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CONTENTS Arranged by Subject Categories

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ADDITIONS AND CORRECTIONS

AUTHOR INDEX

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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A Recombinant Sickle Hemoglobin Triple Mutant with Independent Inhibitory Effects on Polymerization*

(Received for publication, June 13, 1996, and in revised form, July 19, 1996)

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As part of a comprehensive effort to map the most important regions of sickle hemoglobin that are involved in polymerization, we have determined whether two sites previously shown to be involved, Leu-88(β) and Lys-95(β), had additive effects when substituted. The former site is part of the hydrophobic pocket that binds Val-6(β), the natural mutation of HbS, and the latter site is a prominent part of the hemoglobin exterior. A sickle hemoglobin triple mutant with three amino acid substitutions on the β-chain, E6V/L88A/K95I, has been expressed in yeast and characterized extensively. Its oxygen binding curve, cooperativity, response to allosteric effectors, and the alkaline Bohr effect showed that it was completely functional. The polymer solubility of the deoxy triple mutant, measured by a new micromethod requiring reduced amounts of hemoglobin, was identical to that of the E6V(β)/K95I(β) mutant, *i.e.* when the K95I(β) substitution was present on the same tetramer together with the naturally occurring E6V(β) substitution, the L88A(β) replacement had no additive effect on polymer inhibition. The results suggest that Lys-95(β) on the surface of the tetramer and its complementary binding region on the adjoining tetramer are potential targets for the design of an effective antisickling agent.

Sickle cell anemia results from a single point mutation in the gene encoding β-globin, whereby the Glu-6(β) residue in hemoglobin A (HbA) is substituted by Val in sickle hemoglobin (HbS) (1, 2). This hydrophobic side chain initiates a process by which the densely packed deoxyhemoglobin tetramers inside the red blood cells interact through other sites to form long polymer fibers that distort the cells into a characteristic sickle shape. Although the identity of many of these amino acid sites involved in polymer formation and the extent to which they participate is known (3–8), the quantitative contributions to polymerization of many other sites are unknown. A goal of this study was to provide such information for selected polymerization contact sites for which natural mutants either do not exist or have not been reported. Recombinant sickle double and triple mutants are used for this purpose.

Studies describing the hydrophobicity and stereochemistry of deoxy HbS have shown that Val-6(β) binds tightly between Phe-85 and Leu-88 in the acceptor pocket on an adjacent β-chain. According to computer-generated models, the three-dimensional fit of the side chain of Val into the acceptor pocket is much better than that of Ala (7), explaining the inability of Hb Makassar with Ala-6(β) to polymerize (8), even though the hydrophobicity of Ala and Val do not differ drastically. Other studies have suggested that substitutions by larger hydrophobic residues at the position 6, readily promote polymerization (9). These findings point out the complexity of the polymerization process, which cannot be explained simply by the hydrophobicity and stereochemistry of the β-6 site and its corresponding acceptor pocket. Indeed, it has been established that other contact sites in the gelation process reinforce the initial contact (3–5, 9, 10). In addition, studies with noncovalent chemical inhibitors have shown that these compounds do not act as predicted by their hydrophobic nature (11), implying a significant contribution of other interactions.

In our efforts to understand the mechanism of sickle hemoglobin gelation and to identify the critical sites in the gelation process, we use a yeast expression system (6, 12–15) to produce HbS double and triple mutants as an adjunct to chemical modification studies (16–18). Unlike the *Escherichia coli* expression system, the yeast system produces a native hemoglobin molecule, as judged by many biochemical criteria (15). In addition, since yeast incorporates its own heme group into globin, there are no time-consuming manipulations, such as reconstituting hemoglobin with exogenous heme. Thus, it is feasible to study the involvement of any site on the hemoglobin molecule in the gelation process and to judge the significance of any differences between the crystal structure (5) and the electron microscope structure (4, 19) of HbS. For example, we recently determined that the contact site Lys-95(β) on the outside of the tetramer distant from the hydrophobic pocket, which was implicated in one structure (4) but not the other (5), was significantly involved in the gelation process (12). Indeed, its substitution by Ile inhibits gelation twice as much as a mutation at a site in the acceptor pocket, L88A(β) (6). The diverse locations of these two sites prompted us to design a recombinant Hb having both K95I(β) and L88A(β) in addition to the Val-6(β) mutation in order to measure whether the influence of the two substitutions on gelation is additive. Such a study may reveal important details of the gelation process and could influence efforts for developing well targeted clinically effective inhibitors. For these studies, we employ a new method based on the drastic decrease in the solubility of hemoglobin S upon addition of dextran (20).

