Protein & Peptide Letters, 2006, 13, 517-523

Rattlesnake Hemoglobins: Functional Properties and Tetrameric Stability

Fabio Renato Lombardi[1,4], Maristella Conte Anazetti[2], Giovanni César Santos[3], Johnny Rizzieri Olivieri[3], Walter Filgueira de Azevedo Jr.[3] and Gustavo Orlando Bonilla-Rodriguez[1,*]

[1]*Depto. de Química e Ciências Ambientais, IBILCE-UNESP, Rua Cristóvão Colombo 2265, S.J. do Rio Preto SP, Brazil CEP 15054-000,* [2]*Depto. de Bioquímica, IB, UNICAMP,* [3]*Depto. de Fisica, IBILCE-UNESP,.* [4]*Depto de Biologia, Centro Universitário de Jales, Rua Francisco Jalles, 1851, Jales, CEP 15700-000, Brazil*

Abstract: The present work analyzed the tetrameric stability of the hemoglobins from the rattlesnake *C. durissus terrificus* using analytical gel filtration chromatography, SAXS and osmotic stress. We show that the dissociation mechanism proposed for *L. miliaris* hemoglobin does not apply for these hemoglobins, which constitute stable tetramers even at low concentrations.

Keywords: Hemoglobin, rattlesnake, tetramer stability.

INTRODUCTION

Several papers report that, in the absence of organic polyphosphates (stripped form), hemoglobins (Hb) from several species, exhibit high oxygen (O_2) affinity, a lower alkaline Bohr effect than that described for human hemoglobin, and non-cooperative O_2-binding at high pH values. However, in the presence of organic polyphosphates, such as adenosine triphosphate (ATP) or inositol hexaphosphate (IHP), these hemoglobins show an evident oxygen affinity decrease and a simultaneous increase of the Bohr effect and cooperativity [1,2,3]. The initial proposal was based in the hemoglobins from the semi-aquatic South American snake *Liophis miliaris*. It was proposed a mechanism for oxygen transport involving oxy-dimer / desoxi-tetramer reversible transitions [1,4]; deoxygenated Hb would predominate in the tetrameric state ($\alpha_2\beta_2$), splitting into dimers ($\alpha\beta$) when oxygenated.

Subsequently, this mechanism was extended to other snake species, such as, *Bothrops alternatus* [5], *Boa constrictor* [3] and *Helicops modestus* [6]. For *Mastigodryas bifossatus* hemoglobins [7] the authors concluded that there were no clear evidences supporting the dissociation hypothesis, at least for that species.

In order to find a structural explanation for the dissociation hypothesis, Matsuura *et al.* [8] sequenced two globin chains of *Liophis miliaris*. The results showed that in the $\alpha_1\beta_2$ interface region there are two substitutions, which the authors identify as essential to understand why that hemoglobin would dissociate. The residue Glu β 101 (G3) was substituted to Val and Glu β 43 (CD2) was changed to Thr. According to the authors, this loss of two negative charges per beta chain would destabilize the tetramer, favoring its dissociation.

In 1999 it was reported [6] that the stripped hemolysate of the semi-aquatic snake *Helicops modestus* displayed high affinity for O_2-binding, and absence of both Bohr effect and cooperativity, what was interpreted as agreeing with the expected behavior for dimeric Hb. In the presence of organic polyphosphates, the hemoglobin showed low oxygen affinity and O_2-binding became cooperative (at pH below 7.5), functional features compatible with the tetrameric form. At higher pH values, the authors reported a gradual decrease of the cooperativity, suggesting a weakening of the electrostatic interactions between ATP and Hb, and dissociation to dimers. The authors proposed that most of ectothermic animals have hemoglobins that dissociate reversibly between tetramers and dimers.

In the present work, we analyzed the functional properties of O_2-binding and the tetrameric stability of the major hemoglobin from the South American rattlesnake *Crotalus durissus terrificus* by analytical gel filtration chromatography and Small Angle X-Ray Scattering (SAXS). In addition, we investigated, by osmotic stress, the number of water molecules differentially bound to the oxygenated form, using as a probe to check dissociation phenomena. We looked for functional and structural evidences of dissociating hemoglobins, as described for the semi-aquatic snake *Liophis miliaris*.

There is an implicit idea that dissociating hemoglobins would occupy an intermediate evolutionary stage between monomeric Hbs and the tetrameric assemblies found for lower and upper vertebrates, respectively [9].

