FUNCTIONAL BEHAVIOR OF TORTOISE HEMOGLOBIN *Geochelone denticulata*

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(With 4 figures)

ABSTRACT

The hemolysate from *Geochelone denticulata* contains two main hemoglobin components, as shown by ion exchange chromatography and polyacrylamide gel electrophoresis (PAGE). Electrophoresis under dissociating conditions showed three types of globin chains. The apparent molecular mass, as determined by gel filtration on Sephadex G-200, was compatible with tetrameric Hb, which was unable to polymerize. The *G. denticulata* Hb has a P50 value of 9.56 mm Hg at pH 7.4. The Hb oxygenation appears to be under the control of organic phosphates and hydrogen ion since it is strongly affected by those species. In the presence ATP or IHP the P50 values increased to 29.51 mm Hg and 54.95 mm Hg, respectively, at pH 7.4. The n50 was generally lower than 1.5 in stripped Hb, suggesting a dissociation of tetramers. In the presence of organic phosphates n50 values increased to approximately 2.5. The Bohr effect was evident in oxygen equilibrium experiments. The hematocrit (32%) and Hb concentration (5.7 mM as heme) of *G. denticulata* blood were substantially larger than those of *G. carbonaria,* but the methemoglobin levels were similar in both species, approximately 1%. Thus, the oxygen capacity of blood appears to be higher in *G. denticulata* than in *G. carbonaria,* particularly considering the functional properties of their Hbs, which would guarantee the survival of animals.

Key words: hemoglobin, Hb-O_2 affinity, Bohr effect, *Geochelone denticulata*, tortoise.

RESUMO

Comportamento funcional da hemoglobina da tartaruga *Geochelone denticulata*

O hemolisado de *Geochelone denticulata* contém dois componentes principais, de acordo com a cromatografia de troca iônica e PAGE. Eletroforese sob condições dissociantes mostrou 3 tipos de cadeias de globina. A massa molecular aparente, determinada pela filtração em gel sobre Sephadex G-200, foi compatível com Hb tetramérica que foi incapaz de polimerizar. A Hb de *G. denticulata* tem valor de P50 de 9,56 mm Hg em pH 7,4. A oxigenação da Hb parece estar sob controle de fosfatos orgânicos e íons hidrogênio, uma vez que ela é fortemente afetada por essas espécies. Na presença de ATP ou IHP, os valores de P50 aumentaram para 29,51 mm Hg e 54,95 mm Hg, respectivamente, a pH 7,4. O n50 foi geralmente menor que 1,5 na Hb *stripped*, sugerindo dissociação de tetrâmeros. Na presença de fosfatos orgânicos, os valores de n50 aumentaram para aproximadamente 2,5. O efeito Bohr foi evidente nos experimentos de equilíbrio com oxigênio. O hematócrito de 32% e a concentração de Hb de 5,7 mM em heme no sangue de *G. denticulata* foram substancialmente maiores do que da *G. carbonaria*, mas os níveis de metahemoglobina foram similares em ambas as espécies, aproximadamente 1%. Portanto, a capacidade de oxigenação do sangue parece ser maior na *G. denticulata*, particularmente considerando as propriedades funcionais da Hb, que garantiria a sobrevivência dos animais.

Palavras-chave: hemoglobina, afinidade Hb-O_2, efeito Bohr, *Geochelone denticulata*, tartaruga.

INTRODUCTION

In general, the functional properties of hemoglobins (Hbs) appear to be well adapted to meet the different metabolic needs of animals and their environmental constraints. To examine the structure and function of Hb from closely related species of turtle, *G. denticulata* Hb was studied and compared with that of *G. carbonaria* Hb (Torsoni *et al.*, 1997). These two species of turtle belong to the Testudinidae family. *G. denticulata* may be distinguished from *G. carbonaria* by a more drab coloration of its carapace, although coloration alone is sometimes not sufficient to distinguish between the two species. *G. carbonaria* is typically found in grassland or savanna ecosystems while *G. denticulata* is restricted to rain forests (Fretey, 1978; Castaño *et al.*, 1981).

