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# Hb S-São Paulo: A new sickling hemoglobin with stable polymers and decreased oxygen affinity

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ABSTRACT

Hb S-São Paulo (SP) [HBB:c.20A > T p.Glu6Val; c.196A > G p.Lys65Glu] is a new double-mutant hemoglobin that was found in heterozygosis in an 18-month-old Brazilian male with moderate anemia. It behaves like Hb S in acid electrophoresis, isoelectric focusing and solubility testing but shows different behavior in alkaline electrophoresis, cation-exchange HPLC and RP-HPLC. The variant is slightly unstable, showed reduced oxygen affinity and also appeared to form polymers more stable than the Hb S. Molecular dynamics simulation suggests that the polymerization is favored by interfacial electrostatic interactions. This provides a plausible explanation for some of the reported experimental observations.



## Introduction

Hemoglobin S (Hb S, or $\beta^s$ globin) results form a single nucleotide mutation (CTG → GAG) in the sixth codon of the β-globin gene that causes glutamic acid to be substituted by valine on the surface of the variant β chain. This change allows Hb S to polymerize when deoxygenated because the valine residue favors hydrophobic interactions between adjacent globin chains [1].

The presence of this mutation in homozygosis (Hb S/Hb S) and in a number of compound heterozygous conditions with common Hb C, Hb E, Hb D Punjab, Hb O-Arab and beta thalassemia traits, results in a group of common clinical manifestations known as sickle cell disease (SCD).[1] Polymerization of deoxygenated Hb S is the primary indispensable event in the molecular pathogenesis of sickle-cell disease, as it triggers a cascade of vaso-occlusions and hemolysis abetted by elements of inflammation, oxidant damage, deregulated homeostasis of nitric oxide (NO) and endothelial-cell injury [2,3].

Heterozygous individuals (AS) are usually free of clinical symptoms because of the abundance of Hb A in erythrocytes (approximately 60%), which prevents polymerization of Hb S under physiological conditions. The same asymptomatic behavior can be observed in heterozygous carriers of four sickling Hb variants with a double mutation (β6 Glu → Val and other residue substitutions at the same beta-globin): Hb C-Ziguinchor [HBB:c.20A > T p.Glu6Val; c.176C > G p.Pro58Arg], Hb C-Harlem [HBB:c.20A > T p.Glu6Val; c.220G > A p.Asp73Asn], Hb S-Oman (β6 [HBB:c.20A > T p.Glu6Val; c.364G > A p.Glu121Lys] and Hb S-Travis [HBB:c.20A > T p.Glu6Val; c.428C > T p.Ala142Val] [4–7]. However, two other double-mutant variants that cause sickling in heterozygotes, namely, Hb S-Antilles [HBB:c.20A > T p.Glu6Val; c.70G > A p.Val23Ile] and Hb Jamaica Plain [HBB:c.20A > T p.Glu6Val; c.205C > T p.Leu68Phe], cause their carriers moderate and severe anemia, respectively, as well as splenomegaly [8,9]. These mutant proteins differ from others in their decreased affinity for oxygen, which can enhance polymerization of the Hb S variant and lead to manifestations of the sickle cell disease even in simple heterozygotes [8,9].

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[1] *Abbreviations used:* SCD, sickle cell disease; NO, nitric oxide; MD, molecular dynamics; CE-HPLC, cation exchange-high performance liquid chromatography; RP-HPLC, reverse phase-high performance liquid chromatography.

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We describe the structural and functional properties of Hb S-São Paulo (Hb S-SP), a new double-mutant variant with altered functional behavior that displays the sickle mutation β6 Glu → Val (A3) and a new substitution, β65 Lys → Glu (E5). We also carried out molecular dynamics (MD) computer simulations, a molecular modeling technique widely used to study protein structure and dynamics [10], in order to investigate the structural features and conformational adaptations of the new Hb S double mutant and thus gain insights into its functional behavior from a molecular-level perspective.

## Material and methods

An 18-month-old Brazilian boy with clinical features of sickle cell disease who was referred to the School of Medical Sciences Hospital at the State University of Campinas, in Campinas, state of São Paulo, southeastern Brazil, for investigation of hemoglobinopathies. His hematological parameters are shown in Table 1.

### *Protein characterization*

After informed consent had been obtained from the patient's mother, alkaline hemoglobin electrophoresis was performed on cellulose acetate with Tris–EDTA-boric acid buffer at pH 8.9, and acid electrophoresis was carried out in agar gel with sodium citrate buffer at pH 5.8. Isoelectric focusing (IF, RESOLVE TM Systems, Neonatal Hemoglobin Test Kit, Perkin Elmer-Wallace, Akron, OH, USA) was also used to distinguish the anomalous protein from the normal fraction. In addition, cation exchange-high performance liquid chromatography (CE-HPLC) (VARIANT™; Bio-Rad Laboratories, Hercules, CA, USA) was used to quantify hemoglobin concentrations. Hb $A_2$ was measured by elution from cellulose acetate after electrophoresis at alkaline pH [11]. Globin chains were analyzed by reverse phase-high performance liquid chromatography (RP-HPLC) on a Waters Alliance HPLC system (Waters, Milford, MA, USA). Heinz body, stability (heat, isopropanol and *n*-butanol) and solubility tests were carried out using standard procedures [11]. Polymerization of Hb S-SP was monitored by spectrophotometry at 700 nm, as described by Adachi and Asakura [12], for 24 h. The tests were carried out at 30 °C with stripped hemolysate at a concentration of 0.123 g/dL in the presence of 1.8 M phosphate buffer at pH 7.34 in a hermetically sealed tube to avoid contact with oxygen and thus minimize the influence of Hb F on Hb S-SP polymerization. The results were compared with those obtained using the stripped hemolysate of heterozygous Hb S at the same concentration.