It is essential to remember that vertebrate hemoglobins split into dimers at low concentrations, but for a dissociation phenomenon having a physiological meaning, it should be operational at the high Hb concentration found within the red blood cells. On the other side, most of the reported func-

*Address correspondence to this author at the Depto. de Química e Ciências Ambientais, IBILCE-UNESP, Rua Cristóvão Colombo 2265, S.J. do Rio Preto SP, Brazil CEP 15054-000, Brazil; Tel: (5517) 3221-2361; Fax: 32212356; E-mail bonilla@ibilce.unesp.br

Financial Support: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Grants 00/04932-5 and 03/00085-4 (GOBR), 99/01931-9 (MCA) & 98/12807-4 (FRL). This work was also supported by grants from CNPq, CAPES, Fundo Bunka de Pesquisa (Banco Sumitomo), FUNDUNESP, and LNLS (Brazil).

0929-8665/06 $50.00+.00

© 2006 Bentham Science Publishers Ltd.

tional studies are usually performed using diluted (about 3% v/v) Hb solutions.

MATERIAL AND METHODS

Adult rattlesnake (*Crotalus durissus terrificus*) specimens weighing 300–500g were supplied by the Institute Butantan (São Paulo) and the Central Animal Facility of our Campus. The animals were anesthetized with ether, and blood was collected by cardiac puncture, using a syringe containing buffered saline (0.05M Tris-HCl pH 8.0 containing 0.9% NaCl, 0.2% D-Glucose and 1mM EDTA) solution. Red blood cells were washed four times by centrifugation (2,000 xg for 10 minutes each) with buffered saline. Hemolysis was accomplished with buffer 0.03M Tris-HCl pH 9.0 containing 1mM EDTA, followed by centrifugation (8,000 xg for 60 minutes). The stripped form (Hb without phosphates) was obtained by dialysis against the same buffer and concentrated by centrifugation using Amicon Centriprep-30, followed by gel filtration on Sephadex G-25 (Sigma) on a 2.6 x 30 cm column equilibrated with the same buffer.

Hemolysate purification was performed on DEAE-Sepharose Fast Flow (*Pharmacia*) using a linear saline gradient between 0.03M Tris-HCl buffer pH 9.0 and the same buffer, containing 0.04M NaCl. The isolated components were further deionized by several passages through mixed-bed Amberlite MB-1 (*Sigma*), concentrated by centrifugation as described, and stored in aliquots in liquid nitrogen until use. All buffers were prepared using ultra pure water (*ELGA Scientific*). The purity of the samples, stabilized by carbon monoxide, was verified by isoeletric focusing in agarose gels [10]. Human hemoglobin HbAo was also purified from blood collected of a healthy non-smoker adult by ion-exchange chromatography and used in SAXS experiments as a control.

Oxygen equilibrium determinations were performed at 20°C by the tonometric-spectrophotometric method [11] using stripped Hb form and in the presence of anions (ATP and chloride). Protein concentration was about 50-60μM (heme) in suitable 0.05M buffers (Hepes & Tris) covering the pH range from 7.0 to 9.0. The experiments were performed adding catalase and superoxide dismutase in order to decrease methemoglobin formation [12]. Samples containing more than 5% methemoglobin were discarded. The spectra of hemoglobin solutions were collected in the range from 500 to 700nm using a Cary 100 Scan spectrophotometer (*Varian*).

Conformational changes were studied by the osmotic stress method [12,13], estimating the number of water molecules differentially bound to the oxygenated form. These experiments were performed using 0.03M Hepes-NaOH pH 7.5 buffer at 20°C, setting water activity by adding sucrose (analytical grade). Colombo *et al.* [13] demonstrated that binding of sucrose to Hb can be ruled out.

Proton binding per heme (ΔH^+), also known as "Bohr effect" was estimated using equation 1:

$$\Delta H^+ = \Delta \log P_{50} / \Delta pH \quad [14]. \tag{1}$$

The calculation of the binding constants of ATP for both the oxy (K_O) and deoxyhemoglobin (K_D) forms of Hb-II was performed using Equation 2 as a model by non-linear regression based on the Levenberg-Marquardt algorithm,

$$\log(P_{50})_p = \log(P_{50})_a + \frac{1}{4}\log\left(\frac{1+K_D[X]}{1+K_O[X]}\right) \tag{2}$$

where log P_{50p} and P_{50a} refer to the measurements in the presence and in the absence of ATP, respectively, K_D and K_O are the ATP association constants for the deoxy and oxyhemoglobins and X is molar concentration of free ATP [15,3].

The number of oxygen-linked ATP molecules bound per subunit was calculated using Wyman's linkage equation [16]:

$$-\Delta x = \Delta \log P_{50} / \Delta \log[ATP] \tag{3}$$

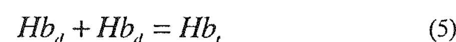
Since the initial slope of $\log P_{50}/\log[ATP]$ showed that a single phosphate binds to each hemoglobin, free ATP was calculated by subtracting Hb concentration from nominal phosphate molarity. These experiments were done using 0.05 M Tris-HCl buffer, pH 7.5 at 20°C.

