Fish hemoglobins

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Abstract

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Vertebrate hemoglobin, contained in erythrocytes, is a globular protein with a quaternary structure composed of 4 globin chains (2 alpha and 2 beta) and a prosthetic group named heme bound to each one. Having myoglobin as an ancestor, hemoglobin acquired the capacity to respond to chemical stimuli that modulate its function according to tissue requirements for oxygen. Fish are generally submitted to spatial and temporal O_2 variations and have developed anatomical, physiological and biochemical strategies to adapt to the changing environmental gas availability. Structurally, most fish hemoglobins are tetrameric; however, those from some species such as lamprey and hagfish dissociate, being monomeric when oxygenated and oligomeric when deoxygenated. Fish blood frequently possesses several hemoglobins; the primary origin of this finding lies in the polymorphism that occurs in the globin loci, an aspect that may occasionally confer advantages to its carriers or even be a harmless evolutionary remnant. On the other hand, the functional properties exhibit different behaviors, ranging from a total absence of responses to allosteric regulation to drastic ones, such as the Root effect.

Introduction

Hemoglobin is surely the most studied of all proteins. Indeed, the molecular analysis of hemoglobin has been the testing ground for many contemporary ideas and concepts in biology, particularly the understanding of the crystallographic structure and structure-function relationship of proteins, ligand binding, structural transitions between conformers, allosteric interactions, and others (1,2).

The ability of the aerobic metabolism of animals to satisfy the demands for oxygen is only possible thanks to the role of proteins such as hemoglobins, contained in red cells or erythrocytes, which facilitate the dissolution of large quantities of gas and transport it to the tissues, where it functions as the final acceptor of electrons originating from oxidative catabolic reactions (3). The function of hemoglobin seems to be adapted to the different metabolic necessities of animals and to constant environmental changes (4).

Among vertebrates, fish are the animals of greatest interest to research on adaptive processes due to their position in the evolutionary chain and to their genotypic characteristics. These features guarantee enormous plasticity in the selection of environments and the capacity of some species to cope with different conditions of temperature, pressure, salinity, and oxygen availability. They are also the group of animals with the highest number of multiple hemoglobins (3).

Numerous studies reporting biochemical adaptations in fish are available in the literature. According to these reports, fish present a high genotypic plasticity, i.e., their genetic material is able to undergo modifications, with its expression varying according to environmental changes. The ability of fish to form hybrids, such as Tambacu "created" by crossing a female Tambaqui with a male Pacu, is confirmed by the high degree of genetic variation detected among the different species, and the existence of species that preserve features closely similar to those of their ancestors (5).

The evolution of vertebrate hemoglobins has been subjected to many selective restrictions. The duplication of the ancestral gene that gave origin to the alpha and beta subunits from a common ancestor occurred at least 450 million years ago, and great changes in the environment have occurred during this period. The evolution of hemoglobins was totally neutral and mainly involved selected adaptations associated with physiological and environmental changes (6).

The hemoglobin structure

Hemoglobin is composed by polypeptide chains, known as globins, each having a prosthetic group called heme, identical in every fish species studied to date. On the other hand, globins differ from species to species and among isoforms. Remarkably,

globins seem to occur in all organisms and tissues, exhibiting a diversity of quaternary structures and a large number of functions apart from oxygen transportation and storage, as illustrated by cytoglobins and neuroglobins (7,8).

The organization of globin genes has been well defined in mammals, birds and amphibians. In mammals, the alpha and beta globin genes are located on different chromosomes; in humans, the alpha globin gene is located on chromosome 16 and the beta globin gene on chromosome 11. In chickens, the alpha and beta globin genes are also on different chromosomes. In amphibians, such as the *Xenopus*, the genes are on the same chromosome. In teleosts, including the Atlantic salmon, carp and zebrafish, the adult alpha globin gene is adjacently linked to the beta globin gene and the embryonary globins are completely different from adult globins (9).

In vertebrates, the hemoglobin molecule includes four globin chains that create a stable tetramer (Figure 1) formed by two alpha-like and two beta-like chains with 141 and 146 amino acid residues, respectively. Each one contains a heme group, which allows the binding of four oxygen (O_2) molecules in a reversible form, according to the scheme below:

$$Hb + {}_4O_2 = Hb(O_2)_4$$
$$\text{deoxy-Hb} \quad \text{oxy-Hb}$$
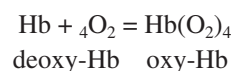
The main function of hemoglobin is to transport oxygen from the gas-exchange organs to peripheral tissues. It must be able to bind oxygen strongly but at the same time to release it when necessary, depending on the partial pressure of the gas (10). Reversible oxygen binding is possible thanks to the heme group, specifically with the participation of an iron atom in the ferrous form, Fe^{2+}.

