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The Root effect - a structural and evolutionary perspective

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Abstract: Haemoglobin carries oxygen from the environment to tissues; in vertebrates, it is contained in specialized cells, called erythrocytes. Over the last century, the study of the chemical properties of this haemoprotein has provided a wealth of information. One of its most important and ancient physiological features is the Root effect, found in many teleost fish (and some amphibians). The Root effect corresponds to an extreme pH sensitivity and can be described as an exaggerated Bohr effect: it dictates to what extent the oxygen tension can be raised in acid-producing tissues. It is likely that the eye choroid rete represents the most ancient anatomical structure associated with the presence of Root effect haemoglobins. This review describes our overall understanding of the molecular properties, biological occurrence, physiological role and evolutionary origin of Root effect haemoglobins. The current knowledge of the structural properties of Root effect haemoglobins is discussed in the light of recent results obtained on the haemoglobins of the cold-adapted notothenioids *Trematomus newnesi* and *T. bernacchii*.

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Introduction

The biochemistry of oxygen transport in polar fish offers a wealth of important indications on physiological adaptations of the organism to the climatic conditions of the habitat and the remarkable functional flexibility of haemoglobin (Hb) in fish physiology. Hence, studies on Hbs of polar fish are helping to improve the general understanding of the extremely complex structure-function relationship in this ancient and versatile protein.

The Hb molecule has evolved structural and functional diversity to adapt and modify its features under selective pressures of all types, but both the predominantly helical structure and a large number of amino acid residues are well conserved. The primary role of Hb, that of carrying oxygen to vertebrate tissues, is probably the origin of its adaptation to widely different environmental conditions. Its specialized function imposes severe structural constraints on the molecule. It is not therefore surprising that only a small fraction of the residues of the polypeptide chains have been allowed to be replaced during evolution. According to the species-adaptation theory of Perutz (1983), the replacement of a few key amino acid residues leads to functional modulation. The first protein crystal structures of myoglobin and Hb provided the basis for understanding the relationship between changes in amino acid sequence and protein structure (Kendrew *et al*. 1958, Perutz *et al*. 1965). The quaternary structure, assembling the four globin subunits, also provided the classic source of theories and structural studies of allosteric conformational transitions (Monod *et al*. 1965, Perutz *et al*. 1987). The main concept of the two-state allosteric model of Monod, Wyman and Changeux (MWC) was that the Hb molecule can exist only in two quaternary states, corresponding to a low-affinity structure called T (tense) and a high affinity structure called R (relaxed) (Monod *et al*. 1965). According to the MWC model, the cooperative O_2 binding arises from a shift in the population from the T to R structure as binding increases. This model further postulates that the heterotropic effects, such as the Bohr effect, are ascribed to shifts of the allosteric equilibrium.

Fish Hbs, like other vertebrate Hbs, are tetrameric proteins consisting of two α- and two β-subunits, each of which contains one O_2-binding haem group. These subunits are paired in two dimers, $\alpha_1\beta_1$ and $\alpha_2\beta_2$. Within different species the transport of O_2 can be modulated by changes in the Hb structure and allosteric-ligand concentration (ATP for most teleost fish), and by changes in the expression of multiple Hbs likely to display different functional features. During evolution, complex and sophisticated molecular mechanisms, e.g. modulation by pH, carbon dioxide, organophosphates and temperature, have been developed to regulate O_2 transport by Hb.

The decreased oxygen affinity of Hb at lower pH values in the physiological range is known as the alkaline Bohr effect, reviewed by Riggs (1988). In many Hbs from teleost fishes, when the pH is lowered, the O_2 affinity decreases to such an extent that Hbs cannot be fully saturated even at very high O_2 pressure. In addition, cooperativity is totally

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lost and the O_2 capacity of blood undergoes reduction of 50% or more of its value at alkaline pH. This feature is known as the Root effect, reviewed by Brittain (2005). Root effect Hbs are so strongly pH dependent that they are able to unload a large amount of bound O_2 at low pH and against a pressure gradient. The structural basis of the Root effect has been addressed in many studies. Nevertheless, it remains a puzzle in several aspects. Originally, it was proposed that inter-subunit salt bridges stabilize the T quaternary structure, lower the O_2 affinity of T relative to R, and are responsible for the Bohr and Root effects (Perutz & Brunori 1982). The ongoing debate on the structural interpretation of the Root effect suggests that the classical model is an oversimplified explanation of the pH modulation in fish Hbs.