The dimer to tetramer association constant (${}^4K_2$) was determined by analytical gel filtration chromatography using the method developed by Ackers [17]. The experiments were performed on a Sephadex G-75 column (1 x 20cm) at room temperature, previously equilibrated with 0.05M Tris-HCl buffer, pH 8.0. The hemoglobin samples, stabilized with carbon monoxide to avoid oxidation, were applied as large volumes in the concentration range from 1 to 60μM (heme). The column flow rate was maintained at about 12 mL/hr with a peristaltic pump P-1 (Pharmacia). Continuous absorbance readings of the elution profile were collected using a quartz flow cell attached to a Cary 100 spectrophotometer (*Varian*). The void volume V_0 was determined with blue-dextran solution, and the column was calibrated with Myoglobin (*Sigma*), Carbonic Anhydrase (*Sigma*) and deoxygenated Human Hemoglobin A. The internal volume V_i was measured with ATP (*Sigma*), reading the absorbance at 260nm. The usual approach involves transforming the elution volumes to partition coefficients using the equation 4:

$$\sigma = (V_e - V_0) / V_i \tag{4}$$

where Ve is the elution volume, V_0 the exclusion volume (dextran blue), and Vi the internal volume (ATP). V_0 and Vi correspond to the limits 0 and 1, respectively. Since we did not find differences between using the partition coefficients or the elution volumes for the fitting, we chose to use these for the estimation of ${}^4K_2$.

Dimers' association can be represented by the expression 5 below:



$$Hb_d + Hb_d = Hb_t \tag{5}$$

where the dimer's fraction f_d will be described by equation 6:

$$f_d = \left(\frac{Hb_d}{Hb_d + Hb_t}\right) \tag{6}$$

The association constant for the bound state ($^4K_2$) is represented by the fraction of tetramers divided by the fraction of dimers, as shown by :

$$^4K_2 = \left(\frac{f_t}{f_d^2} * Hb\right) = \left(\frac{1-f_d}{f_d^2 * Hb}\right) \qquad (7)$$

The total Hb concentration is represented by f_d+f_t , and we ca4n rearrange the previous expression as shown below:

$$^4K_2 * f_d^2 * Hb + f_d - 1 = 0 \qquad (8)$$

obtaining a quadratic equation with f_d=X.

Accordingly, we can calculate the root f_d as shown by equation 9:

$$f_d = \left(\frac{-1+\sqrt{1+{}^4K_2 * Hb}}{2 * {}^4K_2 * Hb}\right) \qquad (9)$$

The association constant $^4K_2$ was estimated using nonlinear regression, applied to equation 10:

$$V_w = (f_d * (V_d - V_t)) + V_t \qquad (10)$$

where V_w refers to the Hb solution, f_d is the fraction of dimers, and V_d and V_t represent the elution volumes for the dimers and tetramers, respectively (18=Nenortas and Beckett 1994), constrained within the upper and lower limits obtained independently as described below. In the model f_d was substituted by equation 9.

V_t was obtained by passing deoxygenated human hemoglobin, and estimatives of V_d were obtained as reported by Perutz *et al.* [19] and Chiancone *et al.* [20]:

$$V_d = (V_{Mb} + V_t)/2 \qquad (11)$$

The equipment used to collect Small Angle X-Ray Scattering (SAXS) was a generator of X-ray with rotating anode Rigaku RU300. For the SAXS experiments was utilized a target of copper and radiation K_α=1,54Å. The x-ray beam was colimated with a block slit system [21] and K_β radiation was eliminated using a Ni filter. The equipment potency was 4,5kW (50kV – 90mA). The contributions to the scattering intensity from the solvent, capillary and air were subtracted from the total intensity.

The SAXS measurements were carried out using Hb-II concentrations 10 mg/mL and collecting time between 6–8h, at room temperature (20°C). The buffer utilized in the experiments was 0.03M Hepes pH 7.0 and 8.0.

Information of the quaternary molecular structure were obtained from the distance distribution function p(r):

$$p(r) = \frac{1}{2\pi^2}\int_0^\infty I(h)(hr)\sin(hr)dh \qquad (12)$$

The p(r) function is proportional to the number of pair of electrons separated by the distance r which are encountered by combinations between all the elements of the macromolecule.

