Novel Mechanism for High-Altitude Adaptation in

Hemoglobin of the Andean Frog *Telmatobius peruvianus*

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Abbreviations used (defined when first-used): DPG, 2,3-diphosphoglycerate; NTP, nucleoside triphosphate; $P_{50}$, half-saturation $O_2$ tension; $P_m$, median $O_2$ tension; $n_{50}$, Hill cooperativity coefficient at $P_{50}$; $n_{max}$, the maximum cooperativity, MWC, Monod, Wyman and Changeux; $K_T$ and $K_R$, $O_2$ association constants of low-affinity (tense) and high-affinity (relaxed) states, respectively, of Hb; $q_H$, the number of interacting $O_2$ binding sites; $\varphi$, Bohr factor ($\Delta \log P_{50}/\Delta \text{pH}$); $\Delta G$, free energy of cooperativity; $\Delta H^{\text{app}}$, apparent heat of oxygenation.
(Abstract)

In contrast to birds and mammals, no information appears to be available on the molecular adaptations for O2 transport in high-altitude ectothermic vertebrates. We investigated hemoglobin (Hb) of the aquatic Andean frog *Telmatobius peruvianus* from 3800 m altitude as regards isoform differentiation, sensitivity to allosteric cofactors and primary structures of the α and β chains, and carried out comparative O2-binding measurements on Hb of lowland *Xenopus laevis*. The three *T. peruvianus* isoHbs show similar functional properties. The high O2 affinity of the major component results from an almost complete obliteration of chloride sensitivity, which correlates with two α-chain modifications: blockage of the N-terminal residues and replacement by nonpolar Ala of polar residues Ser and Thr, at position α131(H14) in human and *X. laevis* hemoglobins, respectively. The data indicate adaptive significance of α-chain chloride-binding sites in amphibians, in contrast to human hemoglobin where chloride appears mainly to bind in the cavity between the β chains. The findings are discussed in relation to other strategies for high-altitude adaptations in amphibians.

Keywords: amphibians, chloride binding, hypoxia, organic phosphates, oxygen transport
HOW IS OXYGEN TRANSPORT to metabolising tissues secured at high-altitude? In contrast to intensive investigations in birds and mammals (6,39,59), the molecular strategies for O₂ transport in high-altitude ectothermic vertebrates remain unexplored, despite greater variations in environmental conditions (temperature, pH, O₂ tension, etc.) and lesser capacities for homeostatic regulation of internal physical and chemical conditions compared to homeothermic vertebrates, and a long-standing interest in high-altitude, aquatic amphibians (1,24).

The anuran genus *Telmatobius* (that variously is referred to as frogs or toads) occurs in the Andes mountains at altitudes from 2000 m to over 4000 m (14) where aerial O₂ tensions fall from approximately 159 mm Hg at sea level to 92 mm Hg. The hypoxic stress is compounded in aquatic species, particularly at night when photosynthetic activity in the ponds ceases (21). *T. culeus* found in lake Titicaca at 3812 m, has reduced, poorly-developed lungs but exhibits compensatory physiological and behavioural adaptations (24) that include an “over-sized”, folded skin, which is penetrated by cutaneous capillaries and ventilated by ‘bobbing’ behaviour under hypoxia, and small erythrocytes and higher erythrocyte counts, blood O₂ affinities and O₂ carrying capacities than anurans living at sea level (24). Subspecies of *Bufo spinulosus* living at sea-level and at 3100 to 4100 m in the Andes analogously exhibit increasing blood-O₂ affinities with altitude (43).

The O₂ affinity of blood is a product of the intrinsic O₂ affinity of the Hb molecules and the erythrocytic effectors that modulate Hb-O₂ affinity. Compared to mammals that use 2,3 diphosphoglycerate (DPG) and fish that use ATP (often in conjunction with the more potent effector guanosine triphosphate) (60) as organic O₂-affinity modulators, amphibian red cells carry both ATP and DPG in widely varying relative concentrations (22). Moreover, as seen in *Rana temporaria* (13), and *R. catesbeiana* (49-51) individual amphibian isoHb components may exhibit functionally-significant interactions.

