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# Understanding the molecular basis of the high oxygen affinity variant human hemoglobin Coimbra

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ABSTRACT

Human hemoglobin (Hb) Coimbra (βAsp99Glu) is one of the seven βAsp99 Hb variants described to date. All βAsp99 substitutions result in increased affinity for $O_2$ and decreased heme-heme cooperativity and their carriers are clinically characterized by erythrocytosis, caused by tissue hypoxia. Since βAsp99 plays an important role in the allosteric α1β2 interface and the mutation in Hb Coimbra only represents the insertion of a $CH_2$ group in this interface, the present study of Hb Coimbra is important for a better understanding of the global impact of small modifications in this allosteric interface. We carried out functional, kinetic and dynamic characterization of this hemoglobin, focusing on the interpretation of these results in the context of a growth of the position 99 side chain length in the α1β2 interface. Oxygen affinity was evaluated by measuring p50 values in distinct pHs (Bohr effect), and the heme-heme cooperativity was analyzed by determining the Hill coefficient (*n*), in addition to the effect of the allosteric effectors inositol hexaphosphate (IHP) and 2,3-bisphosphoglyceric acid (2,3-BPG). Computer simulations revealed a stabilization of the R state in the Coimbra variant with respect to the wild type, and consistently, the T-to-R quaternary transition was observed on the nanosecond time scale of classical molecular dynamics simulations.

## 1. Introduction

Human hemoglobin (Hb) is the hemeprotein found in high concentrations in erythrocytes, and is functionally responsible for the transport of oxygen ($O_2$) from the lungs to peripheral tissues. Hb is a tetramer comprised of two α-like (α or ζ) and two β-like (β, δ, γ or ε) globin chains, each associated with a *heme* group, a protoporphyrin IX with a ferrous iron atom that can reversibly bind molecular $O_2$. The different combinations of globin chains are adapted to distinct stages of human development from the embryonic period to adult life, depending on the $O_2$ demand and environmental availability. During fetal life, for example, fetal hemoglobin (HbF) (α2β2), a variant that has high affinity for $O_2$, due to its decreased affinity for the allosteric effector 2,3-biphosphoglycerate (2,3-BPG), is expressed to compete for the $O_2$ from maternal HbA (α2β2), which is mainly produced during adult life [1].

The stability of the tetramer is controlled by the α1β1/α2β2 contacts, whereas the α1β2/α2β1 interface ensures the stability of the transition between the two conformations with different oxygen affinity, known as the T (Tense) and R (Relaxed) states, with low and high affinity for $O_2$, respectively [2,3]. The α1β2 interface is particularly close to the heme pocket, such that changes in this interface are closely related to $O_2$ binding. One of the most important interactions in the α1β2 interface involves βAsp99, which forms hydrogen bonds with αTyr42 and αAsn97, stabilizing the T state (Fig. 1) [2,4].

Until now, 99 human Hb variants with high oxygen affinity have been reported, and 79 of them involve β-chain residue replacements. The majority of these variants have substitutions at one of the three crucial regions for Hb stability and function: the β-chain C terminus, the α1β2 interface and the 2,3-BPG binding site. To date, seven hemoglobin βAsp99 substitution variants have been detected: Hb Ypsilanti

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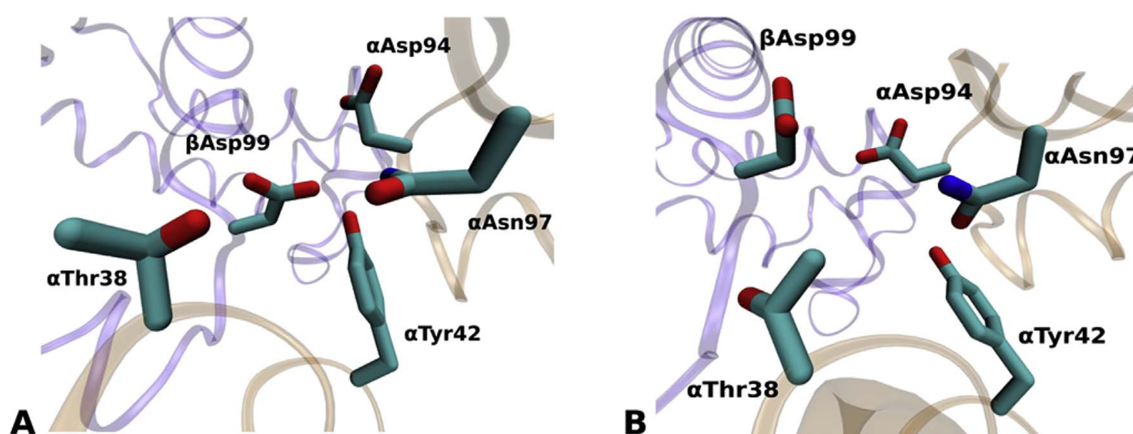


**Fig. 1.** The α1β2 Hb interface; α1 in orange and β2 in purple. βD99 interactions in the (A) HbA deoxy, or T state (PDB ID 2HHB), and (B) the oxy, or R state (PDB ID 1HHO). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(βAsp99Tyr), Hb Kempsey (βAsp99Asn), Hb Yakima (βAsp99His), Hb Radcliffe (βAsp99Ala), Hb Hotel-Dieu (βAsp99Gly) and Hb Coimbra (βAsp99Glu). In all these cases, substitution of βAsp99 resulted in increased $O_2$ affinity and a decreased heme-heme cooperativity. Moreover, individuals with any of these mutations, in heterozygosis, are clinically characterized by erythrocytosis [5]. The mechanism of this compensatory production of red blood cells (also known as polycythemia), mediated by the presence of these hemoglobin variants is, therefore, caused by tissue hypoxia that prompts higher levels of erythropoietin and consequently intensive erythropoiesis [1,6–8].

