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# Influence of the A Helix Structure on the Polymerization of Hemoglobin S\*

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**Hb S variants containing Lys- $\beta$ 132  $\rightarrow$  Ala or Asn substitutions were engineered to evaluate the consequences of the A helix destabilization in the polymerization process. Previous studies suggested that the loss of the Glu- $\beta$ 7-Lys- $\beta$ 132 salt bridge in the recombinant Hb  $\beta$ E6V/E7A could be responsible for the destabilization of the A helix. The recombinant Hb (rHb) S/ $\beta$ 132 variants polymerized with an increased delay time as well as decreased maximum absorbance and Hb solubility values similar to that of Hb S. These data indicate that the strength of the donor-acceptor site interaction may be reduced due to an altered conformation of the A helix. The question arises whether this alteration leads to a true inhibition of the polymerization process or to qualitatively different polymers. The oxygen affinity of the  $\beta$ 132 mutated rHbs was similar to that of Hb A and S, whereas the cooperativity and effects of organic phosphates were reduced. This could be attributed to modifications in the central cavity due to loss of the positively charged lysine. Since Lys- $\beta$ 132 is involved in the stabilization of the  $\alpha$ 1- $\beta$ 1 interface, the loss of the  $\beta$ 132(H10)- $\beta$ 128(H6) salt bridge may be responsible for the marked thermal instability of the  $\beta$ 132 mutated rHbs.**

(7) exhibit an increased propensity to form polymers. Site-directed mutagenesis and expression in heterologous systems allows determination of the contribution of various sites in the polymerization process (8–10). Studies of recombinant Hbs (rHbs)<sup>1</sup> have shown that in Hb S Antilles the polymer fibers were stabilized at the axial contact by the replacement of Val with the more hydrophobic residue Ile (10). We have previously reported studies of the function and polymerization of another rHb,  $\beta$ E6V/E7A (11). In this rHb, the association of Glu- $\beta$ 6(A3)  $\rightarrow$  Val and Glu- $\beta$ 7(A4)  $\rightarrow$  Ala mutations on the same  $\beta$  chain (rHb  $\beta$ E6V/E7A) results in an apparent decrease of the polymer formation. We therefore postulated that this decrease could be due to an instability of the A helix because of the loss of a salt bridge between the A and H helices, namely between the Glu- $\beta$ 7(A4) and Lys- $\beta$ 132(H10) residues (11). Modification of the second partner in the salt bridge (Lys- $\beta$ 132(H10)) may also result in its rupture. This residue participates in several contacts at the  $\alpha$ 1- $\beta$ 1 interface (12) and might also be involved in the stability of the A helix. We have thus engineered two doubly mutated Hbs in which the Glu- $\beta$ 6(A3)  $\rightarrow$  Val mutation is associated with either Lys- $\beta$ 132(H10)  $\rightarrow$  Ala or Asn substitution (rHbs  $\beta$ E6V/K132A and  $\beta$ E6V/K132N, respectively). We have also engineered the single mutant Hbs Lys- $\alpha$ 2 $\beta$ 2132(H10)  $\rightarrow$  Ala and Lys- $\alpha$ 2 $\beta$ 2132(H10)  $\rightarrow$  Asn as controls (rHbs  $\beta$ K132A and  $\beta$ K132N, respectively).

## MATERIALS AND METHODS

The  $\beta$ E6V,  $\beta$ K132A, and  $\beta$ K132N mutations were introduced into the  $\beta$ -globin cDNA by site-directed mutagenesis using synthetic primers (Genset, Paris, France). The mutated  $\beta$ -globin subunits were produced as fusion proteins in *Escherichia coli* using the expression vector pAT-PrclIFX $\beta$  (13). After extraction and purification, the fusion proteins were cleaved by digestion with bovine coagulation factor Xa (14). 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  $\beta$ -subunits were folded in the presence of cyanhemin and the partner  $\alpha$ -subunits (prepared from natural Hb A) to form the tetrameric Hb  $\alpha$ 2 $\beta$ 2 (13–15).

