Steric and hydrophobic determinants of the solubilities of recombinant sickle cell hemoglobins

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Abstract

Models for the structure of the fibers of deoxy sickle cell hemoglobin (Hb S, $\beta 6$ Glu $\rightarrow$ Val) have been obtained from X-ray and electron microscopic studies. Recent molecular dynamics calculations of polymer formation give new insights on the various specific interactions between monomers. Site-directed mutagenesis with expression of the Hb S β subunits in *Escherichia coli* provides the experimental tools to test these models. For Hb S, the $\beta 6$ Val residue is intimately involved in a specific lateral contact, at the donor site, that interacts with the acceptor site of an adjacent molecule composed predominantly of the hydrophobic residues Phe 85 and Leu 88. Comparing natural and artificial mutants indicates that the solubility of deoxyHb decreases in relation to the surface hydrophobicity of the residue at the $\beta 6$ position with Ile $>$ Val $>$ Ala. We also tested the role of the stereospecific adjustment between the donor and acceptor sites by substituting Trp for Glu at the $\beta 6$ location. Among these hydrophobic substitutions and under our experimental conditions, only Val and Ile were observed to induce polymer formation. The interactions for the Ala mutant are too weak whereas a Trp residue inhibits aggregation through steric hindrance at the acceptor site of the lateral contact. Increasing the hydrophobicity at the axial contact between tetramers of the same strand also contributes to the stability of the double strand. This is demonstrated by associating the $\beta 23$ Val $\rightarrow$ Ile mutation at the axial contact with either the $\beta 6$ Glu $\rightarrow$ Val or $\beta 6$ Glu $\rightarrow$ Ile substitution in the same β subunit. In comparison to native Hb S the solubility of these deoxyHb double mutants is decreased two- and fourfold, respectively.

Keywords: polymer formation; protein-protein interactions; site-directed mutagenesis

Sickle cell anemia arises from a point mutation, $\beta 6$ Glu $\rightarrow$ Val ($\beta E6V$), of the hemoglobin (Hb) molecule. The deoxy form of sickle Hb (Hb S) aggregates to form long, rigid rods that distort the erythrocytes into their characteristic sickle shape. The rods possess a highly ordered structure involving seven double strands corresponding to the double strand of the crystals of deoxyHb S (Edelstein, 1981; Padlan & Love, 1985; Rodgers et al., 1987; Carragher et al., 1988). Two types of contacts stabilize the double strand: (1) the axial contacts between tetramers of the same strand, and (2) the lateral contacts, responsible for double strand formation in which the $\beta 6$ Val donor side chain of one tetramer is positioned in the hydrophobic EF acceptor pocket of an adjacent tetramer (Fig. 1). Other natural mutations at the $\beta 6$ position have

been described. These are Hb G-Makassar $\beta 6$ Glu $\rightarrow$ Ala ($\beta E6A$), Hb Machida $\beta 6$ Glu $\rightarrow$ Gln ($\beta E6Q$), Hb C $\beta 6$ Glu $\rightarrow$ Lys ($\beta E6K$) (Blackwell et al., 1970; Harano et al., 1982). None of these isolated purified Hbs lead to polymer formation. Hb G-Makassar is of special interest as the mutation of Glu to Ala not only suppresses the repulsive negative charge of the glutamyl residue, but also creates a hydrophobic environment at the lateral contact region mentioned above. The failure of Hb G-Makassar to form paired filaments clearly shows that the suppression of the charge at the $\beta 6$ position is not sufficient for stabilizing the pairs at the origin of the fibers. There are two ways for enhancing the stability of the monomer-monomer interactions (considering an Hb S tetramer as a monomer). One is to raise the stability of the interaction at the donor-acceptor site; the second is to raise the stability of the contact between monomers at the single-strand interaction interface.

