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Is there a Root effect in *Xenopus* hemoglobin?

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The reaction of *Xenopus* hemoglobin with oxygen and carbon monoxide has been reinvestigated over the pH range 8.5–6.0, in the absence and presence of organic phosphates (2,3-diphosphoglycerate or inositol hexakisphosphate), to establish if the tetramer can be stabilized in a T-quaternary state by protons and polyphosphate; the equilibrium and kinetic data indicate that *Xenopus* hemoglobin does exhibit a Root effect. These new results are discussed with reference to those reported by Bridges et al. [(1985) Resp. Physiol. 61, 125–136] on *Xenopus* blood and, more generally, to the molecular definition and the structural basis of the Root effect as an extreme form of the Bohr effect.

Hemoglobin; Allosteric transition; Bohr effect

1. INTRODUCTION

In 1931 Root [1] reported that the oxygen binding capacity of fish blood equilibrated with air was affected by pH, and that below neutrality the total amount of oxygen bound was significantly less than the total concentration of binding sites. This peculiar type of Bohr effect, whereby the oxygen affinity of the hemoglobin at acid pH values becomes so low such that the protein is only partially oxygenated even in air, has been referred to as the 'Root effect'. The phenomenon has been investigated by respiratory physiologists and physical chemists, who have shown that this pH-linked effect is related to a proton-induced structural change of the hemoglobin molecule which, at low pH, is characterized by an oxygen binding curve that is either non-cooperative, or more often clearly anti-cooperative ($n < 1$ [2–4]).

In 1982 Perutz and Brunori [5] proposed that this extreme type of Bohr effect was related (at least partly) to the presence of a serine residue at position β(F9)93, and provided a structural interpretation to account for the marked drop in oxygen affinity and cooperativity of the hemoglobin at low pH. To substantiate this hypothesis, they reported equilibrium and kinetic data on ligand binding by hemoglobin from the aquatic frog, *Xenopus laevis* (which also has a Ser at position β(F9)93, in place of the Cys normally found in mammalian hemoglobins); the results confirmed their expectations. In fact it was demonstrated that *X. laevis* hemoglobin displays a remarkably low oxygen affinity even in the absence of organic phosphates: below pH 7.5 $p_{1/2}$ is approximately 2–3-times higher than that of HbA and other anuran amphibia hemoglobins [5–8].

The conclusion that *Xenopus* hemoglobin displays (under appropriate conditions) a Root effect has been subsequently challenged by Bridges et al.

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Abbreviations: DPG, 2,3-diphosphoglycerate; IHP, inositol hexakisphosphate; Mb, myoglobin; Hb, hemoglobin; Bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane

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[9], who measured the oxygen binding capacity of *Xenopus* blood and established that even at pH 6 the blood was fully saturated in air ($Y>90\%$). Thus, because *Xenopus* blood displays no Root effect, Bridges et al. [9] concluded that the stereochemical model proposed by Perutz and Brunori [5] is incorrect.

This paper addresses itself to the point in question, and represents an attempt to clarify how far the disagreement is real, and how far it is a matter of experimental conditions and/or of definition. Our new measurements indicate that *Xenopus* Hb does display a Root effect, as defined in molecular terms by Perutz and Brunori; nevertheless, the oxygen binding capacity of *Xenopus* blood is almost, though not completely, pH independent essentially because: (i) the intracellular concentration of polyphosphates is insufficient to stabilize the T-state fully, and (ii) chain functional heterogeneity is absent. The problem is significant, because the prediction that *Xenopus* Hb should display a Root effect was reached on the basis of its amino acid sequence and the three dimensional structure of human hemoglobin. Thus it represented a challenge to the approach successfully used by Perutz and co-workers [10] to account for structure-function relationships of different hemoglobins by applying the 'principle of invariance' of the so called Mb fold, i.e. the idea that the three dimensional structure of mammalian (human) Hb may be used, in conjunction with sequence information, to account for the functional properties of vertebrate hemoglobins purified from species which are distant on the evolutionary scale.

2. MATERIALS AND METHODS

Xenopus blood was obtained from adult animals by cardiac puncture; after washing with physiological saline, red cells were used immediately for oxygen binding experiments, or employed to prepare hemoglobin by a method which took care to minimize rupture of the nuclei; Hb was free from nucleic acid contaminations as demonstrated by UV spectroscopy. The hemoglobin was employed as soon as possible, because ageing leads to the formation of octamers linked by disulfide bridges.

Oxygen equilibria were obtained by the tonometric method of Rossi-Fanelli and Antonini [11]; particular care was taken to control the spectrum of fully saturated hemoglobin, in view of the low oxygen affinity observed in the presence of polyphosphates at low pH. The methemoglobin content was checked after each experiment and never exceeded 4%.

