

Enhanced polymerization of recombinant human deoxyhemoglobin $\beta 6$ Glu→Ile

(hemoglobin S/hydrophobic effect/protein engineering)

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ABSTRACT Polymerization of the deoxy form of sickle cell hemoglobin (Hb S; $\beta 6$ Glu→Val) involves both hydrophobic and electrostatic intermolecular contacts. These interactions drive the mutated molecules into long fibrous rods composed of seven pairs of strands. X-ray crystallography of Hb S and electron microscopy image reconstruction of the fibers have revealed the remarkable complementarity between one of the $\beta 6$ valines of each molecule (the donor site) and an acceptor site at the EF corner of a neighboring tetramer. This interaction constitutes the major lateral contact between the two strands in a pair. To estimate the relative importance of this key hydrophobic contact in polymer formation we have generated a polymerizing Hb with isoleucine at the $\beta 6$ position ($\beta E6I$) by site-directed mutagenesis. The mutated β chains were produced in *Escherichia coli* and reassembled into functional tetramers with native α chains. Compared to native Hb S, the $\beta E6I$ mutant polymerizes faster and with a shortened delay time in 1.8 M phosphate buffer, indicating an increased stability of the nuclei preceding fiber growth. The solubility of the $\beta E6I$ mutant Hb is half that of native Hb S. Computer modeling of the donor-acceptor interaction shows that the presence of an isoleucine side chain at the donor site induces increased contacts with the receptor site and an increased buried surface area, in agreement with the higher hydrophobicity of the isoleucine residue. The agreement between the predicted and experimental differences in solubility suggests that the transfer of the $\beta 6$ valine or isoleucine side chain from water to a hydrophobic environment is sufficient to explain the observations.

Sickle cell hemoglobin (Hb S) has been the subject of intense interest because the replacement of the charged glutamate residue at the sixth position (A3) in the β chains by an uncharged valine leads to a drastic decrease in the solubility of the mutant Hb in its deoxy form. This drives the mutated molecules into long fibrous rods, distorting the erythrocytes into their characteristic sickle shape (1, 2). The thermodynamic behavior and kinetics of polymerization have been studied in detail (for recent reviews see refs. 3 and 4). The long rods in sickled cells possess a highly ordered structure involving 14 helical strands of the mutated tetramers. X-ray analyses of the structure of the polymers (5) as well as molecular imaging studies (6-8) have provided a clear understanding of the mechanisms responsible for the formation of the polymers, which include both hydrophobic and electrostatic interactions between the abnormal tetramers.

Three other natural mutations at the $\beta 6$ position have been described: Hb C (Glu→Lys), Hb Machida (Glu→Gln) (9), and Hb G-Makassar (Glu→Ala) (10). None of these Hbs

exhibit gelling properties similar to those of Hb S (10, 11). These reports strongly suggested that the localized change in hydrophobicity at the surface of the A helix in the β chains of Hb S is a major determinant in producing the oxygen-linked decrease in solubility of deoxy Hb S, leading to the formation of the polymers. Structural analyses of the fibers and computer molecular modeling have revealed the remarkable complementarity between the side chain of the $\beta 6$ valine of one tetramer with an acceptor site constituted by the $\beta 85$ phenylalanine and $\beta 88$ leucine side chains of another tetramer, thereby explaining the primary intermolecular interaction in Hb S fibers. The solubility of the Hb S tetramers may be further decreased in the exceptional double-substituted variants such as Hb S-Antilles ($\beta 6$ Glu→Val, $\beta 23$ Val→Ile) (12) and Hb S-Oman ($\beta 6$ Glu→Val, $\beta 121$ Glu→Lys) (13). The solubility is also influenced in erythrocytes by the presence of other variants in cases of double heterozygosity (1, 2). When present alone, these non-S mutant hemoglobins do not have gelling properties. In the present report we address the question of whether the structural basis for the initiation of the nucleation process in deoxy Hb S is the stereospecificity for the valyl side chain or is determined by the strength of the hydrophobic interaction between the donor site at the $\beta 6$ position and the acceptor site at the EF corner. To answer this question we have produced β -globin chains in *Escherichia coli* with the $\beta 6$ (A3) Glu→Ile mutation.