The C-terminal residue of His in β146 appears to be involved in the Root effect in some Hbs but not in all. Moreover, a fundamental difference between Root effect Hbs and Hbs with normal Bohr effect is that in the former the $\alpha_1\beta_2$ interface remains stable in the T state upon oxygenation, whereas in the latter the switch to the R state occurs.

Recent studies on the Root effect Hb of the spot *Leiostomus xanthurus* have suggested that the destabilization of the R state (and not the stabilization of the T state) at low pH, inducing an R→T transition, may explain the molecular basis for the Root effect (Mylvaganam *et al*. 1996). In the R state of spot Hb, the central cavity is more narrow than in human Hb (Hb A), due to the presence of some specific residues in the β chains. The same features have also been found in the Antarctic notothenioid *Trematomus bernacchii* Hb displaying the Root effect (Ito *et al*. 1995). However, these structural properties by themselves are not sufficient to explain the absence of the Root effect in Hb 1 of the other Antarctic notothenioid, *Trematomus newnesi* (D'Avino *et al*. 1994) that has all the residues believed to be important to evoke the Root effect. Interestingly, the sequence-identity between these two major cold-adapted Hbs is very high.

Recently, O_2-binding experiments under a wide range of medium conditions have brought about a new model of allosteric regulation of Hb, the *Global Allostery Model*, reviewed by Yonetani *et al.* (2002). The Global Allostery Model highlights the role of heterotropic effectors in altering the tertiary structures of both T and R states and consequently in modulating the O_2 affinity and the Bohr/Root effect. These authors used bezafibrate and inositol hexaphosphate (IHP) on human Hb under a wide range of pH, obtaining clear indications that allosteric effectors interact with Hb in both the T and R states whereas, in the absence of the allosteric effectors, the conformational transition seems not to be strictly related to the release of protons. Current evidence, herein reviewed, now supports the view that in Root effect Hbs the steric influences on the tertiary structure act in addition to quaternary transition between R and T conformations. Allosteric effectors (organophosphates and protons) may modulate these steric effects within both conformations. It is feasible that the Root effect does not completely depend on the primary structure, but also on how and to what extent the Hb molecule binds effectors in the T and R states (Yonetani *et al.* 2002).

As pointed out by Brittain (2005), a detailed definition of determinants for the Root effect requires structural information on R and T states at different pH values. Following this line, we review recent advances in understanding the structure, function and evolution of the Root effect in fish Hbs. This review also describes our general knowledge of the structural basis of the Root effect in Hbs of Antarctic notothenioids, e.g. *T. newnesi* Hb C (see below) and *T. bernacchii* Hb.

Bovichtidae, Pseudaphritidae, Eleginopidae, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae and Channichthyidae are the families of the dominant suborder Notothenioidei, thought to have arisen in Antarctica through adaptive radiation of the ancestral stock. Channichthyidae are devoid of Hb, and in the other seven families the concentration of the single major Hb component is highly reduced. In contrast, the nototheniid *T. newnesi* has two functionally distinct major Hbs. The Hb system of *T. newnesi* (D'Avino *et al.* 1994), a semipelagic, active fish, is made of Hb C (20–25% of the total), and Hb 1 (70–75%; it has the α chain in common with Hb C) and Hb 2 (5%). Only Hb C displays pH regulation. Cooperativity of oxygen binding is present in Hb 1 and Hb 2 in the whole physiological pH range, but is completely lost in Hb C at lower pH (a typical feature of Root effect Hbs). This Hb system can ensure oxygen binding at the gills (via Hb 1) and controlled delivery to tissues (via Hb C) also when active behaviour may produce acidosis. Even if Hb C has not been subjected to selection, its expression can nonetheless be activated as a consequence of needs arising from the fish life style. High levels of Hb C, conceivably redundant in other notothenioids (which have only traces of Hb C, but rely on the Root-and Bohr-effects of both Hb 1 and Hb 2), compensate for the lack of regulation of Hb 1 and Hb 2 by protons and other effectors. Unlike the vast majority of Antarctic fish, *T. newnesi* is a cryopelagic fish, not a sluggish bottom feeder, and a correlation may exist between the possession of two major components and a relatively active life style.