### *Molecular analysis of HBB*

The gene mutation was identified using genomic DNA obtained from peripheral leukocytes (Blood Genomic Prep Mini Spin, GE Healthcare, Amersham, UK). Beta-globin gene sequencing was performed with an automated sequencer (ABI PRISM™ 377 DNA Automated Sequencer, Applied BioSystems, Foster City, CA, USA) using primers described elsewhere [13]. The mutations were confirmed by reverse DNA sequencing and by cloning techniques using the pGEM-T Easy Vector kit (Promega, Madison, WI, USA). Concomitant α-thalassemia was excluded by performing multiplex gap-PCR for the most common deletions [14]. Likewise common non-deletional mutations were excluded by a combination of specific PCR assays and restriction enzyme analysis [15].

### *Function tests*

The functional behavior of the erythrocyte hemolysate (in the stripped state and at a concentration of 70 μM/*Heme*) was analyzed by the Rossi-Fanelli and Antonini method [16] at 25 °C. Purification of the total hemolysate was performed using Sephadex

**Table 1**
Hematological, biochemical and molecular data of the patient and his mother and sister.

| | Proband | Mother | Sister |
|---|---|---|---|
| Age (years) | 1.5 (18 months old) | 31 | 10 |
| RBC ($10^6$/mm$^3$) | 3.73 | 4.9 | 5.18 |
| Hb[a] (g/dL) | 8.9 | 13.9 | 13.6 |
| Hct[a] (%) | 29.2 | 38.9 | 39.5 |
| MCV[a](fl) | 78.3 | 79.4 | 76.3 |
| MCH[a] (pg) | 23.9 | 28.4 | 26.3 |
| RDW[a] (%) | 18.5 | 13.4 | 12.4 |
| Reticulocytes (%) | 1.51 | 0.6 | 0.53 |
| Electrophoresis (cellulose acetate, pH 8.9) and CE-HPLC[a] profiles | Hb $A_2$,Hb F, Hb A, Hb X[b] | Hb $A_2$, Hb S, Hb A | Hb $A_2$, Hb S, Hb A |
| Hb $A_2$ (%) | 2.72[d] | 4.4[e] | 4.4[e] |
| Hb F (%) | 12.3 | 0.6 | 0.3 |
| Hb X (%) or Hb S (%) | Hb X = 29.2 | Hb S = 38.6 | Hb S = 37.7 |
| Acid electrophoresis profile (agar gel, pH 5.8) | Hb A, Hb F, Hb X[b] | Hb A, Hb S | Hb A, Hb S |
| RP-HPLC[a] (% of globin-chains) | HEME = 10.44, δ = 2.22%, β = 24.58%, $\beta^X$ = 11.47%, α = 45.79%, $\gamma^G$ = 2.19% $\gamma^A$ = 3.31% | HEME = 10.15,δ = 2.28%, β = 23.66%, $\beta^S$ = 18.32%, α = 45.59% | HEME = 9.99, δ = 2.46%, β = 24.32%, $\beta^S$ = 17.58%, α = 45.65% |
| Solubility test | Reduced solubility | Reduced solubility | Reduced solubility |
| Stability tests | Slightly unstable Hb | Normal | Normal |
| Mutations found in the β-globin genes | codon 6 (β) GAG-GTG[c]<br>codon 65 (β)AAG-GAG[c]<br>codon 2 (β)C-T[c]<br>(in *cis*) | codon 6(β) GAG-GTG[c]<br>codon 2 (β)C-T[c]<br>(in *cis*) | codon 6(β) GAG-GTG[c]<br>codon 2 (β)C-T[c]<br>(in *cis*) |
| α Genotype[a] | αα/αα | αα/αα | αα/αα |

[a] RBC = Red Blood Cells; Hct = Hematocrit (packed red blood cell volume); MCV = Mean Corpuscular Volume of Erythrocytes; MCH = Mean Corpuscular Hemoglobin of Erythrocytes; RDW = Red Cell Distribution Width of Erythrocytes; CE-HPLC = Cation Exchange-High Performance Liquid Chromatography; RP-HPLC = Reverse Phase-High Performance Liquid Chromatography; α Genotype = α-Globin Gene Genotype.
[b] Hb X = Hb S-São Paulo.
[c] Heterozygosis.
[d] Measured by elution from cellulose acetate after electrophoresis at alkaline pH.
[e] These high values do not indicate a beta thalassemia trait but are an artifact due to overlapping of the glycated Hb S with the (normal) Hb $A_2$ [35].

G-25 (Sigma–Aldrich, St. Louis, MO, US) and Hepes (*N*-(2Hydroxyethyl) piperazine-*N*′-2-ethane sulfonic acid) buffer (Sigma–Aldrich, St. Louis, MO, US) without addition of NaCl. Oxygen equilibrium curves in the absence and presence of inositol hexaphosphate were plotted (IHP, 0.1 mM) (Sigma–Aldrich, St. Louis, MO, US). Oxygen affinity (determined by measuring p50), Bohr Effect, and cooperativity (by Hill plot) were analyzed according to previously described methods [16,17]. The results obtained were compared with the results for hemolysates from normal human adult red cells that contained mainly stripped Hb A (95%).

Functional tests at pH 7.34 were also carried out using a Hemox Analyzer (TCS Medical Products Division, Southampton, PA). In these analyses we used 20 μL of red blood cell concentrate stored at −80 °C, to which Hemox buffer solution (TCS Medical Products Division) was added as recommended by the manufacturer. The results for affinity and cooperativity obtained with the hemolysate containing the Hb S-SP were compared with the hemolysate containing the normal standard, the hemolysate containing the control heterozygous Hb S and the hemolysate containing standard 100% Hb F, all of which were also stored at −80 °C for the same time.

In addition, tests with the polymerized Hb S-SP fraction were also carried out in pH 7.34 using the Hemox Analyzer. The polymerized variant was isolated using a solubility test, as previously described [11]. The results obtained were compared with the control heterozygous Hb S fraction isolated in its T conformation by the same method.

It should be stressed that in the case of the Hemox Analyzer no protein purification was carried out, unlike in earlier functional studies. Nevertheless, all the samples were subjected to the same conditions, and the method and reagents recommended by the manufacturer were used.