For some species of primitive fish such as lampreys and hagfish, reversible hemoglobin dissociation occurs in response to oxygen binding. In the case of lampreys, the

Figure 1. Hemoglobin molecule with four globin chains, two alpha-like and two beta-like chains each bearing a heme group, responsible for the reversible binding of oxygen. The figure was generated by Pymol 0.99 (DeLano Scientific LLC, Palo Alto, CA, USA) using a pdb file (1OUU) from trout hemoglobin, *Oncorhynchus mykiss*.
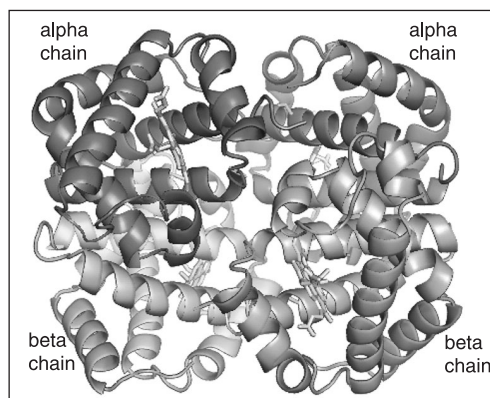
oxygenated form is monomeric, undergoing associations with dimers and tetramers when deoxygenating (11). Figure 2 shows a monomer of lamprey hemoglobin. In *Myxine glutinosa* (hagfish) there are three monomeric hemoglobins in the oxygenated form, but when they release O_2, there is an association to form heterodimers and heterotetramers (12).

Allosteric control

The quaternary structure guarantees the possibility of interaction among the subunits of hemoglobins and the emergence of new properties, known as allostery. This is important when the oxygen saturation curve is considered. The curve has a sigmoidal structure, demonstrating the existence of cooperativity among the globins. In other words, O_2 binding occurs with progressive facilitation between the first and fourth subunits, representing extremes of low and high affinity for the gas, respectively. The property of an O_2-binding subunit modifying the subsequent binding of the same molecule is known as homotropic allosteric interaction (6).

The most accepted model explaining the allosteric properties of hemoglobins is the one proposed by Monod et al., in 1965. According to these investigators, hemoglobin presents two conformations with different affinity for oxygen: the low-affinity state is denominated "tense" or "T" and the high-affinity form is known as "relaxed" or "R". The T and R states predominate in deoxygenated and oxygenated hemoglobin, respectively (6).

Nevertheless, other ligands are involved in the cooperative effects that occur when oxygen binds to hemoglobin. They modulate the affinity for the gas according to physiological necessities. Such mechanisms constitute the "fine tuning" of hemoglobin function, and since they affect O_2 binding while binding to different sites, they represent the heterotropic allosteric interactions

(3). The chemical species capable of causing such alterations are denominated allosteric effectors or agents, and these effectors preferentially bind to one of the conformational states, T or R, stabilizing them, and therefore they reduce or increase O_2 affinity, respectively (13). Binding of the effector may occur to both canonical or classic states as described first for phosphates binding to the hemoglobins from dromedary (*Camelus dromedarius*) (14) and the "matrinxã" fish (*Brycon cephalus*) (15). The heterotropic effectors can induce different sub-states in addition to those previously mentioned, as was determined in hemoglobins from the fish "tamoatá" (*Hoplosternum littorale*) (16).