Physiology and evolution of the Root effect

Physiological function

The physiological role of the Root effect is to secrete O_2 against high O_2 pressures into the swimbladder (when present) and the retina, following local acidification of the blood in a counter-current capillary system. Lactic acid as well as CO_2 are released into the blood stream and acidify

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the blood. Both the swimbladder and the retina possess a small acid producing rete (known as choroid rete, in the eye). Among non-teleosts, the choroid rete is present only in *Amia* (Wittenberg & Haedrich 1974) and evolved just once in fishes, in the clade *Amia* + Teleostei (Berenbrink *et al.* 2005). The choroid rete is well developed in the basal notothenioid families (Eastman 2006). In spite of its physiological importance, the choroid rete is evolutionarily labile and has been frequently lost during fish evolution (Wittenberg & Haedrich 1974). In the phyletically derived Antarctic families, many species have lost the rete, and the discovery of reduced and microscopic retia (Eastman 2006) suggests that this loss might occur gradually. In Notothenioidei, the randomness of its loss remains an open question (Eastman 2006). However, among notothenioids (all lacking the swimbladder), the few species possessing Hbs without a Root effect, as well as Hb-less Channichthyidae (Ruud 1954), lack also the choroid rete.

The molecular phylogenetic studies identifying the Clade X, which groups, among other, notothenioids, zoarcoids and percoids, also infer at least three distinct origins for the Antarctic components of the clade, as well as indicating that the whole Antarctic lineages lack the swimbladder, in contrast with their respective sister groups (yet unidentified), in which this structure is maintained (Dettaï & Lecointre 2004, 2005).

In many fish the swimbladder functions as a hydrostatic organ, filled with gas from the blood to achieve neutral buoyancy. The gas partial-pressure gradients necessary for the diffusional gas transport from the blood to the swimbladder are achieved by a decreased gas carrying capacity caused by blood acidification (Pelster 1997). The latter is produced by specialized epithelial cells, known as gas gland cells. These cells produce lactic acid via the glycolytic pathway, and CO_2 via the pentose phosphate. Under these conditions, the O_2 affinity of Root effect Hb decreases strongly and O_2 is delivered into the swimbladder. The change in O_2 affinity is so strong that O_2 can be secreted into the swimbladder or retina against high opposing pressures. Therefore, the physiological role of Root effect Hbs is to inflate swimbladders at considerable depths or maintain high levels of oxygenation in the poorly vascular retinal tissues.

Interspecific distribution and evolution

The Bohr and Root effects are almost exclusively present in teleosts (Noble *et al.* 1986), and there is currently no explanation for the lack of these physiological characters in some advanced teleost groups. Studies on temperate-elasmobranch Hbs have shown that their functional properties do not always include well developed cooperativity or proton and organophosphate regulation. Elasmobranchs may have diverged from the mammalian line prior to stabilization of the homotropic and heterotropic interactions typical of mammalian Hbs. In *Torpedo marmorata* Hb, the replacement Asp β97→Lys (Huber & Braunitzer 1989) results in the loss of the salt bridge that is crucial for the Bohr effect observed in human HbA (Perutz & Brunori 1982). Hence, this mutation may be responsible for the lack of Bohr and Root effects in *Torpedo nobiliana* Hb, that binds O_2 with low cooperativity and shows no

Table I. Taxa and data source.