The radius of gyration of macromolecule in solution is determined either Guinier approximation or by p(r) function. The advantage to use the p(r) function is that the whole scattering curve is used in the calculation and not only a small fraction of curve, as when Guinier approximation is used. The radius of gyration of the molecule is related to p(r) by:

$$R_g^2 = \left(\frac{\int_0^\infty p(r)r^2 dr}{2\int_0^\infty p(r)dr}\right) \qquad (13)$$

The p(r) function has been determined by indirect Fourier transformation using the ITP program developed by Glatter [22]. This method was shown to be more efficient than the direct Fourier transformation for which termination and background effects are strong [23].This program was also used to determine the desmeared and smoothed scattered intensity I(h), free from smearing collimation effects.

The theoretical p(r) functions were calculated using the program Multibody [24], modified in order to make molecular model building easier [25]. The models used in this program were generated from an arrangement of small spheres that reproduce the desired molecular shape and size. To obtain the theoretical p(r) function, at dimeric form, were utilized atomic coordinates of the human hemoglobin (1HHO), depositing in the data bank of tridimensional structures. We used the coordinates of alpha and beta globins to get a p(r) function of the dimeric form. With these coordinates, building dimer utilizing program Multibody [24] in order calculate radius of gyration and maximum dimension. The same atomic coordinates and procedure were utilized to get a p(r) function to tetrameric form.

RESULTS

The hemolysate of *Crotalus durissus terrificus* showed six components. The analysis of several specimens (not shown here) did not show differences with that pattern. The second component (denominated Hb-II) was the major fraction (about 35%), as shown in Fig. **1**.

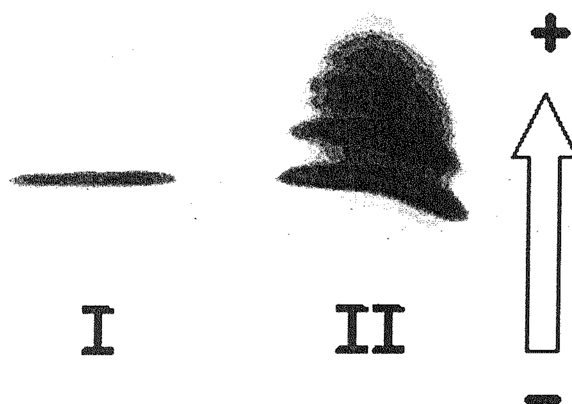


Figure 1. Hb pattern obtained by agarose isoelectric focusing after ion exchange chromatography showing I) purified Hb-II and II) hemolysate. The agarose gel was performed at 4°C, utilizing ampholines covering pH ranges 3-10 and 7-9 (*Pharmacia*).

The hemolysate was submitted to ion-exchange chromatography using DEAE-Sepharose in order to isolate the major (second) component, which was denominated Hb-II. The present work was performed mostly with the isolated Hb-II (Fig. 1), although the data for determination of $^4K_2$ were also obtained with the stripped hemolysate.

Figure **2a** shows the Bohr effect of Hb-II at 20°C. There is a clear biphasic character of the O_2-affinity for the stripped form and in the presence of 0.1M chloride. In the short pH range from 7.0 to pH 7.5, both exhibit a Bohr effect: -0.60 and -0.12 H^+/heme respectively, but proton binding disappears above pH 7.5 for both experimental conditions. The stripped form showed high oxygen-affinity values: $logP_{50}$ = -0.15 (0.7 mm Hg) at pH 7.7. Analysis of the cooperativity (n_{50}), as observed in (Fig. **2b**), shows a small dependence with pH change. At pH 7.0 n_{50}=1.8 and decreased to 1.4 at pH 9.0.