Both H^+ and CO_2 also bind to hemoglobin, affecting its affinity for oxygen and subtly changing the three-dimensional structures. The effect of H^+ on the O_2 affinity is called the Bohr effect. For most hemoglobins, an increase in H^+ concentrations, that is, a pH decrease, lowers the oxygen affinity (3,17), characterizing the alkaline or normal Bohr effect. Under these circumstances, hemoglobin presents a lower oxygen affinity and releases the gas at the tissue level. In
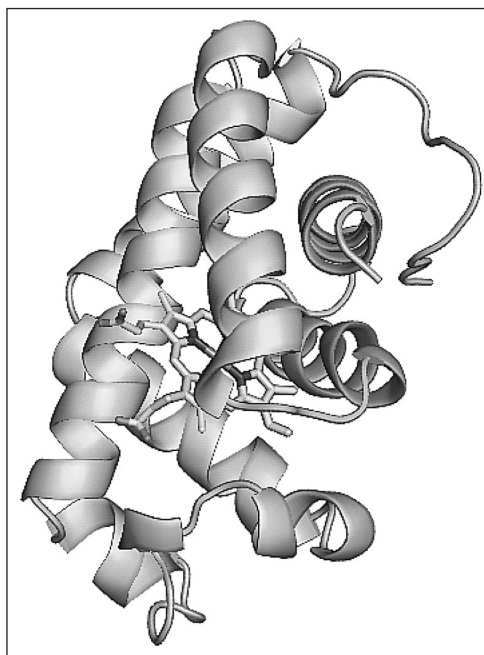

Figure 2. A monomer of lamprey hemoglobin generated by Pymol 0.99 (DeLano Scientific LLC) using the 1F5P.pdb file. The figure shows the globin with its α-chain segments and the heme group.

other words, the protein removes a proton from the environment acting as a base, while the opposite occurs in gas exchange organs, where it releases protons and binds oxygen.

A variation known as the acid or reverse Bohr effect consists of an increase in oxygen affinity with decreasing pH, a phenomenon that occurs even in adult human hemoglobin (Hb A) at pH lower than 6.5 (10).

The alkaline Bohr effect plays an important role, modifying the oxygen affinity of hemoglobin, increasing its saturation in the lungs or gills and facilitating its release in the tissues, particularly when skeletal muscle tissue releases lactic acid during intense exercise (18).

The residues responsible for the normal Bohr effect have been identified: the major contribution is due to His146ß that forms an ionic interaction with Asp94α in the deoxygenated form. Another important residue is Val1ß, whose participation depends on several factors: 1) the presence of chloride, 2) the acetylation or formylation of the residue, and 3) the proportion of CO_2 combined with the N-terminus (3).

Organic phosphates, particularly 2,3-BPG, IPP, ATP, and GTP, preferentially bind to deoxy-hemoglobin, lowering its oxygen affinity. The residues responsible for phosphate binding to deoxy-hemoglobin have positive charges: His2, Lys82 and His143 from the beta chains and the amino group of Val1 from the alpha chains (19).

Blood oxygen affinity depends on the concentration of hemoglobin, its sensitivity in relation to heterotropic effectors, the concentration of these ligands inside the erythrocytes, and the temperature. Changes in these conditions may modify blood oxygen affinity, adjusting the gas transfer according to the physiological necessities and/or environmental conditions (20).

Fish hemoglobins

Vertebrate hemoglobins are contained in specialized cells known as red cells or erythrocytes; they may have a nucleus or not. In the great majority of fish, the erythrocytes are oval, nucleated and larger than in mammals (21) and their numbers range from 800 thousand to 3.5 million/mm^3. Leukocytes are numerous, ranging from 20 to 50 thousand, but some species have levels of up to 100,000/mm^3 (22).

In fish, erythropoiesis starts in the egg sac followed by an intermediate cell mass. These sites have transitory origins, that is, they constitute the primitive generation of red cells which made their first phylogenetic appearance in fish and were subsequently represented in all classes of vertebrates, including mammals. The production of erythrocytes is located on the pre-splenic tissue of the gastrointestinal tract and in the spleen, as seen in Cyclostoma, Sarcopterygii, and Chondrichthyes, whereas in teleosts it is located in the kidneys, with or without splenic participation (23). In rainbow trout (*Oncorhynchus mykiss*), the embryonary globins are initially expressed in the intermediate cell mass located below the notochord in 6- to 7-day embryos, while adult globins are expressed in erythroid cells in the blood, liver and kidney (9).

Hemoglobins are particularly important in fish adaptation as they constitute an interface between the organism and the environment (24). Fish particularly face a very variable environment and temporal and spatial alterations in oxygen availability, in contrast to terrestrial animals.

However, a variety of environmental and physiological adjustments are observed in fish exposed to environmental hypoxia in order to improve O_2 transfer. Many Amazonian fish obtain O_2 directly from the air when submitted to hypoxia, being obligate or facultative air breathers. The anatomical modifications that allow accessory air-breathing include changes in the gills, mouth, stomach, intestine, and vascularization of the swim bladder (25,26). Species that do not have

this capacity are obliged to accomplish metabolic and behavioral changes to deal with limited O_2 availability (25,27). Such adjustments involve ventilation frequency and volume, heart rate, increase in the number of erythrocytes, hematocrit and hemoglobin concentrations, changes in organic phosphate concentrations, presence of iso-hemoglobins with different functional properties, and metabolic depression (25).

