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Functional Studies and Polymerization of Recombinant Hemoglobin Glu- $\alpha_2\beta_2$ 6(A3) \rightarrow Val/Glu-7(A4) \rightarrow Ala*

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In hemoglobin (Hb) S the hydrophobic mutated residue Val- β 6(A3) (donor site) closely interacts with the hydrophobic side groups of Phe- β 85(F1) and Leu- β 88(F4) (EF pocket, acceptor site) of a neighboring tetramer, resulting in decreased solubility and polymerization of the deoxy-Hb. The β 6(A3) residue is followed by two charged residues Glu- β 7(A4) and Lys- β 8(A5). This cluster has no attraction for the hydrophobic EF pocket. We have modified the β 7(A4) residue next to the donor site Val- β 6(A3), replacing the charged Glu by a hydrophobic Ala (rHb β E6V/E7A). The single mutant Glu- β 7 \rightarrow Ala (rHb β E7A) was also engineered. Both rHbs exhibit a heat instability and an increased oxygen affinity compared to Hb A and Hb S. There was a concentration dependence of the ligand binding properties (1–300 μ M in heme) indicating an increased amount of dimers relative to Hb A. The deoxy form of rHb β E6V/E7A polymerizes *in vitro*, with a decreased rate of polymer formation relative to Hb S, while the single mutant β E7A does not polymerize in the same experimental conditions. The Glu- β 7(A4) \rightarrow Ala substitution does not increase the hydrophobic interaction between donor and acceptor site. We speculate that the loss of the normal saline bridge between Glu- β 7(A4) and Lys- β 132(H10) leads to an increased flexibility of the A helix and may account for the difference of the polymerization for this Hb S mutant.

Individuals with sickle cell anemia are homozygous for a mutant form of hemoglobin (Hb S)¹ in which Glu at the β 6 position is replaced by Val. This substitution is responsible for the abnormally low solubility of deoxy-Hb S. Molecules of Hb S in the deoxygenated T structure associate into long helical fibers that deform the erythrocytes and severely diminish their lifetime. Electron microscopy (1, 2) and x-ray crystallographic studies (3) have shown that both fibers and crystals are composed of double strands of Hb S molecules. The formation of the double strands requires the stereochemical contact between complementary surfaces involving specifically the Val- β 6 and a hydrophobic pocket on an adjacent molecule (lateral contact).

In order to further characterize the interactions at the key β 6

site, particularly the role of hydrophobicity, it is useful to consider the effects of other amino acid side chains on fiber formation. Gelation studies of deoxygenated mixtures of S and non-S Hbs have provided considerable information about the location of intermolecular contacts in the polymer fibers (4). More recently, studies of recombinant Hbs modified at the fiber contacts have shown that the presence of the hydrophobic leucine or isoleucine residues at the β 6 position could also initiate the polymerization process (5, 6).

In normal Hb A, the residue Glu- β 6(A3) is followed by two other charged amino acids, Glu- β 7(A4) and Lys- β 8(A5). Two natural Hb variants have been described at the β 7(A4) position, Hb San Jose and Hb Siriraj, in which Glu- β 7 is replaced by Gly and Lys, respectively (7, 8). In mixtures with Hb S (*trans* effect), none of these mutated Hbs has been shown to alter the polymerization process (8). The replacement of Glu- β 7(A4) for Gly in Hb San Jose results in the loss of a charged residue without introducing a nonpolar side chain. We have associated the Glu- β 6(A3) \rightarrow Val and Glu- β 7(A4) \rightarrow Ala mutations on the same β chain (rHb β E6V/E7A) in order to study the interaction of a hydrophobic β 7(A4) side chain, *cis* to the active residue Val- β 6(A3). We have also engineered the single mutant Hb Glu- $\alpha_2\beta_2$ 7(A4) \rightarrow Ala (rHb β E7A) as a control.