### *Molecular dynamics simulation*

MD simulations were performed to probe the structural perturbation introduced by S and SP mutations on Hb structure and polymerization. All simulations were performed using the following protocol: Crystallographic models or computational constructs of the mutants (see below) were solvated with water and sodium and chloride ions with Packmol [18] by defining a 15 Å layer of water and an ionic concentration that neutralizes the system and mimics a physiological salt concentration of ∼0.15 M. The solvated systems were equilibrated and simulated with NAMD using CHARMM parameters [19], periodic boundary conditions, a cutoff of 12 Å for van der Waals interactions with a switching function starting at 10 Å and the particle mesh Ewald method for long-range electrostatic interactions. Simulations were performed under constant pressure using a Langevin piston at 1 atm with a 200 fs period, a 100 fs decay, and 298.15 K temperature. The temperature of the simulated systems was set 298.15 K by velocity rescaling at every 100 steps for equilibration runs, or by the application of a Langevin bath with a damping period of 2 $ps^{-1}$ for production runs. A time-step of 2.0 fs was used, while keeping bonds to hydrogen atoms rigid using SHAKE [20]. Equilibration was performed as follows: First, the total energy of each system was minimized by 2000 steps of Conjugate-Gradients (CG) followed by 200 ps MD, while keeping all protein atoms fixed. Next, the side-chains of the proteins were allowed to move, and 200 steps of CG minimization and 200 ps MD were performed. Finally 5 ns runs using the production run protocols were performed for each system. The final configuration of these last runs were used as starting conditions for 15 ns production runs, which were used for analysis.

Crystallographic models of Oxy and Deoxy Hb were used (PDB ids. 2DN1 and 2DN2 and, respectively, both with 1.25 Å resolution) [21]. Simulations were performed for both native and double (S-SP) mutants, which were constructed by the substitution of the native with the mutant side chain using VMD [22]. The full Hb was simulated (four chains), and the mutations were introduced in each β-chain. Each of these systems had approximately 72,000 atoms. Simulations were also performed with the structure crystallographic model for the Hb S dimmer (eight chains) (PDB. id. 2HBS, 2.05 Å resolution) [23], as well as for the computational construct of the double mutant Hb S-SP dimmer (eight chains), where the K65E mutation was introduced on all β-chains. These systems contained about 168,000 atoms.

Structure figures were done with VMD [22], and home-made software (http://lm-mdanalysis.googlecode.com) was used for analysis. The distances in Fig. 6 were computed considering the side-chain nitrogen atom of Lys residues and the carboxylate carbon atoms of Glu and Asp residues.

## Results

### *Protein characterization*

Alkaline electrophoresis revealed Hb A, Hb F and Hb $A_2$ bands and an abnormal band faster than Hb A (with a pI of 6.68 in the IF) (Fig. 1A), while acid electrophoresis revealed a band with electrophoretic mobility similar to that of Hb S. This abnormal fraction eluted as a peak in the D window in CE-HPLC (Fig. 1B) and represented 29.6% of the total hemoglobin. Globin-chain analysis by RP-HPLC detected an anomalous β-chain that eluted more slowly than the normal β-chain and represented 11.47% of the total hemoglobin (Fig. 1C). The results of the solubility test were compatible with the presence of Hb S and those of the stability tests with the presence of a slightly unstable fraction. Lastly, analysis of Hb S-SP polymerization by spectrophotometry indicated that the delay time for Hb S-SP polymerization was not significantly different from the polymerization time for Hb S (Fig. 2A). However, the absorbance values of the Hb S-SP polymers formed were significantly higher than those of the Hb S polymers even after 24 h, indicating that the Hb S-SP polymer is quite stable (Fig. 2A and B).

### *Molecular analysis of HBB*

Sequencing of the β-globin gene (OMIM ID:141900) revealed both a GAG → GTG transition at codon 6 and an AAG → GAG transition at codon 65, on the same allele, in heterozygosity, corresponding to the Glu → Val replacement at the 6th position and Lys → Glu at the 65th position of the of the β-globin, respectively. The common C → T polymorphism at codon 2 was also observed in *cis* with the double mutation [HBB:c.20A > T p.Glu6Val; c.196A > G p.Lys65Glu; SNP rs713040]. Familial analysis indicated that the patient's mother and sister had sickle cell trait, as well as the C → T polymorphism at CD2. Cloning techniques confirmed that both mutations and the polymorphism were present in the same allele of the proband, who inherited the maternal β6Glu → Val allele in addition to the new mutation (β65Lys → Glu) (Fig. 1D), which probably occurred later on the same chromosome (perhaps in the germ line cells of the mother). Concomitant α-thalassemia was excluded by gap-PCR for the most common deletion and non-deletional mutations, on the proband as well on his mother and sister. The father was not available for the study, however, he was asymptomatic. The hematological, biochemical and molecular data of this family are summarized in Table 1.

### *Function tests*

As shown in Fig. 3A, functional tests performed with the stripped hemolysate revealed reduced oxygen affinity in the absence of IHP although Hb F, which should confer lower p50