Order and species	Root effect (%)	Reference
Coelacanthiformes		
Latimeria chalumnae[d]	0*	Bonaventura *et al.* 1974
Lepidosiren paradoxus[d]	0*	Berenbrink et al. 2005
Scorpaeniformes		
Chelidonichthys kumu[d]	60	this study
Liparis tunicatus[c]	40	this study
Perciformes		
Thunnus thynnus[d]	65	Berenbrink *et al.* 2005
Anarhichas minor[c]	70	this study
Notothenia coriiceps[a1]	60	this study
Notothenia angustata[b1]	50	this study
Pleuragramma antarcticum[a1]	50	this study
Pagothenia borchgrevinki[a1]	30	this study
Gobionotothen gibberifrons[a1]	40	this study
Aethotaxis mitopteryx[a1]	0*	this study
Trematomus newnesi[a1]	16	this study
Trematomus bernacchii[a1]	50	this study
Cygnodraco mawsoni[a2]	60	this study
Gymnodraco acuticeps[a2]	5*	this study
Racovitzia glacialis[a2]	55	this study
Bathydraco marri[a2]	60	this study
Pogonophryne scotti[a3]	25	this study
Artedidraco orianae[a3]	20	this study
Cottoperca gobio[b4]	85	this study
Bovichtus diacanthus[b4]	70	this study
Pseudaphritis urvillii[b5]	50	this study
Eleginops maclovinus[b6]	50	this study
Leiostomus xanthurus[d]	60	Bonaventura *et al.* 1976
Salmoniformes		
Oncorhynchus mykiss[d]	60	
Anguilliformes		
Anguilla anguilla[d]	20*	Berenbrink *et al.* 2005
Cypriniformes		
Cyprinus carpio[d]	60	Berenbrink *et al.* 2005
Carassius auratus[d]	60	Berenbrink *et al.* 2005
Danio rerio[d]		Berenbrink *et al.* 2005
Rajiformes		
Bathyraja eatonii[i]	0*	this study
Raja hyperborea[ii]	0*	this study

[a]Antarctic Notothenioidei, [b]Non-Antarctic Notothenioidei, [c]Arctic species, [d]Temperate freshwater and marine species.
[i]Antarctic cartilaginous fish, [ii]Arctic cartilaginous fish.
[1]Nototheniidae, [2] Bathydraconidae, [3]Artedidraconidae, [4]Bovichtidae, [5]Pseudaphritidae, [6]Eleginopidae.
*Lack of choroid rete.
For all species, the oxygen saturation at atmospheric pressure (Root effect) have been calculated as a function of pH (100 mM HEPES/MES in the range 6.0–8.5) in the absence of effector and in the presence of the physiological organophosphate (3 mM ATP, namely a large excess over tetrameric Hb/hemolysate concentration). The Root effect is expressed as the percentage decrease in oxygen saturation at pH 6.5 in the presence of ATP

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appreciable pH dependence or response to organophosphates (Bonaventura *et al.* 1974). The polar skates *Bathyraja eatonii* and *Raja hyperborea* (both of the class Elasmobranchii, order Rajiformes, family Rajidae) have a single major Hb not displaying Bohr and Root effects (Verde *et al.* 2005). The replacement Asp 94β→Leu in *B. eatonii* Hb and Asp 94β→Thr in *R. hyperborea* Hb causes the loss of salt bridges that are essential for the Bohr effect in human HbA. In general, in sharks, lungfish and tetrapods, the Root effect is strongly reduced as indicated by an over 90% in O_2 saturation also at low pH values.

A recent study on temperate fish (Berenbrink *et al*. 2005) has shown that the Root effect apparently evolved one hundred million years before the appearance of the choroid rete, whereas the swimbladder evolved independently at least four times during evolution. According to these authors, the swimbladder, the choroid rete and the Root effect have been lost and regained several times in some fish groups. Most important, the weakening of the Root effect is noticed in lineages where the choroid rete has been lost, whereas the loss of swim-bladder O_2 secretion does not affect the magnitude of the Root effect (Berenbrink *et al*. 2005).

Table I reports the status of the choroid rete and the level of the Root effect in a number of fish species including cartilaginous, lobe-finned and ray-finned fish. It can be observed that among Notothenioidae, only *T. bernacchii* has the rete, whereas *Pagothenia borchgrevinki*, *Aethotaxis mitopteryx*, and *T. newnesi* have lost it. In *Gobionotothen gibberifrons*, the rete is reduced. Bathydraconidae also lack the rete, but *Racovitzia glacialis* still conserves a vestigial rete (Joe Eastman, personal communication 2006). It must be recalled that all notothenioids have lost the swimbladder, but, similar to other teleosts, they have the pseudobranch.

The Hbs of non-teleost fish, similar to the Hbs of tetrapods, lack the Root effect. Among teleosts, Anguilliformes have a weak Root effect, in contrast to Cypriniformes that display a strong effect. The lineages leading to non-Antarctic notothenioids are also characterised by a strong Root effect; a weakening of the effect is noticed in Antarctic notothenioids, although some species display a strong Root effect.