MATERIAL AND METHODS

Hb S. Purified native Hb S was obtained by preparative isoelectric focusing from the hemolysate of a patient homozygous for Hb S, collected before an exchange transfusion. It was stripped of anions by anion-exchange chromatography and kept in the CO form in liquid nitrogen until use. Before the experiments CO was removed by exposure to intense light under a stream of pure oxygen. The visible spectrum of the oxygenated sample showed that the amount of methemoglobin formed during the complete procedure was less than 5%.

Production of $\beta 6$ Glu→Ile ($\beta E6I$) Globin Chains in *E. coli*. Human β -globin was synthesized by using the expression vector pLCiIFX β described by Nagai and Thøgersen (14). This vector directs the synthesis of a cleavable fusion protein consisting of the 31 amino-terminal residues of the phage λ cII protein, the Ile-Glu-Gly-Arg tetrapeptide (factor Xa will cleave any peptide bond preceded by this peptide), and the complete β -globin polypeptide.

Introduction of the Mutation in the β -Globin cDNA. The mutagenic primer d(CAGACTTCTCTATAGGAGTCAGG) was synthesized by the phosphotriester method to introduce the $\beta E6I$ mutation. The mutagenesis experiment was carried

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Abbreviations: Hb S, sickle cell hemoglobin; $\beta E6I$, $\beta 6$ Glu→Ile; $\beta E6V$, $\beta 6$ Glu→Val.

out as described by Nakamaye and Eckstein (15), using the Amersham kit. The *Sac* I-*Hind*III fragment of M13mp10-clIFX β was inserted into pLmp10 to form pLcIIFX β with the mutated sequence.

Protein Expression and Purification. The fusion protein was expressed in *E. coli* host strain QY13, harboring pLcIIFX β . Then the fusion protein was extracted and purified under denaturing conditions (16). The purified protein was dissolved at a concentration of 5 mg/ml in 8 M urea/50 mM Tris·HCl, pH 8.0, dialyzed against 500 mM urea/50 mM Tris·HCl, pH 8.0/1 mM CaCl₂, and digested with bovine blood coagulation factor X (Diagnostica Stago, France), activated with Russell's viper venom immobilized on cyanogen bromide-activated Sepharose 6B (17). After extensive dialysis against water and lyophilization, the β -globin was folded and $\alpha_2\beta_2$ tetramers were reconstituted in the presence of cyanhemin (Sigma) and CO-saturated native α subunits (16). The reconstituted hemoglobin was concentrated under reduced pressure in dialysis tubing and purified by flat-bed isoelectric focusing on a granular support (Ultradex, Sweden) with a pH gradient of 6.0–8.0. The purified Hb was then reduced with sodium dithionite under 1 atmosphere (atm) of CO (1 atm = 101 kPa). Excess dithionite and KCN were removed by mixed-bed ion-exchange chromatography.

Functional Properties of Hb β E6I. Experiments with Hb solutions at a concentration of 60 μ M (heme) were performed at 25°C in 50 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (bis-Tris), pH 7.2/0.1 M NaCl buffer. Oxygen binding curves were recorded with a continuous method (18). Kinetics of CO recombination after flash photolysis were measured as in ref. 19. Isoelectric focusing (PAGE) of the β E6I variant showed a single band migrating like native Hb S.

Kinetics of Polymerization of Native Hb S and β E6I Mutant. These experiments were performed in dilute Hb solutions in 1.8 M potassium phosphate buffer, pH 7.2, following the technique described by Adachi and Azakura (20) with slight modifications (21). The Hb CO was first converted into HbO₂ and concentrated by centrifugation in the cold with the Centricon 10 microconcentrator (Amicon). Rubber-capped cuvettes (1-cm optical path length) containing 4 mg of dry sodium dithionite were flushed with argon and filled with 2 ml of ice-cold deoxygenated phosphate buffer with a gas-tight Hamilton syringe. Then 50 μ l of the Hb solution was introduced into the optical cell by using a Hamilton syringe and the solution was allowed to equilibrate in ice water for 5 min. To keep the final molarity of the buffer constant in all sets of