As a first step we have substituted Ile for Glu at the $\beta 6$

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Fig. 1. Scheme of the deoxyHb S double strand. A indicates the axial contacts between tetramers of the same strand. These contacts involve the B helix ($1\beta_2$) and the GH corner ($1\beta_1$). The $\beta 23(B5)$ site is located at this interface. The lateral contact (L) between two strands involves $\beta 6$ Val in $2\beta_2$ (donor site) and the hydrophobic pocket in $1\beta_1$ on a neighboring tetramer (acceptor site) (redrawn from Dickerson & Geis, 1983).

position by site-directed mutagenesis. The artificial mutant Hb $\beta E6I$ was found to promote faster polymer formation and to lower the solubility to half the value of the native $\beta E6V$ mutant (Baudin-Chich et al., 1990). These results support the argument that hydrophobic interactions are necessary to stabilize the paired filaments. They also show that the acceptor site at the lateral contact is either flexible or large enough to accommodate a slightly bulkier residue such as Ile.

More recently, we assumed that substituting Ile for $\beta 23$ Val as in Hb S Antilles ($\beta E6V, V23I$) (Monplaisir et al., 1986) enhances the energy of the axial contact, leading to more favorable fiber formation. The results revealed the additivity of the effects of the two mutations on Hb solubility and fiber growth (Pagnier et al., 1990). Subsequently, the question was asked as to whether the solubility could be further decreased if the $\beta 6$ Val in Hb S Antilles was replaced by Ile and if additivity could still be demonstrated.

As a consequence of the above interpretation favoring the role of hydrophobic interactions in enhancing deoxy-Hb S polymer formation we also addressed the question of the role of the stereochemistry at the donor-acceptor site. The acceptor pocket appears to be well suited for medium size residues such as Val and Ile. We have engineered the artificial mutant Hb $\beta E6W$ in order to in-

crease the size and the potential steric hindrance of the residue at the donor site.

Results

The presence of the mutation was verified by DNA sequencing and protein analysis by reverse-phase high performance liquid chromatography (Baudin-Chich et al., 1987). Reassembled tetramers showed visible absorption spectra identical to those of native Hb A and Hb S in their deoxy and liganded forms. The electrophoretic patterns of Hb $\beta E6W$ and Hb $\beta E6I, V23I$ were similar to that of Hb S.

As observed for pure Hb S Antilles and recombinant Hb $\beta V23I$, dilute solutions of the doubly mutated Hb $\beta E6I, V23I$ had a lower oxygen affinity than Hb A. Cooperativity in oxygen binding and the effect of 2,3-diphosphoglycerate (2,3-DPG) (1 mM) on oxygen affinity were within the normal range (Table 1).

Polymerization of dilute deoxyHbs was studied in 1.8 M phosphate buffer, pH 7.2, for different Hb concentrations. The solubility and the kinetics of polymerization of the double mutant $\beta E6I, V23I$ were compared to those of the $\beta E6I$ mutant, native Hb S, and reconstituted Hb S. At similar total Hb concentration (0.14 g/dL) the double mutant $\beta E6I, V23I$ polymerized faster than either the $\beta E6I$ mutant or Hb S ($\beta E6V$) (Fig. 2). Figure 2 also shows that the maximum absorbance at 700 nm was lower for the $\beta E6I, V23I$ mutant than for the $\beta E6I$ Hb while maintaining the expected positive correlation with total Hb concentration (not shown). Similar discrepancies have already been observed by others using the same high concentration potassium phosphate buffer technique (Adachi et al., 1980b). They are probably related to discrete dif-

Table 1. Oxygen-binding parameters for artificial mutants of human HbA at the $\beta 6$ position expressed in *E. coli*^a

	P_{50} (mmHg)	n_{50}	P_{50m}/P_{50c}^b
Control ^c	4.2	2.4	—
+DPG	11.3	2.4	—
$\beta E6I^d$	4.0	2.5	0.95
+DPG	9.5	2.2	0.84
$\beta E6W$	5.2	2.05	1.24
+DPG	13.0	2.5	1.15
$\beta V23I^e$	5.4	2.2	1.285
+DPG	15.5	2.0	1.37
$\beta E6I, V23I$	6.4	2.3	1.52
+DPG	17.2	2.0	1.52

^a Conditions were 100 mM NaCl, 50 mM bis-Tris buffer, 50 μ M EDTA, 20 μ g/mL catalase, 60–80 μ M heme, pH 7.2, 25 °C. DPG, diphosphoglycerate, 1 mM.