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 ^{35}S-labeled dATP was from DuPont NEN. The oligonucleotides were synthesized by Operon Technologies (Alameda, CA). CM-cellulose 52 was from What-

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man, and HPLC[1] columns (C-4 and C-18) from Vydac. L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, dextran, DPG, and IHP were purchased from Sigma. The construction of pGS189 and pGS389 plasmids is described elsewhere (12, 14). All the other reagents were of analytical purity.

Site-directed Mutagenesis—To prepare the E6V(β)/L88A(β)/K95I(β) triple mutant, we used the M13mp18 recombinant phage as a template. The construction of this phage containing the β-globin cDNA with the E6V(β) and the L88A(β) coding mutations has been described earlier (6). The oligonucleotide 5′-ATC CAC GTG CAG GAT GTC ACA GTG CAG-3′ was used to create the Lys-95(β) → Ile mutation by the method of Kunkel (21). The underlined bases were those used to create the desired mutation. The presence of the mutations was screened by partial sequencing of the mutation site. The mutation frequency was increased to 65% by supplementing the reaction mixture with the Gene 32 Protein and by prolonging the reaction time, as described previously.[2] The mutated β-globin region was subcloned to pGS189sickle, which contains the native α-globin and the Glu-6(β) → Val mutated β-globin cDNAs, by digesting with *Sph*I enzyme. Finally, the α- and β-globin gene cassette was isolated as a *Not*I fragment after digesting the newly synthesized pGS189$^{sickle\text{-}Ala\text{-}88\text{-}Ile\text{-}95}$ with *Not*I and *Bgl*I and inserted into pGS389 previously digested with *Not*I. The correct insertional direction was verified by restriction mapping and the entire β-globin gene was sequenced using a fluorescence-based detection system (Perkin-Elmer/Applied Biosystems) to show that the Glu-6(β) → Val, Leu-88(β) → Ala, and Lys-95(β) → Ile were the only mutations in the globin chain.

Yeast Expression System—The yeast cells were transformed by the pGS389$^{sickle\text{-}Ala\text{-}88\text{-}Ile\text{-}95}$ plasmid using the lithium acetate method (22). The transformants were selected and the copy number of the plasmid increased by growing the yeast on a complete minimal medium first without uracil, then without uracil and leucine (14). To express the E6V(β)/L88A(β)/K95I(β) triple mutant hemoglobin, the yeast was grown in YP medium for 4 days with ethanol as the carbon source. The promoter controlling the transcription of the globin genes was induced by adding 3% galactose 20 h prior to the harvesting of the yeast cells. The cells were disrupted in a Bead Beater homogenizer and the Hb triple mutant was purified in the CO form on a CM-Cellulose 52 column as described earlier (12, 15). The average yield was 3 mg of the purified hemoglobin/liter of culture medium. The preparations of the E6V(β)/L88A(β) and the E6V(β)/K95I(β) recombinant hemoglobins were described earlier (6, 12).

Mass Spectrometry Analysis—Electrospray mass spectrometric analysis was performed with a Finnigan-MAT TSQ-700 triple quadrupole mass spectrometer (23, 24). Seventy pmol of the hemoglobin sample was loaded onto a desalting protein cartridge (Michrom BioResources, Inc., Auburn, CA) and washed with 1 ml of deionized water. The sample was eluted from the cartridge using a solution of water/acetonitrile/acetic acid, 30/67.5/2.5 (v/v/v) and electrosprayed directly into the mass spectrometer. The flow of the eluting solution was maintained at 6 μl/min through a 100-μm inner diameter fused silica capillary. The spectrum given in Fig. 1 is an average of 16 scans, obtained at a rate of 3 s/scan.