experiments, variations in Hb concentrations were obtained by dilutions of the stock solutions so that the volume of Hb added into the buffer was always 50 μ l. The polymerization was initiated by the temperature-jump technique (0°C \rightarrow 30°C). Recordings of the absorbance changes at 700 nm due to turbidity were made with a Cary 219 spectrophotometer. The solubility (c_{sat}) was determined by measuring the Hb concentration of the soluble phase after completion of the polymerization process. This was done by centrifuging the turbid solution at 2500 $\times g$ for 20 min at 30°C under mineral oil and sampling an aliquot of the supernatant with a gas-tight syringe. Another fraction of the sample was kept for the measurement of the total Hb concentration. Both samples were then equilibrated with 1 atm CO overnight. Hb concentration was estimated by absorbance at 568.5 nm ($\epsilon = 14.2$ cm⁻¹ per mmol of heme). There was less than 3% difference in total Hb concentration between measurements before and after the polymerization process, indicating that complete solubilization of the polymers without denaturation of the Hb had been achieved. Parameters of the polymerization [delay time τ , total absorbance change (ΔA), and polymer formation rate k (ΔA /final time - τ)] were computed from the recording traces (3, 20, 21). Computer modeling of the donor-acceptor site was performed by using the BRUGEL package installed on a VAX 8700 computer (22).

RESULTS

The analysis of DNA sequence of the noncoding strand after mutagenesis showed the presence of codon ATT (Ile) instead of codon CTC (Glu). The entire coding sequence of factor X- β -globin (β E6I) was determined to confirm the integrity of the sequence. The presence of the mutation was verified by reverse-phase high-performance liquid chromatography (23). Reassembled tetramers made of native α chains and mutated β chains showed visible absorption spectra identical to those of normal Hb A and native Hb S in their deoxy and liganded (O₂ or CO) forms.

Equilibrium and kinetic ligand binding studies served as controls for the purity and functional quality of the mutant Hb. Any nonallosteric contribution can be seen as a perturbation of the lower asymptote of the oxygen equilibrium curve. The kinetic measurements can detect small differences in the rate of ligand (CO) recombination after photolysis. Both techniques are sensitive to the allosteric transition. The only detectable deviation from normal behavior (native Hb A or Hb S) was a slightly increased oxygen affinity and

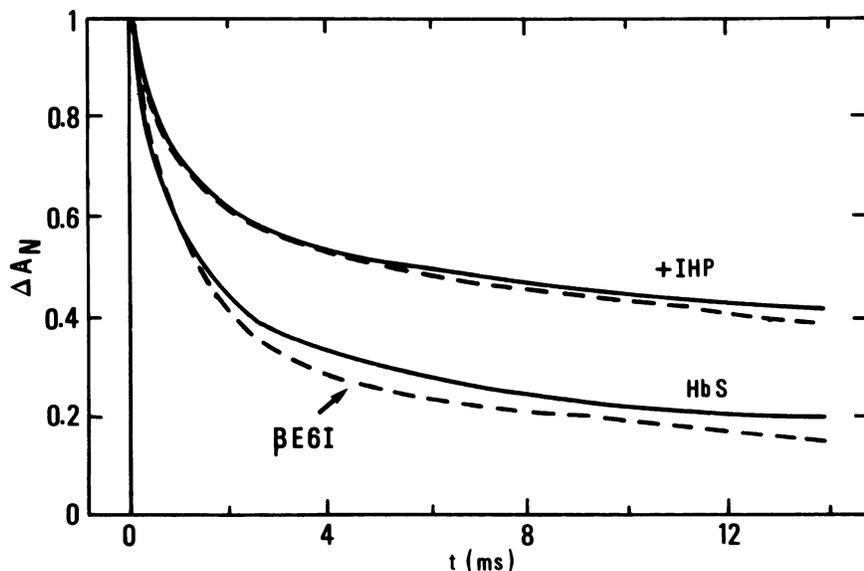


FIG. 1. Bimolecular recombination kinetics of CO (0.1 atm) to Hb β E6I (broken lines) compared to that for native Hb S (solid lines), with (upper pair) and without (lower pair) 1 mM inositol hexakisphosphate (IHP). ΔA_N is the normalized change in absorbance at 436 nm and t is time after photodissociation. IHP increases the slow fraction, characteristic of rebinding to the T-state tetramer. The β E6I mutant shows amounts of the slow phase similar to those of native Hb S, indicating a functional tetramer. Conditions were 0.1 M NaCl/50 mM bis-Tris buffer, pH 7, 25°C, 60 μ M heme.

