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Allosteric water and phosphate effects in *Hoplosternum littorale* hemoglobins

Patricia Peres[1], Walter F. de Azevedo Júnior[1] and Gustavo O. Bonilla-Rodriguez[2]

[1]*Departamento de Física, and* [2]*Departamento de Química e Ciências Ambientais, IBILCE-UNESP, State University of São Paulo, São José do Rio Preto SP, Brazil*

This paper reports the results obtained using the osmotic stress method applied to the purified cathodic and anodic hemoglobins (Hbs) from the catfish *Hoplosternum littorale*, a species that displays facultative accessorial air oxygenation. We demonstrate that water potential affects the oxygen affinity of *H. littorale* Hbs in the presence of an inert solute (sucrose). Oxygen affinity increases when water activity increases, indicating that water molecules stabilize the high-affinity state of the Hb. This effect is the same as that observed in tetrameric vertebrate Hbs. We show that both anodic and cathodic Hbs show conformational substrates similar to other vertebrate Hbs. For both Hbs, addition of anionic effectors, especially chloride, strongly increases the number of water molecules bound, although anodic Hb did not exhibit sensitivity to saturating levels of ATP. Accordingly, for both Hbs, we propose that the deoxy conformations coexist in at least two anion-dependent allosteric states, T_o and T_x, as occurs for human Hb. We found a single phosphate binding site for the cathodic Hb.

Keywords: hemoglobin; osmotic-stress; catfish.

Water plays a unique and ubiquitous role in biomolecules and biochemical reactions; folding, stability, and function of protein molecules are all influenced by interaction with water molecules [1]. A central role for water in determining structure and regulating function of proteins is becoming increasingly evident, as water molecules act as allosteric effectors by preferentially binding to a specific protein conformation [2].

Significant changes in protein hydration are conveniently studied by the osmotic stress method, a simple method [3,4] based on water activity of the solution, which is altered by changing the concentrations of solutes (polyols, sugars and amino acids).

Fish hemoglobins (Hbs), which display a wide range of oxygen binding properties and allosteric effects, and are characterized extensively both structural and functionally, are excellent candidates for such an analysis. In several cases fishes have iso-Hbs with marked functional differentiation in terms of allosteric control and cooperation.

This study analyzed the effect of water on the cathodic and anodic Hbs from *Hoplosternum littorale*, a catfish from the Amazon basin that displays facultative accessorial air oxygenation by air gulping and gas-exchange by using a partially modified intestine when water oxygen falls below a critical concentration [5]. The Hbs present different oxygen affinities and responses to allosteric effectors. Its anodic Hb displays a reverse Bohr effect in the stripped form, changing to a normal response to protons in the presence of ATP. The major component, named cathodic Hb, exhibits a pronounced alkaline Bohr effect and, accordingly, a high response to pH changes [5].

Generally, only one molecule of organic phosphate (NTP) is bound per deoxy-Hb molecule, although additional binding sites for ATP have been proposed [5–8]. For the cathodic Hb from the fish *H. littorale*, Weber *et al.* [5] suggested the possible existence of one additional phosphate binding site.

Materials and methods

Hemolysate preparation

Blood was collected by caudal vein puncture from adult specimens at the Central Animal Facility of the State University of São Paulo (IBILCE-UNESP) at São José do Rio Preto SP (Brazil). The animals were anesthetized using benzocaine (1 g per 15 L of water) and, after blood collection, the specimens showed a fast recovery from anesthesia. Subsequent Hb purification procedures were carried out at low temperature (around 4 °C) using ultrapure water (Elga Sci.). Red blood cells (RBC) were washed by centrifugation four times with buffered saline (containing 50 mM Tris/HCl pH 8.0 and 1 mM EDTA). RBC were frozen in liquid N_2 and hemolysis was accomplished by adding buffer A (30 mM Tris/HCl pH 9.0), followed by clarification by centrifugation (1000 ***g*** for 1 h). Using the same buffer, but containing 0.2 M NaCl, initial purification was performed by gel filtration on Sephacryl S-100 HR (Sigma) on an equilibrated 2.6×30 cm column. The

Correspondence to G. O. Bonilla-Rodriguez, Departamento de Química e Ciências Ambientais, IBILCE-UNESP, State University of São Paulo, Rua Cristovão Colombo 2265, São José do Rio Preto SP, CEP 15054–000. Fax: +5517 2212356, Tel.: +5517 2212361, E-mail: bonilla@qca.ibilce.unesp.br

Abbreviations: 2,3-BPG, 2,3-biphosphoglycerate; Hbs, hemoglobins; RBC, red blood cells.