Analytical Methods—Isoelectric focusing, amino acid analysis, and other procedures were performed as described earlier (12, 15, 25). To isolate the α- and β-globin chains, a Vydac C-4 column was equilibrated with acetonitrile in 0.1% trifluoroacetic acid and eluted as described under "Results." The isolated β-globin chains were digested with trypsin, and the resulting peptides were separated on a Vydac C-18 reversed phase column using an acetonitrile gradient in 0.05% HCl, a modification from previous studies (12, 15, 25).

Functional Studies—The oxygen dissociation curves were determined at 37°C on a modified Hem-O-Scan instrument (Aminco) as described previously in 50 mM bis-Tris, pH 7.4 (26). Before the measurements, the Hb samples were dialyzed, converted from the CO form to the oxy form (27), and concentrated using CentriPrep, Centricon, and MicroCon ultrafiltration devices (Amicon; molecular weight cutoff 10,000). The final protein concentration (0.5–2.2 mM) was established by amino acid analysis on a Beckman 6300 analyzer. The pH dependence of the oxygen affinity (Bohr effect) and the effects of allosteric modulators were determined at a Hb concentration of 0.5 mM in the same buffer.

Determination of C_{sat}—Polymer solubilities of the hemoglobins under study were determined by the "Dextran-C_{sat}" micromethod of Bookchin *et al.* (20), with minor modifications. This method is based on the marked decrease in the solubility of deoxy-HbS on admixture with 70-kDa dextran, at physiological ionic strength and pH (a preliminary account of this method and results is described in Ref. 20; full report in preparation). A comparison of the results found with this procedure with those reported previously using another method is described below. Concentrated solutions of the test hemoglobin in 0.05 M potassium phosphate, pH 7.5, were mixed with concentrated dextran solutions in the same buffer to give a final dextran concentration of 120 mg/ml. The solutions were overlayered with paraffin oil, chilled on ice, and deoxygenated by adding (with a Hamilton syringe) a deoxygenated solution of sodium dithionite to give a final concentration of 50 mM. After stirring and incubation for 30 min in a 37°C water bath, the resulting gel under the oil layer was carefully but vigorously disrupted with a narrow plunger or wire loop, and the tubes were centrifuged at room temperature in a microcentrifuge at 14,000 rpm for 20 min. The gel disruption and centrifugation procedure was repeated twice. After confirming the presence of a solid Hb phase by viewing the tube in front of a bright light, the oil was aspirated. The hemoglobin concentration of the supernatant (C_{sat}) was determined by amino acid analysis in duplicate. Each determination of the gelation concentration was performed three to four times with a precision of +10% or less.

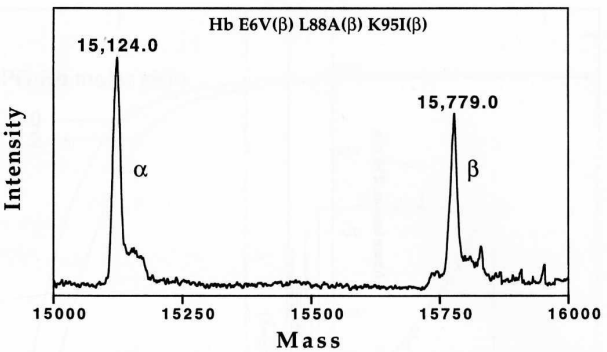

FIG. 1. **Reconstituted electrospray ionization mass spectrum of the α- and β-chains of the recombinant E6V(β)/L88A(β)/K95I(β) triple mutant.** Peaks designated *a* and *b* represent the protonated α- and β-globin chains of the triple mutant and their molecular masses.

RESULTS

Mass Spectrometry—The expected molecular mass (15,779.0 Da) was obtained for the purified E6V(β)/L88A(β)/K95I(β) Hb mutant β-chain by matrix-assisted laser desorption mass spectrometry (Fig. 1). This value agrees well with the calculated value of 15,781.2 for a β-chain having the three substitutions, *i.e.* the difference of 89.3 mass units between the mass of the β-chain of HbA (15,868.3 Da) is within the experimental error of the combined calculated differences of 87.1 mass units for the substitutions Glu → Val, Leu → Ala, and Lys → Ile (30.0, 15.0, and 42.1 mass units difference, respectively). The measured molecular mass for the α-chain of E6V(β)/L88A(β)/K95I(β) (15, 124.0) is in accord with the calculated value (15,126.4 Da) for the natural α-chain of HbA within the experimental error.