MATERIALS AND METHODS

The β E6V and β E7A mutations were introduced into the β -globin cDNA by site-directed mutagenesis using synthetic primers (Genset, France). The mutated β -globin subunit was produced as a fusion protein in *Escherichia coli*, using the expression vector pATPrCIIFX β (9). After extraction and purification, the fusion protein was cleaved by digestion with bovine coagulation factor Xa (10). The presence of the mutation(s) was confirmed by reverse-phase high-performance liquid chromatography of the tryptic digests and amino acid analysis of the abnormal peptides. The purified β -subunits were folded in the presence of cyanhematin and the partner α -subunits, prepared from natural Hb A, to form the tetrameric Hb $\alpha_2\beta_2$ (9, 11). Electrophoretic studies included electrophoresis on cellulose acetate and isoelectrofocusing of the recombinant Hbs. Fluorescence studies of the rHbs were performed at a concentration of 10 μ M on a heme basis, in 10 mM phosphate buffer, pH 7.0, using an SLM 8000 spectrofluorometer. Fluorescence spectra were measured in the region for tyrosine and tryptophan emission, for air-equilibrated samples. The heat stability of the Hbs was determined by incubating the recombinant and native Hbs at 65 °C in 10 mM phosphate buffer pH 7.0 according to Wajzman *et al.* (12) at 0.1 mM heme under 1 atm of CO or 1 atm of O₂.

The oxygen binding curves were recorded at 25 °C with a continuous method using the Hemox Analyzer system (TCS, Huntington Valley, PA) (13). The Hb samples were in 0.05 M bis-Tris buffer, pH 7.2, 0.1 M NaCl, 50 μ M EDTA, 20 μ g/ml catalase. Hb concentration was 60–70 μ M (on a heme basis). Met-Hb content, calculated from the visible absorption spectra was found to be less than 5% at the end of the recordings.

Bimolecular recombination of CO was studied after flash photolysis dissociation with 10-ns pulses at 532 nm. Detection was at 436 nm for samples equilibrated under 0.1 atm of CO (14). Measurements were made at different concentrations to study the dimer-tetramer equilibrium (15). For concentrations above 30 μ M (on a heme basis) 1-mm

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¹ The abbreviations used are: Hb, hemoglobin; rHb, recombinant Hb; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

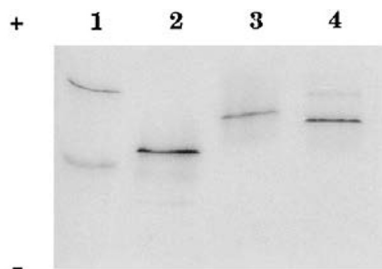


FIG. 1. Isoelectric focusing of rHbs $\beta E7A$ (lane 3) and $\beta E6V/E7A$ (lane 2) compared to Hbs AC (lane 1) and S (lane 4). Mobilities of the Hbs were determined by isoelectric focusing in polyacrylamide gel, with a pH gradient ranging from 6.0 to 8.0.

optical cuvettes were used. Below $30 \mu M$, cuvettes of dimension 4×10 mm were used with detection along the 10-mm axis and dissociation at 90° along the 4-mm axis.

Kinetic studies of the polymerization of mutant deoxy-Hbs were carried out in concentrated potassium phosphate buffer, for different Hb concentrations, following initiation by a temperature jump ($0 \rightarrow 30^\circ C$). The solubility and the kinetics of polymerization of the mutated Hb were compared to those of native Hb S. Under these conditions, the assembly of Hb S into fibers results, after a characteristic lag period, in a cooperative increase in turbidity (measured by the absorbance at 700 nm) until a plateau was achieved. The Hb solubility (C_{sat}) was determined by measuring the concentration of the soluble phase after completion of the polymerization process (16, 17).

Molecular graphics models of deoxy-Hb $\beta E7A$ were performed starting from the crystallographic coordinates of the deoxy structure of Hb A (file 3HHB, Protein Data Bank, Brookhaven National Laboratory) reported by Fermi *et al.* (18). Minimization of the potential energy in the mutant and normal Hbs were performed using the CHARMMTM program (version 22) (19) with a Silicon Graphics Indigo workstation. The mutated residue was initially minimized holding the rest of the structure constant, by starting with a high harmonic constant that was progressively decreased to 0.

RESULTS

Reverse-phase high performance liquid chromatography of the tryptic digest of the purified fusion proteins $\beta E7A$ or $\beta E6V/E7A$ revealed the presence of an abnormal peptide. Amino acid analysis of these two peptides confirmed the presence of the expected mutation(s). Reassembled tetramers showed visible absorption spectra identical to those of native Hb A and S in their deoxy and liganded forms. Fluorescence studies did not show significant differences between the two mutated rHbs and native Hb A, indicating correct refolding of the recombinant Hbs.