Turtle Hbs exhibit many properties, which differ from those of other vertebrates. Svedberg & Hedenius (1934) studied the sedimentation characteristics of various hemoglobins. They found that mammalian, avian and fish hemoglobins were uniform in size with sedimentation coefficient corresponding to molecular weighs of 60-70 kD. Larger molecules were found in hemolysates of amphibians and reptiles red cells and were formed by polymerized Hb. Sullivan & Riggs (1967a, b) demonstrated that many turtle Hbs readily polymerize under oxidizing conditions. The polymeric Hb may be formed by disulfide bonds among tetramers or by the tetramer-tetramer self-association, which was found in chicken Hb (Riggs, 1998). Turtle Hbs also differ from mammalian Hbs in their number of components. Most turtles show two major components and one or more minor Hb (Sullivan & Riggs, 1967a).

The role of organic phosphates as allosteric modulators of oxygen binding to vertebrate Hbs is well established, and is associated with conformational change in the protein (Benesch *et al.*, 1968). The oxygen binding properties of turtle Hbs are also known to be affected to different degrees by organic phosphates, but the physiological significance of this molecular polymorphism is not yet fully understood. Adenosine triphosphate (ATP) appears to be the main organic phosphate allosteric modulator in reptiles and is present in high concentrations in the red blood cells of a number of species. The erythrocytes of birds and reptiles may also contain inositol pentaphosphate (Coates, 1975; Bartlett, 1976; Davis, 1991).

The present paper reports on the Hb-O_2 affinity and cooperation of purified Hb from *G. denticulata*, including the response to pH and organic polyphosphates. The polymerization of Hb by intermolecular disulfide bridges and extensive formation of mixed disulfides (glutathionyl-Hb) has been reported for turtles (Reischl, 1986), and depends on the occurrence of externally positioned reactive thiol groups. Hb from *G. denticulata*, however, does not polymerize. For this reason we also assessed the availability of SH groups in Hb from this species.

MATERIAL AND METHODS

Preparation of Hb solutions

Blood was obtained from adult *G. denticulata*, by cardiac puncture using a cooled syringe containing 100 µl of sodium heparin (5,000 IU/ml) in 0.9% NaCl per 5 ml of blood. Prior to sampling, the puncture site was disinfected with ether and a local anesthetic (lidocaine) was applied. The hematocrit was estimated by standard methods. Red blood cells were separated by centrifugation for 10 min at 1,100 g and washed three times with 10 vol of cold 1.7% NaCl (w/v), containing 1 mM ethylenediamine tetraacetic acid (EDTA) and then lysed in 25 mM Tris-HCl-1 mM EDTA, pH 8.5, as previously described (Ogo *et al.*, 1979). The stroma was removed by centrifugation for 5 min at 15,000 g, and the supernatant was stripped of salts and small molecules by passing the hemolysate through a 2.0 cm x 50 cm column of Sephadex G-25 equilibrated with the Tris-EDTA buffer above described. The purified Hb was used as soon as possible after preparation and was not frozen.

The Hb concentration in hemolysates was estimated spectrophotometrically using the mM extinction coefficient for human Hb A (Antonini & Brunori, 1971) or as cyanometHb using Drabkin reagent (100 mg NaCN and 300 mg K_3FeCN_6 dissolved in 1 L of water). The Hb concentration (mM) was calculated using an εmM of 11.5 mM^{-1} cm^{-1} at 540 nm (Winterbourn, 1990). The methemoglobin concentration (mM) was estimated based on the absorbances at 577 nm and 630 nm (Winterbourn, 1990) as follows:

$$[\text{Methemoglobin}] = 279A_{630} - 3.04A_{577}$$

Hemoglobin concentrations were expressed per heme group.

Ion exchange chromatography

Stripped Hb (about 100 mg of protein) was dialyzed against 25 mM Tris-HCl plus 1 mM EDTA, pH 8.3, and applied to a column (1.8 cm x 20 cm) of DEAE-cellulose equilibrated with the above buffer. The Hb was eluted with the same buffer by decreasing the pH from 8.3 to 6.5, in a gradient fashion. The separation was carried out at 20°C and the elution profile monitored at 541 nm.