Structural studies on the Root effect haemoglobins

Any interpretation of the Root effect should account both for the number of protons released upon oxygenation (n_{H+}), derived from p_{50} (the oxygen partial pressure required to achieve half saturation) as a function of pH, and for the loss of cooperativity measured by the Hill coefficient (n_{Hill}). Therefore a structural analysis should focus on two aspects:

i) the number of residues whose pK_a should shift upon oxygenation, dictating the number of protons released per tetramer, n_{H+},

ii) the reason for the drop in cooperativity at acidic pH (n_{Hill} approaches or falls below 1 at pH < 6.5). Unlike the first aspect, the second one depends on the allosteric model used.

Initially, in the absence of a crystal structure of a Root effect Hb, Perutz & Brunori (1982) proposed a stereochemical interpretation based on the structure of human Hb A. This first interpretation of the Root effect suggested a role for the replacement by Ser of reactive Cys 93β of mammalian Hbs. According to this hypothesis, a hydrogen bond between Ser and C-terminal His is able to stabilize the quaternary deoxy (T) structure. Mutant design and the subsequent structural analysis of Root effect Hbs have confuted this hypothesis.

More recently, two main hypotheses, essentially based on the stereochemical model (structural translation of the concerted model (Perutz *et al*. 1987)), have been proposed to interpret the loss of the oxygen-driven cooperative T→R transition related to the Root effect. The first hypothesis invokes destabilization of the R form at acidic pH, due to a cluster of positive charges at the $\beta_1\beta_2$ interface (Mylvaganam *et al*. 1996), and the second hypothesis suggests stabilization of the low-affinity T state (Ito *et al.* 1995, Mazzarella *et al*. 1999).

Initially, when the crystal structure of deoxy Hb from *T. bernacchii* (Hb–Tb) was resolved at moderate resolution, it revealed a relevant inter-Asp hydrogen bond, at the $\alpha_1\beta_2$ interface, that could stabilize the T state (Ito *et al.* 1995). The hypothesis on pH modulation of the R state was supported by the crystal structure of carbomonoxy Hb of *Leiostomus xanthurus*. Mylvaganam *et al*. (1996) proposed that some protons released in the T→R transition derive from two positively charged clusters formed across the allosteric $\beta_1\beta_2$-interface of the R structure. These clusters would justify both two of the Root protons and the destabilization of the R state at acidic pH. Nevertheless, other Hb structures in the R form do not support this hypothesis. In fact, two Root effect Hbs, Antarctic Hb–Tb (Camardella *et al*. 1992) and tuna Hb (Yokoyama *et al*. 2004), as well as the non-Root effect major component of *T. newnesi*, Hb 1-Tn (Mazzarella *et al*. 1999), have the same cluster in the carbomonoxy form. Relevantly, since Root effect Hb-Tb and Hb 1-Tn (devoid of the Root effect) in the R form display close similarity of the quaternary association, the differences in pH dependence do not seem related to the modulation of the R state (Mazzarella *et al*. 1999). Therefore, the pH modulation of the functional properties of Antarctic fish Hbs was attributed to changes in the tertiary structure within the T state, supporting the global allostery model (Yonetani *et al.* 2002). Five crystal structures of Root effect Hbs in the deoxy state from *T. bernacchii* (Mazzarella *et al*. 2006a), tuna (Yokoyama *et al*. 2004) and cathodic Hb C of *T. newnesi* (Hb C-Tn, (Mazzarella *et al*. 2006b)) are available in the literature.