Note: A website is available at http://www.qca.ibilce.unesp.br/labbioq.html

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fractions containing Hb were pooled and dialyzed overnight against buffer A and subsequently purified on Q-Sepharose using a linear saline gradient between 0 and 100 mM of NaCl. The isolated components were concentrated by centrifugation on Amicon microconcentrators. Analytical isoelectric focusing was performed in agarose gel. The Hb solutions were stored in liquid N_2 in aliquots that were thawed immediately before oxygen binding studies were carried out.

Osmotic stress experiments

Water activity was varied by addition of pure sucrose (Acros Organics). In the osmotic stress method changes in the Hb–oxygen affinity are related to changes in water activity that can be converted to changes in protein hydration by use of linkage equations [2,9]. Oxygen binding experiments were performed with 60 μM (heme) Hb solutions in 30 mM Hepes buffer, pH 7.5 in the presence and absence of ATP and NaCl, as described by Colombo and Bonilla-Rodriguez [4]. All equilibrium measurements were carried out at 20 °C by the tonometric method [10]. The functional parameters P_{50} (O_2 partial pressure at half saturation) and cooperativity (n_{50}) were calculated from Hill plots by linear regression around half saturation. Hemoglobin and methemoglobin concentrations were estimated using the extinction coefficients for human Hb [11]. Data obtained from samples containing more than 5% methemoglobin (final concentration) were discarded.

The Hb solution osmolalities (Osm) were determined after binding experiments from freezing point depression measurements using an Osmette A model 5002 osmometer (Precision Systems Inc.). The osmolality was transformed to the natural logarithm of water activity through the following relationship [4]:

$$\ln a_w = \frac{\Delta}{K_f M_w} = -\frac{-Osm}{M_w} \quad (1)$$

where Δ is the freezing point depression, $K_f = 1.86$ K·kg·mol^{-1} is the cryoscopic constant, and M_w is the molarity of pure water (55.56 mol·L^{-1}).

The effect of water as a single heterotropic ligand on oxygenation is typically analyzed with the following linkage equation [12,13]:

$$\frac{d\ln(P_{50})}{d\ln(a_w)} = \frac{\Delta n_w}{4} = -\left(n_w^{oxy} - n_w^{deoxy}\right) \quad (2)$$

where a_w is the water activity. The slope of the linkage plot $\ln(P_{50})$ vs. $\ln(a_w)$ gives the differential number of water molecules bound in the conformational transition from the deoxy to the oxy structures, Δn_w.

The slopes were compared according to Zar [14] using GRAPHPAD PRISM version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). We tested the null hypothesis (no significant difference between slopes) for paired experiments using a P threshold of 0.05.

Calculation of the association constants of ATP to the forms oxygenated and deoxygenated of the cathodic Hb

The 'x' number of molecules of ATP differentially bound per heme between the deoxy- and oxy-Hb was calculated using the linkage equation of Wyman [12]:

$$x = \Delta \log P_{50} / \Delta \log[\mathrm{ATP}] \quad (3)$$

The association constants with ATP were calculated by a nonlinear regression fitting using the program SIGMAPLOT (Jandel Scientific, San Rafael, CA, USA), according to the equation below [15]:

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

where $\log(P_{50})_p$ is the logarithm of P_{50} measured in the presence of ATP, $\log(P_{50})_a$ is measured in the absence of ATP, K_D and K_O are the association constants to the deoxygenated and oxygenated forms, respectively, and X is the free molar concentration of ATP. O_2 binding experiments were performed at pH 7.5 and at 20 °C.

Results

O_2 equilibria of Hb at various osmolalities

Cathodic Hb. We tested the oxygen affinity of the cathodic Hb as a function of water activity in different experimental conditions: for the stripped Hb (in an ATP and chloride-free buffer solution), in the presence of 0.1 mM and 1 mM of ATP, in a buffer containing 100 mM NaCl, and a last set containing 100 mM NaCl + 1 mM ATP. The plots (Fig. 1) show that $\ln(P_{50})$ varies linearly with changes in the water activity (a_w); this is in agreement with Colombo *et al.* [2] and Hundahl *et al.* [16]. Oxygen-affinity decreased for all the experimental sets containing ATP and/or chloride, in comparison with the stripped Hb.