Aiming to identify the molecular adaptations for O₂ transport in high-altitude amphibians we investigated isoHb differentiation and the interactive effects of pH, temperature, chloride ions, ATP and DPG on O₂ binding in *T. peruvianus* Hb, carried out some comparative measurements on Hb from the
lowland, aquatic toad *Xenopus laevis* and determined the primary structures of the α and β chains of the major *T. peruvianus* isoHb.

**METHODS**

*Animals, Hb preparation and isolation.* *Telmatobius peruvianus* of either sex was collected at 3800 meter’s altitude from small streams near the Andean village Cancosa at the Bolivian boarder in North Chile. Frogs used (n=5) weighed 17.2 (± 2.5) g, and measured 4.9 (± 0.2) cm (snout-vent). Blood samples were taken within 12 hours of descent to the sea-level laboratory at Iquique. Electrophoresis on cellulose acetate strips at pH 8.6 indicated Hbs with the same anodic migration rates in all specimens. Specimens of the lowland African clawed toad *Xenopus laevis* were purchased from Blades Biological, Cowden UK. Animal handling followed the ‘Guiding principles for research involving animals and human beings’ (2).

Hb purification was carried out at 0-5°C as previously described (61). Hb heterogeneity was investigated by isoelectric focussing in 110 ml LKB columns (Bromma, Sweden) in 0.87% ampholines (pH 6.7 - 7.7) (46). Separated isoHb fractions were dialysed against 0.01 M HEPES buffer containing 5·10⁻⁴ M EDTA, pH 7.7 (at 5°C) and stored at –80°C in 0.1 ml aliquots that were freshly thawed for molecular and functional characterization. Hemolysates were stripped of organic phosphates using MB1 mixed ion-exchange resin (BDH Chemicals, Poole, England).

*O₂-equilibrium measurements.* O₂ equilibria of thin (~0.01 mm) layers of Hb dissolved in 0.1 M HEPES buffers were measured using a modified diffusion chamber as previously described (57,58). The \( P_{50} \) [half-saturation O₂ tensions (1 mm Hg = 0.133 kPa)] and \( n_{50} \) (cooperativity coefficient at 50% O₂ saturation) values recorded represent individual data point interpolated from Hill plots (\( \log ([\text{OxyHb}]/[\text{Hb}]) \) vs. \( \log P_{O2} \); correlation coefficient \( r > 0.995 \)) that were generated on the basis of at least 4 equilibration steps between 30 and 70% O₂-saturation. The pH values were measured in oxygenated subsamples equilibrated to the same temperatures (61). O₂-equilibrium curves focusing on extreme O₂ saturations (<2% and >98%) were analyzed by end-weighted fitting (61) of the two-state Monod-Wyman-Changeux (MWC) equation (40) to the data. The overall heat of oxygenation (\( \Delta H^o \)) that includes
contributions from oxygenation-linked reactions) was evaluated from the van’t Hoff equation (4,63).

*Primary structure determinations.* Separation of the α and β chains, enzymatic digestions, and isolation and amino acid sequence analyses of the peptides were carried out as previously described (61).

**RESULTS**

*Hb heterogeneity.* Isoelectric focussing revealed one major component, Hb II, and two minor ones, Hb I and Hb III, with isoelectric points of 7.34, 7.45 and 7.30, and relative abundances of 81:12:7 respectively (Fig. 1). At pH 7.55 *T. peruvianus* isoHbs I, II and III show practically identical P$_{50}$ values (Table 1) that correspond with that of the composite hemolysate (P$_{50}$ = 7.3 at 20°C, pH 7.5) (Fig. 1, inset). In conjunction with corresponding results at pH 7.1 (not shown) this indicates the absence of functionally-significant interactions between the individual isoHbs under the experimental conditions. Cooperativity in O$_2$ binding at half-saturation ($n_{50}$) was pronounced (2.8) and pH independent in Hbs I and II (Fig. 3) but lower (2.2) in Hb III.