Almeida-Val and Val (5), who studied *Prochilodus nigrans*, showed that hemoglobin O_2 affinity varies according to a seasonal variation of the levels of GTP and ATP within the erythrocytes. A low affinity was observed during the summer, when the levels of GTP and ATP were elevated. On the other hand, a higher O_2 affinity was observed during the winter, simultaneously with low levels of GTP and ATP. Val et al. (28) observed a higher oxygen affinity due to changes in the intra-erythrocytic levels of organic phosphates in *Pterygoplichthys mustiradiatus* when exposed to hypoxic conditions. Affonso (29) also reported a seasonal change in the affinity of hemoglobin for oxygen in *Hoplosternum littorale*.

There are decreases in ATP and GTP concentrations in Amazonian fish when exposed to hypoxia. A decrease in organic phosphate concentrations was also observed when an increase in environmental temperature occurred. Weber (30) reported a decrease in the concentration of triphosphate nucleotides in several fish species after they were submitted to hypoxia. When the environmental temperature increases, water oxygen concentration falls. Gender, age, seasons, and the environment affect hemoglobin concentrations (23). For example, Tambaqui (*Colossoma macropomum*) is a fresh water migratory teleost which presents a great tolerance to pH changes, O_2 quantities in the water and resistance to changes in physicochemical parameters occurring in water during daily and seasonal fluctuations (31).

Physiological adaptations involve a depression in metabolic demands for energy obtained through the glycolytic pathway, with the production of lactate. These adjustments are modulated by variable expressions of the enzymes of this pathway, such as lactate dehydrogenase (32). In some species, such as the Crucian carp (*Carassius carassius*), the final product is ethanol (33), providing a greater capacity to resist hypoxia or anoxia over long periods of time during northern European winters.

In some species, blood pH lowering does not induce a complete deoxygenation of all hemoglobins, but a small fraction remains oxygenated, even at low pH. This is partially due to the presence of multiple hemoglobins and their different oxygen-binding properties (34,35).

The several forms of hemoglobins present in fish species are named isohemoglobins (36-40). The primary origin of this variety is the polymorphism that occurs in the constituent globin loci of hemoglobins and two hypotheses may explain the evolution of these polymorphisms. The first is known as the "selectionist" theory, while the second is the "neutralist" theory, describing the diversity of hemoglobin by neutral mutations (40,41). According to Pérez et al. (40), an analysis of data collected on fish hemoglobins from different species supports the second hypothesis as the cause of most sequence variations. Figure 3 shows an example of the isoforms from Tambacu and Tambaqui.

From the selectionist standpoint, it can be supposed that multiple hemoglobins having functional diversity, acting together, may have a better capacity of gas transportation during environmental variations than when there is only one hemoglobin. Thus, hemoglobin heterogeneity would have a selective value in unstable environments. Some research, such as that from Houston and Cyr (42), shows that there is a variation in the hemoglobin pattern with the acclimation to

different temperatures in *Carassius auratus*, reinforcing the hypothesis of polymorphisms as an adaptive mechanism. Generally, the larger number of hemoglobin components, found not just in fish but also in reptiles and amphibians, is significant when compared to mammals and birds. This multiplicity of components generates questions relative to their origin and their possible functional meaning.

The great majority of fish species present symmetric hemoglobins, that is, two pairs of identical globin chains. Nonetheless, some present asymmetric hemoglobins, exhibiting at least three different globin chains in a single-hemoglobin molecule (19,43).

An additional phenomenon, observed in the hemoglobins of some species, is the possibility of dimer exchange between different hemoglobins, leading to asymmetric arrangements as described in carp hemoglobin (44). Thus, the number of isoforms can be significantly elevated by this property, observed both *in vivo* and *in vitro* (43,45-47). Changes in the relative quantities of different hemoglobins, with the corresponding adjustment of oxygen-binding properties may represent adaptations to environ-

mental factors such as temperature (48).

Another explanation of the coexistence of multiple hemoglobins, even when they do not have functional differences, is the fact that deoxyhemoglobin is found at its limit of solubility, and the presence of isoforms avoids precipitation within the erythrocyte (27).