^b m—Mutant Hb; c—control Hb.

^c Control curves for reconstituted human Hb A.

^d From Baudin-Chich et al. (1990).

^e From Pagnier et al. (1990).

Fig. 2. Kinetics of polymerization of deoxyHb $\beta E6I,V23I$ compared to $\beta E6I$ and $\beta E6V$. Polymerization studies were performed in 1.8 M phosphate buffer, pH 7.2, after a temperature jump from 0 to 30 °C. Changes in turbidity of the solutions were measured spectrophotometrically by recording the absorbance at 700 nm. Hb concentrations were 0.145, 0.147, and 0.142 g/dL for $\beta E6I,V23I$, $\beta E6I$, and $\beta E6V$, respectively.

ferences in the structure or nature of the aggregates of the sickling Hbs depending on the rate of the salting-out process as is also observed upon addition of inositol hexaphosphate (Adachi et al., 1980a). The relationship between the delay-time (τ) prior to polymerization and the total Hb concentration is illustrated in Figure 3; the data for the $\beta E6I,V23I$ mutant were shifted toward the left, indicating shorter delay of polymer formation compared to $\beta E6V$ and $\beta E6I$ mutant Hb. Note also that the slope (n) of the plots were different for the three mutants with n values of 2.1, 2.7, and 3.1 for the Hb $\beta E6I,V23I$, Hb $\beta E6I$, and Hb $\beta E6V$, respectively. The lower n value for the $\beta E6I,V23I$ mutant indicates that the mechanism of aggregation may not be the same as that for native Hb S. An equimolar mixture of the $\beta E6I,V23I$ Hb and native Hb A exhibited a delay-time similar to that of pure native Hb S (Fig. 3). The solubility (c_{sat}) value for $\beta E6I,V23I$ deoxyHb was decreased when compared to $\beta E6I$ and native deoxyHb S ($\beta E6V$), two- and fourfold, respectively (Fig. 4).

The mutant Hb $\beta E6W$ exhibits a slight decrease in oxygen affinity with a nearly normal interaction with 2,3-DPG (Table 1). The n_{50} value, an index of cooperativity in oxygen binding at half saturation, was lower than controls but was restored upon addition of 2,3-DPG. Carbon monoxide (CO) recombination after flash photolysis confirmed the equilibrium studies in showing a lower rate of CO bimolecular recombination compared to COHb A. The effect of inositol hexaphosphate (IHP) was similar for the two Hb solutions (not shown).

Fluorescence studies revealed that the emission spectrum for the $\beta E6W$ mutant Hb was shifted to longer wavelengths with a peak at 340 nm compared to the emission peak of the three Trp residues (325 nm) normally present in each Hb $\alpha\beta$ dimer. The fluorescence in-

Fig. 3. Relationship between the log of the reciprocal delay-time (τ , min) and the log of the initial Hb concentration (g/dL). The dotted circle and triangle at the bottom right of the figure are values for equimolar mixtures of the artificial mutants and native Hb A ($\beta E6$). An equimolar mixture of $\beta E6I,V23I$ mutant and native HbA had a delay-time of polymerization similar to that of a pure Hb S solution. Note the difference in the slopes between the curves for the three mutants as discussed in the Results section.

tensity of $\beta E6W$ was twice that of native Hb A. Thus the mutant $\beta E6W$ showed a spectrum indicating a more exposed or less buried Trp, as expected for a surface residue (Fig. 5).