Electrophoretic Studies—Cellulose acetate electrophoresis and isoelectrofocusing (Fig. 1) of the purified rHbs $\beta E7A$ and $\beta E6V/E7A$ showed a single band migrating at isoelectric points of 7.1 and 7.4, respectively. These values are lower than those observed for Hb S and Hb C with pI at 7.2 and 7.5, respectively. The electrophoretic pattern of $\beta E7A$ compared to Hb S was similar to that described by Yamashiro *et al.* (20) for the rHb $\beta E7V$. The natural mutated Hbs Siriraj $\beta E7K$ and San-Jose $\beta E7G$, also exhibited anomalous electrophoretic mobilities compared to those of Hb C $\beta E6K$ and Hb S $\beta E6V$ (8). This may be explained by the fact that the $\beta 7(A4)$ residue is partially buried within the molecule, while $\beta 6(A3)$ is exposed.

Heat stability of the oxy form of the mutated rHbs was compared to those of human Hbs A and S. We did not observe any difference between oxy-Hb A and oxy-Hb S, while both mutants precipitated rapidly. After 10 min incubation at $65^\circ C$, 80% of the mutated oxy-rHbs were denatured, relative to 10–15% for oxy-Hbs A and S. While the CO forms of Hb A and S are stable at this temperature, 15% of the mutated rHbs were denatured after 10 min incubation at $65^\circ C$ under CO. Yamashiro *et al.* (20) also observed a marked instability of the rHb

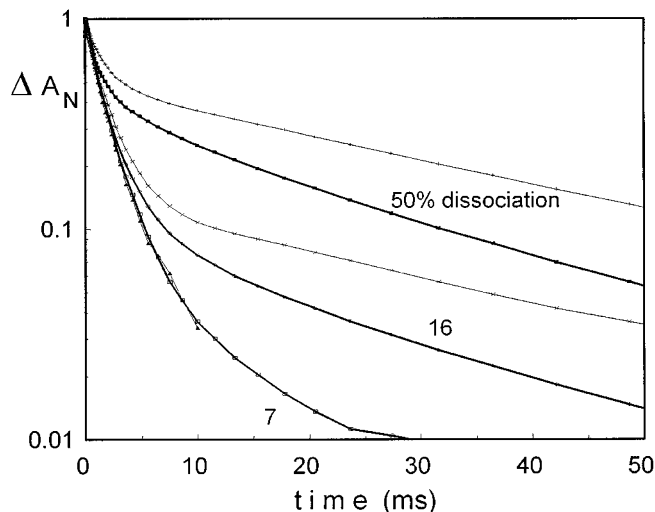


FIG. 2. Recombination of CO to Hb A and rHb $\beta E6V/E7A$ (bold lines). Three levels of photodissociation are shown; all curves are normalized to better show the fraction of slow phase. At the lowest dissociation level, the reaction is mainly from triply to fully liganded forms and occurs with the rapid rate for both Hbs. The slow phase, typical of binding to deoxy-Hb, increases at higher dissociation levels. The double mutant displays less of the slow phase, in part due to an elevated dimer contribution, as described in Fig. 3. Conditions: 50 mM phosphate buffer at pH 7, $25^\circ C$, $100 \mu M$ (in heme) for Hb A, and $300 \mu M$ for the mutant rHb.

$\beta E7V$, either under heat or mechanical stress. The mutant oxy-Hb San Jose, $\beta E7G$, was slightly more heat unstable than oxy-Hb A, while mechanical precipitation rates were similar to those observed for oxy-Hb S (7). The other naturally occurring mutant Hb Siriraj, $\beta E7K$, is expressed at only 30–35% in heterozygous patients, which is likely due to its instability as shown by isopropanol test (8). Thus, all the substitutions described at the $\beta 7$ position, Gly, Ala, Val, or Lys for Glu, result in instability of the mutated Hb tetramer. It is worth noting that the effect of the mutation is independent of the charge or the stericity of the side chain in promoting the Hb instability.

Functional Studies—The oxygen equilibrium curves showed a lower P_{50} (partial pressure for half saturation) and reduced cooperativity for both rHbs $\beta E7A$ and $\beta E6V/E7A$ relative to native Hb A. Since it is known that the ligand-binding properties of Hb S are similar to that of Hb A, the functional abnormalities could be attributed to the $\beta E7A$ mutation. The shift to lower P_{50} values could be due to a change in the tetramer ligand binding parameters, or to an increase in dimer contribution (21). The shape of the oxygen equilibrium binding curves suggested an increased amount of dimers.