The interplay of organophosphates and protons in Root

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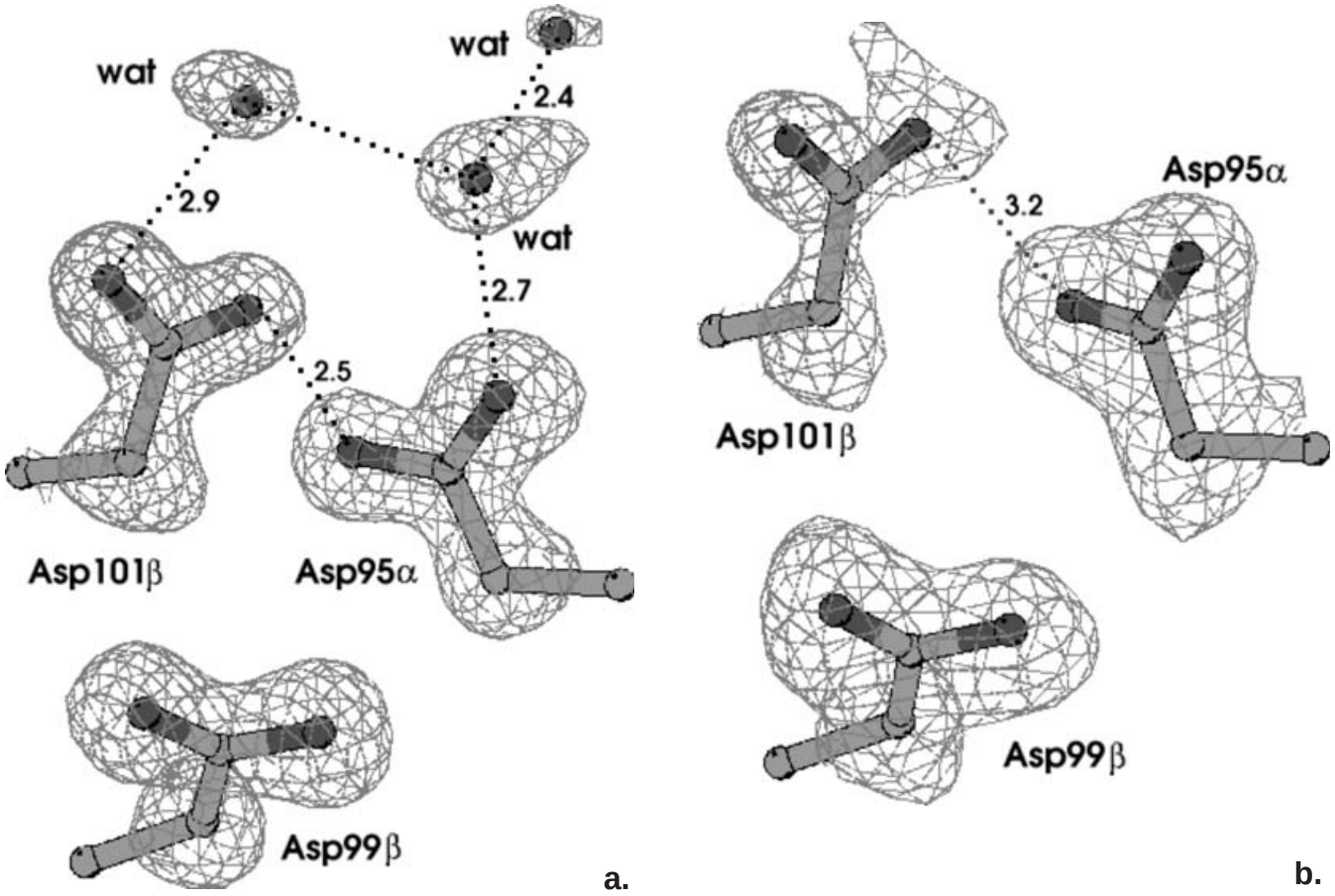


Fig. 1. Electron density map of the $\alpha_2\beta_1$ interface of deoxy *Trematomus bernacchii* Hb at **a.** pH 6.2, and **b.** pH 8.4. Distances are in Å, and "wat" stands for water molecules.

effect Hbs, although observed in functional studies (Camardella *et al*. 1992, D'Avino *et al*. 1994), does not have a clear structural counterpart. Indeed, despite the presence of IHP in the crystallization reactor of deoxy Hb C-Tn (Mazzarella *et al*. 2006a) and Hb-Tb (Mazzarella *et al*. 2006b) at acidic pH, no electron density of IHP has been revealed from their high resolution X-ray structure.

The crystal structure of the deoxy form of Hb C-Tn (endowed with the Root effect), has been particularly helpful in explaining the proton heterotropic effect. The sequence is characterised by an extremely low histidyl content (D'Avino *et al*. 1994), suggesting that these residues do not contribute to pH modulation. Nevertheless, the deoxy structure exhibits a hydrogen bond formed between Asp 95α and Asp 101β, stabilized by Asp 99β (Mazzarella *et al*. 2006b). This structural motif *per se* is sufficient to generate the Root effect, and it can be considered as the minimal structural requirement needed for designing Root effect Hbs. Consistent with the results on *T. newnesi* Hb C, in both tuna and *T. bernacchii* Hbs, two Root protons released upon oxygenation at physiological pH are due to the breakage of an Asp-Asp hydrogen-bond at the $\alpha_1\beta_2$ interface (Fig. 1).