*Effector sensitivities and allosteric interactions.* Strikingly, the O$_2$ affinity of *T. peruvianus* Hb II is almost insensitive to chloride ions, despite pronounced effects of [ATP+Cl$^-$] and [DPG+Cl$^-$] (Figs. 2 & 3, Table 1). The potentiation of the Bohr effect by chloride (associated with increased ionization of the positively-charged sites with falling pH) was correspondingly small ($\varphi$ = -0.16, compared to -0.43 and -0.52, in the presence of Cl$^-$, [ATP+Cl$^-$] and [DPG+Cl$^-$]). The higher O$_2$ affinities and increased anion sensitivities observed at 10° than at 20°C (Fig. 3A) reflect exothermic oxygenation and linked endothermic dissociation of allosteric effactors that reduce $\Delta H^{\text{off}}$ (Table 1). Hb I showed the same O$_2$ affinity trends as the major component (Hb II) but slightly lower anion sensitivities (Table 1, Fig. 3C).

In the absence of anions *X. leavis* and *T. peruvianus* Hbs show almost identical O$_2$ affinities (P$_{50}$ = 7.3 mm Hg at 20°C) (Fig. 2). In the presence of 0.1 M chloride, however, *X. laevis* Hb exhibits a much lower affinity than *T. peruvianus* Hb, revealing preservation of a pronounced chloride effect, and a larger Bohr effect ($\varphi$ = -0.40 compared to –0.16 in *T. peruvianus*) (Fig. 3; Table 1).

Dose-response curves (Fig. 4) show that ATP and DPG exert the same effects on the O$_2$ affinity of *T. peruvianus* Hb. The maximum slope of the log P$_{50}$ vs log [phosphate] plots approximates 0.25,
tallying with O2-linked binding of one phosphate molecule per deoxyHb tetramer. The maximum ATP/DPG-induced logP50 shift is smaller than that for DPG and human Hb (Fig. 4) and the phosphate concentrations required for half the maximum change in log P50 indicates an apparent dissociation equilibrium constant in *T. peruvianus* Hb that is an order of magnitude higher than for human Hb and DPG (*Kd* = 7.9 \* 10⁻⁴ compared to 0.71 \* 10⁻⁴ M; Fig. 4).

Extended Hill plots for *T. peruvianus* Hb II at 10 and 20 °C and in the absence and presence of ATP and the derived MWC parameters are shown (Fig. 5 and Table 2). The number of interacting O₂ binding sites (qH) estimated by fitting this allosteric parameter along with the others was 3.76 ± 0.10 (n=4) indicating a stable tetrameric Hb structure, which also is reflected by the exact superimposition of extended Hill plots obtained at different Hb concentrations (Fig. 5). In contrast to most vertebrate Hbs where organic phosphates primarily reduce the O₂ association constant of the low-affinity, tense state of the Hb molecules (*Kₜ*) (55,62) thus increasing the free energy of cooperativity (∆G), ATP also decreases the association constant of the high-affinity relaxed state (*Kᵣ*), and reduces ∆G (Table 2). However, the *Kᵣ* values need to be viewed with caution due to difficulties in measuring the last few per cent saturation of the oxygenation curve (37).

*Primary structure.* The primary structures of the α and β chains of Hb II are shown (Fig. 6). Whereas the β chain was directly accessible for Edman degradation, N-terminal sequencing of the intact globin chains showed that the α chain was blocked. Attempts to deblock the chain failed to give clear-cut results, so that the four/five N-terminal amino acid residues of this chain are not known. The primary structures of both globin chains were reconstructed from relevant peptides. Each sequence was obtained at least twice. The obtained sequences were aligned unambiguously with known amphibian sequences (Fig. 6).

