7</u> | <u>13</u> |
| Ala | <u>18.6</u> | <u>14.2</u> | <u>21</u> | <u>15</u> |
| Cys | 0.3 | 0 | 1 | 2 |
| Val | <u>11.2</u> | <u>15.4</u> | <u>13</u> | <u>19</u> |
| Met | 0 | 0 | 2 | 1 |
| Ile | 0 | 1.0 | 0 | 1 |
| Leu | 17.8 | 18.2 | 18 | 18 |
| Tyr | 1.8 | 2.0 | 3 | 3 |
| Phe | 6.1 | 6.7 | 7 | 8 |
| His | 7.8 | 7.2 | 10 | 9 |
| Lys | 10.6 | 9.7 | 11 | 10 |
| Trp | 0 | 0 | 1 | 2 |
| Arg | 2.3 | 2.1 | 3 | 3 |

this peptide was consistent with its assignment as a peptide fragment comprising amino acids 83–104 and showed 1 mol of Ile/mol of the peptide (Table II). The correct mutation site was verified by sequencing the peptide isolated in Fig. 4 with a high yield of Ile at the position 95(β) (Table III).

Oxygen Binding—In order to ascertain that the recombinant Hb double mutant had retained the functional properties of hemoglobin, its oxygen binding curve was measured. As shown in Fig. 5, at high Hb concentration (4.1 mM), a typical sigmoidal curve was found; the P_{50} value at this concentration of the E6V/K95I Hb was 33.5 mm Hg, and it was fully cooperative with a Hill coefficient of 3.1 (Fig. 5, inset).

Response to Allosteric Effectors—The response of the E6V/K95I(β) double mutant to chloride, DPG, and IHP is shown in Table IV. At a concentration of 0.7 mM in tetramer, the recombinant Hb had a basal P_{50} value of 10 mm Hg. As with sickle Hb (20), NaCl at concentrations of 20 mM or lower did not significantly change the oxygen binding of the recombinant Hb E6V/K95I(β). With increasing NaCl concentration, the P_{50} value hyperbolically increased, reaching a maximum of 25 mm Hg at a concentration of about 1 M NaCl. Under these conditions, the Hill coefficient decreased from 3.2 to 2.0, especially with NaCl concentrations above 0.5 M. DPG, a much more potent effector of Hb function, showed a marked increase in the P_{50} value of the double mutant at low concentration. Above 1.3 mM DPG, no further increase in the P_{50} value was observed. IHP, at a concentration of 0.7 mM IHP, led to a maximum P_{50} value of 56 mm Hg.

Gelation of the Hemoglobin Double Mutant—To measure the gelation concentration of the recombinant Hb E6V/K95I(β), the P_{50} value of different concentrations of the double mutant was determined in 100 mM potassium phosphate buffer, pH 6.8, as described by Benesch *et al.* (32). In this procedure physiological concentrations of hemoglobin, such as those that occur in red cells, are used. The P_{50} values ranged from 23.5 to 58.5 mm Hg between the Hb concentrations of 5–48 g/dl (Fig. 6). There was a rapid decrease in the oxygen affinity once the concentration of the E6V/K95I(β) double mutant reached a value of 39.5 g/dl. This point was defined by Benesch *et al.* (32) as the concentration of hemoglobin at the onset of gelation (C^*). When com-

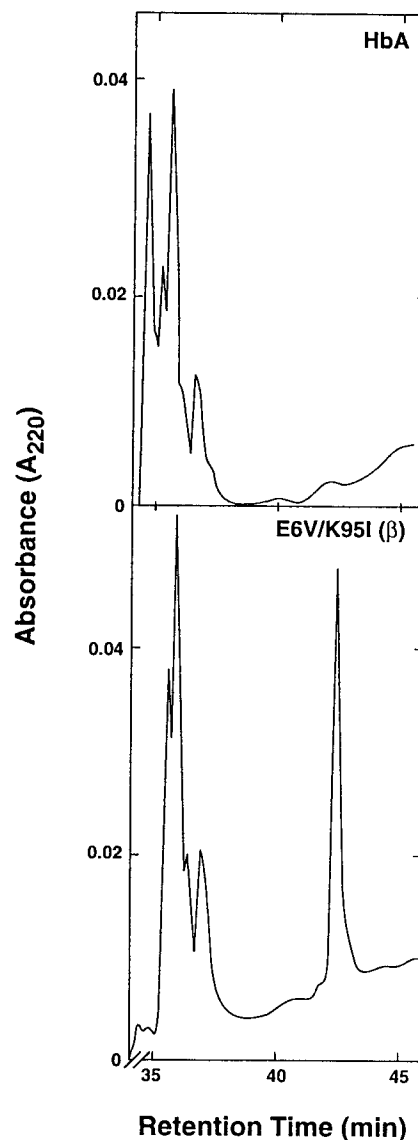


FIG. 4. Tryptic peptide maps of the β -globin chains. The β -globin chains of HbA and K95I(β) Hb were isolated as shown in Fig. 3, carboxymethylated, and digested with trypsin. The resulting peptides were chromatographed on a Vydac C-18 column using a linear acetonitrile gradient of 12–80%.

