Isohemoglobin Differentiation in the Bimodal-breathing Amazon Catfish * 

**Hoplosternum littorale**

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The bimodal gill(water)/gut(air)-breathing Amazonian catfish *Hoplosternum littorale* that frequents hypoxic habitats uses “mammalian” 2,3-diphosphoglycerate (DPG) in addition to “piscine” ATP and GTP as erythrocytic O2 affinity modulators. Its electrophoretically distinct anodic and cathodic hemoglobins (HbAn and HbCa) were isolated for functional and molecular characterization. In contrast to HbAn, phosphate-free HbCa exhibits a pronounced reverse Bohr effect (increased 

O2 affinity with decreasing pH) that is obliterated by ATP, and opposite pH dependences of Kt (O2 association constant of low affinity, tense state) and the overall heat of 

oxygenation. Dose-response curves indicate small chloride effects and pronounced and differentiated phosphate effects, DPG < ATP < GTP < HIP. HbCa-O2 equilibria analyzed in terms of the Monod-Wyman-Changeux model show that small T state bond energy differences underlie the differentiated phosphate effects. Synthetic peptides, corresponding to N-terminal fragment of the cytoplasmic domain of trout band 3 protein, undergo oxygenation-linked binding to HbCa, suggesting a metabolic regulatory role for this hemoglobin. The amino acid sequences for the α and β chains of HbCa obtained by Edman degradation and cDNA sequencing show unusual substitutions at the phosphate-binding site that are discussed in terms of its reverse Bohr effect and anion sensitivities.

The ability of fish to colonize a large variation of biotopes is integrally related with the striking molecular and functional differentiation encountered in their hemoglobin (Hb) systems. Variations in the functional properties of Hb result partly from variations in molecular structure that determine the intrinsic O2 binding properties (1) and partly from regulatory changes in the physicochemical conditions under which they operate in vivo, such as red cell pH (that varies with ventilation rate and catecholamine stimulation) and in the type and concentration of heterotropic effectors like organic phosphates that decrease Hb-O2 affinity (2–6).

In addition to “anodic” Hbs (HbAn) that migrate anodically under normal electrophoretic conditions (pH ~8.6) and have relatively low O2 affinities and marked Bohr effects (decreased O2 affinity that enhances O2 release in the acid tissues) and Root effects (reduction in O2 binding capacity upon acidification that induces O2 unloading in the swim bladder and retina), many fish species express “cathodic Hbs” (HbCa) that have high isoelectric points and lack significant pH effects suggesting that they safeguard O2 transport to tissues under hypoxic and acidic conditions (7–9). Previous studies on the physiological and molecular implications of Hb multiplicity in fish have been concentrated on only a few species, such as rainbow trout, *Oncorhynchus mykiss*, and the eel *Anguilla anguilla* that exhibit radical differences, indicating the existence of diverse molecular strategies among teleosts. Thus, whereas cathodic HbI of trout lacks a Bohr effect and is insensitive to phosphate effectors (10, 11), cathodic eel HbCa shows a reverse Bohr effect in the absence of phosphates and greater phosphate sensitivity than anodic eel HbI (12–14). Also, whereas the NTP pool of trout erythrocytes almost entirely consists of ATP, GTP is the main effector in eels, where it shows a greater effect on Hb-O2 affinity and greater decreases in concentration following hypoxic exposure than ATP (12).

Deoxygenated Hb may also bind the cytoplasmic domain of erythrocytic band 3 proteins (cd-B3) in competition with glycolytic enzymes, as demonstrated for the human proteins (15, 16). The absence of effects of peptides corresponding to N-terminal fragments of trout cd-B3 on O2 affinity of anodic trout HbIV, despite pronounced effects on human Hb (17), calls for closer study of Hb-band 3 interaction in fish.

*Hoplosternum littorale*, a small, heavily armored catfish from the Amazon basin, is an ideal model for investigating molecular adaptations in Hb function to extreme environmental conditions, bimodal breathing and modes of life. While using gills for gas exchange in well aerated water, it surfaces to swallow air in O2-deficient waters and has a thin-walled part of the intestine phase-high-performance liquid chromatography; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol; MES, 4-morpholineethanesulfonic acid.

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The abbreviations used are: Hb, hemoglobin; HbAn, electrophoretically anodic Hb; HbCa, cathodic Hb; DPG, 2,3-diphosphoglycerate; cd-B3, cytoplasmic domain of Band 3 protein; MWC, Monod, Wyman and Changeux; Kt and Kp, O2 association constant of low affinity, tense, and high affinity relaxed states, respectively, of Hb; RP-HPLC, reverse-phase high-performance liquid chromatography; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol; MES, 4-morpholineethanesulfonic acid.

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that is kept devoid of food and appears to be a site for aerial gas exchange (18). The fish constructs floating nests of dead weed that expose the developing embryos to higher O₂ tensions than those prevailing in the water (18). A further peculiarity is that its red cells contain the "mammalian" cofactor DPG as well as "piscine" effectors ATP and GTP in approximately equal concentrations and that the DPG levels vary with environmental temperature (19, 20). It has single anodic and cathodic HbAs (that exhibits and lacks a Root effect, respectively) and shows no evidence for polymorphism in Hb multiplicity (21).

