Novel mechanism for high-altitude adaptation in hemoglobin of the Andean frog Telmatobius peruvianus

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1Department of Zoophysiology, University of Aarhus, 131 C. F. Møllers Alle, DK 8000 Aarhus C, Denmark; 2Clinicum, Laboratorio Automatizado, Iquique, Chile; 3Biochemistry Department, University of Antwerp, Universiteitsplein 1, B-2610 Antwerpen, Belgium; and 4Laboratorio de Transporte de Oxígeno, Universidad Cayetano Heredia, Lima 31, Peru

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Weber, Roy E., Hrvoj Ostojic, Angela Fago, Sylvia Dewilde, Marie-Louise Van Hauwaert, Luc Moens, and Carlos Monge. Novel mechanism for high-altitude adaptation in hemoglobin of the Andean frog Telmatobius peruvianus. Am J Physiol Regul Integr Comp Physiol 283: R1052–R1060, 2002. First published August 22, 2002; 10.1152/ajpregu.00292.2002.—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 Hb of the aquatic Andean frog Telmatobius peruvianus from 3,800-m altitude as regards isoform differentiation, sensitivity to allosteric cofactors, and primary structures of the α- and β-chains, and we 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 NH2-terminal residues and replacement by nonpolar Ala of polar residues Ser and Thr found at position α131(H14) in human and X. laevis Hbs, respectively. The data indicate adaptive significance of α-chain chloride-binding sites in amphibians, in contrast to human Hb 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.

amphibians; chloride binding; hypoxia; organic phosphates; oxygen transport

HOW IS OXYGEN TRANSPORT to metabolizing tissues secured at high altitude? In contrast to intensive investigations in birds and mammals (7, 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 with 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 2,000 to over 4,000 m (14) where aerial O2 tensions fall from ~159 mmHg at sea level to ~92 mmHg. 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 3,812 m has reduced, poorly developed lungs but exhibits compensatory physiological and behavioral adaptations (24) that include an “oversized,” folded skin, which is penetrated by cutaneous capillaries and ventilated by “bobbing” behavior 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 3,100 to 4,100 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 with 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 (6) 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, and carried out 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.

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Oxygen equilibrium characteristics of isolated Telmatobius Hb I and Hb II and stripped Xenopus Hb

Table 1. Oxygen equilibrium characteristics of isolated Telmatobius Hb I and Hb II and stripped Xenopus Hb

<table>
<thead>
<tr>
<th></th>
<th>Telmatobius Hb II</th>
<th>Telmatobius Hb I 20°C</th>
<th>Xenopus Hemolysate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>20°C</td>
<td>10°C</td>
<td>20°C</td>
</tr>
<tr>
<td></td>
<td>P50 (7.5)</td>
<td>P50 (7.0)</td>
<td>δH (7.5)</td>
</tr>
<tr>
<td>Stripped</td>
<td>7.33</td>
<td>7.83</td>
<td>-0.06</td>
</tr>
<tr>
<td>Cl</td>
<td>8.22</td>
<td>9.90</td>
<td>-0.16</td>
</tr>
<tr>
<td>ATP</td>
<td>26.67</td>
<td>16.5</td>
<td>-0.29</td>
</tr>
<tr>
<td>Cl + ATP</td>
<td>14.0</td>
<td>22.9</td>
<td>-0.43</td>
</tr>
<tr>
<td>Cl + DPG</td>
<td>14.4</td>
<td>26.1</td>
<td>-0.52</td>
</tr>
</tbody>
</table>

P50 values in mmHg; δH values in kJ/mol; δ, Bohr factor (Δ log P50/Δ pH).