Isoelectric Focusing—This analysis was performed for the triple mutant by the method described previously (15). The triple mutant showed a similar pI value as the E6V(β)/K95I(β) mutant (12) in agreement with the expected mutations, since the L88A(β) mutation has previously been shown not to have an altered pI value (6).

HPLC Separation of Globin Chains—The α- and β-globin chains were separated by reversed phase HPLC on a Vydac C4 column using acetonitrile in 0.1% trifluoroacetic acid as the mobile phase. With an acetonitrile gradient from 40 to 45%, the α- and β-chains did not separate due to the combined effects of the slightly increased elution of the β-chain having the L88A(β) mutation (6) and the considerably decreased elution of the β-chain with the K95I(β) mutation (12). The chains were suc-

[1] The abbreviations used are: HPLC, high performance liquid chromatography; DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

[2] J-P. Himanen, A. M. Popowicz, and J. M. Manning, submitted for publication.

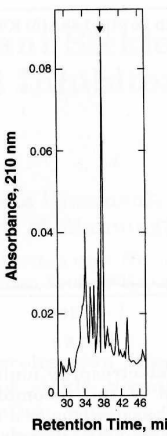

FIG. 2. **Tryptic peptide map of the β-globin chain of the triple mutant.** The isolated β-globin chain of E6V(β)/L88A(β)/K95I(β) was carboxymethylated and digested with trypsin as described in the text. The resulting peptides were chromatographed on a Vydac C-18 column using a linear acetonitrile gradient of 12–44% in 0.05% HCl.

cessfully separated by using an isocratic elution with 38.8% acetonitrile. Each had the expected amino acid composition relative to Pro (set at 7.0): Asx (12.1), Thr (6.4), Ser (5.3), Glx (10.0), Gly (12.7), Ala (15.6), Val (18.3), Ile (0.9), Leu (16.7), Tyr (1.9), Phe (6.4), His (8.0), Lys (9.5), Arg (3.1). Cys, Met, and Trp are destroyed partially or completely during acid hydrolysis in non-evacuated hydrolysis tubes.

Peptide Mapping—Tryptic peptide mapping of the isolated β-chain was performed as described previously (6, 15, 25, 26) to verify the expected mutations. The peptides were separated on a Vydac C-18 column using a shallow gradient of acetonitrile (from 12 to 44% in 50 min) in 0.05% HCl (Fig. 2). The major peak eluting at about 38 min was collected and its amino acid composition was found to be consistent with that of the expected 22-residue mutant peptide comprising β 83–104 within experimental error. The values relative to Val (set at 1.0) were: Asx (3.0), Thr (1.3), Ser (1.8), Glx (1.6), Gly (1.9), Ala (1.5), Cys (0.5), Ile (0.5), Leu (1.9), Phe (2.6), His (0.7); however, Pro and Arg were not detected.

Tetramer-Dimer Dissociation Constant—Considerable dimerization may occur in hemoglobin even when the Hill coefficient shows high cooperativity, as discussed by Forsen and Linse (28). Using a method recently developed in our laboratory (29), we determined that the tetramer-dimer dissociation constant for the triple mutant was 0.4 μM as compared with the K_d value of 0.7 μM for HbS, indicating that the newly produced mutant hemoglobin did not undergo increased dissociation, *i.e.* at the Hb concentrations used for the functional studies (0.5–2.2 mM) the hemoglobin is predominantly tetrameric.

Functional Properties—The oxygen affinity of the triple mutant at a hemoglobin concentration of 0.5 mM showed an average P_{50} value of 10 mm Hg with a Hill coefficient of 2.7, indicating that the triple mutant retained full cooperativity (Fig. 3). DPG at a 1.2:1 ratio to Hb shows a significant response. The effects of two other anionic effectors, chloride and inositol hexaphosphate, were comparable with those measured earlier for HbS (15), for E6V(β)/L88A(β) (6), and for E6V(β)/K95I(β) (12): the maximum P_{50} value was 22 mm Hg in the presence of 1000 mM Cl−, and 58 mm Hg using an [IHP]:[Hb] ratio of 1.2 (Table I).