Carbon monoxide kinetics was followed using a Gibson-Durrum stopped flow instrument with a 2 cm observation chamber, or a flash photolysis apparatus similar to that described by Brunori and Giacometti [12]. Data analysis was carried out using an HP 87 desk computer.

3. RESULTS AND DISCUSSION

The experimental data on red blood cell suspension were obtained only for oxygen, and involved oxygen binding isotherms and total oxygen saturation in air at different (external) pH values. The oxygen equilibria are indeed very similar to those reported by Bridges and collaborators [9], who showed that Hill's coefficient depends on pH, being $n=2.2$ at pH 7.47 and 1.8 at pH 6.64 (at an intracellular concentration of polyphosphates of approx. 1.4 mol per tetramer). Table 1 provides a summary of total saturation in air (easily correlated to oxygen binding capacity) as a function of pH for fresh *Xenopus* red blood cells and hemoglobin; the corresponding oxygen saturation values for HbA are not indicated to avoid overcrowding, but they are never lower than 95% even in the presence of 3 mM IHP.

In red blood cells exposed to air it may be seen that saturation is significantly below 100% (e.g. $Y=87\%$), provided the pH is low and the intracellular concentration of DPG is high (i.e. cells have been freshly prepared). Furthermore after 24 h incubation of *Xenopus* blood with a 'rejuvenating' solution, which is known to increase the total content of DPG [13] in mammalian red blood cells, a significant decrease in oxygen affinity is observed: $p_{1/2}$ of red cells in isotonic phosphate buffer, pH 6.0, is increased from 37 to 43 mmHg and fractional saturation in air is descreased from 94 to 80% (table 1).

The differences between our results on blood at low pH and those of Bridges et al. ([6]; $Y=90$–100%) are small; we attribute their failure

Table 1

Summary of oxygen equilibria of *X. laevis* blood and purified hemoglobin, stripped and in the presence of organic phosphates

pH	Sample	Phosphate (mM)	log $p_{1/2}$	$n_{1/2}$	Oxygen saturation in air
5.5	red cells	–	1.57	1.5	87
6.0	red cells	–	1.57	2.0	94
6.0	red cells	[a]	1.63	1.8	80
6.8	red cells	–	1.45	2.2	95
7.4	red cells	–	1.25	2.3	100
8.5	red cells	–	0.80	2.0	100
6.0	hemoglobin	no	1.59	1.3	86
6.5	hemoglobin	no	1.51	1.7	90
7.5	hemoglobin	no	1.0	2.6	100
8.6	hemoglobin	no	0.59	2.3	100
6.0	hemoglobin	DPG(5)	1.91	1.08	63
6.5	hemoglobin	DPG(5)	1.81	1.4	75
7.5	hemoglobin	DPG(5)	1.47	1.6	100
8.6	hemoglobin	DPG(5)	0.59	2.3	100
6.0	hemoglobin	IHP(3)	2.10	0.94	54
6.5	hemoglobin	IHP(3)	2.10	1	65
7.5	hemoglobin	IHP(3)	1.63	1.7	95
8.6	hemoglobin	IHP(3)	0.9	2.5	100

[a] Incubated with rejuvenating solution [10] for 24 h

Temperature 20°C. Buffers: isotonic (64 mM) phosphate for erythrocytes and 0.1 M Bis-tris or Tris + 0.1 M NaCl for hemoglobin

to detect less than complete saturation in air to the formation of metHb, which in their experiment at pH = 5.8 reaches a value of 10% (see fig.6 of [9]). As shown by Darling and Roughton [14], and confirmed by others [15], auto-oxidation increases the oxygen affinity because of the random distribution of ferric hemes among the tetramers.

The behaviour of pure *Xenopus* Hb, however, is a different matter. Our new measurements in the absence of organic phosphates (stripped *Xenopus* Hb) and in the presence of DPG or IHP included: (i) oxygen binding and (ii) CO combination kinetics, over the pH range 8.5–6.0. Some of the data are summarized in table 1, and are also shown in figs 1 and 2. The trend is clear-cut; at every pH below 8, addition of organic phosphates brings about a marked decrease in affinity and cooperativity. At pH 7.5, the binding curve is cooperative ($n = 2.5$) as reported before [5]: at pH 6 and 6.5 plus 3 mM IHP, cooperativity is lost ($n = 1 \pm 0.1$), as shown in fig.1. Similar behavior is induced by DPG (3–5 mM), and at pH = 6 cooperativity is marginal for a tetramer ($n = 1.1$).