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  $\mu$ 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, at 100  $\mu$ M heme under 1 atm of CO or 1 atm of O<sub>2</sub> (16).

The oxygen binding curves were recorded at 25 °C with a continuous method using the Hemox Analyzer system (TCS Medical Products, Huntington Valley, PA) (17). 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 (18). Measurements were made at different protein concentrations to study the concentration dependence of the ligand binding kinetics to estimate the dimer-tetramer equilibrium compared with natural Hb A

The substitution of valine for the  $\beta$ 6 glutamic acid residue in human Hb results in the abnormally low solubility of deoxy-Hb S. Under physiological conditions, sickle Hb aggregates upon deoxygenation to form a gel composed of long helical fibers that deform the erythrocytes and severely diminish their life-span. Electron microscopy (1, 2) and x-ray crystallographic studies (3) have shown that both fibers and crystals are composed of double strands of deoxy-Hb S molecules. The formation of the double strands requires stereochemical contact between complementary surfaces involving specifically Val- $\beta$ 6 and a hydrophobic pocket on an adjacent molecule (lateral contact).

The capacity of some Hb variants to facilitate or impair the polymerization process of Hb S is well documented (4). The use of binary Hb mixtures (Hb X + Hb S) has allowed a mapping of the residues involved in areas of contact in the polymer (5). The consequences of mutations associated with Val- $\beta$ 6 on the same  $\beta$  chain are less well known. Six naturally occurring Hb variants have been described with two mutations on the same  $\beta$  chain, one of them being the Hb S substitution. Among them, Hb S Antilles Val- $\beta$ 6/Ile-23 (6) and Hb S Oman Val- $\beta$ 6/Lys-121

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<sup>1</sup> The abbreviations used are: rHb, recombinant Hb;  $C_{\text{sat}}$ , Hb solubility; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

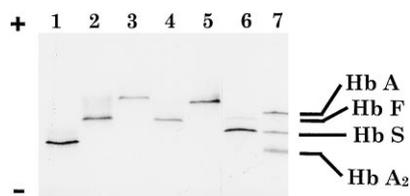


FIG. 1. Isoelectric focusing of rHbs  $\beta$ E6V/K132A (lane 2),  $\beta$ K132A (lane 3),  $\beta$ E6V/K132N (lane 4), and  $\beta$ K132N (lane 5) compared with mixtures of Hbs A and F and Hbs A, A<sub>2</sub>, and S (lanes 6 and 7) and with the rHb  $\beta$ E6V/E7A (lane 1). Mobilities of the Hbs were determined by isoelectric focusing in polyacrylamide gel with a pH gradient ranging from 6.0 to 8.0.

(19). For concentrations above 30  $\mu$ M (on a heme basis), 1-mm optical cuvettes were used. Below 30  $\mu$ M, 4  $\times$  10 mm cuvettes were used with detection along the 10-mm axis and dissociation at 90° along the 4-mm axis.

Kinetics of polymer formation for mutant deoxy-Hbs were carried out in 1.8 M potassium phosphate buffer, pH 7.2, for different Hb concentrations, following initiation by a temperature jump (0 to 30 °C). The solubility and kinetics of polymerization of the mutated Hb were compared with those of native Hb S. Under these conditions and after a characteristic lag period, the assembly of Hb S into fibers resulted in a cooperative increase in turbidity at 700 nm until a plateau was achieved. The Hb solubility ( $C_{sat}$ ) was determined by measuring the Hb concentration of the soluble phase after completion of the polymerization process (20, 21).