Research on the presence of several isohemoglobins and the expressive structural and functional variability observed among them has increased, generating a significant volume of data in this area. According to De Young et al. (39), fish hemoglobins allow the study of functional properties present in normal or exaggerated forms, or even absent.

Usually, publications related to fish hemoglobins classify these proteins as cathodic or anodic according to their electrophoretic behavior. Cathodic hemoglobins (also called class II), with the highest isoelectric point, have low affinity for O_2 and absence of the Bohr effect. The anodic hemoglobins (class I), however, have lower affinity and elevated sensitivity to proton binding (48). Some cathodic hemoglobin, however, such as those of "tamoatá" (*Hoplosternum littorale*), exhibit a reverse or acid Bohr effect and significant binding to phosphates at low pH (40,48).

The analytical method used can also influence the number of isohemoglobins detected. For example, the blood of carp (*Cyprinus carpio*) exhibits three bands of hemoglobins in starch gel and four in isoelectric focusing (38). Regarding trout blood (*Oncorhynchus mykiss*), there are controversies about the number of hemoglobin forms; depending on the analytical method 6 to 9 fractions were reported. Fast performance liquid chromatography showed 9 fractions, identifying five alpha and four beta chains. This multiplicity would allow a better pH buffering within the erythrocyte and a higher hemoglobin concentration.

Trout hemoglobin I (20% of the total)

Figure 3. Hemoglobins from the hybrid fish Tambacu (sample 1) and Tambaqui (*Colossoma macropomum*, samples 2 and 3), analyzed by isoelectric focusing.
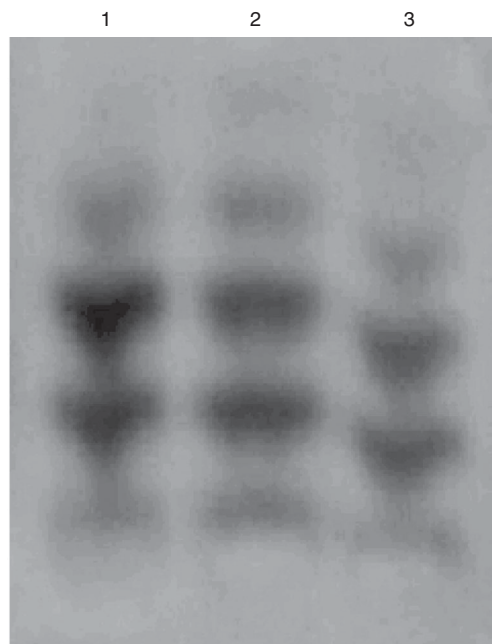
does not present a Bohr effect and is not sensitive to organic phosphates, whereas hemoglobin IV (60% of the total) has a Bohr effect and is sensitive to organic phosphates, also exhibiting a loss of cooperativity at low pH, showing the presence of a Root effect (49). Hemoglobin IV is the major O_2 carrier, consisting of three types of beta chains in equal quantities and three types of alpha chains. Hemoglobin I is less complex because it contains only one type of beta chain and two types of alpha chains, which differ in several residues. The alpha and beta chains of hemoglobin I differ considerably from the ones in hemoglobin IV (46,50).

Trout hemoglobin I does not exhibit a Bohr effect; in other words, oxygen affinity does not depend on pH, but exhibits cooperativity. Hemoglobin IV shows a large Bohr effect and a noticeable sensitivity to organic phosphates. At low pH it displays a Root effect causing oxygen release into the swim bladder and the retina. At pH 7.8, hemoglobin IV is totally oxygenated, while at pH 6.0, it is fully deoxygenated (49,50).

The main function of hemoglobin I would be to guarantee tissue oxygenation, providing a normal gas supply in emergencies, whereas the basic role of hemoglobin IV would be to liberate oxygen against high pressures within the swim bladder. Circular dichroism studies demonstrate great structural differences between hemoglobins I and IV, which are related to the different physiological roles they perform as gas carriers (50).

Many fish species have been investigated in relation to adequate supply of oxygen to the tissues during environmental variations. Several studies with the fresh water species *Labeo capensis* have reported the effects of temperature and oxygen consumption rates, showing a high-aerobic metabolism at low-oxygen tensions associated with high O_2 affinity of the stripped hemoglobin. As is also the case for other ectothermic vertebrates, fish can adapt to changes in environ-

mental temperature and hypoxia, increasing their total hemoglobin content or causing changes in the intrinsic oxygenation characteristics, involving the structure or the relative concentrations of individual hemoglobin components (51).