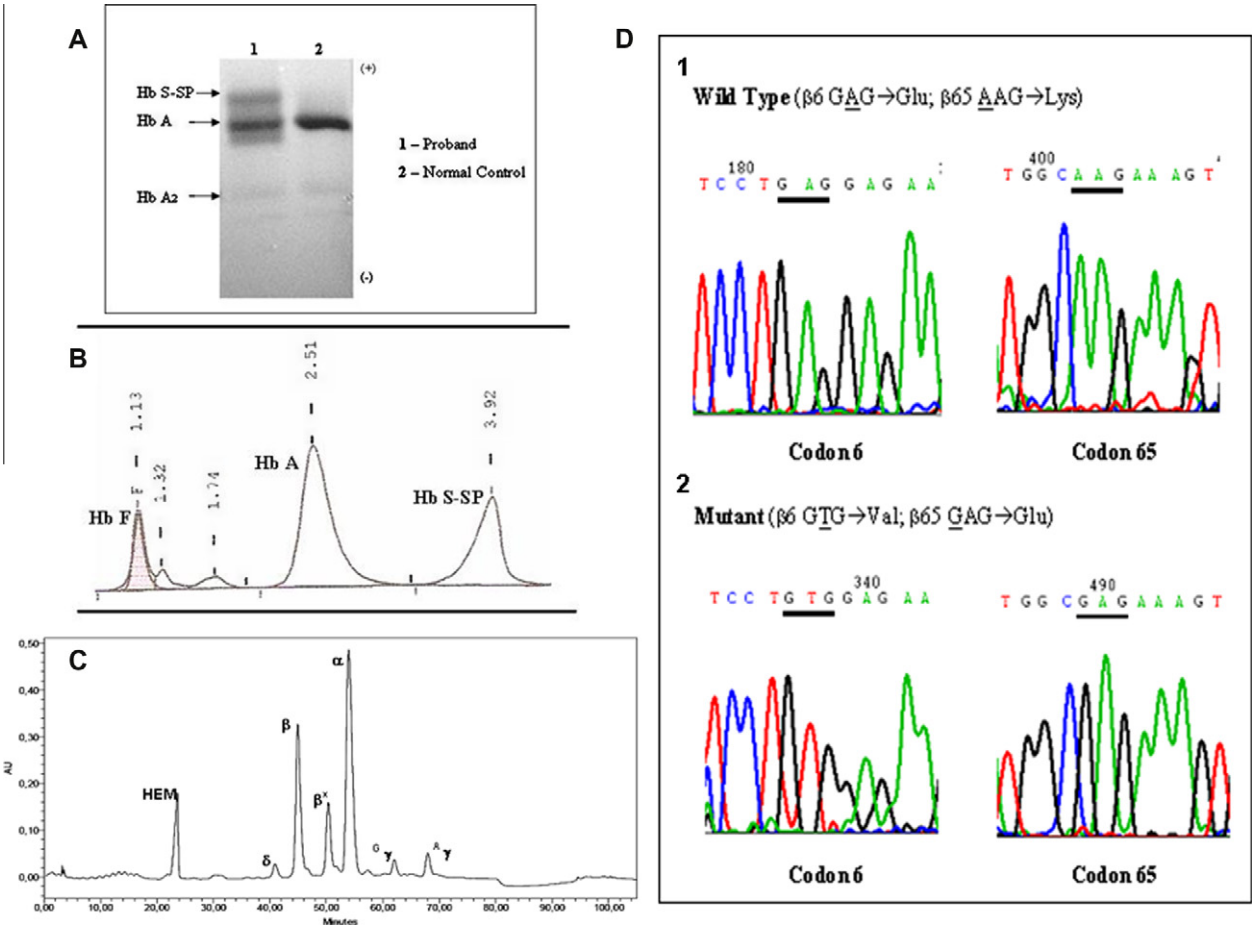



**Fig. 1.** Protein and molecular characterization of Hb S-SP. (A) Electrophoresis at alkaline pH of patient's hemolysate revealed Hb S-SP, Hb A and traces of Hb F and Hb $A_2$; (B) Cation exchange-high performance liquid chromatogram (CE-HPLC) of Hb S-SP; (C) Reverse phase-high performance liquid chromatogram (RP-HPLC) of Hb S-SP. δ, β, α, $\gamma^G$, $\gamma^A$ correspond to delta, beta, alpha, $^G$gamma and $^A$gamma chains, respectively. $\beta^x$ corresponds to the mutant chain [β6 (A3) Glu → Val; β65 (E5) Lys → Glu]; (D) Sequencing chromatograms revealing two clones derived from the patient's DNA: (1) Wild Type Clone; (2) Clone containing the sickle mutation β6 GAG → GTG and the spontaneously arising mutation β65 AAG → GAG, indicating that both mutations were on the same chromosome.

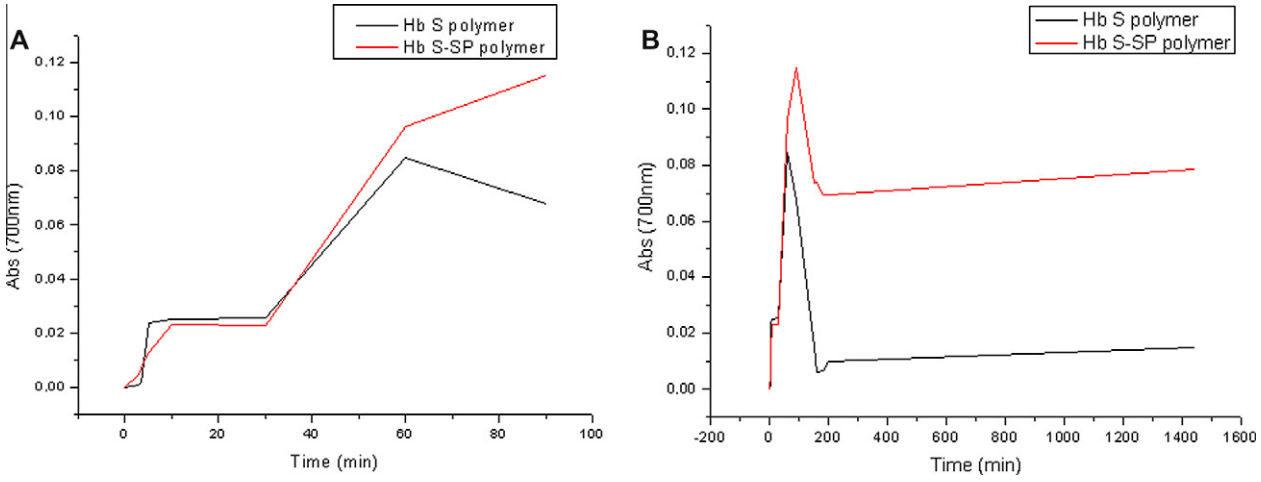



**Fig. 2.** Polymerization kinetics and stability of Hb S polymers (in black) and Hb S-SP polymers (in red), in 1.8 M $PO_4$ buffer (pH 7.34) at 30 °C, indicating that Hb S-SP polymers have significantly greater stability than the Hb S control for (A) 90 min; (B) 24 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

especially in the presence of organic phosphates, was present at a concentration of 12.3% [24]. When IHP was added to the stripped hemolysate we observed a small increase in the affinity of the variant for oxygen in alkaline pHs (>8), resulting in a small increase in the Bohr effect (Fig. 3B). The increase in affinity may be a consequence of the increased concentration of Hb F, as this protein