Molecular graphics models of the deoxy-Hbs  $\beta$ E7A and  $\beta$ K132N 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.* (22). Minimization of the potential energy in the mutant and normal Hbs was performed using the CHARMM<sup>®</sup> program (version 22) (23) with a Silicon Graphics Indigo work station. Holding the rest of the structure constant, the mutated residue was initially minimized by starting with an additional high harmonic constant that was progressively decreased to 0.

## RESULTS

Reverse-phase high performance liquid chromatography of the tryptic digest of the purified mutated fusion proteins  $\beta$ K132A,  $\beta$ K132N,  $\beta$ E6V/K132A, or  $\beta$ E6V/K132N revealed the presence of one or two abnormal peptides. Amino acid analyses of these 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 dilute solution and liganded forms. The fluorescence intensity is sensitive to the small quantities of apoproteins. The intensity for the rHbs was similar to that for natural Hb A, indicating a correct reconstitution to the holoprotein form.

**Electrophoretic Studies**—Cellulose acetate electrophoresis and isoelectrofocusing (Fig. 1) of the purified rHbs  $\beta$ K132A,  $\beta$ K132N,  $\beta$ E6V/K132A, and  $\beta$ E6V/K132N showed a single band migrating at isoelectric points of 6.85, 6.85, 7.10, and 7.10, respectively, relative to 6.95 and 7.20 for Hb A and Hb S and 7.40 for the rHb  $\beta$ E6V/E7A. These values are higher than expected for the replacement of the positively charged Lys with Ala or Asn. This may be explained by the location of the  $\beta$ 132 residue, which is at least partially buried in the central cavity of the Hb tetramer.

Heat stability of the oxy and carboxy forms of the mutated rHbs was compared with that of the natural Hbs A and S and that of the rHb  $\beta$ E6V/E7A. As shown in Fig. 2, we did not observe significant differences between Hb A and S. The stability of the  $\beta$ 132 mutants was dramatically decreased under the same conditions. The doubly mutated rHbs  $\beta$ E6V/K132A and  $\beta$ E6V/K132N were the most unstable, more so than the rHbs containing only the  $\beta$ 132 mutation. The rHbs  $\beta$ E7A and  $\beta$ E6V/E7A were the least unstable mutants (Fig. 2).

**Functional Studies**—The oxygen equilibrium curves (not shown) showed that the oxygen affinity of the rHbs K132N and

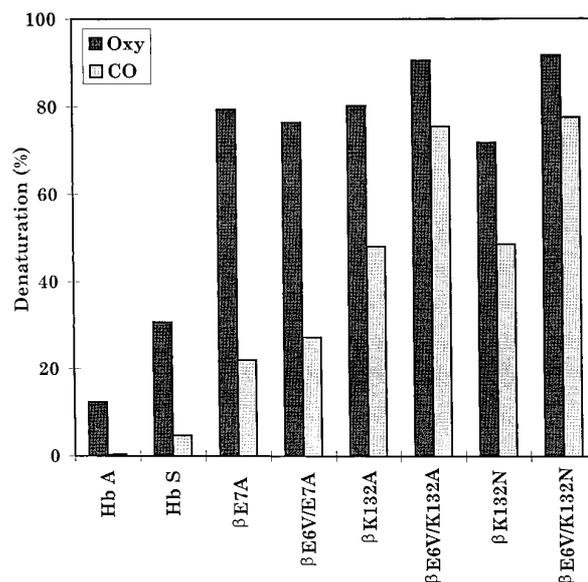


FIG. 2. Thermal stability for the rHbs after 10 and 20 min of incubation (oxy and CO forms, respectively) compared with natural Hbs A and S. Percentage of denatured Hbs was determined as a function of time by incubating the recombinant and native Hbs in 10 mM phosphate buffer, pH 7, at 65 °C.