Polyacrylamide gel electrophoresis (PAGE)

Identification of the Hb components was achieved by slab PAGE at room temperature using stacking gels of 3.5% acrylamide and running gels of 7.5% acrylamide. The gels were run at 1.5 mA/sample for about 4 h using 50 mM Tris-glycine, pH 8.9, in the upper buffer reservoir and 50 mM Tris-HCl, pH 8.1, in the lower buffer reservoir (Davis, 1964; Ornstein, 1964). The samples for electrophoresis were prepared by mixing 20 µl of Hb solution (about 50 µg of protein), 20 µl of upper electrode buffer, 20 µl of glycerol and 20 µl of lower electrode buffer. The Hb bands were detected using 0.05% Coomassie brilliant blue G in acetic acid:methanol:water (1:2:4, v/v) and destained in a solution containing 7% acetic acid and 14% methanol.

Globin chains from 30 µg samples of Hb were separated by PAGE under dissociating conditions (Alter *et al.*, 1980) using 12% polyacrylamide gels containing 6 M urea and 2% Triton X-100 in 5% acetic acid. The gels were run at 8 mA for 18 h and then stained for 24 h in 0.05% Coomassie blue followed by destaining in a 7% acetic acid-30% methanol solution.

Gel filtration

The apparent molecular masses of the Hbs were determined by gel filtration on a column (2.5 cm x 100 cm) of Sephadex G-200 equilibrated at 20°C with 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA. Samples of Hb (2.0 ml, 12 mg/ml) were applied and the absorbance of the eluate was read in a Hitachi U2000 spectrophotometer at 541 nm. The void volume (Vo) was determined using blue-dextran. The column was calibrated with dimeric *L. miliaris (snake)* Hb, 32 kDa (Matsuura *et al.*, 1987), tetrameric human Hb (64 kDa) and catalase (240 kDa). The parameters Vo, Vt and Ve were used to calculate the constant Kav, as previously described (Laurent & Killander, 1964). The data were plotted as Kav of the marker proteins and unknown samples vs. molecular mass.

Oxygen equilibrium studies

Oxygen equilibria of stripped hemolysates were carried out at 25°C by the tonometric method (Riggs & Wolbach, 1956). Hb solutions (50-60 µM as heme) were brought to 0.05 M ionic strength in Tris buffer containing 1 mM EDTA in a pH range of 7.0 to 8.4. Absorbance measurements were made in a Hitachi U2000 spectrophotometer over a wavelength range of 500-600 nm. Oxygen binding equilibrium curves were determined in the absence and presence of 2 mM inositol hexaphosphate (IHP) or 2 mM ATP to test for allosteric effects. IHP (dodecasodium salt from corn) and ATP (disodium salt, from yeast) were purchased from Sigma (St. Louis, USA). The Bohr effect ($\Delta \log P50/\Delta$ pH) was estimated by the difference in log P50 values from pH 7.0 to pH 8.0. The Hill coefficient, n50, was determined using Hill plots at saturation values between 30% and 70%. Hb solutions containing more than 7% of methemoglobin were not used for oxygen equilibrium experiments.

Thiol titration

Titration of the reactive sulphydryl groups of Hb was performed at 25°C using 2,2'-dithiobis (5-nitropyridine) (DTNP). To 1.5 ml of recently collected stripped Hb solution (5-15 µM, as heme) in 0.1 M phosphate buffer pH 8.0 was added 30 µl of 5 mM DTNP (3.1 mg/ml in ethanol) and after 20 min, the A386 nm was measured against a blank containing Hb at the same concentration. The absorption of DTNP in phosphate buffer was subtracted and the number of thiol groups was then calculated using an εmM of 14.0 mM^{-1} cm^{-1} at 386 nm (Winterbourn, 1990).

RESULTS

Hematocrit, blood Hb concentration and methemoglobin levels were determined for 9

specimens of *G. denticulata*. The hematocrit value of $32 \pm 2\%$ v/v corresponded to a Hb concentration of 5.7 ± 0.2 mM (as heme) or 9.1 g%. The methemoglobin content in freshly collected hemolysate was $1.0 \pm 0.1\%$ of the total Hb.