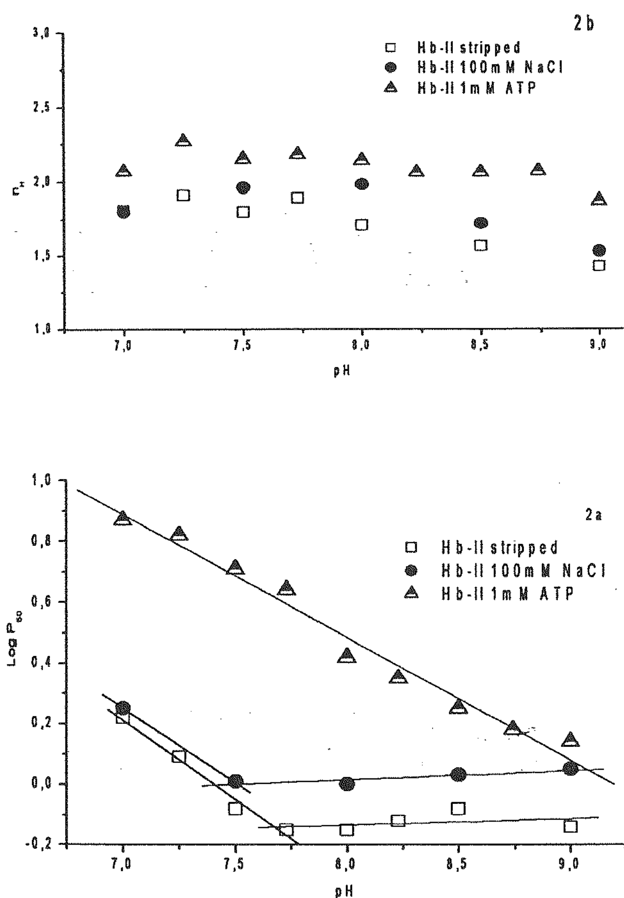


Figure 2. Cooperativity (n_{50}) and oxygen affinity (Log P_{50}) of Hb-II from *Crotalus durissus terrificus* in different pH values at 20°C, in the absence and in the presence of anions (1mM ATP and 0.1M Chloride). Hemoglobin concentration was 60μM (heme).

In the presence of 0.1M NaCl, we verified a small decrease in the affinity of Hb-II in comparison to the stripped form, more clear above pH 8.0. In the presence of chloride most of the data showed a slight increase of cooperativity compared to the stripped Hb.

In the presence of 1mM ATP, the oxygen affinity of Hb-II decreased sharply to log P_{50}=0.64 (P_{50}=4.4 mm Hg) at pH 7.7, and the Bohr effect (-0.44 ± 0.06 H^+/heme) can be observed for the whole pH range tested. Under the same experimental conditions, cooperativity (n_{50}) did not show dependence with varying pH, remaining always around 2.0, higher than for the stripped form.

Figure **3** shows the effect of ATP concentration on oxygen-affinity in the range from 5μM to 5mM. The results showed a differential binding of ATP between the deoxygenated and oxygenated forms (-Δx) of 0.25 per heme, as expected for a 1:1 relationship (ATP:Hb tetramer). The values for the ATP association constants for deoxyhemoglobin were K_D=5.13±0.85x10^5 M^{-1} and K_O=1.92±0.63x10^2 M^{-1}.

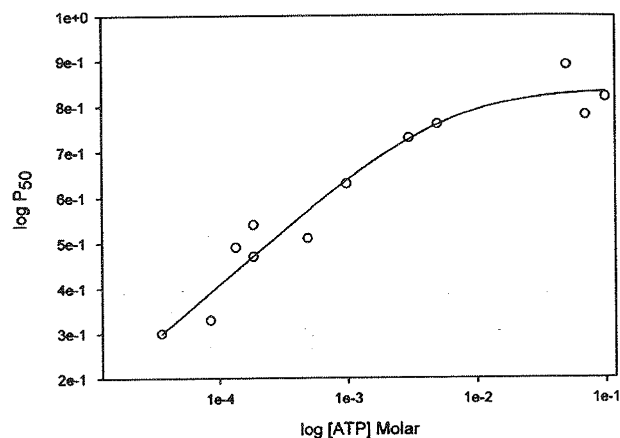


Figure 3. Effect of ATP concentration on the oxygen affinity. The slope of the straight portion of the line indicates the amount of ATP bound per subunit. The experiments were performed with 0.05M Tris-HCl buffer pH 7.5, at 20°C. Hemoglobin concentration was 60μM/heme.

Since Hb dissociation would increase significantly the number of water molecules differentially bound between the oxygenated and deoxygenated forms, since dimers would expose the usually buried area between dimers, we used the osmotic stress approach as a probe to check the existence of that phenomenon. Figure 4 shows the data gathered for three experimental sets: stripped form, in the presence of 0.1M chloride, and in the presence of ATP. The results show that the number of water molecules differentially bound to the oxygenated form (Δn_w), was 36±7 water molecules in the stripped form, increasing to 52±10 water molecules in the presence of chloride. In the presence of 1mM ATP, Δn_w decreased to 45±11 water molecules.

A direct measurement of the tetrameric stability is measurement of the Dimer-Tetramer equilibrium Association Constant ($^4K_2$). Figure **5** shows the relationship between hemoglobin concentration and the centroid elution volume

for *Crotalus* Hb-II. The results obtained after the elution of the stripped form, by analytical gel filtration chromatography, had an elution volume of 8.7±0.08mL, which is very similar to human hemoglobin (Hb A_0): 8.67±0.01 mL between 30-40 μM (heme). At lower concentration (between 5-10 μM) the elution volume was also very similar to human hemoglobin (8.85±0.01mL): 8.84±0.05 mL for Hb-II. We also analyzed the behavior of the hemolysate from *Crotalus*. We did not observe significant differences between the hemolysate (stars) and purified Hb-II (down triangles and squares). The estimated Dimer-tetramer association constant of Hb-II was $1.5\pm0.24\times10^7$ M^{-1}