pared with a value of 23.7 g/dl for sickle Hb (Fig. 6, dashed line) (15, 16), it is obvious that the substitution of Lys-95(β) by Ile has had a major effect on the process of aggregation. The cooperativity was not markedly affected by the concentration of Hb used, because the Hill coefficients varied between 2.5 and 3.3 throughout the concentration range studied. Our data indicate that Lys-95(β) is an important site in the aggregation process in agreement with the conclusions of Bookchin and Nagel (8), of Josephs and co-workers (11), and of Edelstein and co-workers (13, 14).

DISCUSSION

If a therapeutic intervention for sickle cell disease is directed at the sickle Hb molecule itself, it would be advantageous to know the strengths of the various side chain interactions between the deoxyhemoglobin S tetramers in order to choose the most effective target site. The solution of the structure of the sickle hemoglobin aggregate (3) revealed many contact sites in the aggregate but did not provide an indication of their relative strengths. Our objective in this communication was to measure such a value for Lys-95(β), a prominent site on the exterior of

the molecule. From the results in this communication, the contribution of this site to the aggregation process appears to be even greater than that of the initial hydrophobic interaction involving Val-6(β) and the Phe-85/Leu-88(β) region, because the Hb concentration for the E6V/K95I double mutant at the

onset of gelation is increased much more (39.5 g/dl) than that for the E6V/L88A(β) double mutant (31.2 g/dl) compared with 23.7 g/dl for HbS (17). Considering that the E6V/K95I double mutant retains the original Leu-88(β) (Table III), this effect on gelation is especially dramatic.

Hofmann *et al.* (34) have reported the considerable formation of a sulfur adduct with heme, sulfheme, produced in a yeast expression system beginning about 16 h after the start of the galactose induction period and increasing for the subsequent 3 days. In our studies, we do not exceed a 20-h induction period with galactose (15–17, 26), and we find the production of only one major hemoglobin with the correct spectral properties, indicating the absence of sulfheme. The minor heme-containing proteins produced were less than 10% of the total hemoglobin and were separated from the main hemoglobin component on the HPLC-column (16). The K95I(β) Hb double mutant described in this manuscript displayed the normal spectral profile of ferrous Hb and had the correct functional properties of hemoglobin as shown by a Hill coefficient of 3.1 and a normal response to the allosteric regulators DPG, chloride, and IHP.

In considering the earlier approach of using mixtures of different mutant hemoglobins with substitutions at various positions to assess possible points of interaction in the sickle Hb aggregate (8), the argument could be raised that, because the hemoglobins had the substitutions on different tetramers,

TABLE II

Amino acid composition of mutant tryptic peptide of K95I(β)

The value found for Ile was used to normalize the values for the other amino acids. The mutant peptide was isolated as described in the legend to Fig. 4. There is some destruction of Ser during acid hydrolysis.

| | Found | Expected (fragment 83–104) |
|-----|-------|----------------------------|
| Asx | 3.4 | 3 |
| Thr | 1.9 | 2 |
| Ser | 1.9 | 1 |
| Glx | 2.7 | 2 |
| Pro | 1.1 | 1 |
| Gly | 2.3 | 1 |
| Ala | 1.5 | 1 |
| Cys | 0.2 | 1 |
| Val | 1.2 | 1 |
| Ile | 1 | 1 |
| Leu | 3.1 | 3 |
| Phe | 2.3 | 2 |
| His | 1.8 | 2 |
| Lys | 0.4 | 0 |
| Arg | 1.2 | 1 |

TABLE III

N-terminal sequence of the mutant peptide of K95I(β)

The underlined Ile shows the target site of mutagenesis. The numbers correspond to the amount of PTH-derivative in picomoles found at each cycle of Edman degradation. This peptide was isolated as described in the legend to Fig. 4.