The analysis of the data according to the Wyman equation (Table 1) shows that the cathodic Hb in the stripped form, binds 41 ± 9 extra water molecules in the

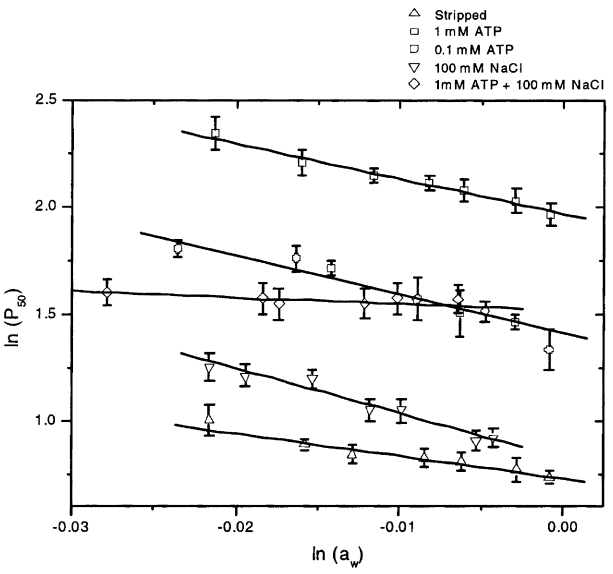

Fig. 1. Relative shift in Hb^{ct} ln(P_{50}) as a function of water activity (a_w). The different conditions were: stripped Hb, 0.1 mM and 1 mM of ATP, 100 mM NaCl and 100 mM NaCl + 1 mM ATP. The straight lines are a linear fit of the data using the integrated form of Wyman linkage equation (Eqn 2). Experimental conditions: 30 mM Hepes buffer, pH 7.5 and 20 °C.

Table 1. Change in the number of water molecules (Δn_w) ± SD bound to the cathodic Hb in the transition from fully deoxy to fully oxy forms, measured by tetramer in different experimental conditions. Experiments for the cathodic Hb were performed in 30 mM Hepes buffer pH 7.5 and 20 °C. The slopes were compared using the stripped condition as a reference.

Sample	Experimental condition	Δn_w ± SD Wyman	Statistical analysis	Correlation coefficient
Hb^{ct}	Sucrose, stripped Hb	41 ± 09	Reference	0.972
	Sucrose + ATP 1 mM	66 ± 12	*	0.988
	Sucrose + ATP 0.1 mM	73 ± 08	**	0.905
	Sucrose + NaCl 100 mM	85 ± 12	*	0.911
	Sucrose + ATP 1 mM + NaCl 100 mM	4 ± 16	**	0.893

*$P = 0.001 < P < 0.01$, **$P < 0.001$.

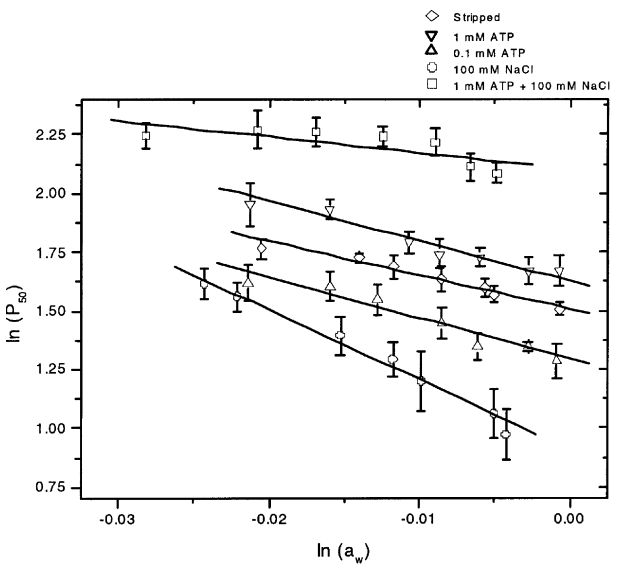

Fig. 2. Relative shift in Hb^{an} ln(P_{50}) as a function of water activity (a_w). The different conditions were (stripped Hb, 0.1 mM and 1 mM of ATP, 100 mM NaCl and 100 mM NaCl + 1 mM ATP). The straight lines are a linear fit of the data using the integrated form of Wyman linkage equation (Eqn 2). Experimental conditions: 30 mM Hepes buffer, pH 7.5 and 20 °C.