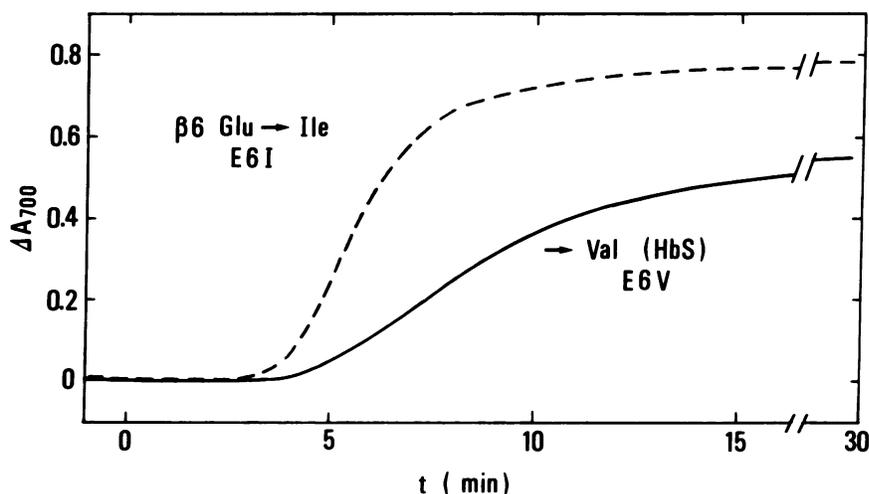


Fig. 2. Kinetics of polymerization of deoxy Hb β E6I and native Hb S (β E6V). Studies were carried out in 1.8 M phosphate buffer, pH 7.2, after a temperature jump from 0°C to 30°C. The turbidity of the solutions was measured spectrophotometrically by recording the absorbance changes due to scattering at 700 nm. Initial Hb concentrations were 1.37 and 1.4 g/dl for the β E6I and β E6V mutants, respectively.

lower Hill coefficient ($n = 2.5$ versus 2.8). The kinetic results show the usual rates and fraction R (relaxed) and T (tense) after photodissociation (Fig. 1). Both techniques indicated a normal interaction with organophosphate effectors. The heat stability of the variant was similar to that of Hb A or native Hb S.

Because of the small amount of the β E6I mutant available, polymerization of the deoxy form was studied with dilute solutions of the mutant Hb in 1.8 M phosphate buffer. Fig. 2

shows that after a temperature jump from 0°C to 30°C the mutant Hb polymerizes with a shorter delay time and larger ΔA_{700} than native Hb S. Fig. 3A shows the dependence of the delay time of polymerization on the total concentration of tetrameric Hb. The values for the β E6I mutant are shifted to the left. The slopes of the lines relating $\log(1/\tau)$ to $\log(c)$ are 3.4 and 2.9 for the β E6I mutant and for native Hb S, respectively, indicating a similar nucleation process for the two Hbs. Fig. 3B shows that the rate (k) was faster for the β E6I mutant at all values of Hb concentration. Fig. 4A illustrates the variations of ΔA_{700} versus Hb concentration for the two mutants. Values for the β E6I mutant are higher than those for native Hb S, indicating a larger polymer fraction for the β E6I mutant. However, a simple comparison of ΔA is not valid unless the polymers are of the same size, which can be checked by measuring the wavelength dependence of the absorbance (scattering) of the turbid suspension. The β E6I mutant exhibits a stronger wavelength dependence, possibly due to a smaller size of the polymers. Fig. 4B shows that the c_{sat} value for the β E6I mutant is half that of native Hb S. Since c_{sat} is lower for the β E6I mutant, ΔA_{700} should be proportionally greater than for the β E6V mutant at the same Hb