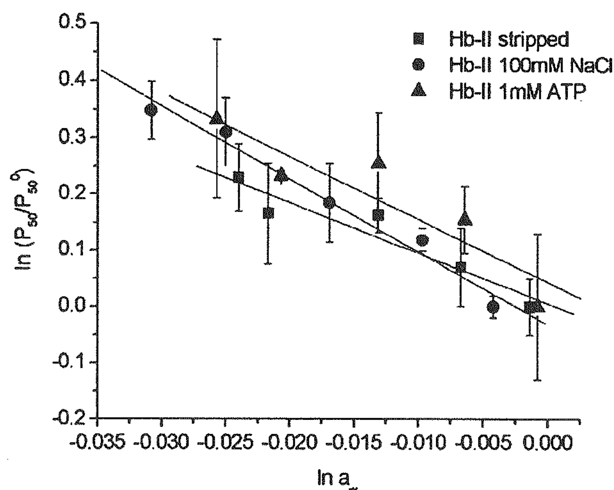


Figure 4. Dependence of ln P_{50} of Hb-II from *Crotalus* on water activity ($\ln a_w$) at different sucrose concentrations. The buffer utilized was 0.03M Hepes-NaOH pH 7.5, at 20°C, in the presence and absence of anions. The sample concentration was 60μM/heme.

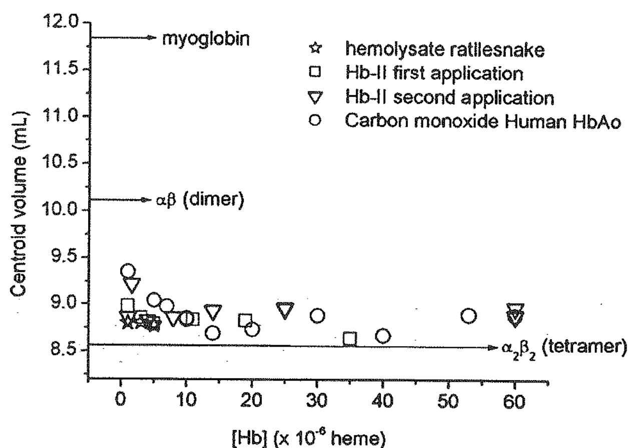


Figure 5. Comparison between the centroid elution volume of *Crotalus* Hb-II and human hemoglobin (HbA_0) (both stabilized with CO), as a function of hemoglobin concentration. The analytical gel filtration chromatography was done in Sephadex G-75, using 0.05M Tris-HCl pH 8.0, at room temperature. Crotalus Hb-II was analyzed in two different sets (applications I and II).

Concerning SAXS experiments, (Fig. 6) exhibits a plot of the distance distribution function p(r) for Hb-II compared to human hemoglobin.

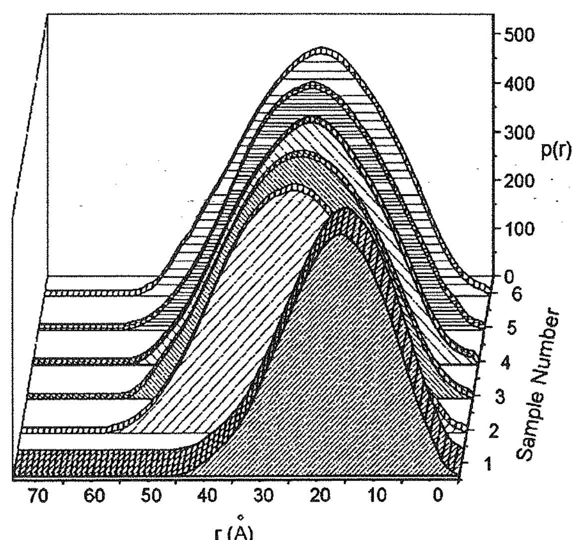


Figure 6. Comparison between the distance distribution functions p(r), considering the curve number (lower right axis): **1.** Dimeric human hemoglobin (theoretical data), **2.** Tetrameric human hemoglobin (theoretical data), and experimental data: **3 and 4.** Experimental data for human hemoglobin at pH 7.0 and 8.0 respectively, **5 and 6.** Experimental data of Hb-II from *Crotalus durissus terrificus* at pH 7.0 and 8.0, respectively. All experiments were performed in 0.03M Hepes buffer pH 7.0 and 8.0, at 20°C. The sample concentration was 0.6mM/heme.