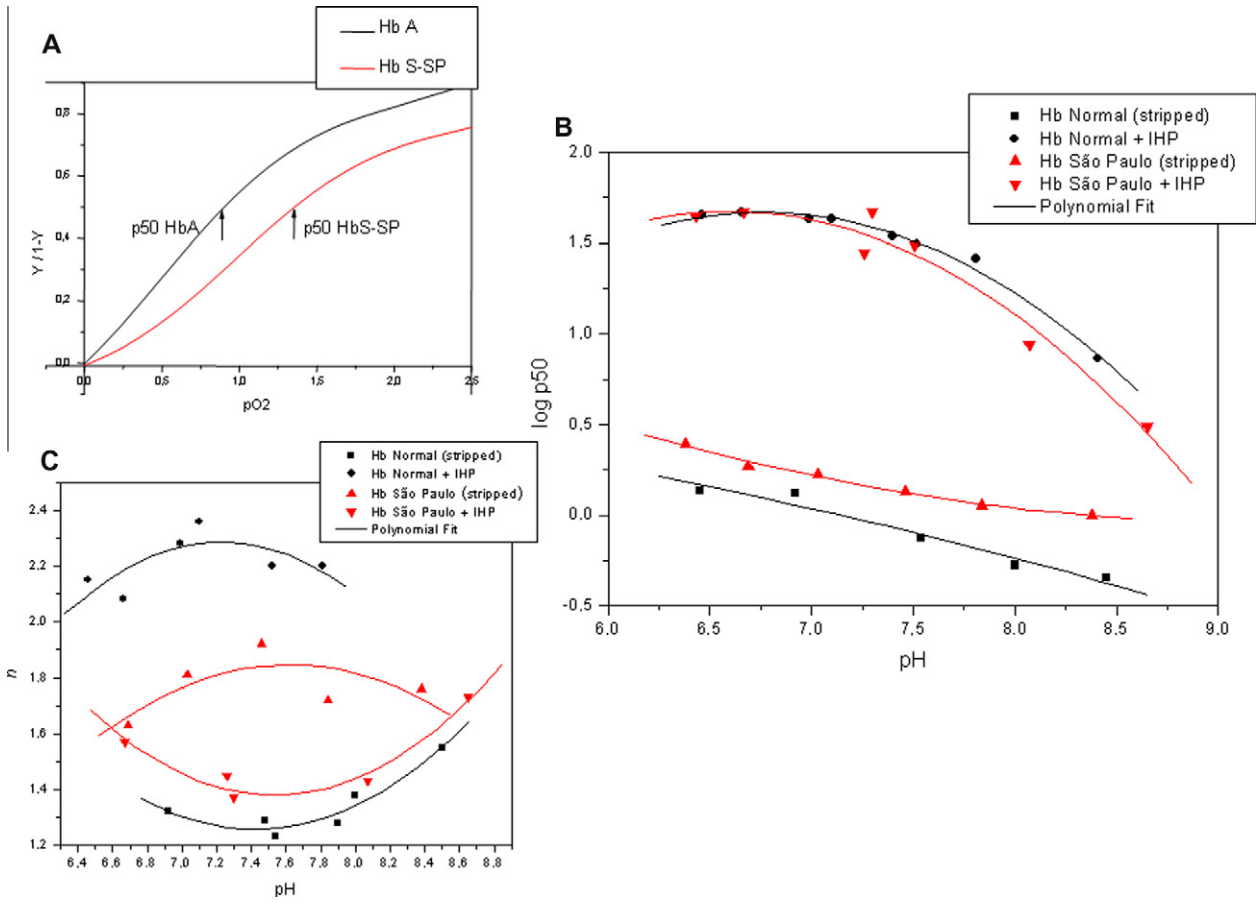



**Fig. 3.** Functional characterization. (A) Higher partial pressure of oxygen ($pO_2$) at pH 7.5 of stripped hemolysate containing Hb S-SP compared with the wild type Hb A; (B) Bohr effect of Hb S-SP in relation to the total hemolysate of a human adult, made up mainly of Hb A, showing the partial affinity of the hemoglobin (p50) as a function of pH in the presence and absence of IHP; (C) Variation in cooperativity (Hill plot) with pH in the presence and absence of IHP.

shows less affinity for 2,3 BPG (substituted here by IHP, which occupies the same active site as 2,3 BPG and has greater chemical stability [25,26]). However, The Hill's coefficient ($n$), which is indicative of *heme–heme* cooperativity, was higher in the absence of IHP and considerably lower in the presence of IHP when compared with the Hb A values (Fig. 3C).

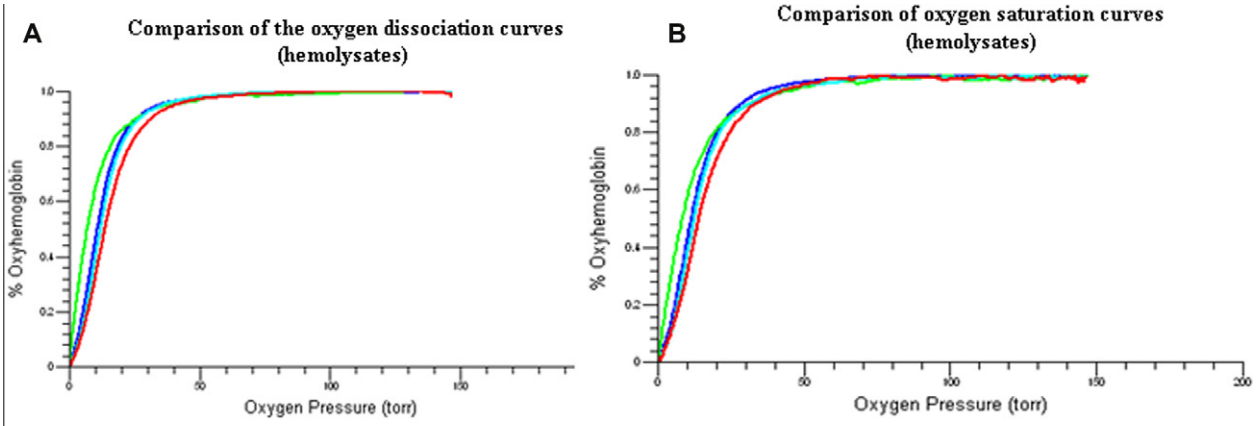
Functional studies in a Hemox Analyzer using red blood cell concentrate confirmed the reduced $O_2$ affinity of Hb S-SP observed in the previous experiments (in its stripped state). In addition, these analyses once again showed the increased $O_2$ affinity of Hb F as well as values of $O_2$ affinity considered normal for Hb S in heterozygosis (Fig. 4A and B) [27] The p50 values obtained from the curves are shown in the table of results in Fig. 4A and B.

