

Description of electrophoretic and chromatographic hemoglobin profile of *Rhinoclemmys punctularia*

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ABSTRACT. Studies of the hemoglobin pattern in Brazilian reptiles are important for determining ecological and phylogenetic relationships, but they are scarce. Peripheral blood samples were obtained from 7 males and 18 females of *Rhinoclemmys punctularia*. The hematological profile was based on the total hemoglobin and hematocrit values. The hemoglobin profile was obtained using electrophoretic procedures at different pH, isoelectric focusing, globin chain electrophoresis, and HPLC. The hematocrit ($31 \pm 2\%$) and total hemoglobin (7.5 ± 0.2 g/dL) values did not indicate gender variations. Alkaline pH electrophoresis of the total blood samples treated with 1% saponin demonstrated the presence of four well-defined hemoglobin fractions, one major component (fraction I), showing cathodic migration and three others faster than fraction I with anodic migration. When the samples were precipitated with chloroform, only two hemoglobin fractions were observed, similar to fractions I and III from the

first procedure. Isoelectric focusing and HPLC showed the same pattern. With acid and neutral pH electrophoresis, two fractions with anodic migration were observed. The globin chain identification at alkaline pH showed two fractions, but four fractions were observed at acidic pH, suggesting that different polypeptide chains are involved in the hemoglobin molecule. The chromatographic separation of the total blood sample demonstrated that the major fraction comprised 81.9% and the minor 18.1%. The results obtained demonstrated a similarity between these hemoglobin components and those of some Chelidae reported in the literature for both land and aquatic animals, reflecting the adaptation to environmental conditions.

Key words: Hemoglobins, *Rhinoclemmys punctularia*, Electrophoretic profile, Hemoglobin polymorphism

INTRODUCTION

Reptiles have scales, breath-using lungs and with the exception of crocodilians have a three-chambered heart. They are ectothermic and the majority is ovoviviparous, such as snakes, lizards, crocodiles, and Chelonia (Orr, 1986). *Rhinoclemmys punctularia*, popularly known as the spot-legged turtle, belongs to the Reptilia class and composes the order Testudinata, along with the land turtles, fresh-water turtles and marine turtles (Usinger et al., 2000). In general, they are excellent divers and can stay under the water for long periods. During the long dives, the blood is redirected to the tissues that have a good tolerance to low oxygen levels such as the muscles and nerves. The muscles of some marine turtles, such as the leatherback turtle (*Dermochelis coriacea*), possess high concentrations of myoglobin, a protein that stores and transports oxygen in the muscle tissues (Sea Turtles, 1994).

The spot-legged turtle lives in or around rivers or lakes, feeding on small toads, frogs and fish. Their reproduction habits are not well known; the males can grow to 28 cm and the females to 30 cm. They have small orange spots on the head and live in South America including Brazil, where the water has a pH of about 5.2 to 6.0 (Gans, 1980; Ernest and Barbour, 1989).

Hemoglobin (Hb) in vertebrates is a globular protein with a molecular mass of 64,500 kDa, containing two alpha and two beta globin chains, where each one is associated with a prosthetic group, in which the iron atom is found in the ferrous state (Fe^{2+}). The alpha globin chain has 141 amino acid residues and the beta chain has 146 residues (Lehninger et al., 1998). X-ray diffraction analysis shows a spherical molecule of approximately 5.5 nm in diameter. Each one of the four globin chains has a characteristic tertiary structure, and they have a tetrameric arrangement, forming the quaternary structure of the molecule (Perutz et al., 1960). Both the tertiary and the quaternary structures of hemoglobin in the different species of vertebrates show similarities when analyzed by X-ray diffraction and by chemical methods (Lehninger et al., 1998). They are considered homologous proteins with marked heterogeneity, frequently related to phylogeny, indicating

that very close species show similarities in the electrophoretic properties of these proteins, which can be utilized as indicators of a phylogenetic relationship (Dessauer et al., 1957).

Hb is responsible for the transportation of oxygen and part of the carbon dioxide. The structural and functional diversity of hemoglobin reflects the extraordinary variations in the morphology and adaptation of animal kingdom members. A great variation of Hb isoforms occurs in vertebrates, and this diversity may represent the necessary adaptation to cope with a changing environment. The electrophoretic pattern of elasmobranchiates, reptiles and amphibians is difficult to interpret owing to the polymerization caused by the reaction between the sulfhydryl groups forming disulfide bridges.

Differences in the Hb properties of different animals have been reported and reflect biological adaptations, which suggest that such studies may be utilized in taxonomy (Gratzer and Allison, 1960; Sullivan and Riggs, 1967).