Chromatographic separation of the total hemolysate on DEAE-cellulose resulted in two main components labeled Hb1 (75%) and Hb2 (25%), based on their order of elution (Fig. 1). Identification of the Hb components was performed by PAGE (Fig. 2).

Gel filtration studies indicated that *G. denticulata* Hb was not capable of forming polymers and eluted as a single peak corresponding to an apparent molecular mass of about 64 kDa. Aged Hb solutions (7 days after blood collection) exhibited a similar elution profile.

The globin composition of total hemolysate and of the individual components was analyzed by PAGE under dissociating conditions. The total hemolysate consisted of three globin chains named as G1, G2 and G3: component Hb1, consisted of G1 and G3 and component Hb2 contained G1 + G2 globin chains (Fig. 3).

The content of sulphydryl groups/tetrameric Hb as determined in freshly collected stripped hemolysate was 1.7 ± 0.3 (n = 5) available thiol groups based on titration with DTNP.

The oxygen binding properties of *G. denticulata* Hb were investigated in stripped hemolysate at pH values ranging from 7.0 to 8.4 and in the presence of 2 mM ATP and 2 mM IHP (Table 1, Fig. 4). The partial pressure of oxygen at half saturation (P50) at 25°C was 9.56 mm Hg at pH 7.4.

The influence of the heterotropic effectors ATP and IHP on oxygen affinity is illustrated by the decrease in O_2 affinity: the values of P50 increased to 29.51 and 54.95 mm Hg, respectively. Minimal oxygen affinity was observed at the lower pH values.
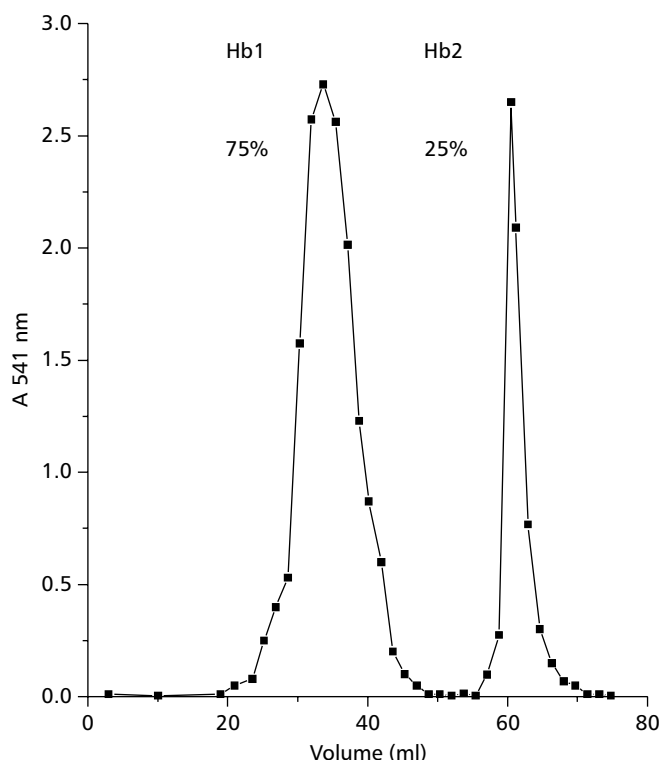