CO recombination kinetics for the rHb $\beta E6V/E7A$ are shown in Fig. 2. As for Hb A, two bimolecular phases were observed, corresponding globally to the two tetramer conformations. The slow phase is unique to deoxy or *T*-state tetramers, since the dimers show only the rapid *R*-like phase. The slow phase is best observed at high dissociation levels where a significant amount of deoxy and singly liganded forms are produced. Under these conditions, the rate of recombination for the *T*-like phase was faster by a factor of 1.3 for the double mutant relative to Hb A. As the laser intensity is lowered in order to decrease the fraction dissociation, the kinetic curves show more of the rapid phase, since the reaction is then dominated by the transition R_3 to R_4 . The curves in Fig. 2 are normalized to better show the change in the fraction of slow recombination. Compared to Hb A, the double mutant shows less slow phase; even after correction for dimers, there is a slight decrease in the amount of slow phase. The rapid phase had the same recombination rate for

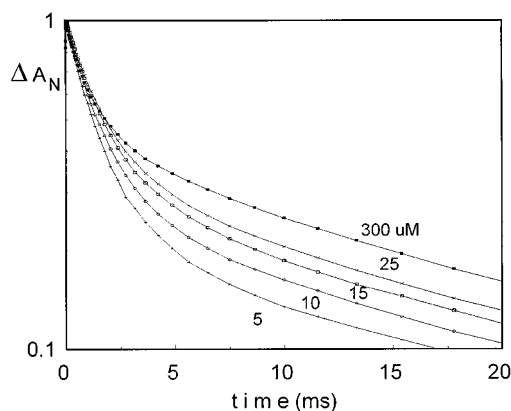


FIG. 3. Recombination of CO to rHb β 6V/E7A at different protein concentrations, shown on a heme basis in μ M. As the protein concentration is lowered, a lower fraction of slow phase is observed, since dimers show only the rapid phase. Conditions: 50 mM phosphate buffer at pH 7, 25 $^{\circ}$ C.

Hb A and the double mutant. The oxygen equilibrium curves were therefore analyzed with a model including the dimer contribution (22). The resulting parameters for the tetramer fraction of the rHb β 6V/E7A are then more similar to those of Hb A. There is still a decrease in K_T and therefore in P_{50} . This is consistent with the kinetic results which showed a faster rate of recombination for the slow (T -state) phase.

As the protein concentration is lowered, the dimer contribution increased as evidenced by a loss of the slow (T -like) kinetics. This normally occurs near 1 μ M (total heme) for Hb A at pH 7 and it is therefore difficult to observe a full dimerization of Hb A due to the small signal size. However, at higher pH values, or for systems with higher dimer levels, this technique allows one to rapidly demonstrate a concentration dependence of the ligand binding properties (15). The results for the double mutant β 6V/E7A are shown in Fig. 3. Compared to the maximum concentration of 300 μ M, the amount of slow phase decreases by a factor of 2 near 15 μ M. This would correspond to a value of $K_{42} = (\text{dimer})^2/(\text{tetramer})$ of about 10 μ M, an order of magnitude larger than for Hb A.

Polymerization of rHb β 6V/E7A and rHb β E7A in the deoxy form was studied *in vitro* by the temperature jump method (0 to 30 $^{\circ}$ C) in 1.8 M phosphate buffer at pH 7.2 and compared to that of native Hb S. Fig. 4 illustrates the variations of ΔA_{700} as a function of time after the temperature jump. In these conditions, the rHb β E7A did not polymerize at concentration up to 2.7 g/liter. The maximum absorbance at 700 nm for the β 6V/E7A mutant was lower than for Hb S, while the delay time was slightly longer. The C_{sat} value for the double mutant was not significantly different from that of natural Hb S (Fig. 5). The dependence of the delay time of polymerization on the total concentration of deoxy-Hb was also similar for both rHb β 6V/E7A and Hb S, indicating a similar nucleation process for the two Hbs. For both Hbs, the aggregation process was reversible in the presence of CO and in ice water. The variation of the turbidity as a function of the initial Hb concentration for the β 6V/E7A mutant was constantly lower than that recorded for Hb S. This is possibly due to a smaller polymer fraction or to qualitatively different polymers or aggregates.