Bohr Effect—A plot of the change in P_{50} *versus* pH for the triple mutant had a slope of 0.37 (correlation coefficient, r = 0.972) (Fig. 4), compared with the value of 0.41 (r = 0.995) found for HbA. Thus, within experimental error, the alkaline Bohr coefficient was unchanged compared with HbA, consistent with the native structure of the recombinant hemoglobin.

Polymerization—Comparison of the new micromethod used for direct measurement of gelation in these studies with the previous procedure using oxygen affinity changes (6, 12) indicates similar effectiveness of each mutation on inhibition of polymerization, even though the absolute values differ. Thus, ratios of 1.36 and 1.27 for the gelation concentrations of K95I/L88A recombinant hemoglobins were calculated for the oxygen affinity method and the present method, respectively. The dextran-C_{sat} determinations of the following deoxyhemoglobins are shown in Fig. 5, natural HbS, the double mutant E6V(β)/L88A(β), the double mutant E6V(β)/K95I(β) and the triple mutant E6V(β)/L88A(β)/K95I(β). The results shown are an average of three to four determinations with a precision of + 10% or less. At initial concentrations below the C_{sat}, the final concentrations of each Hb at equilibrium, when plotted as a function of the initial concentrations, fell on a line with a slope of 1.03, indicating that the procedure itself did not result in precipitation (denaturation) of the hemoglobins. When initial Hb concentrations exceeded the C_{sat}, the final supernatant Hb concentrations (C_{sat} values) remained constant, independent of further increases in initial Hb concentrations over the ranges tested. The mean dextran-C_{sat} value of the triple mutant was 91 mg/ml, indicating that it requires a considerably higher concentration than deoxy-HbS (mean: 34 mg/ml) for polymerization. The C_{sat} of the triple mutant was not significantly different from the value found for E6V(β)/K95I(β) double mutant (90 mg/ml); the value for the E6V(β)/L88A(β) (67 mg/ml) was between those of deoxy-HbS and those containing the Lys-95(β) substitutions. Deoxy HbA remained soluble at concentrations up to 149 mg/ml with no evidence of precipitation during the procedure. Thus, the new micromethod described here measures the true gelation of deoxy HbS.

DISCUSSION

In this study the recombinant triple mutant E6V(β)/L88A(β)/K95I(β) produced in yeast was shown to have the predicted amino acid composition, molecular mass, isoelectric point, and trypsin cleavage sites. Its oxygen affinity, cooperativity, response to negatively charged effectors, alkaline Bohr effect, and the tetramer/dimer dissociation constant were the same as those for HbS. These results, together with extensive characterization of recombinant hemoglobins by a variety of biochemical criteria (15, 25, 26, 29), are consistent with the expression by the yeast system of a native hemoglobin molecule with the correct N-terminal processing. Thus, we have no evidence for any misfolding of the triple mutant as reported for other recombinant hemoglobins made using *E. coli* as a production host (30). Hence, the gelation of the native HbS and the recombinant double and triple mutants by the procedure described here can be taken as reliable measurements of the gelation concentrations.

We reported previously that Lys-95(β), which is distant from the hydrophobic pocket in the region of Phe-85(β)-Leu-88(β) comprising the acceptor site for Val-6(β), inhibits gelation much more than the substitution of a residue in the pocket itself (6, 12). Our results agreed with some previous reports implicating Lys-95(β) in the gelation process (33) and as an intermolecular contact site in the polymer (3, 4), although this site was not involved in the Wishner-Love double strand crystal of deoxy-HbS (5). The strong influence of the β-95 site, which is located on the exterior of the tetramer at the lateral contact site of the HbS tetramer, on gelation strongly suggests that the K95I(β) mutant of HbS has different protein self-assembly properties than HbS itself (32). The role of the Val-6(β) and its hydrophobic acceptor pocket may be to provide a