Reptiles possess two or more major Hb components and other minor components with different mobility. The correlation of the positions in electrophoretic migration of these components with taxonomic findings suggests the synthesis of components under an independent control and a biologically adapted efficiency for oxygen transport, thereby guaranteeing the survival of the species (Sullivan and Riggs, 1967; Torsoni et al., 2002).

Studies of Hb characterization in the Chelidae began in 1957, but there have been very few investigations in Brazil and even fewer for *Rhinoclemmys punctularia*, a species endemic to the Amazon region (Romero et al., 2002). The objective of the present study was to identify the electrophoretic and chromatographic Hb profiles of *Rhinoclemmys punctularia* with the aim of contributing to the understanding of this protein in regard to habitat characteristics.

MATERIAL AND METHODS

Blood samples

Blood samples were collected in tubes with anticoagulant (EDTA) from 18 females and seven males of *Rhinoclemmys punctularia* by puncture of the dorsal venous sinus. Blood samples were obtained using the usual procedures for health evaluation, and, after blood-drawing, they were returned to the environment. The animals were from Pará State, maintained in captive conditions, with adequate amounts of food and controlled water.

Preparation of the hemoglobin solution

A part of the erythrocytes was separated by centrifugation and washed in a 0.8% NaCl solution three times, and one part was lysed with water and precipitated using chloroform. Approximately 100 μ L of the washed red blood cells was lysed with 1% saponin. The Hb solution was stored by refrigeration until use, but no more than 24 h (Bonini-Domingos, 2006).

Hematological profile

The hematocrit and total Hb concentration were estimated by previously described conventional methods. Hb concentration was determined by spectrophotometry utilizing the extinction coefficient of human Hb A, and cyano-methemoglobin was assayed with Drabkin's

solution, where its concentration was calculated using coefficients previously described for turtles (Torsoni et al., 2002).

Hemoglobin electrophoresis

The identification of the Hb components was made by electrophoretic procedures on cellulose acetate with Tris-EDTA-boric acid (TEB) buffer at pH 7, at acid pH on agar gels (Oxoid) and in neutral phosphate buffer in cellulose acetate as previously described by Bonini-Domingos (2006) and Torsoni et al. (2002).

Isoelectric focusing

The isoelectric pattern of the Hb fractions was obtained on 3% agarose gels (Pharmacia) with an ampholyte range of pH 3 to 10, as described by Naoum (1999) for Hb analysis and using human Hb as control.

Globin chain analysis

The globin chains were identified by electrophoretic methods on cellulose acetate at pH 8.7 in TEB-urea-2 mercaptoethanol buffer and on a 12% polyacrylamide gel with 5% acetic acid buffer. After the gel runs, amide black and Coomassie blue stain were used for each gel, respectively, followed by destaining with 7% acetic acid and 30% methanol solution (Bonini-Domingos, 2006).

High-performance liquid chromatography

The VARIANT II (Bio-Rad Laboratories), automated equipment, was utilized with a cationic column and a buffer system with different ionic charge pre-established for Hb analysis, and the procedure was carried out as described by the manufacturer.

RESULTS

The hematocrit results obtained did not indicate significant differences with respect to gender, and the mean value was $31 \pm 2\%$. The mean total Hb was 7.5 ± 0.2 g/dL and did not show significant individual differences in all specimens analyzed.

Sample preparation for the electrophoretic procedures was carried out in two ways. Initially, some tests were performed to standardize the quantity of the sample to be applied and the voltage used in each electrophoretic system, and to adapt them for animal blood analysis. In this procedure, the Hb profile in alkaline pH electrophoresis was different for different hemolysate preparations. The samples of total blood treated with 1% saponin showed the presence of four well-defined Hb fractions. Fraction I was the major component and had a cathodic electrophoretic character and fractions II, III and IV were faster than fraction I and were anodic in nature.

With the samples treated with chloroform, two Hb fractions were demonstrated corresponding to fractions I and III from the first electrophoretic procedure. As chloroform denatures unstable Hb, it is probable that the other two components (II and IV) observed in the preparation with saponin showed molecular instability and were lost in the process of prepara-

tion with chloroform, thus giving a cleaner electrophoretic trace. No individual variations or variations related to the gender of the animal were observed among the samples studied. The same electrophoretic pattern was observed using isoelectric focusing, and the pI of each fraction was established at 7.4 for fraction I and 5.0 for fraction III.

For acid electrophoresis using agar gel and phosphate buffer, the Hb of the different samples displayed a migration pattern similar to that of human Hb S, i.e., anodic character. In cellulose acetate and neutral buffer, the samples showed a migration pattern with two Hb fractions, the major one faster than the minor. After staining, small sub-fractions could be seen, which was probably caused by the polymerization process of the samples, which is very common in reptile Hb.