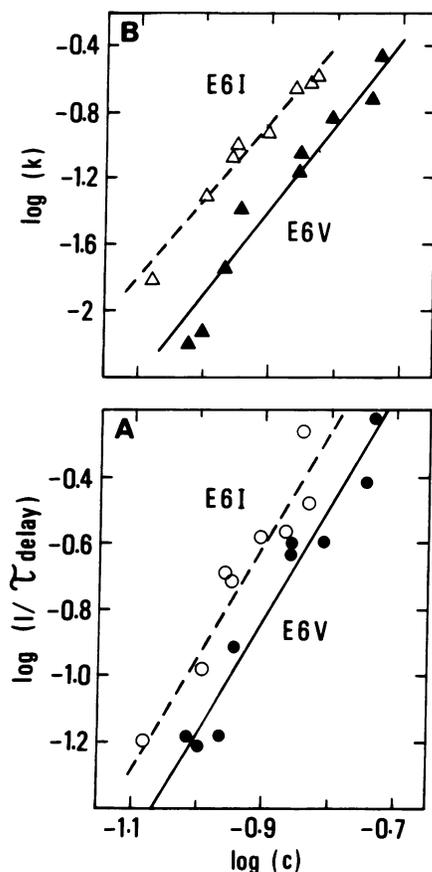


FIG. 3. (A) Relationship between the \log_{10} of the reciprocal of delay time (min) and \log_{10} of the initial Hb concentration c (g/dl) for Hb β E6I (○) and native Hb S (●). Kinetic studies of polymerization of the deoxy forms for the two Hbs were carried out in 1.8 M phosphate buffer, pH 7.2, after a temperature jump from 0°C to 30°C. (B) Relationship between the \log_{10} of the rate of polymerization k (min^{-1}) and $\log_{10} c$ for the two mutants. k was calculated as $\Delta A_{700} \text{ total} / (\text{final time} - \tau)$.

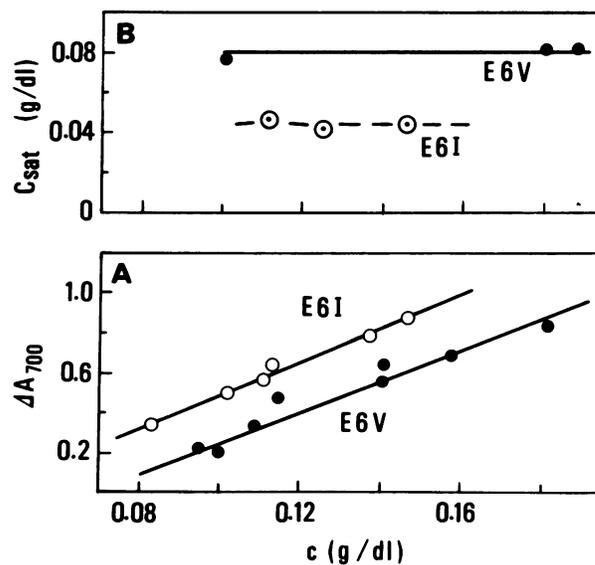


FIG. 4. (A) Relationship between the maximal turbidity (ΔA_{700}) and initial Hb concentration for the two mutants. (B) Supernatant Hb concentration at the plateau of polymerization (c_{sat}) for Hbs β E6I and β E6V as a function of the initial Hb concentration. Conditions were as described in the legend of Fig. 2.

concentration. Since initiation is facilitated for βE6I , the average length of the polymers could well be smaller.

In our calculations of the buried surface at the lateral contact, replacement of valine by isoleucine increased the contribution of the $\beta 6$ position by about 10% (from 125 Å² to 137 Å²). Additional pairwise interactions generated by the presence of isoleucine at the $\beta 6$ position were also observed. Although effectively the same residues are involved in the lateral contact with either isoleucine or valine, after minimizations we observed increased van der Waals contacts with isoleucine compared with valine.

DISCUSSION

We have engineered an artificial hemoglobin mutant (βE6I) which does not occur naturally, since two point mutations would be required in the same codon. The substitution at $\beta 6$ of isoleucine for glutamate results in polymerization of the deoxy form by a nucleation process similar to that of native Hb S but with an about 2-fold decrease in solubility.