The mutant deoxyHb $\beta E6W$ did not polymerize in 1.8 M phosphate buffer after 2 h of observation and for Hb concentrations up to 0.3 g/dL, i.e., twice that used for native Hb S. We noticed a slight temperature-induced instability observed as an immediate turbidity change at 700 nm after the temperature jump from 0 to 30 °C, possibly due to incorrect refolding. When mixed with an equimolar concentration of native Hb S, the $\beta E6W$ mutant inhibited polymerization of Hb S in a way very similar to that observed in equimolar mixtures of Hb S and Hb A.

Discussion

We have engineered four artificial Hbs with β -chain mutation(s): $\beta E6I$, $\beta V23I$ (Baudin-Chich et al., 1990; Pag-

Fig. 4. Supernatant Hb concentration (solubility in g/dL) as a function of initial Hb concentration. The solubility C_{sat} was determined by measuring the Hb concentration in the CO form of the soluble phase after completion of the polymerization process and centrifugation for 60 min at 30 °C under mineral oil. Open triangles and squares are values for native and reconstituted Hb S ($\beta E6V$), respectively (see Materials and methods). The values for the $\beta E6I$ mutant are from Baudin-Chich et al. (1990). Values for equimolar mixtures of native Hb S and Hb A (upper points) are shown for comparison.

Fig. 5. Fluorescence emission spectra of the Hb S and $\beta E6W$ artificial mutant. Note the right shift of the emission spectrum for the $\beta E6W$ mutant and the large increase in fluorescence intensity. The spectrum for Hb S was identical to that of native Hb A. Conditions were 10 mM phosphate buffer, pH 7.2, 25 °C. Heme concentration was 10 μM .

nier et al., 1990), $\beta E6W$, and the double substitution $\beta E6I, V23I$.

Pure Hbs $\beta V23I$ and $\beta E6W$ do not polymerize. The substitution at $\beta 6$ Ile for Glu, and the association of both mutations $\beta 6$ Ile and $\beta 23$ Ile on a same β subunit result in polymerization of the deoxyHbs, with about twofold and fourfold decreases in solubility, respectively, when compared to deoxyHb S.

Fibers of deoxyHb S are made of helical bundles of filaments linked together in pairs (Padlan & Love, 1985). $\beta 6$ Val plays no part in the formation of single filaments. These are the structural units of crystals of both deoxyHb A and S and the Hb S fibers, with nearly identical contacts joining molecules (axial contacts). The paired filaments are found only in deoxyHb S (Fig. 1); they are held together by one of the symmetrically related $\beta 6$ Val residues of each molecule of one filament adhering to $\beta 85$ Phe and $\beta 88$ Leu of a molecule of the partner filament. The combination can be compared to that between antigen and antibody or protease and inhibitor, except that the subunit interfaces in oligomeric proteins such as Hb tetramers are usually larger and more hydrophobic than functional recognition sites in antigen-antibody and protease-inhibitor complexes (Janin & Chothias, 1990; Padlan, 1990). The contribution of hydrophobicity to the stability of these interactions has been estimated from the correlation between the accessible surface of residues and the free energies associated with their transfer from water to organic solvent (Eisenberg & McLachlan, 1986). When polypeptide subunits associate to form oligomers, external residues become buried if involved in the contact area. The free energy lost by such hydrophobic side chains can be calculated from the free energy lost upon their transfer from water to a hydrophobic solvent. In the mutants Hb $\beta E6V$, Hb $\beta E6I$, and Hb $\beta E6I, V23I$, the external $\beta 6$ and $\beta 23$ residues become buried on polymerization of the protein. In other words, the hydrophobic side chains of Val or Ile are transferred from water to the hydrophobic environment with simultaneous loss of free energy. The loss of free energy associated with the change from Val to Ile, estimated to $\Delta\Delta G = 400$ cal/mol (Eisenberg & McLachlan, 1986), is related to the ratio of the solubilities (S) of the two forms deoxyHb $\beta E6V$ and deoxyHb $\beta E6I$ such that $\log(S_{Val}/S_{Ile}) = \Delta\Delta G/2.3RT$ with $2.3RT = 1,340$ cal/mol at 30 °C. Calculating the value for S_{Val}/S_{Ile} gives 2, comparable to the observed ratio of solubilities in 1.8 M phosphate buffer. In the double mutant $\beta E6I, V23I$, assuming that each mutation Val $\rightarrow$ Ile contributes equally, the loss of free energy is twice as much as in the simple mutant, $\Delta\Delta G = 800$ cal/mol. This results in a ratio of solubilities of 4, which is identical to the experimental value. These results confirm the additivity of the contributions of $\beta 6$ and $\beta 23$ Ile side chains to the energy of intermolecular contacts.