As shown in table 1, oxygen saturation of *Xenopus* Hb in air at pH 6 and in the presence of 5 mM DPG, is significantly less than unity ($Y = 63\%$), in agreement with the enhancement of the Bohr effect brought about by polyphosphates [5]. In fig.1 the oxygen binding isotherms of *X. laevis* and human hemoglobins at pH 6.5 and 20°C are compared, to illustrate the relatively low oxygen affinity and the large decrease in cooperativity induced by polyphosphates in *Xenopus* Hb.

Under the same conditions, the kinetics of CO combination at pH 6.5–6.0 and 1 mM IHP is pseudo-first order, with a combination rate constant corresponding to k(T) (binding to the T-state) and no evidence for chain heterogeneity (fig.2).

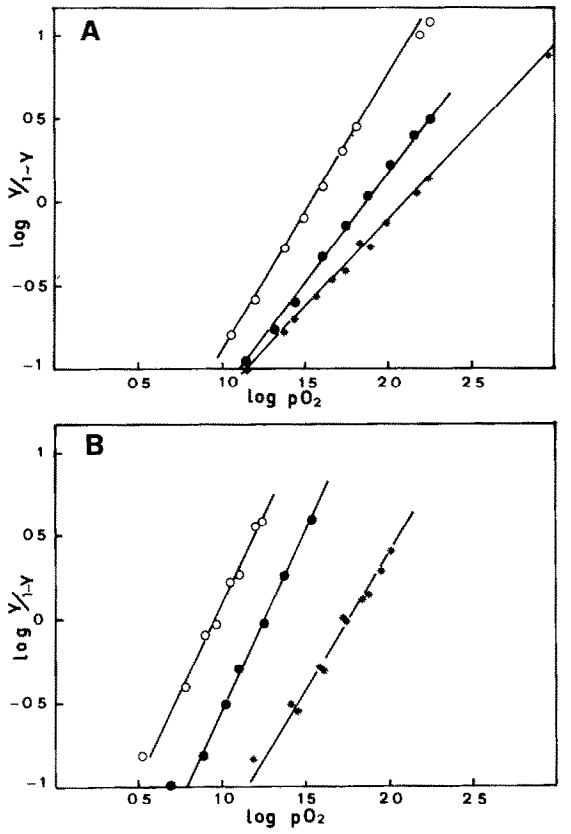

Fig.1. Oxygen binding equilibria for *X. laevis* (A) and human hemoglobin (B), in 0.1 M Bis-tris buffer + 0.1 M NaCl, at pH = 6.5, in the absence and presence of organic phosphates (DPG or IHP). Temperature 20°C. Hb concentration approx. 5 mg/ml. Complete saturation has been controlled by shifting pH to 8.5 at the end of the experiment, the solution being equilibrated with oxygen at 1 atm. (○) Experimental points with stripped Hb; (●) the same + 5 mM DPG; (*) the same + 3 mM IHP.

Moreover, as shown by Perutz and Brunori [5], small flash experiments (approx. 15% photolysis) indicate the absence of a quickly reacting species in *X. laevis* Hb at low pH (< 6.5) and in the presence of IHP, whereas HbA under the same conditions is largely quickly reacting, showing that *X. laevis* hemoglobin is 'frozen' in the T-state and HbA is not.

On the other hand, at higher pH the time course of CO combinaion by stopped flow is clearly autocatalytic, and in fact identical to that of HbA [15] (fig.2). The kinetics can be computer

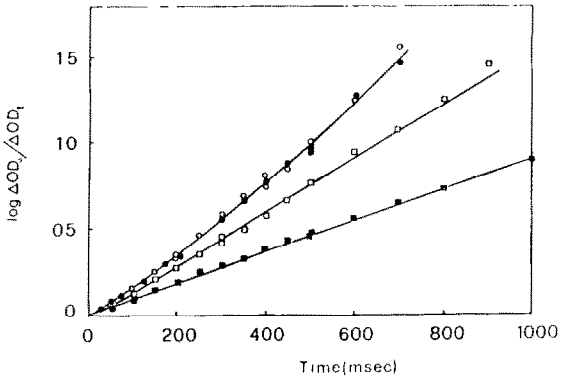

Fig.2. Time course of CO combination to *X. laevis* Hb (●,■) and human Hb (○,□) at two pH values [i.e. 8.5 (○,●) and 6.0 (□,■)] in the presence of 1 mM IHP. Buffer: 0.1 M Bis-tris or Tris-HCl + 0.1 M NaCl. CO concentration (50 μM) and Hb concentration (2 μM); both after mixing. Temperature 20°C.

simulated satisfactorily with an Adair kinetic scheme [16,17].

The results confirm that the combined effect of low pH (6–6.5) and polyphosphates (DPG or IHP) stabilize the low affinity quaternary T-state of *Xenopus* Hb to a much greater extent than that of HbA, in good agreement with partial photolysis experiments [5] for *X. laevis* and [15] for HbA).