Initially the data concerning the radius of gyration and maximum dimension obtained for human hemoglobin were determined from atomic coordinates (1HHO) of this protein deposited in the Protein Data Bank. The radius of gyration and maximum dimension obtained for this first data set were 23 and 17Å, respectively.

Subsequently, we performed a data collection for a second data set of Hb human, at pH 7.0 and 8.0, in order to confirm the theoretical data obtained in the first data set. The radius of gyration measurements, at pH 7.0 and 8.0, were 23.2 and 22.9Å respectively. These results validated our theoretical calculations.

The data show that Hb-II have a radius of gyration of 22.6Å and maximum dimension of 63Å. These data are very similar to the values observed for human hemoglobin, as discussed above.

DISCUSSION

The number of hemoglobins found for *Crotalus durissus terrificus* is higher than the reported number for other snakes like *Liophis miliaris*, *Bothrops alternatus*, *Mastigodryas bifossatus*, and *Laticauda laticaudata* , having only two components [1,5, 3, 26]. Intra specific variations in the hemolysate pattern could be ascribed to sex, age, season, etc. Although we analyzed blood from several specimens, did not find variations of that pattern.

high pH values [7].

trol, obtaining

agreement with those values reported in the literature [37] ($1.0x10^6M^{-1}$).

The data obtained for Hb-II ($^4K_2$=1.5±0.24x10^7M^{-1}) showed that the assembly, checked both for isolated Hb-II and the stripped hemolysate, contains stable tetramers, being less dissociated than human hemoglobin (Fig. 5). If the hemoglobin had the behavior proposed for *Liophis miliaris* hemoglobin, the elution values would reach those indicated by the "αβ dimer" label in figure 5, or at least close to it.

The data allow us to estimate that, under the experimental conditions used for the functional studies (60μM/heme), Hb-II exhibits 97% of tetramers in solution. Dumolin et. al., [38] analyzed the dissociation constant of human hemoglobin, in the range of concentrations between 0.02 and 4μM. They found that human hemoglobin, at 1μM, possess about 60% tetramers in solution, whereas under similar conditions, Hb-II possess 78% as tetramers.

In order to check the previous data, we carried out experiments of SAXS for Hb-II in solutions at pH 7.0 and 8.0, since the hypothesis concerning Hb dissociation has always proposed a physiological role, what implies that it must occur even at high Hb concentrations.

The radius of gyration and maximum dimension of a macromolecule are important parameters that characterize molecular species with different molecular mass, such as the hemoglobin tetramer (64kDa) and its dimeric form (32kDa).

The data (figure 6) show that Hb-II possess a radius of gyration of 23.6Å at pH 7.0 and 23.8Å at pH 8.0. The maximum dimension of this protein was 63Å.

These results agree with reported values for human hemoglobin, whose radius of gyration is 22.8Å at pH 7.0 and 23.2Å at pH 8.0. The maximum dimension for human hemoglobin and Hb-II was 63Å. However, these data are very different compared to the dimeric hemoglobin, which has a radius of gyration of 17Å and maximum dimension of 50Å.

These results also confirm the presence of tetramers of in solution. Therefore, the idea proposed [6] that most of ectothermic animals have dimeric hemoglobins in the oxygenated form, as proposed for *Liophis miliaris* and *Helicops modestus* is not valid, and should be supported by experimental evidences.

ACKNOWLEDGEMENTS

We thank Dr. Arif Cais for kindly supplying specimens, Mr. J. R. Brandão Neto and Dr. I. Polikarpov (LNLS) for their help in the synchrotron data collection, and Ms. Eliani I. N. Ohira for technical help.

REFERENCES

[1] Focesi, Jr. A., Ogo, S.H., Matsuura, M.S.A. and Say, J.C. (1987). *J. Med. Biol. Res. 20,* 861-864.

[2] Matsuura, M.S.A., Ogo, S.H. and Focesi, Jr. A., (1987) *Comp. Biochem. Physiol. 86,* 683-687.

[3] Bonilla, G.O. Oyama, Jr. S., Nagatomo, C.L., Matsuura, M.S.A. and Focesi Jr., A. (1994) *Comp. Biochem. Physiol. 109A,* 701-707.

[4]. Focesi, Jr. A., Ogo, S.H. and Matsuura, M.S.A. (1990). *Comp. Biochem. Physiol. 96,* 119-122.

[5] Oyama, Jr. S., Nagatomo, C.L., Bonilla, G.O., Matsuura, M.S.A. and Focesi, Jr. A. (1993) *Comp. Biochem. Physiol. 105,* 271-275.

[6] Bonafé, C. F., Matsukuma, A.Y. and Matsuura, M.S.A. (1999) *J. Biol. Chem. 274,* 1196-1198.