DISCUSSION

In native Hb A, the Glu- β 7(A4) residue forms an intrachain salt bridge with the Lys- β 132(H10) in both R - and T -state structures (23). One may attribute the high oxygen affinity of the two mutated rHbs to the loss of this salt bridge, resulting in the formation of a cavity between the A and H helices, as illustrated in Fig. 6. These structural changes may induce a

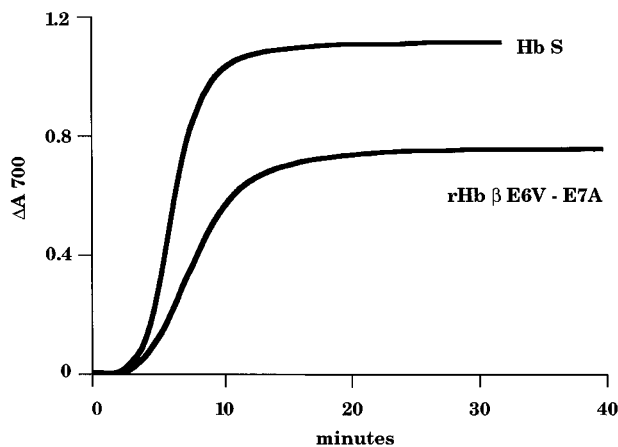


FIG. 4. Polymerization of deoxy-Hb S and deoxy-rHb β 6V/E7A. Kinetics of the polymerization of the deoxy-Hbs in 1.8 M phosphate buffer, pH 7.2, were studied by the temperature jump method at 30 $^{\circ}$ C. Initial concentrations of both Hbs were 1.8 g/liter.

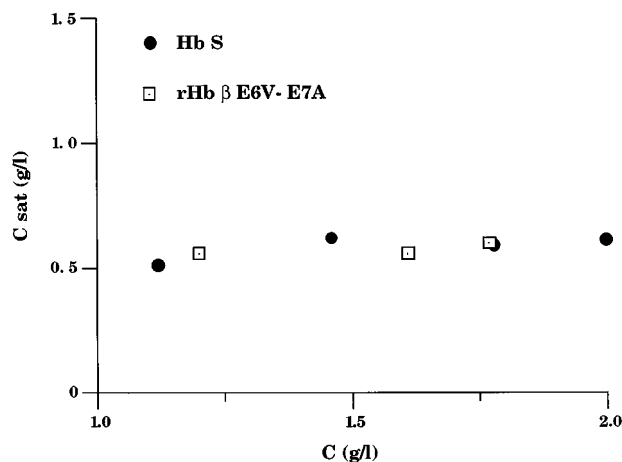
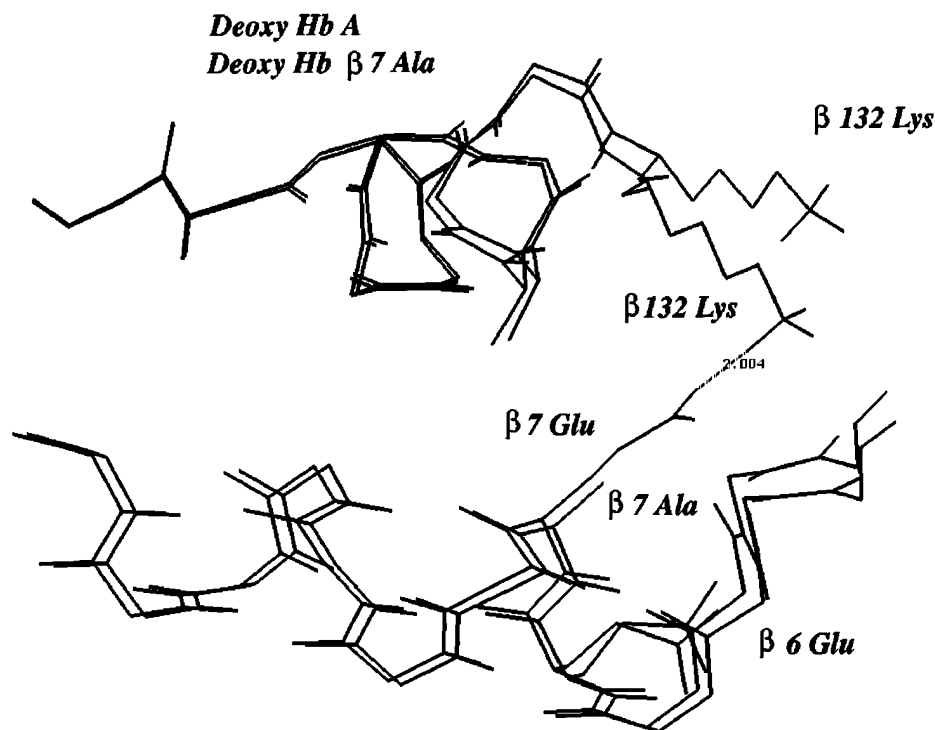


