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

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Hb variants containing Lys-β132 → 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-β7-Lys-β132 salt bridge in the recombinant Hb βE6V/E7A could be responsible for the destabilization of the A helix. The recombinant Hb (rHb) S/β132 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 β132 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-β132 is involved in the stabilization of the α-β interface, the loss of the β132(H10)-β28(H6) salt bridge may be responsible for the marked thermal instability of the β132 mutated rHbs.

The substitution of valine for the β6 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-β6 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-β6 on the same β chain are less well known. Six naturally occurring Hb variants have been described with two mutations on the same β chain, one of them being the Hb S substitution. Among them, Hb S Antilles Val-β6/Ile-23 (6) and Hb S Oman Val-β6/Lys-121 (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) 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, βE6V/E7A (11). In this rHb, the association of Glu-β6(A3) → Val and Glu-β7(A4) → Ala mutations on the same β chain (rHb βE6V/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-β7(A4) and Lys-β132(H10) residues (11). Modification of the second partner in the salt bridge (Lys-β132(H10)) may also result in its rupture. This residue participates in several contacts at the α-β 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-β6(A3) → Val mutation is associated with either Lys-β132(H10) → Ala or Asn substitution (rHbs βE6V/K132A and βE6V/K132N, respectively). We have also engineered the single mutant Hbs Lys-α18β132(H10) → Ala and Lys-α18β132(H10) → Asn as controls (rHbs βK132A and βK132N, respectively).

MATERIALS AND METHODS

The β6V, βK132A, and βK132N mutations were introduced into the β-globin cDNA by site-directed mutagenesis using synthetic primers (Genset, Paris, France). The mutated β-globin subunits were produced as fusion proteins in Escherichia coli using the expression vector pAT-PreIFXp (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 β-subunits were folded in the presence of cyanohemins and the partner α-subunits (prepared from natural Hb A) to form the tetrameric Hb α2β2 (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 μM on a heme basis in 10 mM phosphate buffer, pH 7.0, using an SLM 8000 spectrophotometer. 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, using an SLM 8000 spectrophotometer. 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, using an SLM 8000 spectrophotometer. Fluorescence spectra were measured in the region for tyrosine and tryptophan emission (for air-equilibrated samples).

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-nm pulses at 532 nm. Detection was at 436 nm for samples equilibrated under 0.1 atm of CO or 1 atm of O2 (16). 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.

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1 The abbreviations used are: rHb, recombinant Hb; C50, Hb solubility; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.
Polymerization of Hb S Lys-β132 → Ala and Lys-β132 → Asn

RESULTS

Reverse-phase high performance liquid chromatography of the tryptic digest of the purified mutated fusion proteins βK132A, βK132N, βE6V/K132A, or βE6V/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 tryptic digest of the purified mutated fusion proteins and liganded forms. The fluorescence intensity is sensitive to the presence of one or two abnormal peptides. Amino acid analyses respectively, relative to 6.95 and 7.20 for Hb A and Hb S and the tryptic digest of the purified mutated fusion proteins (20, 21).

Molecular graphics models of the deoxy-Hbs βE7A and βK132N 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 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 overlap constant that was progressively decreased to 0.

Heat stability of the oxy and carboxy forms of the mutated Hbs was compared with that of the natural Hbs A and S and that of the rHb βE6V/E7A. As shown in Fig. 2, we did not observe significant differences between Hb A and S. The stability of the β132 mutants was dramatically decreased under the same conditions. The doubly mutated Hbs βE6V/K132A and βE6V/K132N were the most unstable, more so than the rHbs containing only the β132 mutation. The rHbs βE7A and βE6V/E7A were the least unstable mutants (Fig. 2).

The oxygen equilibrium curves (not shown) showed that the oxygen affinity of the rHbs K132N and E6V/K132N was similar to that of native Hb A. When Lys-β132 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 β132 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 μM indicate that the rHbs do not present a significant increase in the amount of dimers.

Polymerization of the rHbs βE6V/K132A and βE6V/K132N in the deoxy form was studied in vitro by the temperature jump method and compared with that of the rHb βE6V/E7A and natural Hb S. Fig. 3 illustrates the variations of ΔA<sub>700</sub> as a function of temperature after the temperature jump. Under these conditions, the rHbs βK132A 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 it was observed for native Hb S. The maximum absorbance at 700 nm was lower than for Hb S, whereas the delay time (τ) 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 βE6V/K132A and βE6V/K132N, indicating longer delay times compared with Hb S and the rHb βE6V/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 βE6V/E7A on the one hand and for the rHbs βE6V/K132N and βE6V/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 β132 mutations than when associated with Glu-β7 → Val. The C<sub>α</sub> values for the double mutants were not significantly different from those of natural Hb S and rHb βE6V/E7A (Fig. 6). For the two double mutants, the aggregation process was reversible in the presence of CO and in ice water.
DISCUSSION