On the basis of the available literature, it can be assumed that, in the past, there was a selection of adaptive factors as the final product of the evolutionary process, which allowed the survival of certain species in adverse environments. In response to environmental pressures, changes in the physiology, the number of isohemoglobins and the presence or absence of the Bohr and Root effects, act in different ways among different species, allowing a better exploitation of unfavorable environments, and, therefore, resulting in adaptability to different locations (5).

The Root effect

The Root effect is a functional property of fish hemoglobins found until now only in teleost species. This property consists of a drastic reduction in hemoglobin O_2 affinity, causing a decrease in the oxygen transportation capacity when pH decreases and oxygen tensions are high. Differences in the expression of both the Bohr and Root effects are related to the primary structures of isohemoglobins (19,52). The Root effect may become more prominent with the addition of ATP or GTP (19). One example is the major hemoglobin from "matrinxã" (*Brycon cephalus*), whose saturation falls to 45% in the presence of ATP at pH lower than 7.0 (15). Hemoglobins with a Root effect do not present the acid Bohr effect and at low pH O_2 binding tends to be non-cooperative (6); therefore, it would not be correct to classify this behavior as an "exaggerated Bohr effect", as postulated in some publications (53).

The fish swim bladder is an organ that helps to adjust fluctuation, being filled with O_2 obtained from blood by diffusion. The

acidification that produces the Root effect is not produced by the erythrocytes but by a special anatomical structure. According to Pelster (54), the Root effect is necessary to fill the swim bladder and also to supply oxygen to the retina, which does not have capillaries. The partial pressure of the gas, which is necessary for diffusion from the blood to the swim bladder to occur, is achieved through a reduction of the gas transportation capacity of the blood, obtained with the acidification of blood. Epithelial cells from the swim bladder, the gas glands, produce lactic and carbonic acids necessary for blood acidification. Although having an excellent oxygen supply, they have a very low-oxidative metabolism and produce lactic acid through the glycolytic pathway, as well as CO_2 by the pentose phosphate shuttle. Both metabolites reduce blood capacity to transport oxygen thanks to the Root effect. This causes an increase in the partial oxygen pressure of blood, which is followed by diffusion of this gas to the swim bladder (55).

Another anatomical structure that requires the Root effect is the fish retina. The Root effect is triggered by the release of acids from retina cells generating the gradient of partial pressure needed to cover the long diffusion distance to the retina (55). This seems to be the most important physiological function of the Root effect, since it exists in some species that do not have a swim bladder (52).

Perutz and Brunori (56) postulated that ßF9 Ser is fundamental for the Root effect. However, Nagai et al. (57), *apud* Brittain (6), demonstrated that this substitution is not essential.

It is not known if these interactions affect the hemoglobin T or R states (52). Theoretically, a direct stabilization of the T state or the destabilization of the R state may pro-

duce the Root effect, with some important residues and alterations in the secondary structure; possibly, there is no single combination able to generate the Root effect (53).

In the field of certainties, according to what was demonstrated in tuna fish hemoglobin, a particular interaction is responsible for half the Root effect. A proton is shared between two aspartate residues: Asp95 (alpha) and Asp101 (beta), which approach the T state by dimer rotation (6,58). The same hemoglobin shows two addtional interactions, one involving the distal histidine and the heme group and the other involving ßHis69 and ßAsp72 (6). The analysis of the hemoglobin from another species (*Leiostomus xanthurus*), which has been used as a classic example of the Root effect, proposes the existence of a positively charged region between the beta chains, which would turn the R state unstable when protonated (59).

According to Brittain (6), there have probably been several combinations of key residues and secondary alterations capable of granting some hemoglobins an extreme sensitivity to blood acidification during evolution.

Fish hemoglobins offer enormous structural and functional variations to researchers, with hemoglobins that do not bind classic allosteric agents, or display drastic responses; others bind oxygen cooperatively or not, even displaying negative cooperativity. For sure, in spite of the existence of numerous publications on fish hemoglobin, there are still several questions remaining to be elucidated and the definitive answers depend on even more multidisciplinary approaches with the contribution of biochemists, chemists, physicists and biologists. Twenty years have passed since Perutz (1) formulated a series of questions and they still remain unanswered.

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