$\beta$ E6V/K132N was similar to that of native Hb A. When Lys- $\beta$ 132 was replaced with Ala the oxygen affinity was slightly increased (Table I). The cooperativity in ligand binding and the 2,3-diphosphoglycerate effect were decreased for all mutants. Since it is known that the oxygen binding properties of Hb S are similar to those of Hb A, the functional abnormalities could be attributed to the presence of the  $\beta$ 132 mutation. CO recombination kinetics for the rHbs (not shown) were similar to those observed for Hb A, indicating that the mutant rHbs retain allosteric function. At low protein concentration, loss of the allosteric form is normally observed due to the increased fraction of dimers. Results at 1  $\mu$ M indicate that the rHbs do not present a significant increase in the amount of dimers.

Polymerization of the rHbs  $\beta$ E6V/K132A and  $\beta$ E6V/K132N in the deoxy form was studied *in vitro* by the temperature jump method and compared with that of the rHb  $\beta$ E6V/E7A and natural Hb S. Fig. 3 illustrates the variations of  $\Delta A_{700}$  as a function of time after the temperature jump. Under these conditions, the rHbs  $\beta$ K132A and K132N did not polymerize at all concentrations studied (up to 2.0 g/liter). The kinetic curves of polymerization of the doubly mutated Hbs were sigmoidal, as was observed for native Hb S. The maximum absorbance at 700 nm was lower than for Hb S, whereas the delay time ( $\tau$ ) was longer at all concentrations studied (1.6–3.0 g/liter). A logarithmic plot of the reciprocal of the delay time *versus* initial Hb concentration (Fig. 4) showed straight lines shifted toward the right for the rHbs  $\beta$ E6V/K132A and  $\beta$ E6V/K132N, indicating longer delay times compared with Hb S and the rHb  $\beta$ E6V/E7A. Plotting the log of the aggregation rate as a function of log C showed straight lines with similar slopes for Hb S and rHb  $\beta$ E6V/E7A on the one hand and for the rHbs  $\beta$ E6V/K132N and  $\beta$ E6V/K132A on the other (Fig. 5). The time required to reach maximum polymerization depends on Hb concentration. At equivalent initial concentration that time was longer when the sickle mutation was associated with the  $\beta$ 132 mutations than when associated with Glu- $\beta$ 7  $\rightarrow$  Val. The  $C_{sat}$  values for the double mutants were not significantly different from those of natural Hb S and rHb  $\beta$ E6V/E7A (Fig. 6). For the two double mutants, the aggregation process was reversible in the presence of CO and in ice water.

TABLE I  
Functional studies of the rHbs  $\beta$ K132A,  $\beta$ K132N,  $\beta$ E6V/K132A, and  $\beta$ E6V/K132N

Experimental conditions: 0.05 M bis-Tris buffer, 20  $\mu$ g/ml catalase, 50  $\mu$ M EDTA, 60–80  $\mu$ M heme, 100 mM NaCl, 25  $^{\circ}$ C, 1 mM 2,3-diphosphoglycerate. The percentage of MetHb at the end of the oxygen equilibrium curve recording was between 1.5 and 5%.

Hb	- 2,3-Diphosphoglycerate		$n_{50}$	+ 2,3-Diphosphoglycerate	
	$P_{50}$	$\Delta \log P_{50}^a$		$P_{50}$	$\Delta \log P_{50}^b$
	mm Hg			mm Hg	
A	4.4		2.5–2.8	14.0	0.50
Recombinant $\beta$ K132A	3.5	-0.10	1.9	Not determined	
Recombinant $\beta$ K132N	4.7	0.03	2.1	10.3	0.34
Recombinant $\beta$ E6V/K132A	3.1	-0.15	1.7	7.4	0.38
Recombinant $\beta$ E6V/K132N	4.3	0.01	1.9	9.1	0.33

<sup>a</sup> Shift in  $P_{50}$  expressed as  $\Delta \log P_{50}$  relative to Hb A.

<sup>b</sup> Expressed as  $\Delta \log P_{50} \pm 2,3$ -diphosphoglycerate.