FIG. 5. Supernatant Hb concentration at the plateau of polymerization (C_{sat}) for deoxy-Hb S and deoxy-rHb β 6V/E7A, as a function of the initial concentration, after centrifugation of the turbid solution at $2500 \times g$ for 30 min, at 30 $^{\circ}$ C, under mineral oil. Conditions were as described in the legend of Fig. 4.

relaxation of the T structure when Glu is replaced by Ala. However, this interpretation is not consistent with the fact that Hb San Jose exhibits functional properties identical to those of Hb A (7). In this natural mutant Hb, the presence of a Gly residue at the β E7 position is likely responsible for similar structural consequences compared to what is observed in the presence of Ala; none of these residues allows the formation of a salt bridge, and Gly is smaller than Ala. Thus, these structural modifications are not sufficient to account for the high oxygen affinity of the rHb β E7A. The data of Yamashiro *et al.* (20) also do not support the importance of the small size of the β 7 residue such as Gly or Ala, because the substitution of Val- β 7 for Glu slightly increased the oxygen affinity. The authors suggested that "the hydrophobic side chain of Val- β 7 may cause a conformational change in helix A and/or affect the linkage between helices A and H compared with Gly- β 7" (20). Our results are in agreement with this interpretation. One may speculate that due to the location of the β 7(A4)- β 132(H10) normal salt bridge, close to the α 1- β 1 interface, its rupture may favor the dissociation of the tetramer. This has been confirmed by kinetics of CO rebinding to the photodissociated mutant rHb β 6V/E7A.

Combinations of Hb S with another α or β chain variant are

FIG. 6. Schematic representation of the region delimited by the A and H helices in the deoxy structures of the minimized Hb mutant $\beta E7A$ and Hb A. These structures were obtained after potential energy minimization using the CHARMM™ program. In deoxy-Hb A, the oxygen atom of the $\beta 7$ glutamic carboxylate group is located at 2.0 Å from the lysine $\beta 132$ ammonium group. This allows the formation of an intrachain salt bridge between the A and H helices. The replacement of the glutamic residue by alanine does not allow the formation of the salt bridge and induces a displacement of the lysine $\beta 132$ away from the A helix. This image was obtained using the Quanta 3.3 program (Molecular Simulations Inc.) with a Silicon Graphics 4D25G workstation.



responsible for a variety of clinical patterns (24). Information on the location of intermolecular contacts in the polymer have been obtained by studying the *in vitro* and *in vivo* interactions of Hb S and natural Hb mutants (4). Both Hbs San Jose and Siriraj behave like deoxy-Hb A when interacting with deoxy-Hb S (7, 8). These results excluded either the inhibition or the potentiation of polymerization by the substitutions Gly- $\beta 7$ or Lys for Glu in the *trans* position, which is compatible with the fact that the $\beta 7$ residue in *trans* is not involved in the specific lateral contact stabilizing the deoxy-Hb S polymer. Our results show that when associated in *cis* to the sickle mutation the substitution Glu- $\beta 7(A4) \rightarrow$ Ala does not increase the hydrophobic interaction between donor and acceptor site, but leads to a decrease in the maximum change in absorption at 700 nm (Fig. 4). The fact that the value of C_{sat} is the same for the rHb $\beta E6V/E7A$ and Hb S suggests that the polymer size is different. The positioning of the A helix is of crucial importance in the adjustment of the donor and acceptor sites. The comparison of the backbone of the β subunit of deoxy-Hb S which contains the Val- $\beta 6$ involved in the lateral contact with that of deoxy-Hb A, shows that the main difference is a shift in the A helix (24, 25). It has been shown that Hb Leiden, in which one of the Glu-A3 or -A4 residues is deleted, inhibits the polymerization of deoxy-Hb S; shortening the A helix and moving the Pro- $\beta 5$ may perturb the adjustment of the donor and the acceptor sites (26). The $\beta E7A$ mutation results in the loss of the Glu- $\beta 7$ -Lys- $\beta 132$ salt bridge which would render the A helix softer (Fig. 6). As a result the $\beta 6$ Val residue would not fit well in the EF pocket and the formation of the polymers would be delayed. In this regard, the role of the Glu- $\beta 7$ -Lys- $\beta 132$ salt bridge in the properties of the two rHb $\beta E7A$ and $\beta E6V/E7A$ described in this report will be of major interest for the development of therapeutic agents.

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