Automated functional tests with Hb SP-P isolated in a solubility test revealed more clearly the reduced $O_2$ affinity of the variant (Fig. 4C and D), and showed reduction in *heme–heme* cooperativity compared with the total hemolysate, as the Hb S polymer (Fig. 5).

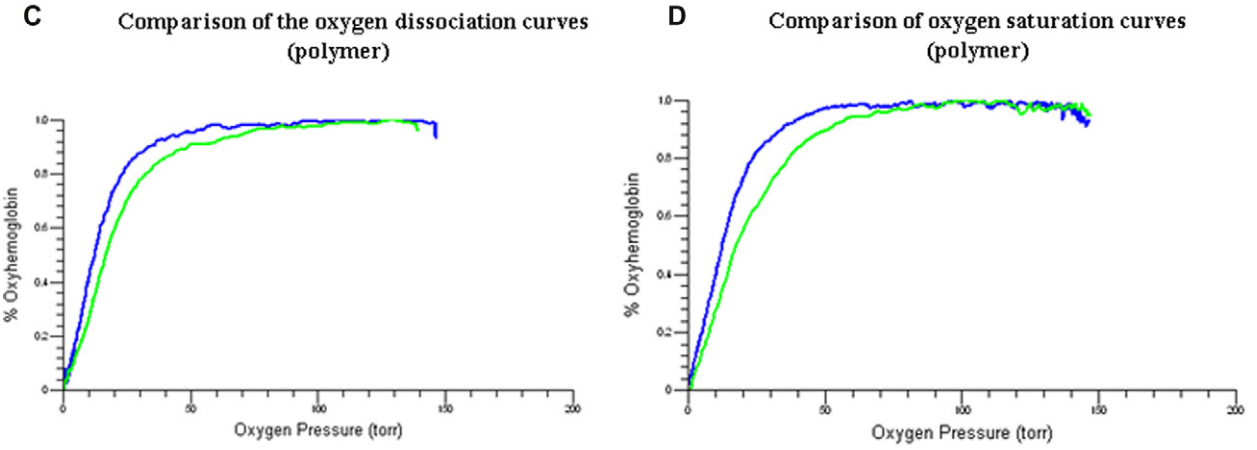
### *Molecular dynamics simulation*

We performed simulations of Oxy-Hb and Deoxy-Hb, in the native and double-mutant (Hb S-SP) forms. Each simulation was 20 ns long, being equilibrated in the first 5 ns run. The residue in position 65 of the Hb β-chains is located in Helix E oriented to the solvent. In both native Oxy-Hb and Deoxy-Hb, it participates in an effective electrostatic interaction with residue Asp21, as shown in Fig. 6A. Since the SP mutation inverts the electrostatic charge of Lys65, it may readily affect this interaction. Indeed, our simulations show that in both Oxy-Hb and Deoxy-Hb the distance between residue in position 65 (Lys or Glu) and Asp21 increases, as shown by the displacement of distribution curves in red in Fig. 6B and C. The shift of these electrostatic bonds allow for the favorable approach of mutant Glu65 with the side chain of Lys66, as shown by distance distributions in black in Figs. 6B and C. Since the Helix E residues discussed above are located close to the active site, they could have some direct implication in oxygen affinity [28,29]. However, within the time-scale of our simulations, no perturbation in His63 was observed (Supplementary Fig. S1), thus this possibility is not supported by the present study.

In addition, we probed the possible effect of the β65 Lys → Glu mutation on Hb polymerization, which is known to take place in Hb-S alone. We simulated the crystal structure of the Hb S dimer and a computational construct of the double mutant dimer (Fig. 7A). This crystallographic dimer putatively displays the dimerization surface leading to Hb S polymerization. The β65 Lys → Glu mutant is located in the proximity of the dimer interface, thus possibly affecting dimer stability. In Fig. 7A the residue in position 65 is depicted with van der Waals spheres. The residues of the upper dimer that are closer to the mutant residue are shown by a surface representation, and belong to a β-chain. In Fig. 7A and B, residues that interface with position 65 are colored by type; positively charged residues are shown in blue, and negatively charged residues in red. There are two negatively charged residues (Glu7 and Asp79), and four positively charged ones (Lys8, Lys82, Lys132, and N-terminal Val1). Therefore, there is a net positive charge at this surface, and the β65 Lys → Glu substitution at the neighboring




| Key: | Comparison of p50 values (mmHg): | A | B |
|---|---|---|---|
| | Hb A control hemolysate | 10.74 | 11.45 |
| | Hb F control hemolysate | 6.83 | 8.30 |
| | Hb A ; Hb S control hemolysate | 11.86 | 12.35 |
| | Hb A ; Hb F ; HbS-São Paulo hemolysate | 13.41 | 13.91 |




| Key: | Comparison of p50 values (mmHg): | C | D |
|---|---|---|---|
| | Hb S polymer | 11.41 | 11.67 |
| | Hb S-SP polymer | 16.37 | 17.04 |

**Fig. 4.** Automated functional tests. (A) Comparison of the oxygen dissociation curves and values corresponding to p50 (mmHg) of the total hemolysates. (B) Comparison of the hemoglobin oxygen saturation curves and values corresponding to p50 (mmHg) of the total hemolysates. (C) Comparison of the oxygen dissociation curves and values corresponding to p50 (mmHg) of the Hb S and Hb S-SP fractions isolated in their T form by the formation of polymers obtained from the solubility test. (D) Comparison of the hemoglobin oxygen saturation curves and values corresponding to p50 (mmHg) of the Hb S and Hb S-SP fractions isolated in their T form by the formation of polymers obtained from the solubility test.