FIG. 3. Polymerization of deoxy-rHb  $\beta$ E6V/K132A and  $\beta$ E6V/K132N compared with deoxy-Hb S and deoxy-rHb  $\beta$ E6V/E7A. Kinetics of 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 the Hbs were 1.8 g/liter.

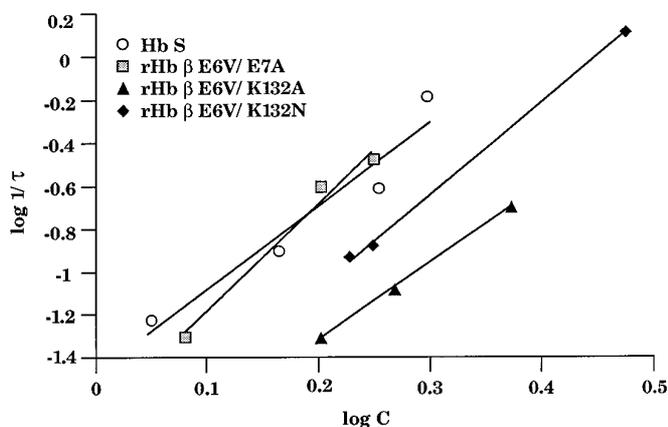
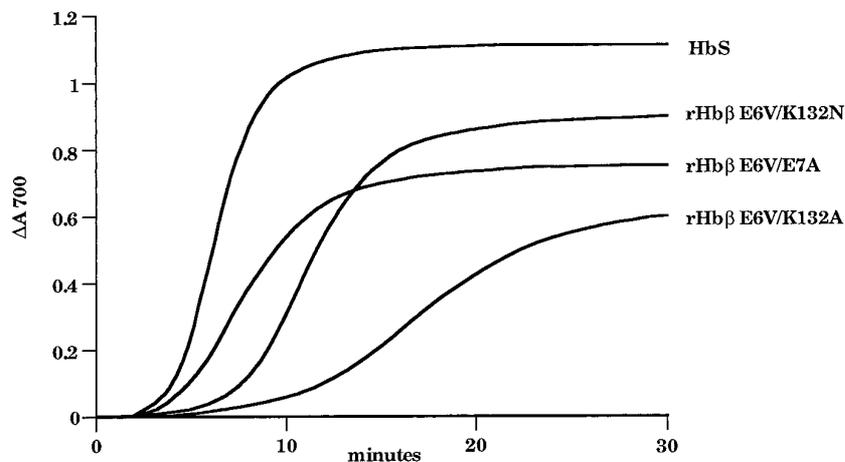


FIG. 4. Relationship between the log of the reciprocal of delay time  $\tau$  ( $\text{min}^{-1}$ ) and the log of initial concentration  $C$  (g/liter) for deoxy-rHb  $\beta$ E6V/K132A and  $\beta$ E6V/K132N compared with deoxy-Hb S and deoxy-rHb  $\beta$ E6V/E7A.

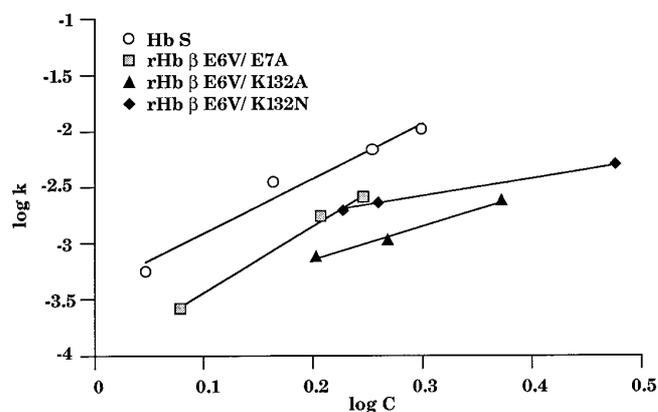


FIG. 5. Relationship between the log of the rate of polymerization  $k$  ( $\text{s}^{-1}$ ) and the log of initial concentration  $C$  (g/liter) for deoxy-rHb  $\beta$ E6V/K132A and  $\beta$ E6V/K132N compared with deoxy-Hb S and deoxy-rHb  $\beta$ E6V/E7A.  $k$  was calculated as  $\Delta A_{700}/(\text{final time} - \tau)$ .