In contrast to *Rana esculenta* and *R. catesbeiana* β chains that lack the first six N-terminal residues compared to most other vertebrate Hbs (5,16), *T. peruvianus* β chains consist of 145 amino acid residues. Of these 93 (64.13 %) are identical with *X. laevis* β-1 chains and 87 (56.55 %) with human Hb. The differences in *T. peruvianus* compared to *X. laevis* are concentrated in the N-terminal region where only 8 of the 24 N-terminal β chain residues are identical. Although common in fish Hbs, acetylation of
the "-amino group of the " chains as found in T. peruvianus HbII is rare in amphibians and has only been reported in β - III larval (56), α-III larval (38) and α-C chains (49) of R. catesbeiana.

**DISCUSSION**

The hypoxic challenge at altitude where O2 loading may be critical is compounded in aquatic habitats – as indicated by increased blood O2 affinities encountered in lowland amphibians with increasing reliance on water as the respiratory medium (32) and the higher blood-O2 affinities in predominantly aquatic T. culeus and X. laevis than in predominantly terrestrial Rana and Chiromantis species (Fig. 7A). These inter-species correlations accord with observations that hypoxic exposure increases blood-O2 affinity by decreasing red cell DPG levels in the salamander Ambystoma tigrinum (66) and raises plasma catecholamine levels (that may increase O2 affinity through red cell swelling (29,42,42)) in the toad Bufo marinus (3). However, 10-11 days’ hypoxic acclimation did not change blood-O2 affinity in the salamander Desmognathus quadramaculatus (36).

The ATP-induced Hb-O2 affinity shifts (Fig 2) and the difference in affinities between stripped Hb and whole blood in T. peruvianus and X. laevis (Fig. 7B) reveal pronounced capacities for effector modulation in both species. The similar effects of ATP and DPG on the O2 affinity of T. peruvianus Hb (Fig. 4) tally with similar magnitudes of ATP and DPG binding constants in human Hb (25) and suggest that the differences in erythrocytic NTP/DPG ratios (~3.0 and ~0.8, respectively, in Lake Titicaca T. culeus and X. laevis (22)) do not contribute to the species differences in blood O2 affinity. The observation that ATP alone decreases the O2 affinity of T. peruvianus Hb slightly more than ATP+Cl' (Fig. 3) may be attributed to competition of the two anions for the same sites in the central cavity (19,44) and neutralization of the positively-charged phosphate binding sites by chloride.

The anuran Hbs show distinctive structure-function relationships. In comparison to human Hb, T. peruvianus HbII shows a less tight binding of ATP and DPG (Fig.4), despite conservation of the positively-charged organic phosphate binding sites in the cavity between the β chains, viz., N-terminal Val, β2(NA2)His, β82(EF6)Lys, and β143(H21)Lys that replaces histidine in human Hb. In X. laevis the deletion of the first β-chain residue (Fig. 6) could bring the N-terminus closer to the bound cofactor and
preserve phosphate sensitivity despite the loss of His(NA2). The Bohr effect of the stripped *T. peruvianus* HbII is small despite the presence of β146His(HC3) and a negatively-charged residue (Glu) in position β94(FG1) that contribute about half of the anion-independent Bohr effect in human Hb (30,47). In contrast to the majority of vertebrate Hbs, where allosteric effectors decrease the affinity of the T state of the deoxygenated molecule (53,54,62), frog Hbs may also be modulated in the R state, as evident from the ATP sensitivity of *T. peruvianus* HbII (Fig. 4 and Table 2) and the pH effect in *Rana temporaria* Hb (13). The molecular mechanism underlying these effects must await the elucidation of the crystal structures of deoxy and oxy forms of amphibian Hbs.