We have previously demonstrated that the association of the βE7A and βE6V mutations on the same β chain leads to a decreased polymer formation; the Glu-β7(A4) for Ala substitution in human Hb resulted in heat instability and in an increased oxygen affinity of the rHbs βE7A and βE6V/βE7A (11). In human Hbs A and S, Glu-β7(A4) forms an intrachain salt bridge with Lys-β132(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 βE7A and βE6V/βE7A 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 modifications of Lys-β132(H10), which also participates in the salt bridge. In contrast with the data obtained with the rHbs βE7A and βE6V/βE7A, CO rebinding to the photodissociated β132 mutants did not reveal an increased dissociation into dimers, and the rHbs modified at the β132(H10) site did not exhibit high oxygen affinity (Table I). Note that naturally occurring Hb Yamataga Lys-β132 Asn is described as having a slightly decreased oxygen affinity (25). In human deoxy-Hb, the β132 residue interacts with the N-terminal β-chain residues Val-β1(NA1), His-β2(NA2), and Leu-β3(NA3) (12). Two of these residues participate in the 2,3-diphosphoglycerate binding. The loss of a positive charge when the Lys-β132 is replaced by either Ala or Asn may result in the destabilization of the contacts in the central cavity. These structural modifications

| Hb                  | - 2,3-Diphosphoglycerate | + 2,3-Diphosphoglycerate |
|---------------------|--------------------------|--------------------------|
|                     | $P_{S0}$ | Δ log $P_{S0}$ | $n_{S0}$ | $P_{S0}$ | Δ log $P_{S0}$ |
| A                   | 4.4     | 2.5–2.8       | 14.0     | 0.50     |
| Recombinant βK132A  | 3.5     | 0.03          | 2.1      | 0.34     |
| Recombinant βK132N  | 4.7     | –0.15         | 1.7      | 0.38     |
| Recombinant βE6VK132A | 3.1    | 0.01          | 1.9      | 0.33     |
| Recombinant βE6VK132N | 4.3   |              |          |          |

Table I

Functional studies of the rHbs βK132A, βK132N, βE6VK132A, and βE6VK132N

Experimental conditions: 0.05 M bis-Tris buffer, 20 μg/ml catalase, 50 μM EDTA, 60–80 μM heme, 100 mM NaCl, 25 °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%.

a Shift in $P_{S0}$ expressed as Δ log $P_{S0}$ relative to Hb A.

b Expressed as Δ log $P_{S0}$ – 2,3-diphosphoglycerate.
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-β132(H10) is not only linked to Glu-β7(A4) but may also interact with Ala-β128(H6), which is involved in the a1-β1 contacts, and with Gly-β136(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 β7(A4)-β132(H10) salt bridge near the a1-β1 interface and of β132(H10)-β128(H6) that directly participates in the a1-β1 stabilization may induce a heat instability of the mutated tetramers (Fig. 2). Among the three natural β132 mutants, only Hb Cook (Lys-β132 → Thr) has been shown to be slightly unstable (25).

Combinations of Hb S with another a or β 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-β132(H10) → Gln behaves like deoxy-Hb A when interacting with deoxy-Hb S, demonstrating that the β132 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 β chain, neither the Lys-β132 → 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 β6E/V/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-β132 is replaced by Ala than when replaced by Asn, the inhibition being immediate with the rHb β6E/V/E7A. The β132 site is sterically near the acceptor pocket involving Phe-β85 and Leu-β88. One may speculate that substituting the β132 residue modifies the acceptor pocket and the fitting between the donor and acceptor sites. In the rHb β6E/V/K132A, two phenomena are susceptible to interfere with the polymerization process. The absence of the salt bridge as in the rHb β6E/V/E7A would render the A helix softer and would modify the acceptor pocket.

As a result Val-β6 would not fit well in the acceptor pocket, and the formation of the polymers would be delayed. When Lys-β132 is replaced by Asn the consequences seem to be less important. Molecular graphic modeling studies indicate that the position of the Asn-β132 residue makes it possible to be hydrogen-bonded to Glu-β7 (Fig. 7), thus maintaining better donor-acceptor site contacts. Alternatively, although Val-β6 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

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**Fig. 6.** Supernatant Hb concentration at the plateau of polymerization (C_sat) for deoxy-rHb β6E/V/K132A and β6E/V/K132N compared with deoxy-Hb S and deoxy-rHb β6E/V/E7A as a function of the initial concentration. The C_sat was determined after centrifugation of the turbid solution at 2500 × g for 30 min at 30 °C under mineral oil. Conditions of polymerization were as described in the Fig. 3 legend.

**Fig. 7.** Schematic representation of the region delimited by the A and H helices in the deoxy structures of the minimized Hb mutants βE7A (A) and βK132N (B) compared with Hb A. These structures were obtained after potential energy minimization using the CHARMm™ program. In deoxy-Hb A, the oxygen atom of the β7 glutamic carboxylate group is located at 2.0 Å from the lysine β132 am- monium 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 β132 away from the A helix. When replacing the lysine residue by asparagine the loss of the salt bridge Glu-β7- Lys-β132 could be compensated by two interactions, Glu-β7-Asn-β132 and His-β2-Asn-β132, 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.
nucleus. Note that the value of $C_{sat}$ 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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Polymerization of Hb S Lys-$\beta$132 $\rightarrow$ Ala and Lys-$\beta$132 $\rightarrow$ Asn