ATP-induced TetramORIZATION and Cooperativity in Hemoglobin of Lower Vertebrates*

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The importance of intracerythrocitary organic phosphates in the allosteric control of oxygen binding to vertebrate hemoglobin (Hb) is well recognized and is correlated with conformational changes of the tetramer. ATP is a major allosteric effector of snake Hb, since the absence of this nucleotide abolishes the Hb cooperativity. This effect may be related to the molecular weight of about 32,000 for this Hb, which is compatible with the dimeric form. ATP induces a pH-dependent tetramerization of deoxyHb that leads to the recovery of cooperativity. This phenomenon may be partially explained by two amino acid replacements in the β chains (CD2 Glu-43 → Thr and G3 Glu-101 → Val), which result in the loss of two negative charges at the αβ interface and favors the dissociation into dimers. The ATP-dependent dimer ↔ tetramer may be physiologically important among ancient animal groups that have similar mutations and display variations in blood pH that are governed by these animals’ metabolic state. The enormous loss of free energy of association that accompanies Hb oxygenation, and which is also observed at a much lower intensity in higher vertebrate Hbs, must be taken into consideration in allosteric models. We propose that the transition from a myoglobin-like protein to an allosteric one may be of evolutionary significance.

In vertebrates, hemoglobin (Hb) exists as a tetramer in its intracerythrocitary environment, and it is this form that is involved in the classic structural change from a low to a high O2 affinity molecule in the presence of increasing O2 concentrations. This phenomenon, known as cooperativity, is reflected in the sigmoidal shape of the O2 saturation curve.

Protons and organic phosphate are important in the physiologic transport of O2 in most vertebrate groups, since they stabilize the low affinity form of Hb (1, 2).

Previous studies have demonstrated an oxygen-induced dissociation of snake Hb at physiologic pH and Hb concentration, as well as in the presence of high levels of organic phosphate (3). The structural basis of this phenomenon is the replacement of amino acid residues β-CD2–43 and β-G3–101 at the αβ interface which is responsible for tetramer stabilization. These key residues, normally both glutamic acid, are replaced by threonine and valine, respectively, in snake Hb (4). This loss of negative charges would disturb the interface contact, leading to a pronounced tendency of Hb to dissociate into dimers. Since these residues are also replaced in most hemoglobins from ectothermic animals (5–9), this suggests that a dissociation of Hb occurs during oxygenation. The physiologic role of such Hb dissociation is considered in the present investigation.

EXPERIMENTAL PROCEDURES

Hemoglobin Preparation—Adult snakes of both sexes weighing 200–400 g were obtained from the Instituto Butantã (São Paulo) and were kept in the laboratory until bleeding. The hemolysate was prepared as described by Rossi-Fanelli and Antonini (10) and was freed of salts and small organic molecules by passage through a Sephadex G-25 column (2.0 × 90 cm) equilibrated with 1 mM Tris-HCl, pH 9.0 (11), to produce "striped" Hb.

Measurement of Redox Potentials—The redox titrations were carried out according to Antonini et al. (12). Five milliliters of Hb solution (140 μM as heme) were deoxygenated in a tonometer and then transferred anaerobically, with continuous flush of N2, to the titration half-cell, which contained 0.1 M Tris-HCl plus 0.1 M NaCl (pH range: 7.0–8.0). Thiobin was added as a mediator in a molar ratio to protein of 2–4%. The oxidation of deoxyHb was performed by the stepwise addition of a degassed solution of 5 mM potassium ferricyanide. The measured electrode potentials were referred to the normal hydrogen electrode (13). The oxidation-reduction potential at 50% of oxidation provided the midpoint potential (Ep/2).

O2-Hb Equilibrium—The experiments were performed at 20 °C in 0.1 M Tris-HCl buffer of different pH values containing 0.1 M NaCl, using a penometric-spectrophotometric method (14). The protein concentration was 80 μM (as heme).

RESULTS AND DISCUSSION

To gain insight into the possible physiologic role of pH and ATP in the subunit assembly of snake (Helicops modestus) Hb, we investigated the Hb-O2 equilibrium as a function of proton concentration in the presence or absence of ATP (Fig. 1). Stripped snake Hb showed a high affinity for O2 and no allosterism, in accordance with a molecular mass compatible with the dimeric form (3, 10, 15). In the presence of organic phosphates, the molecule became cooperative (nH = 2) with a low O2 affinity at a pH up to 7.4. With increasing pH, the Hb gradually lost cooperativity, suggesting a weakening of the electrostatic interaction between ATP and Hb. As a result, the latter tended to assume the properties of stripped Hb. The pH sensitivity cannot be attributed exclusively to a classic Bohr effect in view of the dimerization process that is also present.