Fig. 1 — Elution profile of stripped *G. denticulata* Hb from a column of DEAE-cellulose eluted with 25 mM Tris-HCl containing 1 mM EDTA using a pH gradient of 8.3-6.5. The absorbance was determined at 541 nm. Hb1 and Hb2 indicate the two main fractions obtained (based on the order of elution).
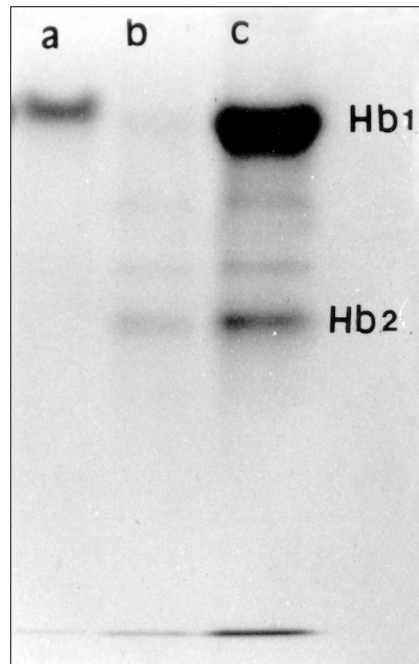
Fig. 2 — PAGE profile of *G. denticulata* Hb at pH 8.9. The gels were stained with Coomassie blue. (a) and (b) Hb1 and Hb2, respectively, (c) total hemolysate.
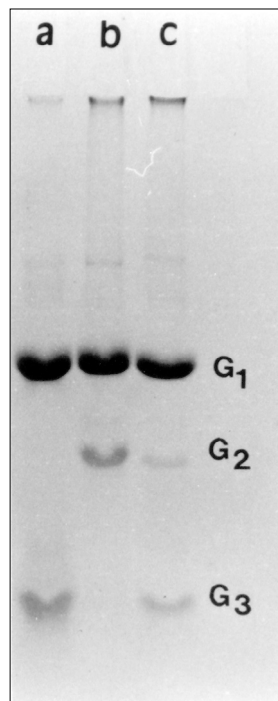

Fig. 3 — PAGE of the globin chains of *G. denticulata* Hb in the presence of Triton X-100 and urea, at acid pH. The gels were stained with Coomassie blue. (a) and (b) Hb1 and Hb2, respectively, (c) total hemolysate.

The Bohr effect ($\Delta \log P50/\Delta pH$) for stripped Hb measured at pH 7.0 to 8.0, was –0.57; the addition of 2 mM ATP or 2 mM IHP resulted in values of –0.66 and –0.69, respectively (Fig. 4). The apparent cooperativity between subunits suggested by the Hill coefficient (n50) is shown in the inset of Fig. 4. In stripped Hb, the Hill coefficient was little influenced by pH; the n50 values varied 1.0-1.5 at pH from 7.0 to 8.4. In the range of pH studied the addition of 2 mM ATP or 2 mM IHP induced an increase in n50 values to approximately 2.0-2.5.

DISCUSSION

The complexity of chelonian Hbs compared to that of mammalians is well demonstrated by the variety Hb components observed in hemolysates from several turtle species (Sullivan & Riggs, 1967a; Rucknagel *et al.*, 1984; Torsoni *et al.*, 1997). The chromatographic profile (Fig. 1) and electrophoretic pattern (Fig. 2) of fresh total hemolysate from *G. denticulata* showed two main components (Hb1 and Hb2). An identical pattern was obtained for aged Hb solutions on PAGE. This finding contrasts with that for *G. carbonaria* in which the fresh Hb presented a complex electrophoretical pattern and varied with the duration of storage, demonstrating its polymerization capacity (Torsoni *et al.*, 1997). The polymerization may be formed by disulfide bridges among tetramers. Disulfide bonds may also yield mixed disulfide between Hb and non-protein SH groups that are demonstrated by the high electrophoretical heterogeneity of *G. carbonaria* Hb (Torsoni *et al.*, 1996).
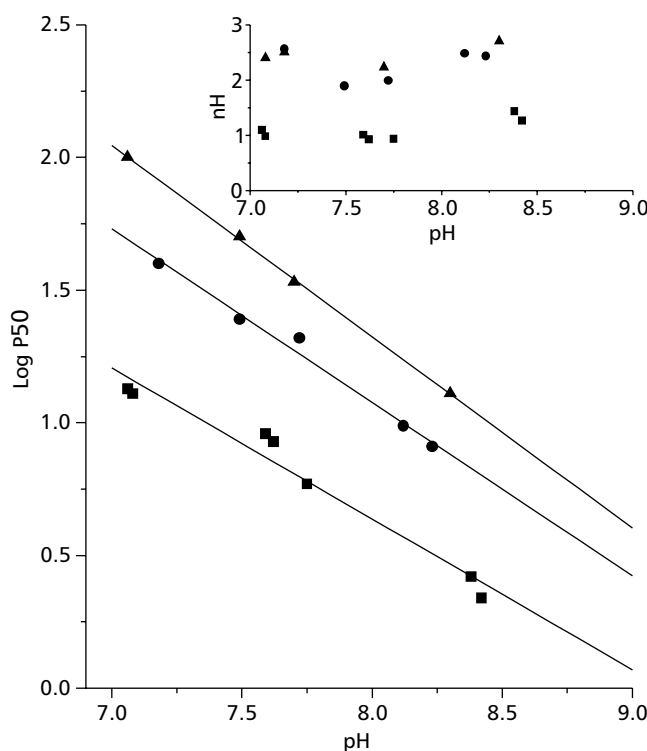