It is interesting that these deleterious mutations can be as subtle as that of the Asp99Glu mutation in Hb Coimbra, and still significantly affect Hb function. The difference between HbA and Hb Coimbra is, at a molecular level, the insertion of a methylene group in the α1β2 interface. Nevertheless, this variant is characterized by an increased affinity for $O_2$ and subsequent erythrocytosis, as well as other clinical complications caused by the hyperviscosity of the blood in its carriers. The study of Hb Coimbra is, therefore, key to a better understanding of the underlying mechanisms of the T-to-R transition [9,10], because it is an example of how a small change in the α1β2 interface can have a dramatic impact on protein function.

## 2. Materials and methods

The samples studied here were obtained from peripheral blood of three carriers of Hb Coimbra, who were referred to the Hematology and Hemotherapy Center, University of Campinas, in Campinas, State of São Paulo, Brazil, for investigation of hemoglobinopathies. Ethical aspects and inform consent involved in this project were approved by the Brazilian research ethics committee (CAAE.0036.0146.000–07; Project 061/2007). The results obtained here were compared to six control samples: three samples collected from adults, mainly containing HbA; three samples from newborn individuals, mainly composed of HbF).

The erythrocytes were isolated, washed in cold saline and the Hb content were extracted by lysis in deionized water and the debris were removed by centrifuging.

### 2.1. Functional assays

Oximetry tests at pH 7.4 were carried out in a Hemox Analyzer system (TCS Scientific Corporation, New Hope, PA, USA) using 25 ⎡L of red blood cell concentrate, stored at −80 °C, and following the standard protocol and reagents recommended by the manufacturer (TCS Scientific Corporation). The results of oxygen affinity and heme-heme cooperativity of the Hb Coimbra in the lysates (containing Hb Coimbra, HbA, as well as $HbA_2$ – approximately 2.5%, and HbF – approximately 1%), were compared to three standard hemolysate mainly containing HbA (95%), as well as three newborn erythrocytes lysates (with levels of HbF varying from 63.8% to 87.4%). No protein purification was performed for these analyses and all samples were treated and stored under the same conditions and period.

The function of Hb Coimbra from cell lysates were also studied after purification with Sephadex G-25 gel chromatography (Sigma–Aldrich, St. Louis, MO, US), at pH 8.2 and pH 6.2, in Hepes buffer (*N*-(2Hydroxyethyl) piperazine-*N′*-2-ethane sulfonic acid) buffer– 50 mM (Sigma–Aldrich, St. Louis, MO, US), without addition of NaCl. In order to guarantee the elimination of 2,3-BPG and other organic phosphates from the samples, an Amberlite Mixed Bed column (MB 150; Sigma–Aldrich, St. Louis, MO, US) was also used. Hb and globin chain integrity was verified by Native-PAGE and SDS-PAGE (data not shown). The stripped lysates (Hb concentration of 70 ⎡M/heme) were submitted to spectrophotometric method at 25 °C, pH range 6.5–8.5, as previously described by Rossi-Fanelli and Antonini [2,11]. The equilibrium curves were performed in the absence and the presence of inositol hexaphosphate (IHP, 1 mM) (Sigma–Aldrich, St. Louis, MO, US). Sigmoidal fitting was performed using OriginPro 8.0. Oxygen affinity (determined by p50), Bohr effect and heme-heme cooperativity (determined by the Hill coefficient) from the lysates with Hb Coimbra were compared to stripped lysate mainly containing HbA [2,11,12]. The $O_2$ affinity of Hb Coimbra was also determined under the influence of 2,3-Biphosphoglyceric acid (2,3-BPG, 1 mM) (2,3-Biphospho-D-glyceric acid pentasodium; Sigma–Aldrich, St. Louis, MO, US), pH 7.6, 25 °C, and compared to that of HbF and HbA, from newborn and adult stripped samples, respectively.

### 2.2. Kinetics

Kinetic studies were performed using stopped-flow spectrophotometry in a stopped flow module SFM-300 (Bio-Logic Science Instruments, Seyssinet-Pariset, France) and the UV–vis traces were recorded using the Bio-kine 32 software (Bio-Logic Science Instruments). An integration time of 0.8 ms was used for all measurements (monitoring Hb-$O_2$ dissociation and Hb-CO ligation in the range of 400–700 nm), at 25 °C. Hb concentration (from stripped hemolysate) used here was 80 μM/heme in 100 mM phosphate buffer (J. T. Baker – Fisher Scientific, Pittsburg, PA, US), adjusted to pH 7.4. The dissociation rate constant $k_{off}$ was obtained from the reaction of Hb with Sodium dithionite (DTS - Sigma–Aldrich, St. Louis, MO, US) 6 mM, as reducing agent [13–15], in the ratio of 1:3 (Hb:DTS). Absorbance at 430 nm (absorbance maxima of the deoxi-Hb in the Soret region) as a function of time was used, and the kinetic trace was adjusted monoexponentially, to obtain one characteristic unimolecular constant for the process (Table 2). The same experimental conditions were used to determine $k_{off}$ in presence of 1 mM IHP (Sigma–Aldrich, St. Louis, MO,

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