[7] Bonilla, G.O., Focesi, Jr. A., Bonaventura, C., Bonaventura, J. and Cashon, R.E. (1994) *Comp. Biochem. Physiol. 109B,* 1085-1095.

[8] Matsuura, M.S.A., Fushitani, K. and Riggs, A.F. (1989) *J. Biol. Chem. 264,* 5515-5521.

[9] Bonilla, G.O. (1992) PhD Thesis, State University of Campinas (UNICAMP). Brazil.

[10] Naoum, P.C. (1999) Eletroforese: técnicas e diagnósticos. Livraria Santos Editora, ed. *2,* 93-100.

[11] Rossi-Fanelli, A. and Antonini, E. (1958) *Arch. Biochem. Biophys. 77,* 478-492.

[12] Colombo, M.F. and Bonilla-Rodriguez, G.O. (1996) *J. Biol. Chem. 271,* 4895-4899.

[13] Colombo, M.F., Rau, D.C. and Parsegian.V.A. (1992) *Science, 256,* 655-659.

[14] Giardina, B. Mosca, D. and De Rosa, M.C. (2004) *Acta Physiol. Scand. 182,* 229-244.

[15] Colowich, S.P. and Kaplan, N.O. (1981) *Methods in Enzymology*: Hemoglobins, Academic Press, New York, *76,* 545-547.

[16] Amiconi, G., Bertollini, A., Bellelli, A., Coletta, M., Condó, S.G. and Brunori, M. (1985) *Eur. J. Biochem. 150,* 387-393.

[17] Ackers, G. K. (1975) *The Proteins I,* 1-94.

[18] Nenortas, E. and Beckett, D. (1994) *Analytical Biochemistry, 222,* 366-373.

[19] Perutz, M.F. Shih D.T. and Williamson, D. (1994) *J. Mol. Biol. 239,* 555-560.

[20] Chiancone, E. (1968) *J. Biol. Chem. 243,* 1212-1219.

[21] Glatter, O. and Kratky, O. (1982) *Small Angle X-ray Scattering.* Glatter O, Kratky, O. (eds) Academic Press, London.

[22] Kratky, O. (1982) *Small Angle X-Ray Scattering.* Glatter O, Kratky, O. (eds) Academic Press, London.

[23] Glatter, O. (1977) *J. Appl. Cryst. 10,* 415-421.

[24] Glatter, O. (1980) *Acta Phys. Aust. 52,* 243-256.

[25] Olivieri, J.R. (1992) PhD Thesis. University of São Paulo. Brazil.

[26] Eguchi, Y. and Eguchi, T. (2002) *J. Protein Chem. 21,* 215-221.

[27] Weber, R.E. (1990) Animal Nutrition and Transport Processes. 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects. *6,* 58-75.

[28] Powers, D. (1980) *Am. Zool. 20,* 139-162.

[29] De Rosa, M.C., Sanna, M.T., Messana, I., Castagnola, M., Galtieri, A., Tellone, E., Scatena, R., Botta, B., Botta M. and Giardina, B. (1998) *Biophysical Chemistry 72,* 323-335.

[30] Bonaventura, C., Tesh, S., Faulkner, K.M., Kraiter, D. and Crumbliss, A.L. (1998) *Biochemistry 37,* 496 – 506

[31] Perutz, M.F., Shih, D.T. and Williamson, D. (1994) *J. Mol. Biol. 239,* 555-560.

[32] Fronticelli, C., Pechik, I., Brinigar, W.S., Kowalczyk, J. and Gilliland, G.L. (1994) *J. Biol. Chem. 269,* 23965-23969.

[33] Colombo, M.F. and Seixas, F.A.V. (1999) *Biochemistry 38,* 11741-11748.

[34] Hundahl, C., Fago, A., Malte, H. and Weber, R.E. (2003) *J. Biol. Chem. 278,* 42769-42773.

[35] Peres, P., Azevedo Jr., W.F., Bonilla-Rodriguez, G.O. (2004) *Eur. J. Biochem. 271,* 4270-4274.

[36] Kodkjaer, P., Wang, T., Taylor, T.W. and Abe, A.S. (2000) *Comp. Biochem. Physiol. A 127,* 49-54.

[37] Valdes, Jr. R., Vickers, L.P., Halvorson, H.R. and Ackers, G.K. (1978) *Proc. Natl. Acad. Sci. 75,* 5493-5496.

[38] Dumoulin, A., Manning, L.R., Jenkins, W.T., Winslow, R.M. and Manning, J.M. (1997) *J. Biol. Chem. 272,* 31326-31332.

Received: July 22, 2005 Revised: February 07, 2006 Accepted: February 07, 2006