The similar O₂ affinities in Hbs I, II and III and in the stripped hemolysate (Table 1, Fig. 1) indicate the absence of functionally-significant interactions between the individual isoHbs under the tested conditions. This contrasts with *R. catesbiana* where aggregation of the major tetrameric components B and C, to form a low-affinity BC₂ trimer-of-tetramers is manifested at corresponding pH and Hb concentrations as tested here (50,51).

What, if any, are the distinguishing molecular adaptations to altitude in *T. peruvianus* Hb? Comparison with lowland *X. laevis* Hb shows that the major difference resides with the effects of anions. Although stripped Hbs from the two species show almost identical O₂ affinities and pronounced [ATP+Cl⁻] effects, *T. peruvianus* Hb shows a drastically suppressed chloride sensitivity (Δlog Pₑ₀ = 0.10 compared to 0.32 in *X. laevis* and >0.4 in human Hb, Figs. 2 & 7B). In the absence of other changes this will enhance O₂ loading under hypoxia without the need for reducing erythrocytic organic phosphate levels and thus allosteric regulatory capacity. In contrast to short-term hypoxic challenges that evoke adaptive changes in erythrocytic phosphate levels (41), obligate residence at high-altitude appears to be associated with the presence of high-affinity (iso)Hbs - as previously illustrated in homeothermic vertebrates. However, in contrast to the bar-headed goose and Rüppell’s griffon (7,12,45) that may fly at 9000 and 11300 m above sea level, where the high intrinsic O₂ affinity is attributed to amino acid substitutions located at the α₁β₁ or α₁β₂ interface (26,33,63) and llama Hbs, where high blood affinity is achieved through loss of β-chain phosphate-binding residues, the high affinity in *T. peruvianus* HbII results from a loss of anion sensitivity that correlates with α subunit amino acid substitutions.
Two schools of thought exist as regards O₂-linked chloride binding to human Hb, which has been proposed to occur either at ‘localized’ (19) or at ‘delocalized’ (44) sites. The ‘localized’ binding sites are an α-chain site [lying between the α1Val-NH₃⁺ group and β-OH of α131(H14)Ser and the side chain of α131Ser(H14)] and a β-chain site [between β1Val and the ε-NH₃⁺ group of β82Lys] (47). Evidence for their involvement comes from X-ray diffraction studies of crystallized human Hb specifically carboxymethylated at α1Val (18) and the crystal structure of the human Hb mutant, β(V1M+H2) [where β1Val(NA1) is exchanged for Met and β2(NA2)His is deleted] that document the implication of the N-terminal residues in O₂-linked chloride binding and the chloride-dependent Bohr effect (19). The ‘delocalized’ mechanism proposed by Perutz et al. (44) builds on the view (9) that excess positive charges in the water-filled cavity between the β chains destabilize the T-state, and that chloride ions diffusing into the cavity of deoxygenated human Hb reduce O₂ affinity by partially neutralizing the repulsion between these charges, thus reducing the free energy of the T-structure. This mechanism is supported by observations that amino acid substitutions that increase central cavity electropositivity cause a proportional increase O₂ affinity and vice versa (44).

Whereas the mechanism of chloride binding in human Hb remains unresolved, our data indicate predominant importance of specific (‘localized’) α-chain sites in amphibian Hbs. Thus the α-chain residues (1Val and 131Ser in humans) are conserved in X. laevis Hb (where polar Ser at 131 is substituted by polar Thr and the N-terminal residues are free) that shows pronounced chloride sensitivity, but eliminated in T. peruvianus Hb (where 131 is occupied by nonpolar Ala and the α chain N termini are acetylated) that shows strongly reduced chloride sensitivity. That chloride additionally may bind in the central cavity between the β chains (in competition with organic phosphates) is indicated by the observation that ATP alone has a greater effect on O₂ affinity of T. peruvianus Hb than ATP in the presence of 0.1 M chloride (Fig 3).