RESULTS

Hb heterogeneity. Isoelectric focusing 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 P50 values (Table 1) that correspond with that of the composite hemolysate.
binding at half-saturation ($n_{50}$) was pronounced (2.8) and pH independent in Hbs I and II (see 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 and 3 and 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 with $-0.43$ 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°C than at 20°C (Fig. 3A) reflect exothermic oxygenation and linked endothermic dissociation of allosteric effectors that reduce $\Delta H$ (Table 1). Hb I showed the same $O_2$ affinity trends as the major component (Hb II) but slightly lower anion sensitivities (Table 1 and Fig. 3C).

In the absence of anions, *X. laevis* and *T. peruvianus* Hbs show almost identical $O_2$ affinities ($P_{50} = 7.3$ mmHg 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 with $-0.16$ in *T. peruvianus*) (Fig. 3 and 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 $O_2$-linked binding of one phosphate molecule per deoxyHb tetramer. The maximum ATP/DPG-induced log $P_{50}$ shift is smaller than that for DPG and human Hb.

**Fig. 2.** $O_2$ equilibrium curves of stripped *T. peruvianus* Hb II (top) and *X. laevis* Hb (bottom) 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 $P_{50}$ and $n_{50}$ 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°C (dashed lines) and 20°C (continuous lines); ○, no effectors; △, Cl$^-$/; ○, ATP; ●, ATP + Cl$^-$/; ★, 2,3-diphosphoglycerate (DPG) + Cl$^-$/; [Heme], 0.15 mM (Hb II) and 0.09 mM (Hb I); other details as in Fig. 2.
phosphates primarily reduce the O2 association constant of Hb. In contrast to most vertebrate Hbs where organic phosphates are known to modulate Hb binding affinity, tense state of the Hb molecules (37) indicates that the 

![Fig. 4. Dose-response curves showing effects of ATP (■) and DPG (□) on P50 of T. peruvianus Hb II (pH 7.0); [Cl-], 0.10 M; [Heme], 0.15 mM. • Effect of DPG on human Hb (pH 7.3); [Cl-], 0.05 M, see Ref. 9; temperature, 20°C. Arrows and dotted lines show maximum phosphate-induced changes in P50. Dashed straight lines indicate slopes of 0.25.](image)

Extended Hill plots for T. peruvianus Hb II at 10°C 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 is also 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 (Kp) (55, 62) thus increasing the free energy of cooperativity (ΔG), ATP also decreases the association constant of the high-affinity relaxed state (Kd) and reduces ΔG (Table 2). However, the Kd values need to be viewed with caution due to difficulties in measuring the last few percent saturation of the oxygenation curve (37).

**Primary structure.** The primary structures of the α- and β-chains of Hb II are shown in Fig. 6. Whereas the β-chain was directly accessible for Edman degradation, NH2-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 NH2-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 NH2-terminal residues compared with 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 with *X. laevis* are concentrated in the NH2-terminal region where only 8 of 24 NH2-terminal β-chain residues are identical. Although common in fish Hbs, acetylation of the α-amino group of the α-chains as found in T. peruvianus Hb II 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 interspecies correlations are in accordance 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)] in the toad *Bufo marinus* (3). However, 10- to 11-day 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. Compared with human Hb, *T. peruvianus* Hb II 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 α2-chains, e.g., NH₂-terminal Val, α82(NA2)Lys, and α143(H21)Lys, which replaces histidine in human Hb. In *X. laevis*, the deletion of the first α1-chain residue (Fig. 6) could bring the NH₂-terminus closer to the bound cofactor and preserve phosphate sensitivity despite the loss of His(NA2). The Bohr effect

### Table 2. MWC and derived parameters for *Telmatobius* Hb II at 10 and 20 °C in the absence and presence of saturating ATP concentrations

| °C | pH | Effector | P₅₀ | Pₙ₅₀ | n₅₀ | nₘ₅₀ | Kᵣ | SE | Kᵣ | SE | L | ΔG | rms |
|----|----|----------|-----|------|-----|------|-----|----|-----|----|----|---|-----|-----|
| 10 | 7.082 | ATP     | 14.45 | 13.44 | 2.73 | 2.79 | 0.016 | 0.0014 | 1.78 | 0.917 | 3.3×10⁻¹³ | 10.58 | 0.060 |
| 20 | 7.185 | ATP     | 9.59 | 9.09 | 2.87 | 2.91 | 0.019 | 0.0016 | 2.13 | 0.703 | 1.4×10⁻¹³ | 11.23 | 0.068 |
| 20 | 7.082 | ATP     | 22.73 | 21.37 | 2.76 | 2.81 | 0.0094 | 0.00077 | 0.88 | 0.285 | 1.3×10⁻¹³ | 10.73 | 0.061 |