Fig. 4 — Oxygen affinity (log P50) and n50 values for *G. denticulata* Hb at 25°C as a function of pH. The Hb solutions were buffered against 50 mM Tris-HCl + 1 mM EDTA. (■—■), stripped Hb; (●—●), Hb + 2 mM ATP; (▲—▲) Hb + 2 mM IHP. The lines were obtained by linear regression.

In order to examine the structural differences between the main Hb components (Hb1, Hb2) of *G. denticulata* PAGE was performed under dissociating conditions. Three polypeptide subunits (G1, G2 and G3) were obtained from the total hemolysate. The globin chains of each component purified by ion exchange chromatography were G1 + G3 for Hb1 and G1 + G2 for Hb2 (Fig. 3). Thus, the two main Hb components possess the G1 globin chain, with the second globin chain dissimilar, showing a structural difference between them, as it also occurs *in G. carbonaria* (Torsoni *et al*., 1997). A similar situation has been observed in the turtles *C. picta belli* and *P. hillarii,* (Rucknagel *et al*., 1984) in which the two Hb components possess the same β chain.

Gel filtration studies indicated that *G. denticulata* Hb did not polymerize, although polymerization is frequently seen in turtle Hbs (Sullivan & Riggs, 1967a; Reischl, 1989), including that of *G. carbonaria* (Torsoni *et al*., 1997), which is able to form aggregates larger than tetramers. Polymerization of turtle Hb may occur *in vivo* under conditions of environmental stress and *in vitro*, after hemolysis (Riggs *et al*., 1964; Sullivan & Riggs, 1964). The ability of vertebrate Hbs to polymerize depends on the availability of cysteinyl residues. Only 1.7 SH groups per Hb (tetramer) were observed for *G. denticulata.* This value of 1.7 per Hb tetramer would reflect the different number of available SH group in different polypeptide chain. The free SH probably corresponds to cysteine β93, a highly conserved residues also found in human Hb; the latter also does not form disulfide bonds. In contrast*, G. carbonaria* Hb consists of four SH groups/Hb (tetramer) (Torsoni *et al*., 1996)*,* which would explain the ability of this protein to polymerize.

The Hb-O_2 affinities of *G. denticulata* Hb (expressed as log P50, i.e., the logarithm of oxygen partial pressure required to half-saturate Hb) in the absence and presence of allosteric effectors (ATP, IHP) are shown in Table 1 and Fig. 4. The oxygenation properties of Hbs from different animals vary and are related with the metabolism and environment of the animal from which the Hb derives (Riggs, 1964). The intrinsic Hb-O_2 binding properties of *G. denticulata*, yielded P50 values higher than those of *G. carbonaria* under the same experimental conditions (Torsoni *et al*., 1997). Indeed, *G. denticulata* Hb showed a low O_2 affinity with P50 about 9.56 mm Hg, versus 6.30 mm Hg for *G. carbonaria* Hb, at pH 7.4. In the presence of saturating concentrations (2 mM) of the allosteric effectors ATP and IHP, higher P50 values for *G. denticulata* Hb oxygenation (29.51 and 54.95 mm Hg, respectively versus 14.10 and 20.12 mm Hg, respectively, for *G. carbonaria* at pH 7.4) were observed, suggesting that the release of oxygen from Hb to tissue occurs at a higher O_2 pressure in this species than in *G. carbonaria* (Tosoni *et al*., 1997).