In contrast to evidence for ‘localized’ chloride-binding (above), there is no evidence from the central cavity amino acid exchanges for greater ‘delocalized’, oxygenation-linked chloride binding in X. laevis than in T. peruvianus Hb. Perutz et al. (44) list 5 α-chain and 14 β-chain polar residues in the central cavity of human Hb that may affect O₂ affinity by increasing or reducing the excess positive
charge. Compared to *X. laevis* Hb, *T. peruvianus* Hb II shows one α-chain and six β-chain exchanges at these positions. These are (the helix notation refers to human Hb): α133(H16)Ser→Gly (that represents loss of a polar site), β1(NA1)<no residue>→Val (that does not affect charge), β2(NA2)Gly→His (that increases positive charges), β104(G6)Lys→Val (that reduces positive charge), β135(H13)Asp→Gly (that reduces negative charge and thus increases net positive charge) and β101(G3)Leu→Ala, β132(H10)His→Lys and β136(H14)Ala→Gly (that are electroneutral). Assuming equivalence of structural factors, these exchanges may thus be expected to increase the number of positive charges in the central cavity and consequently the intrinsic O₂ affinity and the chloride effect in *T. peruvianus* compared to *X. laevis* Hb. Such effects are not evident from our data.

Other evidence indicates that ‘localized’, α-chain chloride binding also may be implicated in adaptations encountered in some mammalian Hbs. The high O₂ affinities of Hb from Andean camellid vicuna (31) and of embryonic pig Hbs Gower I and Heide I that have ζ₁(α₁)-type chains (64) are associated with a α130Ala→Thr replacement, which introduces a hydroxyl group that may interfere with chloride binding at neighboring α131Ser. Also, the almost complete lack of chloride effects in human embryonic Hbs Gower I and Portland (ζ₂v₂ and ζ₂v₂) (68) correlates with an analogous α131Ser→ζ₁31Val substitution to that here reported for *T. peruvianus* Hb II.

In conclusion this study shows a novel molecular mechanism for high altitude adaptation in ectotherm vertebrates that involves a reduction in chloride modulation of Hb-O₂ affinity via loss of specific chloride binding sites on the α-chains and still allows for phosphate modulatory capacity. It should, however, be borne in mind that the molecular adaptations supporting tissue O₂ supply are but part of a symphony of organismic, cellular and molecular adjustments expressed in high altitude animals (39,63). As has become well-established, hypoxia elicits a fall in (preferred) body temperature, which in anurans, appears to be adenosine- and lactate-mediated (10,11,67). The low body temperatures – that are naturally experienced by *Telmatobius* living in cold streams of melted snow – impart a range of possible advantages. Apart from raising the O₂ content of the water, it increases blood O₂ affinity, as dictated by the exothermic nature of the Hb-oxygenation reaction. Also, it decreases metabolic rate and lowers tissue
O₂ demands, which in cold-submerged *Rana temporaria* is associated with increased reliance on carbohydrate metabolism and maintenance of homeostatic ATP levels (17). Hypoxia may, however, also have beneficial effects under certain conditions (48). Several studies show that O₂ deprivation may protect tissues of homeo- as well as ectothermic vertebrates against subsequent hypoxic/ischemic episodes (15,20,34). In *Rana pipiens* and goldfish anoxic exposure moreover induces changes in the antioxidant system that minimize subsequent effects of oxidative stress (23,34). *Telmatobius* may be an excellent model for studying adaptations to chronic hypoxemia.

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Figure Legends

Fig. 1. Preparative isoelectric focussing of *T. peruvianus* hemolysate, showing one major (II) and two minor (I and III) isoHb components. o, absorption values at 540 nm; Δ, pH at 25°C. *Horizontal bars*, fractions pooled for analyses. *Right inset*, diagram of column at the end of focusing. *Left inset*, O₂ equilibrium curves of o, stripped hemolysate; j, Hb I; ▽, Hb II; △, Hb III; Buffer, 0.1 M HEPES, [KCl], 0.10 M; [heme], 0.15 mM; 20 °C.