#### DISCUSSION

We have previously demonstrated that the association of the  $\beta$ E7A and  $\beta$ E6V mutations on the same  $\beta$  chain leads to a decreased polymer formation; the Glu- $\beta$ 7(A4) for Ala substitution in human Hb resulted in heat instability and in an increased oxygen affinity of the rHbs  $\beta$ E7A and  $\beta$ E6V/E7A (11). In human Hbs A and S, Glu- $\beta$ 7(A4) forms an intrachain salt bridge with Lys- $\beta$ 132(H10) in both R- and T-state structures (24). The loss of this salt bridge may modify the conformation of the A helix, which could account for the alteration of the polymerization process. We attributed the increased oxygen affinity of the rHbs  $\beta$ E7A and  $\beta$ E6V/E7A to an increased dissociation of the tetramer into dimers demonstrated by the concentration dependence of the ligand binding kinetics. In the present work we have studied the consequences of modifica-

tions of Lys- $\beta$ 132(H10), which also participates in the salt bridge. In contrast with the data obtained with the rHbs  $\beta$ E7A and  $\beta$ E6V/E7A, CO rebinding to the photodissociated  $\beta$ 132 mutants did not reveal an increased dissociation into dimers, and the rHbs modified at the  $\beta$ 132(H10) site did not exhibit high oxygen affinity (Table I). Note that naturally occurring Hb Yamataga Lys- $\beta$ 132  $\rightarrow$  Asn is described as having a slightly decreased oxygen affinity (25). In human deoxy-Hb, the  $\beta$ 132 residue interacts with the N-terminal  $\beta$ -chain residues Val- $\beta$ 1(NA1), His- $\beta$ 2(NA2), and Leu- $\beta$ 3(NA3) (12). Two of these residues participate in the 2,3-diphosphoglycerate binding. The loss of a positive charge when the Lys- $\beta$ 132 is replaced by either Ala or Asn may result in the destabilization of the contacts in the central cavity. These structural modifications

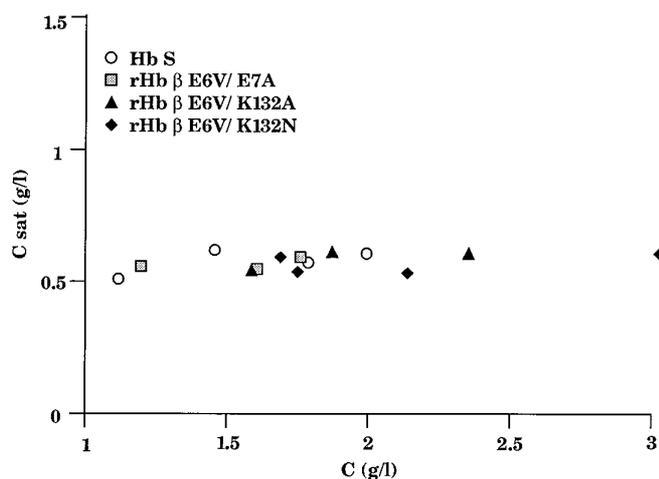


FIG. 6. Supernatant Hb concentration at the plateau of polymerization ( $C_{\text{sat}}$ ) for deoxy-rHb  $\beta$ E6V/K132A and  $\beta$ E6V/K132N compared with deoxy-Hb S and deoxy-rHb  $\beta$ E6V/E7A as a function of the initial concentration. The  $C_{\text{sat}}$  was determined after centrifugation of the turbid solution at  $2500 \times g$  for 30 min at  $30^\circ\text{C}$  under mineral oil. Conditions of polymerization were as described in the Fig. 3 legend.

are able to prevent or modify the binding of the allosteric effector.