P₅₀ and Pₙ₅₀ (half-saturation and median O₂ tension values) in mmHg; Kᵣ, Kᵣ [O₂ association constants of low-affinity (tense) and high-affinity (relaxed) states, respectively] and SE in (mmHg); n₅₀ and nₘ₅₀, cooperativity coefficients at half-saturation and maximal values, respectively; L, allosteric constant [T/R] in the absence of O₂; ΔG (free energy of heme-heme interaction) in kJ/mol; rms, root mean square error. The Monod-Wyman-Changeux (MWC) model was fitted with the number of interacting binding sites (q₃) fixed at 4. (Other details as in Fig. 4 legend.)
of the stripped \textit{T. peruvianus} Hb II 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 \textit{T. peruvianus} Hbs II (Fig. 4 and Table 2) and the pH effect in \textit{Rana temporaria} Hb (6). The molecular mechanism underlying these effects must await the elucidation of the crystal structures of deoxy and oxy forms of amphibian Hbs.

The similar \textit{O}_2 affinities in Hbs I, II, and III and in the stripped hemolysate (Table 1 and Fig. 1) indicate the absence of functionally significant interactions between the individual isoHbs under the tested conditions. This contrasts with \textit{R. catesbeiana} where aggregation of the major tetrameric components B and C to form a low-affinity BC\textsubscript{2} 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 \textit{T. peruvianus} Hb? Comparison with lowland \textit{X. laevis} Hb shows that the major difference resides with the effects of anions. Although stripped Hbs from the two species show almost identical \textit{O}_2 affinities and pronounced [ATP + Cl\textsuperscript{−}] effects, \textit{T. peruvianus} Hb shows a drastically suppressed chloride sensitivity (Δlog \textit{P}_50 = 0.10 compared with 0.32 in \textit{X. laevis} and >0.4 in human Hb; Figs. 2 and 7B). In the absence of other changes, this will enhance \textit{O}_2 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 homothermic vertebrates. However, in contrast to the bar-headed goose and Rippell’s griffon (8, 13, 45) that may fly at 9,000 and 11,300 m above sea level, where the high intrinsic \textit{O}_2 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 \textit{T. peruvianus} Hb II results from a loss of anion sensitivity that correlates with α-subunit amino acid substitutions.

Two schools of thought exist as regards \textit{O}_2-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-NH\textsubscript{3} 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-NH\textsubscript{3} 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+H23) [where β1Val(NA1) is exchanged for Met and β2(NA2)His is deleted], which document the implication of the NH2-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 (10) 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 in 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 NH2-terminal residues are free), which shows pronounced chloride sensitivity, but eliminated in T. peruvianus Hb (where 131 is occupied by nonpolar Ala and the α-chain NH2-termini are acetylated), which shows strongly reduced chloride sensitivity. That chloride may additionally 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, 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 with 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), Val inserted at β1(NA1) (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 with X. laevis Hb. Such effects are not evident from our data.

Other evidence indicates that “localized,” α-chain chloride binding may also be implicated in adaptations encountered in some mammalian Hbs. The high O2 affinities of Hb from Andean camelid vicuna (31) and of embryonic pig Hbs Gower I and Heide I that have ζ(α-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 (ζE2 and ζG2) (68) correlates with an analogous α131Ser→α131Val 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 (11, 12, 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, low temperature 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 are 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- als 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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