Interestingly, this interaction at the $\alpha_1\beta_2$ site of Hb-Tb is very well preserved even at pH 8.4, despite the fact that distances between O^{δ} of Asp 95α and Asp 101β side chains increase from 2.5 Å (at pH = 6.0) to 3.2 Å (at pH 8.4). This hydrogen bond is not formed in either human HbA (probably due to the substitution of Asp 101β by Glu), or in deoxy trout Hb I, devoid of the Root effect (Tame *et al*. 1996).

The role played by the histidyl residues in modulating the strength of the Root effect has been highlighted by the crystal structures of tuna (Yokoyama *et al*. 2004) and *T. bernacchii* (Mazzarella *et al*. 2006a) Hbs, both elucidated at two different pH values, 5.0 and 7.5, and 6.2 and 8.4, respectively. The structure of tuna Hb revealed a strong effect of structural heterogeneity between α and β chains at pH 5.0, expected also from kinetic and functional studies on several fish Hbs (Noble *et al.* 1970, Brunori *et al.* 1973, Bonaventura *et al*. 1976, Morris *et al*. 1981, Fago *et al*. 1997). In particular, at pH 5.0, the two conformations of the α and β distal His are different; in fact, His 60α swings out of the haem pocket and replaces His 46α as hydrogen bonding partner with a propionate group of the haem. This heterogeneity could justify n_{Hill} even lower than 1 in tuna. In

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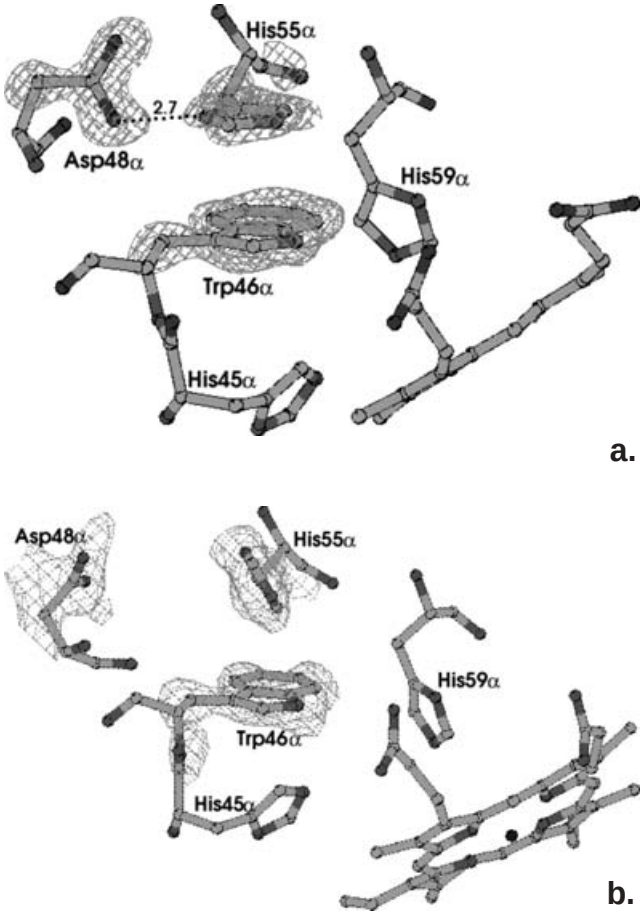


Fig. 2. Electron density map of residues Asp 48α, His 55α and Trp 46α in deoxy *Trematomus bernacchii* Hb at **a.** pH 6.2, and **b.** at pH 8.4. The proximal α His are reported as well. Distances are in Å.

contrast, at acidic pH, both Hb-Tb and Hb C-Tn do not exhibit this heterogeneity associated with such a movement of distal His, which is consistent with $n_{Hill}>1$ in Antarctic fish Hbs (Camardella *et al.* 1992, D'Avino *et al*. 1994).