Based on these unusual findings, we investigated the redox potential of snake Hb under the same conditions as those used for the O2 equilibrium curves in order to better understand the Hb properties in the presence of ATP at different pH values. This approach, applied to either tetrameric Hbs or myoglobins, has been employed to show the conversion of the deoxy to the met form and its close correlation with oxygenation equilibrium curves, since the potentiometric curves share similarities with the equilibrium ligand binding curves for Hb (12, 16–20).Fig. 2A illustrates that snake Hb had a peculiar behavior in this experiment. The redox potential of stripped Hb did not
change with a pH of up to 7.6, but decreased at higher pH values. The resulting curve was similar to that of myoglobin and corroborated our expectation that stripped Hb is dissociated even in the deoxygenated form. The progressive decrease in $E_h$ observed with increasing pH in both stripped and ATP-Hb is correlated to the extend of water ionization on the sixth coordinate of heme iron (18). ATP dramatically changed the redox equilibrium profile. In the presence of ATP, the $E_h$ value at pH up to 7.22 was constant and higher than in the absence of the nucleotide. However, the $E_h$ value decreased sharply in the pH range of 7.22–7.38. This observation is consistent for a tetrameric Hb in which the classic allostERIC model is found. The redox equilibrium curve at pH > 7.80 superposed the stripped Hb curve, indicating the complete release of ATP from its binding site. In the pH range of 7.38–7.80, the redox potential presented a curve compatible with equilibrium between dimers and ATP-bound tetramers. From Fig. 2A we estimated the quantitative contribution of the different molecular forms of Hb (Fig. 2B). The dissociation of tetrameric Hb into dimers was observed primarily between pH 7.38 and 7.55 and varied from 0 to 80%.

The inset in Fig. 2A shows the corresponding Hill plots of the redox equilibrium. At pH 7.0, the oxidated Hb retained its tetrameric form, indicating that ATP remains bound independently of the degree of oxidation. At pH 7.80, the Hb dissociated and had the same $n_H$ values as stripped Hb. However, at pH 7.38, the biphasic behavior indicated that above 50% of oxidation, R-met Hb became very unstable and immediately dissociated into dimers. The substantial differences in the Hb properties described above assume a great significance when the physiological state of ectothermic vertebrates are considered. Several studies have reported large blood pH changes when ectothermic animals are subjected to different temperature or stress conditions (21–23). In such situations, the proton concentration would be particularly important in influencing the binding of ATP to Hb, thereby altering the protein’s $O_2$ affinity. These functional properties may be present in a large array of animals from related groups, since the replacement of amino acid residues at key positions of $\alpha_1\beta_2$ contact is present (5–9).

Fig. 3 proposes a general model for $O_2$ transport by snake and other related vertebrate Hbs in which the ATP plays a central role. In the dormancy state, when low $O_2$ transport is required and the blood pH is increased, the Hb exists in a dimeric form that acts as a reserve supply of $O_2$ in a manner similar to myoglobin. In stress or high activity, the decrease in pH promotes ATP-induced tetramerization and allostery, thereby resulting in a significant $O_2$ release. Thus in this dynamic interchange, ATP and pH changes serve to integrate the physiology of $O_2$ supply. This novel model provides new insight into $O_2$ transport when compared with higher vertebrates in which the cooperative ligand binding of Hb is based on a switching between quaternary states of the Hb tetramer with different $O_2$ affinities (24).

From a thermodynamic aspect, the classic T-R model of Monod-Changeux-Wyman (MCW model) does not take into ac-
Hemoglobin Tetramerization by ATP in O₂ Transport

![Diagram](https://example.com/diagram.png)

**Fig. 3. In vivo model of O₂ transport by snake Hb.**

**Fig. 4. Gibbs free energy levels of Hb subunit association and oxygen binding by human (A) and snake (B) Hb.** D = dimer; T = tetramer; X = O₂. ∆G(n) = free energy of subunit association with "n" molecules of O₂. ∆G(2,n) and ∆G(4,n) = free energy of O₂ binding of dimer and tetramer, respectively, with "n" − 1 molecules of O₂.

...count the free energy of association between αβ dimers (25). This situation was considered by Weber (26), who demonstrated that O₂ binding to human Hb is a first order reaction that is inconsistent with the two-state model. It is noteworthy that the dissociation of snake Hb is an extreme example of decreasing the Gibbs energy of association between dimers, since progressive oxygenation is linked with dissociation into more reactive dimeric species. Thus, the presence of the classic R state is theoretical and difficult to detect experimentally (Fig. 1), except in Hb obtained by redox potential experiments (Fig. 2A). In Fig. 4, we propose a diagram of the Gibbs free energy of O₂ binding with snake ATP-Hb in comparison with stripped human Hb. The most striking feature is the inversion of the free energy of association between oxygenated dimers despite the presence of ATP. Moreover, the binding of the first/second O₂ molecule results in a much higher affinity of tetrameric snake Hb to further O₂ binding than is the case with human Hb.

The evolutionary adaptation, study of Hb structure and function, has been extensively discussed (27, 28). The Agnatha, lampreys, and hagfish, the most primitive group of vertebrates, have monomeric Hb in which the O₂ transport mechanism is accomplished by a dimer ↔ monomer transition. This disaggregation leads to a higher affinity state and allows cooperative behavior (29–31). The singular properties of snake Hb reported here may point to the origin of a stable dimeric molecule that could have important evolutionary implications for heme-heme interactions. The αβ dimeric form may represent an intermediate evolutionary stage of the classic allosterism of vertebrate Hbs, where ATP serves as a central allosteric mediator. The molecular properties of such Hbs may reflect the physiological functions of ectothermic Hb, particularly the adaptations to exogenous and endogenous factors such as ambient hypoxia, temperature, activity, and dormancy. Finally, the mechanism of dimer-tetramer transitions during O₂ transport may represent an intermediate stage of evolution to the stable tetrameric Hb found in higher vertebrates.

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