β-chain can lead to an enhanced dimer stabilization relative to the Hb S dimer. We have performed simulations of the Hb S and the computational construct of Hb S-SP dimer, each lasting 20 ns. The structure of the dimer is stable in this time scale, but the interaction energy profiles of residue in position 65 with the nearby β-chain surface indicate that this interaction may facilitate polymerization. Because of the net positive charge of the interface, the interaction of the interfacial residues with Glu65 (left side of graph, black line) is strongly attractive, whereas interactions with Lys65 (right side, black) are strongly repulsive, as shown in Fig. 7C. These highly different interaction profiles result from the interaction with charged residues (Fig. 7C, red lines), but were also reinforced by a favorable shift in interaction profiles for neutral residues (Fig. 7C, blue). Not unexpectedly, these pronounced shifts in electrostatic interactions may be softened by solvent reorientation and ion placement. However, these interactions also suggest that the dimer of Hb S-SP could

A

| Hemoglobin variant | DC* | SC* |
|---|---|---|
| Hb A (hemolysate) | 2.22 | 2.27 |
| Hb F (hemolysate) | 1.42 | 1.51 |
| Hb A ; Hb S (hemolysate) | 2.43 | 2.48 |
| Hb S polymer | 1.87 | 1.89 |
| HbA;HbF; HbS-SP (hemolysate) | 2.40 | 2.38 |
| Hb S-SP polymer | 1.86 | 1.79 |

*DC = Dissociation Curve ; SC = Saturation Curve

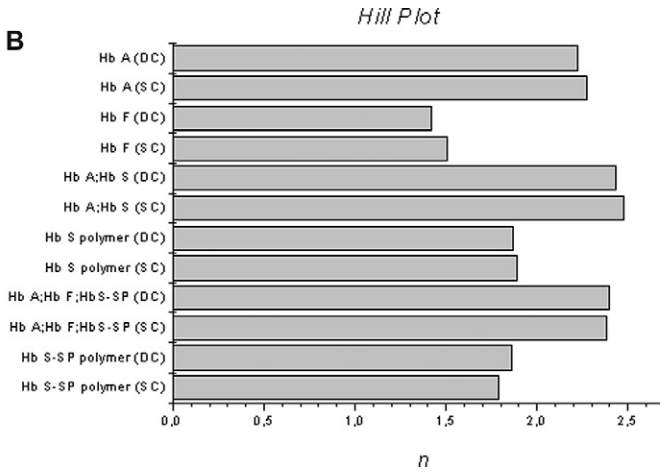



**Fig. 5.** *Heme–heme* cooperativity at pH 7.34 measured by the automated method. (A) Hill's coefficient obtained from the oxygen dissociation and saturation curves. (B) Graphical representation of the Hill's coefficients ($n$) obtained in this way.

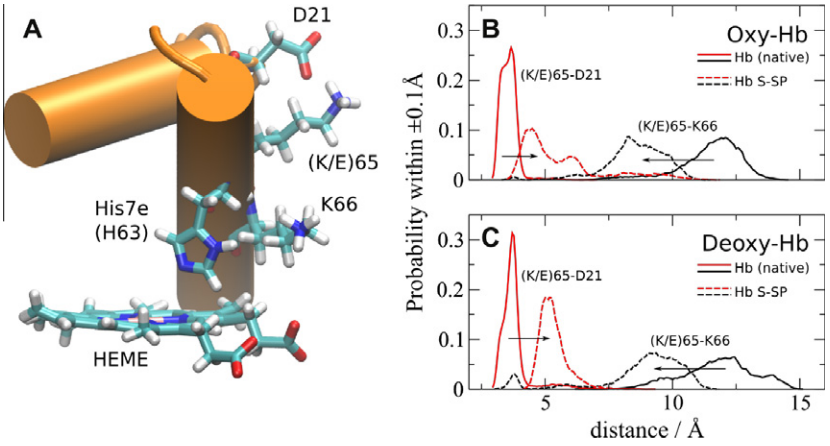



**Fig. 6.** Intramolecular interactions of the Hb S-SP and their possible perturbation of His7e conformation. (A) Residue in position 65 can interact either with Asp21 or Lys66, and is located on Helix E, which contains His7e. (B and C) Mutation of positively charged Lys65 to Glu disrupts the K65-D21 interaction and leads to the approach of the Glu65 side chain to Lys66, both in Oxy-Hb (B) and Deoxy-Hb (C) simulations; arrows indicate the shift in distance distributions promoted by mutations.

form additional salt bridges at this interface and stabilize the dimer, thus facilitating the nucleation process that leads to protein polymerization.

## Discussion

Hb S-São Paulo is a novel double-mutant human hemoglobin that results in moderate anemia in its carrier. This slightly unstable variant has two substitutions in the same β-chain [β6 (A3) Glu → Val and β65 (E5) Lys → Glu] and has reduced oxygen affinity. Furthermore, we suggest that Hb S-SP polymers formed in erythrocytes are more stable than Hb S polymers. This hypothesis was reinforced by the considerable stability exhibited by the Hb S-SP polymers in the spectrophotometric and molecular dynamic analysis (Figs. 2 and 7).

Both the functional tests carried out by the spectrophotometric method, with stripped hemolysate in the absence of IHP, and the automated method using a Clark electrode with total hemolysate in physiological conditions, revealed a reduced $O_2$ affinity in hemolysate containing Hb S-SP (Figs. 3A and B and 4A and B). However, in the presence of IHP, the functional behavior of the hemolysate containing Hb S-SP was similar to that of Hb A at a pH below 8 (Fig. 3B). It is possible that the Hb F concentration (12.3%) in the stripped hemolysate studied here minimized the reduced oxygen affinity of Hb S-SP, since it is well known that fetal human hemoglobin has lower affinity for 2,3 BPG and is therefore more readily available for oxygen interaction [24] (Fig. 3B). Automated functional tests support the hypothesis that Hb F interferes with the affinity of the total hemolysate (Fig. 4A and B), as when Hb S-SP was isolated from the other fractions by polymerization in a low-oxygen-content environment (in the solubility test) it showed quite reduced $O_2$ affinity (Fig. 4C and D) compared with Hb A hemolysate, the Hb A/Hb F/Hb S-SP hemolysate, the Hb A/Hb S hemolysate and the Hb S polymer resuspended in Hemox solution.