Fig. 2. O₂ equilibrium curves of stripped (A) *T. peruvianus* Hb II and (B) *X. laevis* Hb at pH 7.0 and 20°C in the absence of added anions (△) and in the presence of Cl⁻ (○) and Cl⁻+ATP (●). Buffer, 0.1 HEPES; [Heme], 0.15 mM (Hb II); ATP/Hb ratio: >100.

Fig. 3. Effects of pH on P₅₀ and n₅₀ values of stripped *T. peruvianus* and *X. laevis* Hbs in the absence and presence of 0.1 M chloride and saturating phosphate concentrations. A, *T. peruvianus* Hb II at 10°C; B, *T. peruvianus* Hb II at 20°C; C, *T. peruvianus* Hb I at 20°C, and D, *X. laevis* Hb at 10 and 20°C; △, no effectors; ▲, Cl⁻, ′, ATP; ●, ATP+Cl⁻; †, DPG+Cl⁻; dotted lines, 10°C; continuous lines, 20°C; [Heme], 0.15 mM (Hb II) and 0.09 mM (Hb I); Other details as in Fig. 2.

Fig. 4. Dose-response curves showing effects of ATP (△) and DPG (◇) on P₅₀ of *T. peruvianus* Hb II. pH, 7.0; [Cl⁻], 0.10M; [Heme], 0.15 mM. ‣, effect of DPG on human Hb (pH, 7.3; [Cl⁻], 0.05M, after ref. (8)); Temperature, 20 °C. Arrows and dotted lines show maximum phosphate-induced changes in P₅₀. Dashed straight lines indicate slopes of 0.25.

Fig. 5. Extended Hill plots of *T. peruvianus* Hb II at 10°C (◇, □, ‣) and 20°C (△,▲,▲) in the absence (open symbols) and presence (closed symbols) of saturating ATP concentrations (ATP/Hb 134). Y, fractional O₂ saturation; [Heme], 0.26 mM (◇, □, ▲), and 0.16 mM (□).
Fig 6. Amino acid sequences of the α and β chains of *Telmatobus* Hb II compared with those of human, *X. laevis* and *Rana* Hbs. *X. laevis laevis* β, major adult Hb β1 chain (Ac.no.P02132); *Rana catesbeiana* β, adult Hb β-chain (Ac.no. P02135); *X. laevis* α, major adult Hb α1 chain (Ac.no. P02012); *Rana catesbeiana* Ba, adult Hb αB chain (Ac. P51465); *Rana catesbeiana* Ca, adult Hb αC chain (Ac.no. P55267). The protein sequence data for the α and β chains of *Telmatobius peruvianus* Hb II here reported are registered in the Swiss-Prot Protein Data Bank under accession numbers P83113 and P83114, respectively.

Fig. 7. O$_2$ affinities of anuran blood and Hbs. A, P$_{50}$ values for whole blood (cross-hatched columns), stripped hemolysates (hatched columns) and isoHbs (open and speckled columns) in the presence of Cl$, showing the log P$_{50}$ shifts induced by saturating ATP concentrations (stacked solid columns). Experimental conditions for *T. peruvianus* and *X. laevis* Hbs, pH 7.5 and 20°C. Conditions for blood measurements: *Rana temporaria*, pH 7.65, 20°C (35,65); *R. catesbeiana*, pH 7.55, 24°C (35); *R. brevipoda*, pH 7.72, 25°C (52); *Chiromantis petersi*, pH 7.65, 25°C (27); *X. laevis laevis*, 7.6, 25°C (28); [Cl$^-$] in Hb solutions, 100 mM (0.05 M for *C. petersi*). For *Telmatobius*, blood values (*) pertain to *T. culeus* at pH 7.54 and 18°C (24) and Hb values to *T. peruvianus*, 20°C. B, Comparison of the P$_{50}$ values of stripped Hbs in the absence of effectors (open columns), in the presence of 100 mM Cl$^-$ (speckled columns), Cl$^-$$^+$ATP for *T. peruvianus* and *X. laevis* Hbs and DPG+Cl$^-$ for human Hb (solid columns) at the indicated temperature and pH values. The data for human Hb are from Table 6.2 in ref. (25). [Heme], 0.14 mM (*T. peruvianus*), 0.50 mM (*X. laevis*) and 0.60 mM (human Hb).
Table 1. Oxygen equilibrium characteristics of isolated *Telmatobius* HbI and HbII and stripped *Xenopus* Hb.