T to R transition. In the presence of 0.1 mM and 1 mM of ATP these numbers increase to 73 ± 8 and 65.6 ± 12, respectively, and in the presence of 0.1 M chloride this rises to 85 ± 12 water molecules. In the simultaneous presence of 100 mM NaCl and 1 mM of ATP Δn_w decreased drastically to 4 ± 16 water molecules, but oxygen affinity, measured by P_{50}, was higher than in the presence of 1 mM ATP, a finding also reported by Weber *et al.* [5]. All the Δn_w values obtained in the presence of ATP and/or Cl^- were significantly different than that from the stripped form.

Anodic Hb. The other fraction studied here has a similar behavior concerning a_w when compared with the cathodic Hb and other fish Hbs [16], also indicating preferential binding of water molecules to the R state. The allosteric effectors significantly affect water and O_2 binding (Fig. 2), and both chloride and 0.1 mM ATP induced an increase of O_2 affinity, also described also by Weber *et al.* [5]. Linear fitting of the data (Table 2) showed a Δn_w of 58 ± 8 water molecules for the stripped form, increasing to 68 ± 12 in the presence of 0.1 mM and 1 mM of ATP. In the presence of NaCl, Δn_w rose to 116 ± 16 water molecules, the only significant difference when compared to the stripped Hb. In the presence of 1 mM of ATP and 100 mM of NaCl, Δn_w decreased to 28 ± 8. This value was not found to be significant, probably due to the poor linearity of the data with a higher a_w. In contrast to the cathodic Hb, the combined effect of Cl^- and ATP induced the largest decrease of O_2 affinity.

Calculation of the association constants of ATP to the oxygenated and deoxygenated forms of the cathodic Hb

Using Eqn (3) (Fig. 3), we calculated the slope, a Δx of $0.23 \pm 8 \times 10^{-5}$ ATP molecules/heme to Hb, which confirms the binding of a single ATP molecule per Hb tetramer.

Table 2. Change in the number of water molecules (Δn_w) ± SD bound to the anodic Hb in the transition from fully deoxy to fully 'oxy' forms, measured by tetramer in different experimental conditions. Experiments for the anodic Hb were performed in 30 mM Hepes buffer pH 7.5 and 20 °C. The slopes were compared using the stripped condition as a reference.

Sample	Experimental condition	Δn_w ± SD Wyman	Statistical analysis	Correlation coefficient
Hb^{an}	Sucrose, stripped Hb	58 ± 08	Reference	0.965
	Sucrose + ATP 1 mM	68 ± 12	ns	0.891
	Sucrose + ATP 0.1 mM	68 ± 12	ns	0.888
	Sucrose + NaCl 100 mM	116 ± 16	*	0.993
	Sucrose + ATP 1 mM + NaCl 100 mM	28 ± 08	ns	0.873

ns = $P > 0.05$, *$P < 0.001$.

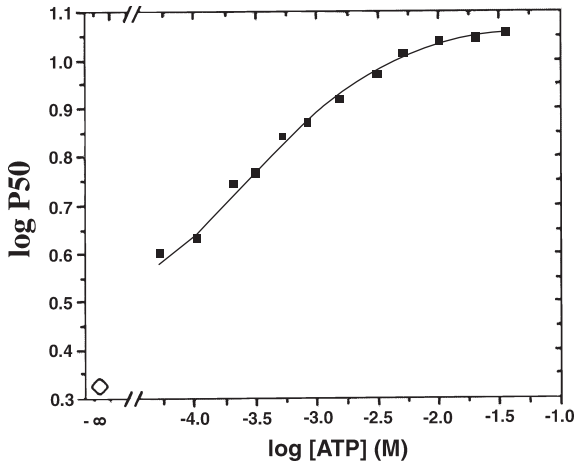

Fig. 3. Variation of Hb^{ct} oxygen affinity vs. free ATP concentration at pH 7.5. The concentration of ATP, varied from 0 to 35 mM. The symbol o represents value of logP_{50} in absence of ATP.

It was possible to calculate the ATP association constants to the oxygenated and deoxygenated Hb according to Eqn (4). The value of the binding constant in the deoxygenated form (K_D) was $2.2 \times 10^5 \pm 1.3 \times 10^4$ M^{-1}, and for the oxygenated form (K_O) was $2.6 \times 10^2 \pm 3.3 \times 10^1$ M^{-1}.