ATP is the major allosteric cofactor present in the adult turtle erythrocytes (Coates, 1975; Bartlett, 1976; Davis, 1991). IHP, which is considered one of most powerful effectors of Hb affinity (Arnone & Perutz, 1972) showed greater effect on the reduction of oxygen affinity of turtle Hb. Nevertheless, IHP, which was used as a substitute for inositol pentaphosphate (IPP) to test the allosteric effect on *G. denticulata* Hb, is not a physiological phosphate within turtle red blood cells; instead, several authors (Bartlett, 1976; Bartlett, 1980; Lutz & Lapenas, 1982; Davis, 1991) found IPP in the erythrocytes of adult turtles, usually at a concentration of 5-10 times lower than ATP. Therefore, *in vivo,* the Hb oxygen affinity would be lower than in stripped Hb.

TABLE 1

Summary of oxygen equilibria for *Geochelone denticulata* Hb.

Samples	P50 (mm Hg)			Log P50/ΔpH (7.0-8.0)
	pH 7.0	pH 7.4	pH 8.0	
Stripped Hb	15.85	9.56	4.37	–0.57
Hb + 2 mM ATP	53.70	29.51	11.75	–0.66
Hb + 2 mM IHP	104.71	54.95	21.38	–0.69

Data obtained from Fig. 4.

A large Bohr effect (Δlog P50/ΔpH) for O_2 binding was observed for *G. denticulata* Hb (Table 1, Fig. 4) and showed a similar amplitude with purified Hb alone and in the presence of ATP or IHP, indicating that the pH dependence of the oxygen affinity was little affected by the presence of organic phosphates. The large Bohr effect and the sensitivity of P50 values to phosphate modulations suggest that the regulation of the Hb-O_2 binding may involve factors which control the blood pH and the concentrations of organic phosphates in red blood cells as occurs in almost all vertebrate Hb. The Bohr effect values were similar to those displayed by *G. carbonaria* Hb, and were somewhat higher than for *Testudo graeca*. In the latter species, the Bohr shift apparently plays only a small role in modifying the Hb-O_2 affinity in situations of apnoea (Burggren *et al.*, 1977).

The oxygen binding process for *G. denticulata* Hb did not appear to be auto-catalytic as was also the case for *G. carbonaria* (Torsoni *et al.*, 1997) and most other turtle species (Burggren *et al.*, 1977). The Hill coefficient was generally lower than 1.5, suggesting a dissociation of tetramers to dimers, as occurs in some species of snakes accompanying the oxygenation in the absence of but not in the presence of ATP or IHP (Matsuura *et al.*, 1987; Focesi *et al.*, 1990; Bonafé *et al.*, 1999). In the presence of the physiological allosteric effector, ATP, the n50 values increased to approximately 2.0-2.5 (inset Fig. 4). Similar values were found in the presence of IHP. It seems, therefore, that the oxygen binding property of *G. denticulata* Hb is cooperative *in vivo*.

As far as these results could be extended to *in vivo* conditions, the oxygen discharge to the tissues and the binding in the pulmonary level would be greatly facilitated both by decrease of pH and by change of ATP level. The decrease of pH would make the nucleotide bind more strongly to the hemoglobin and, therefore, easily release the gas due to the high decrease of the oxygen binding constant to the protein.

The volume of oxygen carried to the tissue by a unit of blood depends upon a number of factors, including the amount of Hb present in the blood and the proportion of Hb that is actually functioning in oxygen transport. Thus, the hematocrit, Hb concentration and methemoglobin levels are important parameters for evaluating the oxygen capacity of the tortoise blood. The high

methemoglobin levels described for certain turtle species (Reischl, 1989), may have considerable physiological importance since oxidized Hb does not bind oxygen reversibly.

The hematocrit (32%) and Hb concentration (5.7 mM as heme) of *G. denticulata* blood were substantially larger than those of *G. carbonaria* (Torsoni *et al.*, 1997), but the methemoglobin levels were similar in both species, approximately 1%. Thus, the oxygen capacity of *G. denticulata* blood appears to be higher than of *G. carbonaria*, particularly considering the functional properties of Hb, which provide the release of oxygen from Hb to the tissue at higher O_2 pressure in *G. denticulata.*

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