Studying the free energy of sickling through molecular dynamics simulation, Kuczera et al. (1990) have recently pointed out that because the interactions with the nonpolar valyl residue at the lateral contact are small, the free energy difference between deoxyHb A and deoxyHb S was predominantly due to the loss of the large electrostatic interactions of the glutamate residue with the solvent. These data are not in conflict with the results presented here: once the $\beta 6$ Glu contribution is lost, van der Waals stabilization at the lateral contact, however weak, may be significantly increased with the $\beta 6$ Ile mu-

tation, and still enhanced when these weak interactions are further stabilized by the presence of Ile instead of Val at the axial contact. The solubility of proteins depends on the electrostatic and hydrophobic properties of the solvent. Due to the inverse relationship between the two effects, it is likely that in our conditions of high salt concentration, the hydrophobic nature of the interaction between monomers becomes predominant in determining the changes in solubility. In their recent report, Kuczera et al. (1990) have also presented important information about residues of the acceptor site that may contribute significantly to the overall calculated free energy difference. Mutations of these residues are under experimental investigation in our laboratory.

The β E6W mutation failed to induce polymer formation in spite of the larger hydrophobicity of Trp relative to that of Val or Ile. A likely explanation for this counter example is that the acceptor site cannot accommodate the Trp residue, due to the size and rigidity of the latter. Among the numerous compounds that inhibit gelation of pure deoxyHb S, Trp is known as a potent inhibitor (Poillon, 1982). Recently, De Cross et al. (1990) have demonstrated that 5-bromotryptophan (5-BrTrp) or 5-BrTrp-5-BrTrp derivatives were even more potent inhibitors and suggested that these inhibitors could interact at the surface of the hydrophobic EF pocket and hinder the binding between the donor and the acceptor sites of Hb S. However, when Trp is placed at the β 6 position, the complementarity between the donor and the acceptor sites is not compatible with a stable interaction. Preliminary modeling studies of the "fit" of the Trp side chain in the acceptor pocket were carried out and suggest that the Trp side chain is too bulky to be accommodated. A systematic search of the conformational space of the Trp torsion angle was performed with the Brugel software package (Delhaise et al., 1984) after replacing the β 6 Val by Trp in the Hb S coordinates system at the lateral contact (Padlan & Love, 1985). With all other side chains maintained in their initial position, the Trp cannot enter the acceptor pocket due to steric hindrance. The most stable conformation found places the indole ring parallel to the A helix oriented toward the β 5 Pro (Schaad, 1991). These results are also supported by the fluorescence studies suggesting that the additional Trp residue in the β subunits is exposed at the surface of the protein and not buried in a hydrophobic environment.

In conclusion, among the hydrophobic substitutions for Glu at the β 6 location, Ala and Trp are not able to induce the formation of the deoxyHb polymers under the present experimental conditions: Ala because it is too small or not sufficiently hydrophobic to interact tightly with the acceptor site, and Trp because it does not fit into that site due to steric hindrance. Only Val and Ile (and most probably Leu) satisfy both essential conditions of hydrophobic interaction and stereospecific adjustment between donor and acceptor sites at the lateral contact.