Discussion

Hemoglobin O_2 equilibria as a function of water activity

The analysis of conformational changes by the osmotic stress approach [2] has proven to be reliable, despite its experimental simplicity, as direct measurements of water binding by a crystal quartz microbalance [17] showed agreement with the calculated Δn_w. Using water activity as a probe allows, accordingly, to analyze conformational changes induced by allosteric effectors that would be difficult or expensive to follow by other methods, and this possibility has been used by other authors to study Hbs [16,19]. Because *H. littorale*'s anodic and cathodic Hbs have been functionally well described by Weber *et al.* [5], we decided to focus our analysis on their conformational transitions, and secondarily on phosphate binding.

Although having very different oxygen-binding properties, both Hbs respond to an increase in water activity with an increase on oxygen affinity, indicating preferential binding of water molecules to the R state, also reported for other vertebrate Hbs [16,19], despite their functional differences.

Concerning the values found for Δn_w during oxygenation, for the cathodic Hb, in the stripped condition the value is smaller than in the presence of saturating levels of Cl^- or ATP, suggesting that in the absence of anions, the Hb assumes a new conformational state, different from the classical T state (T_x), adopting the intermediary state, denominated T_0, more hydrated than the T_x. This fact is in agreement with the findings reported by Colombo and Seixas [3] for human Hb, and it shows that this Hb, although showing a significant reverse Bohr effect, follows a pattern that has already been described for human Hb, which has a normal response to proton binding. Hemoglobins with a reverse Bohr effect appear to have some relationship with air breathing, as they appear in fishes and amphibians with adaptations, as pointed out by Weber *et al.* [5]. Interestingly, the anodic Hb showed a distinct behavior, with chloride exerting the only significant effect on its conformation. This high value is close to that reported for the anodic eel Hb in the presence of KCl and GTP ($\approx$ 118), although the authors did not test chloride alone [16].

The fact that the O_2 affinity from the anodic Hb increased in the presence of chloride or low phosphate concentrations was first reported by Weber *et al.* [5], using data gathered in the presence of low concentrations of NaCl and 2,3-biphosphoglycerate (2,3-BPG) at pH 7.5. The unexpected increase in the O_2 affinity could be interpreted as a result of binding to the R state, as proposed by the previous authors, but could also suggest an excess of negative charges in the Hb central cavity (α_1–β_2 interface), and anion binding to this region would destabilize the interdimeric interface. The large Δn_w obtained for this Hb is probably related to the role exerted by chloride binding, but its explanation would require primary sequence determination and crystallographic analysis or at least molecular modeling, using a crystallized Hb as a template.

When we compare Δn_w values obtained in the presence of ATP and chloride, however, for both Hbs, the last anion induces larger conformational changes, evidence that phosphate at high concentrations can lock the Hb structure in a T-like conformation, similar to previous findings from Caôn [18].

Calculation of the ATP association constants to the oxygenated and deoxygenated forms of the cathodic Hb

The value obtained for the association constant of ATP to the deoxygenated form (K_D) of the cathodic Hb is about 10 times larger regarding 2,3-BPG binding to human Hb ($K_D = 3.6 \times 10^4$ M^{-1}) [20], showing that ATP binds to the cathodic Hb more strongly than 2,3-BPG to human Hb. The obtained value is similar to found for Hb-II of the fish *Piaractus mesopotamicus* (3.1×10^5 M^{-1}) [21]. Concerning the estimative for the ATP association constant to the oxygenated form (K_O), this agrees with that reported for oxygenated Hb human ($K_o = 3.5 \times 10^2$ M^{-1}), and greater than for *P. mesopotamicus* Hb (2.7×10^1 M^{-1}). This strong phosphate binding, combined with a reverse Bohr effect, would ensure effective control of O_2 uptake with high affinity, as well as its delivery by the interplay of the pH and phosphate concentration within the red blood cells.

In conclusion, we showed that both Hbs investigated here respond to an increase in water activity by stabilizing the R state conformation, and that the presence of an intermediate conformational state controlled by anion binding in the oxygenation process is similar amongst Hbs, similarly as found by other authors [3,16]. We did not observe evidences of the presence of additional phosphate binding sites to the cathodic Hb, as suggested by Weber *et al.* [5].

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