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<td>Cl+ATP</td>
<td>14.0</td>
<td>22.9</td>
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<td>9.52</td>
<td>15.1</td>
<td>-0.40</td>
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<tr>
<td>Cl+DPG</td>
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<td>26.1</td>
<td>-0.52</td>
<td>8.97</td>
<td>15.6</td>
<td>-0.48</td>
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*\(P_{50}\) values in mm Hg;  \(\Delta H\) values in kJ mole\(^{-1}\)
Table 2. MWC and derived parameters for *Telmatobius* HbII at 10 and 20 °C in the absence and presence of saturating ATP concentrations. The MWC model was fitted with the number of interacting binding sites ($q_H$) fixed at 4. (Other details as in Legend of Fig. 4).

<table>
<thead>
<tr>
<th>°C</th>
<th>pH</th>
<th>Effector</th>
<th>$P_{50}$</th>
<th>$P_m$</th>
<th>$n_{50}$</th>
<th>$n_{max}$</th>
<th>$K_T$</th>
<th>SE</th>
<th>$K_R$</th>
<th>SE</th>
<th>$L$</th>
<th>$\Delta G$</th>
<th>rms</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>7.082</td>
<td>-</td>
<td>5.64</td>
<td>5.36</td>
<td>3.03</td>
<td>3.07</td>
<td>0.028</td>
<td>0.0018</td>
<td>4.93</td>
<td>1.46</td>
<td>4.9x10^5</td>
<td>11.97</td>
<td>0.045</td>
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<tr>
<td>10</td>
<td>7.070</td>
<td>ATP</td>
<td>14.45</td>
<td>13.44</td>
<td>2.73</td>
<td>2.79</td>
<td>0.016</td>
<td>0.0014</td>
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<td>0.917</td>
<td>3.3x10^5</td>
<td>10.58</td>
<td>0.060</td>
</tr>
<tr>
<td>20</td>
<td>7.185</td>
<td>-</td>
<td>9.59</td>
<td>9.09</td>
<td>2.87</td>
<td>2.91</td>
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<td>2.13</td>
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<td>0.295</td>
<td>1.3x10^5</td>
<td>10.73</td>
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$P_{50}$ and $P_m$ (half-saturation and median O$_2$ tension values) in mm Hg; $K_T$, $K_R$ [O$_2$ association constants of low-affinity (tense) and high-affinity (relaxed) states respectively] and SE (standard error) in (mm Hg)$^{-1}$; $n_{50}$ and $n_{max}$, cooperativity coefficients at half-saturation and maximal values, respectively; L, allosteric constant [T]/[R] in the absence of O$_2$; $\Delta G$ (free energy of heme-heme interaction) in kJ/mol; rms, root mean square error.
Fig. 2. Weber et al.

Panel A: Telmatobius
20°C, pH 7.0

- A: no added anion
- O: 0.1 M Cl
- ▲: 0.1 M Cl + ATP

Panel B: Xenopus

- ▲: 0.1 M Cl + ATP
Fig. 5: Weber et al.

Telmatobius
Hbil

<table>
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<th>°C</th>
<th>ATP</th>
<th>pH</th>
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<tr>
<td>10</td>
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<tr>
<td>10</td>
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<td>7.185</td>
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<tr>
<td>20</td>
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</table>
Fig. 6; Weber et al.

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<th>B</th>
<th>C</th>
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