| | | | | | | | | | | | | | | | | | | | |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|---------------------|-------------------|--------------------------|-------------------|-------------------|-------------------|-------------------|--------------------|--------------------|--------------------|
| Gly ⁸³ | Thr ⁸⁴ | Phe ⁸⁵ | Ala ⁸⁶ | Thr ⁸⁷ | Leu ⁸⁸ | Ser ⁸⁹ | Glu ⁹⁰ | Leu ⁹¹ | His ⁹² | CmCys ⁹³ | Asp ⁹⁴ | <u>Ile</u> ⁹⁵ | Leu ⁹⁶ | His ⁹⁷ | Val ⁹⁸ | Asp ⁹⁹ | Pro ¹⁰⁰ | Glu ¹⁰¹ | Asn ¹⁰² |
| 336 | 137 | 289 | 279 | 106 | 253 | 46 | 142 | 180 | 85 | 176 | 88 | 118 | 129 | 70 | 121 | 67 | 90 | 53 | 60 |

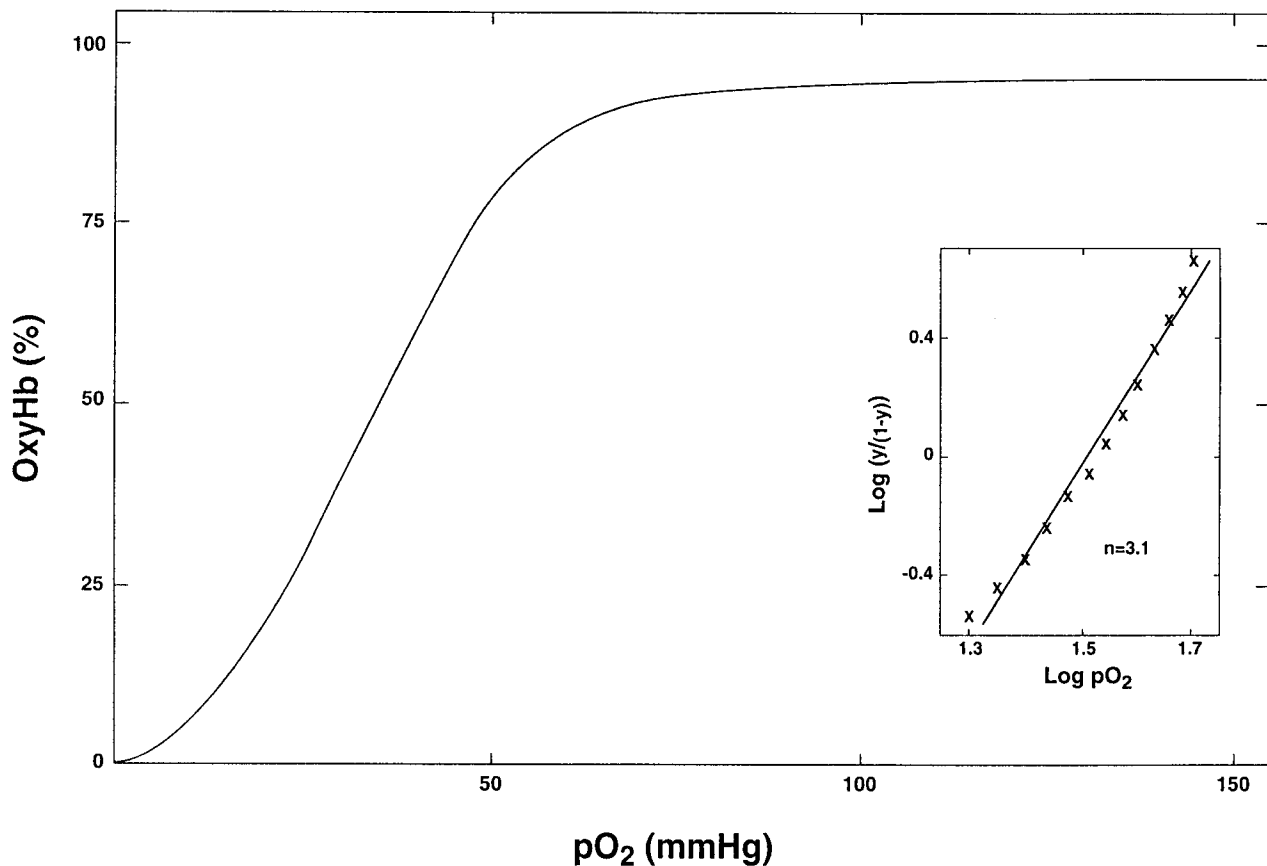


FIG. 5. Oxygen binding curve of K95I(β) Hb. The purified double mutant (4.1 mM in tetramer) in 50 mM bis-Tris buffer, pH 7.4, was converted from the CO to the oxy form (26, 27), and the oxygen binding was measured at 37 °C using a modified Hem O Scan instrument. The inset shows the cooperativity with an n value of 3.1 calculated from the slope of the line.