4. CONCLUDING REMARKS

For many years the dramatic effect of pH on the oxygen binding capacity of fish blood, discovered by Root [1], has been of interest mainly to respiratory physiologists. Some of them, such as Scholander and Johanson, were intrigued by this phenomenon, and investigated the physiological significance of this exterme 'Bohr effect' [18,19], and correlated it to the function of pumping oxygen either into the swim bladder (a hydrostatic organ of teleost fish) or into the eye (see also [20,21]).

Following elucidation of the structure of human and horse Hb [10,22] and the introduction of the allosteric model [23], the phenomenon has been re-investigated with purified fish hemoglobins using combined equilibrium and kinetic methods. Noble and collaborators [2,24] employing carp Hb, Brunori, Giardina and collaborators [3,25,26] working with trout Hb, and other authors, came to

the conclusion that acid pH and organic phosphates (often in combination) stabilize the low affinity state of the macromolecule; thus carp and trout hemoglobins have been used to probe structural, spectroscopic or kinetic properties typical of a fully liganded T-state hemoglobin (T4). Viewed from this perspective, the Root effect is merely an extreme form of Bohr effect; the absence of cooperativity at acid pH, which indicates inhibition of the quaternary transition from the T to the R structure, is now widely used as a diagnostic for Root effect hemoglobins.

The structural basis of this effect is puzzling. Making use of sequence data on several fish hemoglobins and applying the 'invariance principle' of the Mb fold for hemoglobins from different species [27], Perutz and Brunori [5] proposed that the amino acid at position β(F9)93 plays an important role. This residue, which is a Cys in HbA, is replaced in fish hemoglobins by Ser, whose side chain is capable of forming two H-bonds that contribute to the stabilization of the T-state when His β(HC3) 146 is protonated (i.e. at low pH). The hypothesis led them to propose that *Xenopus* Hb (which has a Ser at β 93; [28]) should also display a Root effect; this was supported by experiments and verified by the data reported above, which indicate that at low pH, in the presence of DPG or IHP, *Xenopus* Hb binds oxygen non-cooperatively, with low affinity, and displays the kinetic features of a T-state hemoglobin (see [15,16]).

As regards the significance of the Root effect 'in vivo', our data on *Xenopus* red blood cells are, by and large, in agreement with those of Bridges et al. on blood [9], and the debate reduces, in our opinion, to a problem of definitions. Thus although oxygen affinity at pH 6.5 is indeed very low, the significance of the Root effect in vivo is probably marginal for *Xenopus*.

In the case of purified *Xenopus* Hb, a complete stabilization of the T-state is only observed in the presence of saturating amounts of polyphosphates, and in fact the partial photolysis experiment reported by Perutz and Brunori [5] as decisive evidence in its favor was carried out in the presence of 1 mM IHP at pH 6. Nevertheless there are important differences between the behavior of *Xenopus* and fish hemoglobins which help to clarify this issue. Work by several authors [3,18,29,30] has indicated that the very low ligand affinity of fish hemoglobins at acid pH (and thus the partial saturation in air at $pH < 7.0$) is often associated with a marked chain heterogeneity, which is ligand and/or protein dependent [31]. The classical Root effect of fish blood is therefore the result of two pH-linked conformational effects brought about by protons and enhanced by polyphosphates, i.e. (i) the stabilization of the tetramer in the low affinity quaternary state and (ii) the induction of a functional difference between the two types of chain, possibly of tertiary origin. The latter effect is (in some cases) dominant over the former; in trout HbIV careful oxygen binding equilibria carried out with a high-pressure spectrophotometric cell [3] showed both effects to be important. However some fish hemoglobins which are considered for all intents and purposes 'bona fide' Root effect hemoglobins, display only minor chain heterogeneity, if any, even for oxygen, as indicated for example by carp hemoglobin, which has been investigated in great detail [2,24,29,32]. In the case of *X. laevis* hemoglobin chain heterogeneity is not detected and therefore its low oxygen affinity and partial oxygen saturation in air are determined solely by the T-state properties. In spite of the fact that the affinity of the T-state is not identical for different hemoglobins (e.g. the low asymptote of the oxygen isotherms is pH dependent even in HbA [33], it is satisfying that the oxygen affinity of the low pH, IHP-stabilized T-state of *Xenopus* Hb is similar to that carp Hb (log $p_{1/2} = 2$) under similar conditions.

In conclusion, our results confirm that in *Xenopus* Hb acid pH and polyphosphates inhibit the ligand linked transition from the T to the R structure and allow us to conclude that *Xenopus* Hb does exhibit a Root effect, although the lack of chain heterogeneity in the binding of oxygen is different from the behavior of blood from some teleost fishes.

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