Structural crystallographic studies revealed that in both the R- and T-state Lys- $\beta$ 132(H10) is not only linked to Glu- $\beta$ 7(A4) but may also interact with Ala- $\beta$ 128(H6), which is involved in the  $\alpha$ 1- $\beta$ 1 contacts, and with Gly- $\beta$ 136(H14) located in the central cavity (12). The known natural substitutions described at these two latter sites are responsible for thermal or isopropyl alcohol instability (25). The rupture of the  $\beta$ 7(A4)- $\beta$ 132(H10) salt bridge near the  $\alpha$ 1- $\beta$ 1 interface and of  $\beta$ 132(H10)- $\beta$ 128(H6) that directly participates in the  $\alpha$ 1- $\beta$ 1 stabilization may induce a heat instability of the mutated tetramers (Fig. 2). Among the three natural  $\beta$ 132 mutants, only Hb Cook (Lys- $\beta$ 132  $\rightarrow$  Thr) has been shown to be slightly unstable (25).

Combinations of Hb S with another  $\alpha$  or  $\beta$  chain variant are responsible for a variety of clinical patterns (26, 27). Information on the location of intermolecular contacts in the polymer has been obtained by studying the *in vitro* and *in vivo* interactions of Hb S and natural Hb mutants (4, 5, 27). Hb K-Woolwich Lys- $\beta$ 132(H10)  $\rightarrow$  Gln behaves like deoxy-Hb A when interacting with deoxy-Hb S, demonstrating that the  $\beta$ 132 residue is not directly involved in the interactions stabilizing the deoxy-Hb S polymer (5, 28). Our results show that when associated with the sickle mutation on the same  $\beta$  chain, neither the Lys- $\beta$ 132  $\rightarrow$  Ala nor the Asn substitution increases the hydrophobic interaction between donor and acceptor sites. They both lead to a decrease in the maximum change in absorption at 700 nm (Fig. 3) comparable to what was observed for the rHb  $\beta$ E6V/E7A without significant modification of the solubility of the rHbs (Fig. 6). The question arises whether these data account for an inhibition of the polymerization process and/or for different geometry or size of the polymers. It should be pointed out that the “apparent” inhibition of the polymerization process is more important when Lys- $\beta$ 132 is replaced by Ala than when replaced by Asn, the inhibition being intermediate with the rHb  $\beta$ E6V/E7A. The  $\beta$ 132 site is sterically near the acceptor pocket involving Phe- $\beta$ 85 and Leu- $\beta$ 88. One may speculate that substituting the  $\beta$ 132 residue modifies the acceptor pocket and the fitting between the donor and acceptor sites. In the rHb  $\beta$ E6V/K132A, two phenomena are susceptible to interfere with the polymerization process. The absence of the salt bridge as in the rHb  $\beta$ E6V/E7A would render the A helix softer and would modify the acceptor pocket.