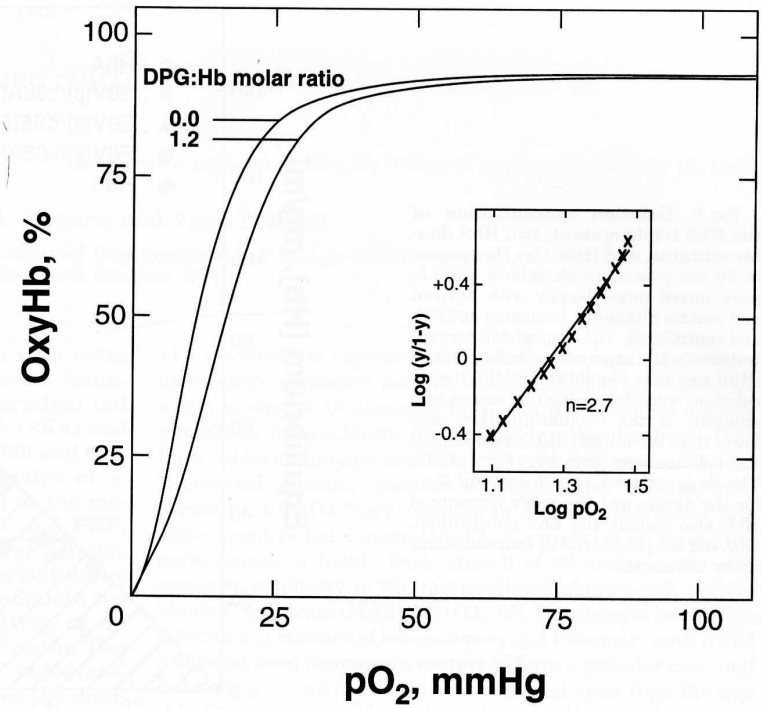

FIG. 3. **The oxygen binding curve of E6V(β)/L88A(β)/K95I(β).** The oxygen binding of the triple mutant (0.5 mM in 50 mM bis-Tris buffer, pH 7.4) in oxy form in the presence or absence of DPG was measured at 37°C using a modified Hem-O-Scan instrument. The *n* value is an average of the two determinations.

TABLE I
The influence of allosteric effectors on the oxygen affinity of E6V(β)/L88A(β)/K95I(β) triple mutant

The Hb concentration was 0.5 mM in 50 mM bis-Tris, pH 7.4.

	P_{50}, E6V/L88A/K95I	HbS[a]
[Cl^-] (mM)		
0	11	10
50	14	
100		15
200	17	16
500	19	21
1000	22	25
[IHP]/[Hb]		
0.4	13	16
0.8	39	37
1.2	58	80
1.6	55	80
2.0	55	

[a] The values for the effect of Cl^- on HbS are from Ref. 15.

molecular switch to turn the gelation either on or off. If this position is mutated to Ala (Hb Makassar), no gelation occurs because Ala prevents sufficient stabilization of the primary nuclei. Our results on the gelation of E6V(β)/L88A(β) mutant (6, 34) also suggest that the Leu to Ala substitution in the acceptor pocket mainly affects the initial nucleation process, but once nucleation has taken place other residues stabilize the polymer. These findings also further emphasize the importance of certain ionizable surface amino acids. Their potential importance as well as that of their complementary sites on adjacent tetramers lies in the possible development of clinical intervention against sickle cell disease. The results presented here demonstrate that two sites on the HbS tetramer exert significantly different and independent effects on the inhibition of polymerization.

Since the polymer solubility of the triple mutant was the same as that of the double mutant without the L88A(β) substitution, *i.e.* E6V(β)/K95I(β), the present results demonstrate that the inhibitory effects of the two β-chain substitutions (L88A and K95I) on HbS, are not additive. Although the

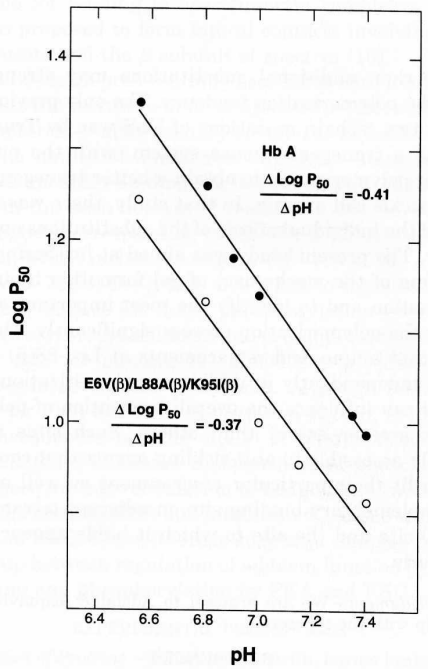

FIG. 4. **The alkaline Bohr effect of E6V(β)/L88A(β)/K95I(β).** The purified triple mutant in oxy form was diluted with bis-Tris buffers of different pH values to a final concentration of 0.5 mM Hb in 50 mM bis-Tris, and the P_{50} values were determined.