Materials and methods

The β E6I, β E6W, and β V23I mutations were introduced into the β -globin cDNA by site-directed mutagenesis (Nakamaye & Eckstein, 1986) using primers synthesized by the phosphotriester method. The β -globin chains were synthesized by using the expression vector pAT PrcIIFX β . This vector directs the synthesis of a fusion protein cIIFX β -globin under the control of λ Pr promoter. *Escherichia coli* strain CAG 1139, harboring pAT PrcIIFX β , was grown at 30 °C in M9 medium containing 2 g of glucose, 20 g of yeast extract, and 0.5 g of vitamin B1 with 10 mg tetracycline per liter. Synthesis of the cIIFX β -globin fusion protein was induced by inactivation of the temperature-sensitive repressor cI₈₅₇ at 42 °C. The cells were harvested after 3–4 h incubation. The protein was extracted, purified, and cleaved with bovine coagulation factor Xa to liberate the β -globin (Nagai & Thogersen, 1984, 1987). The tetrameric Hb $\alpha_2\beta_2$ molecules were reconstituted in the presence of cyanhemin- and carbonmonoxy-saturated native α subunits. The reconstituted Hbs were reduced with sodium dithionite in the presence of CO and purified by ion exchange chromatography on CM-cellulose and DEAE-Sephacel columns.

Oxygen-binding curves were recorded with a continuous method (Kister et al., 1987). Kinetics of CO recombination were obtained after flash photolysis using a 10-ns laser pulse (Yag 585 Quantel, France) providing 160 mJ at 532 nm (Marden et al., 1988). Solutions of Hb A or Hb β E6W (100 μ M on a heme basis) in 0.1 M NaCl, 50 mM bis-Tris, pH 7.0, were deoxygenated under argon and then equilibrated under 0.1 atm CO.

Fluorescence studies were performed in 10 μ M (on a heme basis) Hb A and Hb β E6W solutions, 10 mM phosphate buffer, pH 7.0, using an SLM 8000 spectrofluorometer. Optical cuvettes were 4 $\times$ 10 mm with excitation at 280 nm along the 10-mm axis. Emission spectra were recorded for both buffer and Hb samples. The data presented are the difference spectra with the buffer spectrum subtracted. Spectra were also recorded for deoxyHb β E6W in closed cuvettes under argon.

Polymerization experiments were performed in dilute Hb solutions in 1.8 M phosphate buffer, pH 7.2 (Adachi & Asakura, 1979; Poyart et al., 1981). Kinetics of turbidity changes were recorded at 700 nm, after a temperature jump from 0 °C to 30 °C, with a Cary 219 spectrophotometer. Equimolar mixtures of Hb S or Hb β E6I, V23I with either Hb A or Hb β E6W were prepared in the oxy form and allowed to stand for 60 min in ice water before deoxygenation. The delay time τ and the maximum change in turbidity ΔA_{max} at 700 nm were calculated from the kinetics traces. The solubility (C_{sat}) was determined by measuring the Hb concentration of the soluble phase at completion of the polymerization process, after centrifugation of the turbid solution for 60 min at 30 °C under mineral oil (Poyart et al., 1981). Total Hb concentration and the soluble fraction after polymerization were

estimated spectrophotometrically from the absorbance of the CO forms at 568.5 nm using a millimolar extinction coefficient of 14.5 cm^{-1} .

Control values were obtained from native Hb A and Hb S and reconstituted Hb S. Native Hb A and Hb S were purified by preparative electrofocusing. Hb S was also reconstituted and purified under the same conditions as for the artificial mutants, starting from native apo- β S globin chains separated by carboxymethylcellulose chromatography (Clegg et al., 1966).

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