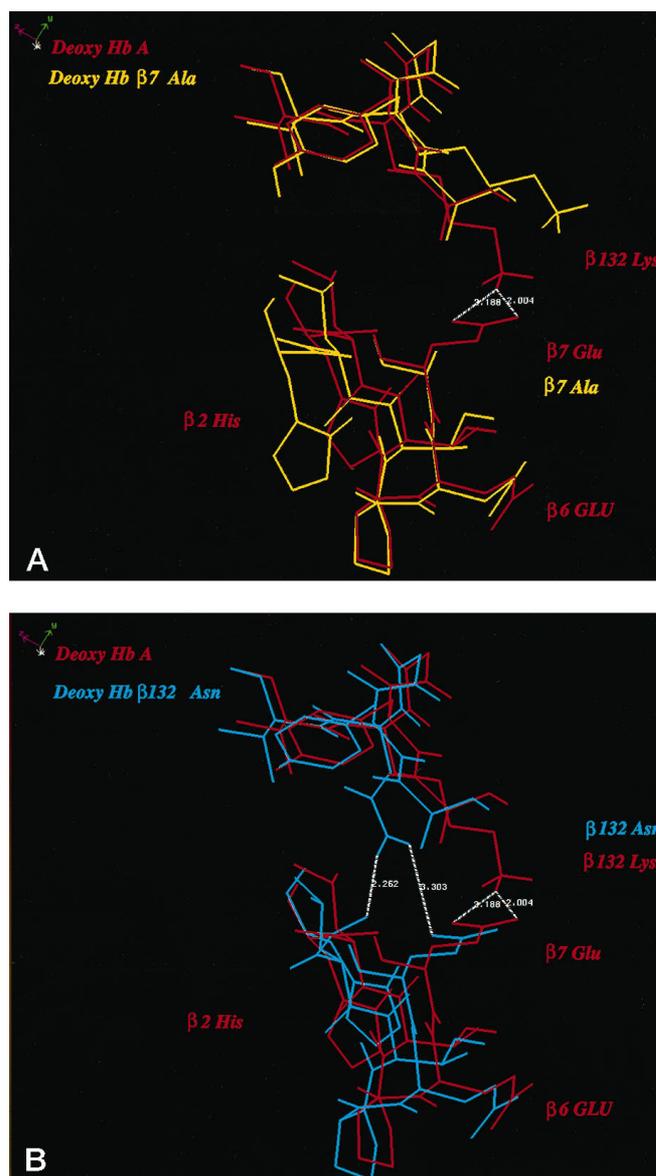


FIG. 7. Schematic representation of the region delimited by the A and H helices in the deoxy structures of the minimized Hb mutants  $\beta$ E7A (A) and  $\beta$ K132N (B) compared with Hb A. These structures were obtained after potential energy minimization using the CHARMM<sup>™</sup> program. In deoxy-Hb 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. When replacing the lysine residue by asparagine the loss of the salt bridge Glu- $\beta$ 7-Lys- $\beta$ 132 could be compensated by two interactions, Glu- $\beta$ 7-Asn- $\beta$ 132 and His- $\beta$ 2-Asn- $\beta$ 132, maintaining the linkage between the A and H helices. This image was obtained using the Quanta 3.3 program (Molecular Simulations Inc.) with a Silicon Graphics 4D25G work station.

As a result Val- $\beta$ 6 would not fit well in the acceptor pocket, and the formation of the polymers would be delayed. When Lys- $\beta$ 132 is replaced by Asn the consequences seem to be less important. Molecular graphic modeling studies indicate that the position of the Asn- $\beta$ 132 residue makes it possible to be hydrogen-bonded to Glu- $\beta$ 7 (Fig. 7), thus maintaining better donor-acceptor site contacts. Alternatively, although Val- $\beta$ 6 is essential to the interaction with the hydrophobic acceptor pocket, other critical residues are involved in the stabilization of the nuclei. Modifying the conformation of the A helix may prevent these secondary contacts, resulting in an unstable

nucleus. Note that the value of  $C_{\text{sat}}$  is similar to that found for Hb S as also observed with rHb  $\beta$ E6V/E7A. The differences observed in the aggregation rates for the  $\beta$ 132 mutated rHbs (Fig. 5) relative to Hb S and to the rHb  $\beta$ E6V/E7A may not only reflect quantitative but also qualitative differences in the polymer formation.

Increasing the delay time (as observed for rHbs  $\beta$ E6V/K132A and  $\beta$ E6V/K132N) would be of major interest to a therapeutic approach to prevent Hb S polymerization in the microcirculation vessels. These studies may help to determine critical target sites for antisickling agents while preserving normal function and stability of the molecule, which remains a challenge.

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