L88A(β) mutant, in which the substitution is in the hydrophobic acceptor pocket, has a gelation concentration about midway between the K95I(β) mutant and HbS itself, it does not appear to influence the overall behavior of the triple mutant.

The results of recent studies on recombinant mutants are consistent with the notion that once the initial contact site is established by the Glu-6 → Val substitution in the sickle Hb

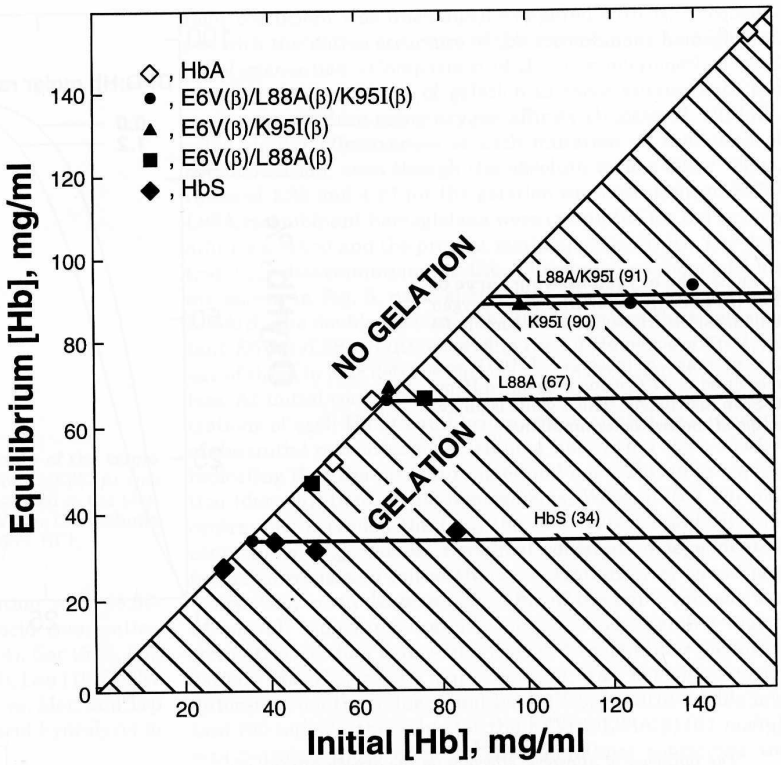

FIG. 5. **Gelation concentration of the HbS triple mutant, two HbS double mutants, and HbS.** Oxy Hb samples in 50 mM potassium phosphate, pH 7.5, were mixed anaerobically with dextran and sodium dithionite, incubated at 37°C, and centrifuged. The hemoglobin concentrations in the supernatant before (initial [Hb]) and after (equilibrium [Hb]) the incubation were determined by amino acid analysis. If the equilibrium [Hb] was lower than the initial [Hb], it represented the gelation concentration (C_{sat}) of Hb. The designations on the *horizontal lines* for the double and the triple mutants of HbS also include the E6V substitution. HbA did not polymerize at concentrations up to 149 mg/ml.

tetramer, then additional substitutions may strengthen or weaken the polymerization tendency. The only previous study involving two β-chain mutations of HbS was by Trudel *et al.* (13) using a transgenic mouse system, with the purpose of promoting polymerization to obtain a better transgenic mouse model of sickle cell anemia. In that study, there was no quantitation of the individual effects of the substitutions on polymer solubility. The present study was aimed at furthering our understanding of the mechanism of gel formation by inhibiting polymerization and to identify the most important sites that influence the polymerization process significantly. The results indicate that amino acid replacements at Leu-88(β) and Lys-95(β) act independently in inhibiting polymerization, *i.e.* certain sites can influence the overall prevention of polymerization to a greater extent than others. Such sites might be potentially accessible to anti-sickling agents that could be designed to fit their particular environment as well as that of their complementary binding site on adjacent tetramers. The Lys-95(β) site and the site to which it binds appear to fulfill such criteria.

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