Most hydrophobic side chains of globular proteins are buried in their interior, where they stabilize the native conformation by the hydrophobic effect, which is mainly entropic, and by dispersion forces, which are mainly enthalpic. When a polypeptide chain folds from the denatured to the native conformation, the free energy lost by one of its hydrophobic side chains can be calculated from the free energy lost on its transfer from water to a hydrophobic solvent. The same applies when a residue such as that at $\beta-6$ of Hb that is external in the native protein gets buried on polymerization of that protein. The free energy ΔG of transfer of amino acids from water to organic solvents has been measured by Fauchère and Pliska (24) and calculated by Eisenberg and McLachlan (25). The observed values of ΔG , in cal/mol (1 cal = 4.18 J), are Ala, -420; Val, -1660; and Ile, -2460 (the reason for discussing Ala will become clear in the next paragraph). The calculated cal/mol values are Ala, -670; Val, -1500; and Ile, -1900. This gives observed and calculated values of $\Delta\Delta G$ for Val \rightarrow Ile of 800 and 400 cal/mol, respectively, and $\Delta\Delta G$ for Val \rightarrow Ala = -1240 and -830 cal/mol, respectively.

If the ratios of the solubilities S of two deoxyhemoglobins reflected the differences in free energy $\Delta\Delta G$ of transfer of the residue $\beta 6$ from water to a nonpolar solvent at 20°C, then $\log(S_{\text{Val}}/S_{\text{Ile}}) = \Delta\Delta G/1340$. Substituting the above predicted values of $\Delta\Delta G$ gives calculated values $S_{\text{Val}}/S_{\text{Ile}} = 2-4$, comparable to the observed ratio of 2 in 1.8 M phosphate buffer. Similarly, the observed and predicted values of $\Delta\Delta G$ for Val \rightarrow Ala give calculated values of $S_{\text{Val}}/S_{\text{Ala}} = 0.1-0.24$. The solubility of Hb G-Makassar ($\beta 6$ Ala) is about the same as that of Hb A, which is 10 times more soluble than Hb S in concentrated phosphate buffer (26). The rough agreement between predicted and observed differences in solubility suggests that the transfer of the $\beta 6$ Val or Ile side chain from water to a hydrophobic environment is sufficient to explain the changes in solubility.

Fibers of Hb S are made up of helical bundles of filaments linked together in pairs (5, 6) with Hb molecules in the members of each pair running in the same direction. $\beta 6$ Val plays no part in the aggregation of deoxy Hb S to form single filaments; these form the structural unit of crystals of both deoxyhemoglobins A and S and the Hb S fibers, with nearly identical contacts joining molecules stacked end-to-end. On the other hand, the paired filaments are seen only in deoxy Hb S; they are held together by one of the symmetrically related $\beta 6$ valines in each molecule of one filament adhering to $\beta 85$ Phe and $\beta 88$ Leu in a molecule of the partner filament. The combination is similar to that between antigen and antibody, because the contact between the two molecules covers a wider area than that of the valine alone. The burying

of a single uncompensated charge has been shown to be sufficient to inhibit the combination antigen and antibody (27). Similarly, the potential burying of the uncompensated charge of $\beta 6$ Glu inhibits the pairing of filaments of deoxy Hb A, but the question was whether the absence of that charge is sufficient to stabilize the pairs. The failure of Hb G-Makassar ($\beta 6$ Ala) to form paired filaments shows that it is not. The striking drop in solubility observed in going from alanine to valine and then to isoleucine at the $\beta 6$ position proves that pairing requires the loss of free energy that takes place when larger hydrophobic side chains are transferred from water to a hydrophobic pocket in the protein, and the loss of free energy increases with the size of the side chain. The dominance of the hydrophobic effect demonstrated here is consistent with the well-known inverse temperature coefficient of the solubility of deoxy Hb S.

The large changes in solubility of deoxyhemoglobins induced by the small changes in free energy loss between the valine and isoleucine side chains show how delicate is the equilibrium between the soluble and insoluble phases in the erythrocyte. Site-directed mutagenesis is the only technique with which to probe such mechanisms. These studies may open a long-term prospect to refine drug design aimed at inhibiting sickling and at improving the clinical course of the disease. All attempts to use valyl peptides to inhibit the gelling of deoxy Hb S have failed. Our results suggest that isoleucyl peptides or peptides containing unnatural amino acids with even longer hydrophobic side chains might raise the solubility of deoxy Hb S and could conceivably form the starting point to the development of new antisickling compounds.

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