The globin chain separation at alkaline pH demonstrated two major fractions, and at acidic pH four fractions were demonstrated with the higher concentration coinciding with the migration of beta A globin and the others similar in migration to human gamma globin fractions. When the different Hb fractions isolated from a separated gel were analyzed, we observed similar migration for different components of the alpha and beta globins, involved in the formation of the Hb molecule. The analysis of globin chain in comparison with *Geochelone carbonaria* and *Phrynosops geoffroanus* showed a similar migration for one or the other globin chain for each species.

The chromatographic cathode separation of the hemolysate demonstrated that the major Hb fraction comprised 81.9% with a retention time of 5.17 min and the minor Hb fraction 18.1% with a retention time of 4.14 min.

DISCUSSION

The turtle Hb samples in previous studies demonstrated the ease of subunits to form polymers through disulfide bridges. However, under reducing conditions the fractions observed could be correlated with taxonomic findings. It has been suggested that Hb fractions with a faster electrophoretic pattern are tetramers, while those with slower patterns are polymers (Frische et al., 2001). The formation of polymers does not seem to be associated with the variation in oxygen balance, but rather hunger or malnutrition modify the control of protein synthesis and favor the appearance of slower fractions (Sullivan and Riggs, 1967).

In this study, the Hb electrophoretic profile of the samples treated with chloroform demonstrated a smaller number of better-defined fractions, probably due to the denaturation of some previously identified fractions or polymers. The process of polymerization can also be observed in the electrophoretic procedure under neutral conditions, in which the heating of the electrophoretic process and the interaction of Hb with the buffer allow the appearance of other sub-fractions of Hb formed by the denaturation of tetramers. The animals examined in this study were held in captivity under controlled hypoxia and feeding conditions. Nevertheless, the profile observed in the preparations using chloroform coincides with the electrophoretic trace seen in isoelectric focusing and the chromatographic profile obtained by HPLC, which show two components, one major and one minor, coinciding with the findings in the literature for other Chelidae (Gratzer and Allison, 1960; Sullivan and Riggs, 1967; Torsoni et al., 2002).

The differences in Hb properties of diverse animals have been reported as reflecting biological adaptation. According to Torsoni et al. (1998), the formation of Hb isoforms

presents a possible role as an auxiliary mechanism in protection against reactive oxygen species and other oxidizing molecules, increasing the resistance of the animal in states of hypoxia, functioning as a redox buffer in the red blood cells and reflecting adaptations to different environments.

The Hb profile of *Rhinoclemmys punctularia* was similar to that of aquatic and land Chelidae, and the analysis of the globin chains may support the hypothesis of Gratzer and Allison (1960), that states that synthesized components under independent control are efficient in biological adaptation to transporting oxygen.

The duplication of the globin genes during the evolution of reptiles may be one of the explanations about the independent control of protein synthesis with respect to its function in transporting gases, as well as the evidence of embryonic and adult Hb, and of a common ancestor shown by molecular studies (Wells and Baldwin, 1994; Hamada et al., 2002).

Under hypoxic conditions, the Hb of these animals shows a certain resistance enabling cardiovascular and hematological adjustments, mainly observed in marine turtles which can remain submerged for long periods of time. Very little is understood about the ontogeny of oxygen transport, but embryonic Hb has been proposed to play a fundamental part in the process (Wells and Baldwin, 1994).

Petruzzelli et al. (1996) determined the sequence of the alpha and beta globins and showed that there are differences between the $\alpha 1\beta 1$ and $\alpha 1\beta 2$ when compared with humans, thereby affecting oxygen affinity and increasing the susceptibility to metabolic effectors which reflect the physiological requisites of the animal and adaptation to the environment.

In birds, reptiles, fishes, and amphibians, the red blood cells are nucleated with a high rate of aerobic metabolism and a capability of synthesizing protein during circulation, showing that they are therefore more complex than mammalian erythrocytes. The distribution of the different Hb among the erythrocytes is one of the factors that contribute to the variability of these cells. Depending on the preparation of the solutions for the analysis of Hb isoforms, other proteins, nucleoproteins and salts may be found. The interaction of the Hb with these components identifies fractions with altered electrophoretic mobility.

Erythropoetic activity differs with the age of the circulating erythrocytes and biochemical characteristics of the cells such as composition of the Hb and whether or not there is aerobic activity. The shape of the red blood cells influences the hematocrit as well as the age of the cells. After physical exercise, the hematocrit and total Hb concentrations tend to be reduced in Chelonia, which may reflect biological adaptation.

The volume of oxygen transported to the tissues per unit of blood depends on several factors, including Hb concentration. Thus, the hematocrit and Hb level are parameters that enable the assessment of the oxygen transport capacity in these animals. The values obtained were similar to those reported in the literature for *Geochelone carbonaria* and *Geochelone denticulata* (Torsoni et al., 1998, 2002); however, other studies of species similar to the spot-legged turtle were not found.

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