The results for *heme–heme* cooperativity of Hb S-SP obtained by spectrophotometry and the automated method were also concordant. An increase in the Hill's coefficient was observed in both analyses (Figs. 3C and 5). This is probably due to the presence of the Val6 substitution, which confers increased *heme–heme* cooperativity before the stable polymer is formed [30,31]. However, the Hill's coefficient observed in the presence of IHP was considerably decreased than that for Hb A. Possibly the high concentration of Hb F was responsible for the decreased *heme–heme* cooperativity of Hb S-SP as a result of its known reduced affinity for 2,3 BPG and lower *heme–heme* cooperativity [24,27]. Another interesting finding is that, as with the Hb S polymer, the Hill's coefficient of the Hb S-SP polymer was reduced, indicating non-cooperative $O_2$ bind-

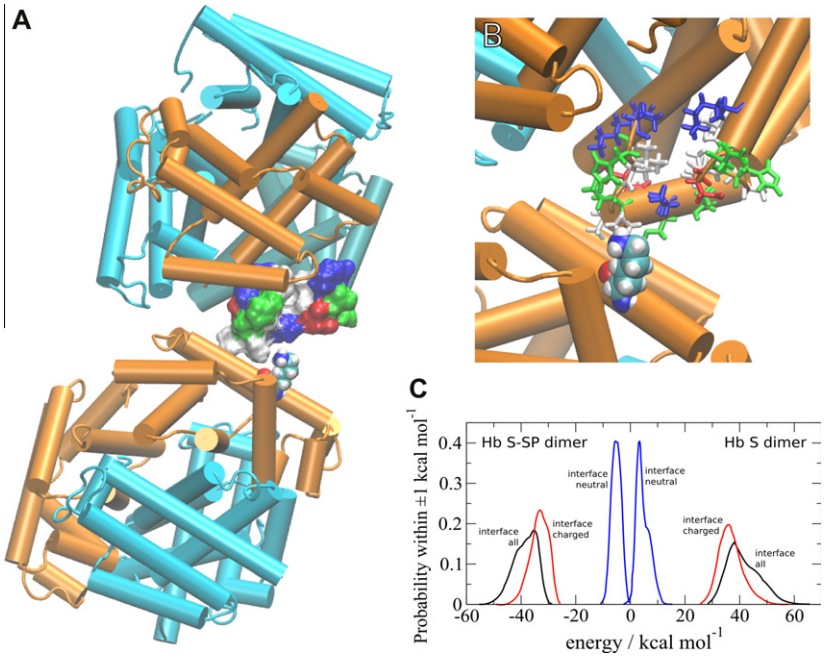



**Fig. 7.** The effect of the SP mutation on the Hb S dimer and its possible role on Hb polymerization. (A) The SP mutation is located at the vicinity of the dimer interface of Hb S, believed to be implied in the polymerization in cells. (B) Residues at the β-chain of the upper Hb S are electrically unbalanced. Four positively charged residues (blue) are close to position 65 of the Hb S, while only two negatively charged residues (red) belong to this interface. (C) The unbalanced charged promote strongly favorable (negative) electrostatic interactions of the interfacial residues with mutant Glu65, and strongly repulsive interactions with native Lys65, suggesting that the SP mutant might provide an additional contribution for dimer stabilization and thus Hb S-SP polymerization. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ing, as expected [31] (Fig. 5). However, more detailed studies are needed to confirm the results for cooperativity and correlate them to the polymerization properties of Hb S-SP (Figs. 2 and 7).

Our MD simulations suggest that the Hb S-SP mutant affects the residue side-chain dynamics in Helix E and could. The most relevant perturbation appears to be an important shift in electrostatic interactions of the residue in position 65: while native Lys65 interacts strongly with Asp21 of the same β-chain, charge inversion on Glu65 leads to repulsion and a tendency of the mutant residue to interact with Lys66. The SP mutant is close to the dimer interface of crystallographic Hb S, which is believed to represent the interaction leading to sickle-cell Hb S polymerization, as shown by a pioneering molecular dynamics simulation study [32]. The residues in the dimer interface region in the proximity of the SP mutation have a positive net charge, as a result of which mutation of β65 Lys → -Glu causes a significant shift in the profile of electrostatic interactions in this region. This suggests that Glu65 may induce an additional stabilization of the dimer, thus promoting nucleation and the subsequent polymerization of the protein.

In summary, we have identified a new double-mutant hemoglobin in heterozygosis showing moderate anemia in an 18-month-old Brazilian male. A similar clinical picture is produced by Hb S-Antilles [HBB:c.20A > T p.Glu6Val; c.70G > A p.Val23Ile] and Hb Jamaica Plain [HBB:c.20A > T p. Glu6Val; c.205C > T p.Leu68Phe], variants that also have reduced oxygen affinity and greater polymerization potential [8,9]. Like Hb S-SP, Hb S-Antilhes also produced abnormal results in polymerization tests [8,9]. However, whereas Hb S-Antilles had higher polymerization speeds than Hb S in tests lasting 50 min [33], the Hb S-SP stripped hemolysate had the same polymerization speed as Hb S when tested for 60 min but greater stability, and polymerized continuously up to 90 min (Fig. 2). These findings are supported by computer simulations of Hb S-SP, which suggest that the substitution of Lys by Glu at residue 65 of the β-chains leads to the formation of more stable dimers than those in Hb S polymers.

This study allowed us to assess the deterioration in the clinical picture of this Hb S-SP carrier caused by the second mutation. In Brazil, it is important to bear in mind the possibility of combined mutations because of the high prevalence of hemoglobinopathies due to the extensive miscegenation and racial diversity of the Brazilian population [34]. The concomitant appearance of Hb S-SP with other variants (such as Hb S or even Hb C, which in heterozygosis result in an asymptomatic phenotype) or even beta thalassemia can very possibly result in a more severe clinical picture.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2012.01.001.

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