In both tuna and Hb-Tb, other elements of tertiary structure contribute to the remaining part of Root protons. A detailed survey of the His modifications in Hb-Tb (with $n_{H+}= 4$), caused by the change in pH, indicates that several hot regions of the molecule are modified (helix Eβ, Cβ tail, CDα corner) and can be considered to be involved at various levels in the release of the Root protons.

His 69β, in Hb-Tb as well as in tuna Hb, can be recognised as a potential contributor to the Root effect. Indeed, at pH 8.4, disruption of the salt bridge Asp 72β-His 69β occurs. The Hb-Tb-deoxy crystal structure cannot exclude a contribution of the Bohr-like protons (coming from a salt bridge between Asp 94α and His 146β) to the Root effect in the absence of chloride. The other source of human Bohr-like protons released in the presence of chloride must be excluded in teleost fish Hbs because the residue at the N terminus in the α chains is always acetylated. In Hb-Tb, at the CDα corner, the break of the salt bridge Asp 48α-His 55α allows the description of a detailed mechanism that transmits the modification from the CDα corner away from the α haem (Fig. 2).

It is well known that the CDα region contributes to the formation of the $\alpha_1\beta_2/\alpha_2\beta_1$ interface and plays an important role as switch region in the allosteric transition of mammalian Hbs (Baldwin & Chothia 1979, Paoli *et al.* 1996). In this region, at acidic pH in the T-state a stabilizing interaction occurs between the side chains of His 55α and Trp 46α. Specifically, a parallel orientation of the two aromatic side chains (π-stacking) is observed. The role of π-stacking in modulating the active site has been also reported in a study on mutants of lysozyme (Harata *et al.* 1993). Upon deprotonation of His 55α, the salt bridge with Asp 48α is lost and the π-stacking interaction with Trp 46α is weakened, leading to a more stable T-shaped configuration of the two aromatic rings (Gervasio *et al*. 2000). In Hb-Tb the proton of His 55α acts as a hook for the three side chains reported in Fig. 2.

A general explanation of the Root effect in Hbs of all fish (both temperate and Antarctic) has still not been achieved. From the structural analyses it appears that the Root effect is a diffuse structural phenomenon, linked to the interplay of a number of factors in the protein architecture. Nevertheless, regarding Antarctic fish Hbs, the current hypothesis on the Root effect is based on overstabilization of the T state, mainly induced by the inter-Asp hydrogen bond at the $\alpha_1\beta_2$ interface (Mazzarella *et al*. 2006b), possibly modulated by salt bridges (Fig. 2) involving histidyl residues (Mazzarella *et al*. 2006a, 2006b). The pH-induced changes of tertiary structure within the T state, as herein reviewed for tuna Hb and Hb-Tb at Eβ and CDα, can also trigger a change in T-state affinity, consistent with the range of functionally distinct high- and low-affinity T states also observed in experiments on human Hb (Samuni *et al.* 2004, 2006).

Concluding remarks

The findings summarized in this review outline the importance of integrating i) phylogeny with Hb structure/function relationships, and ii) morphological and ecological data, as necessary tools to clarify and if necessary revise some aspects of the overall knowledge on Hb features.

Despite more than three decades of studies, it is still virtually impossible to ascribe the presence or absence of the Root effect to substitutions of a few amino-acid residues, or to a single explanation. Indeed, the situation is highly complex, and is probably linked to the combination and interplay of a number of factors in the architecture of the globin tetramer. To date, this multidisciplinary approach has not been sufficiently exploited. The new structural and functional information (Yokoyama *et al.* 2004, Mazzarella *et al.* 2006a, 2006b) will help us, in general, to understand

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the evolution of the Root effect in fish Hbs, and to question whether the maintenance of the effect in Antarctic notothenioid Hbs is also related to environmental conditions. It ought to be considered that a large number of notothenioids has lost (or is perhaps losing) the choroid rete, namely the morphological structure thought to be linked to the Root effect (Eastman 2006). We suggest that the Hb phenotype of notothenioids is undergoing dynamic changes in response to thermal adaptation over evolutionary time, and that the fish morphological changes and the Hb functional changes do not seem to be synchronised.

The identification of the sister group of the suborder Notothenioidei will probably constitute a most important and useful tool to study the evolutionary dynamics of the gain and loss of the Root effect.

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