We report on the interactive effects of pH, the naturally occurring effectors ATP, GTP, DPG, and Cl⁻ and of IHP on O₂ binding of Hoplosternum HbAn and HbCa, and on the oxygenation-linked interaction with a synthetic peptide corresponding to the N terminus of trout cd-B3. In order to understand the structural and allosteric basis for its distinctive functional characteristics, we also analyzed the O₂ equilibria of HbCa in terms of the two-state model for allosteric transitions (22), and we determined the primary structures of its globin chains.

**EXPERIMENTAL PROCEDURES**

Adult H. littorale (65) (14–16 cm, 45–66 × g) locally known as tamoata were collected by throw net in the Solimoes river near Marchantaria, Brazil. Blood was taken in heparinized syringes from the caudal blood vessels. Saline-washed red cells were frozen at −80 °C until use.

Hb was prepared as described previously (23) and dialyzed against 0.02 M Tris-HCl buffer, pH 8.4 (at 5 °C). Electrophoresis on cellulose acetate strips revealed only two Hb components that were separated by anion exchange chromatography on a 27 × 2 cm DEAE-Sephacel column equilibrated with the dialysis buffer and eluted in a 0–0.1 M NaCl gradient. Separated fractions were dialyzed for 24 h against three changes of CO-equilibrated 0.01 M HEPES, pH 7.7, containing 5×10⁻⁴ M EDTA. All preparative steps were carried out at 0–5 °C. The Hb was frozen at −80 °C in 90–150-μl aliquots that were individually thawed immediately before experimentation. Stripped human Hb for control measurements was prepared as described previously (24) from blood of a non-smoking adult.

**O₂ Binding—**O₂ binding equilibria were measured using a modified diffusion chamber, where ultrathin layers of Hb solution were equilibrated with pure (>99.99%) N₂ or O₂ or stepped mixtures of these and air prepared with Wusthoff pumps to ensure full equilibration at each step (23, 25). The pH of Hb solutions was adjusted using HEPES buffers for pH 6.5–8.2, MES buffers for lower, and glycine buffer for higher pH values (final buffer concentration, 0.10 M). The pH was measured in oxygenated (air-equilibrated) Hb samples using a BMS2 Mk2 Blood Micro system and PHM 64 Research pH meter (Radiometer, Copenhagen, Denmark). Chloride was added as KCl and measured using a Radiometer CMT10 chloride titrator. ATP, GTP, and DPG concentrations in stock samples were assayed using Sigma test chemicals. The effects of anions on O₂ equilibria were measured at pH near 7.5 and 7.0, thereafter the P50 (half-saturation O₂ tension) and n₅₀ (Hill cooperativity coefficient at P₅₀) values at these exact pH values were interpolated from linear regressions. The overall heat of oxygenation ΔHₘ, which includes the heat of solution of O₂ (∼13 kJ·mole⁻¹) and the heats of processes linked to O₂ binding such as proton and anion dissociation, was evaluated as: ΔH_m = R(TΔlnP₅₀/ΔT), where R is the gas constant. The effects of synthetic peptides corresponding to the first 10 and 20 amino acid residues of trout cd-B3 on HbO₂ affinity were examined as earlier described (17). The sequence of the 20-mer peptide is Met-Glu-Asn-Asp-Leu-Ser-Phe-Gly-Glu-Asp-Val-Met-Ser-Tyr-Glu-Glu-Glu-Ser-Asp-Ser (the 10-mer comprises the first 10 residues) (26).

To analyze the allosteric interactions, precise O₂ equilibria measured with focus on extreme (low and high) saturation values were analyzed in terms of the MWC model (22), evaluating with focus on extreme (low and high) saturation values were analyzed in terms of the MWC model (22), evaluating with focus on extreme (low and high) saturation values were analyzed in terms of the MWC model (22), evaluating with focus on extreme (low and high) saturation values were analyzed in terms of the MWC model (22), evaluating with focus on extreme (low and high) saturation values were analyzed in terms of the MWC model (22), evaluating with focus on extreme (low and high) saturation values were analyzed in terms of the MWC model (22), evaluating with focus on extreme (low and high) saturation values were analyzed in terms of the MWC model (22), evaluating with focus on extreme (low and high) saturation values were analyzed in terms of the MWC model (22), evaluating with focus on extreme (low and high) saturation values were analyzed in terms of the MWC model (22), evaluating with focus on extreme (low and high) saturation values were analyzed in terms of the MWC model (22). Samples were acidified with trifluoroacetic acid and applied to a ProSphere RP C4 5-μm column (300 Å; 4.6 × 250 mm; Alltech Associates, Inc.) equilibrated with 5% acetonitrile in 0.1% aqueous trifluoroacetic acid. The samples were eluted with a linear gradient of 5–75% acetonitrile in 0.1% trifluoroacetic acid over 45 min at a flow rate of 1 ml/min. Absorbance of the eluate was monitored at 280 nm.

**Enzymatic Digestion and Peptide Isolation**—Globin chains were digested with trypsin at an enzyme:substrate ratio of 1:50 in 200 mM NH₄HCO₃, pH 8.3, at 37 °C for 6 h. The digested products were isolated by RP-HPLC as described for the globin chain isolation. The amino acid sequence of peptides was determined with an automated protein sequence analyzer ABI 471 B (Applied Biosystems), according to the manufacturer’s recommendations.

**Primer Design and cDNA Sequencing**—By using the amino acid sequence of the β chain, the degenerated primer HOPLO F1, TGGGGNAARATHCAYATHGA, a 20-mer with 144 redundancies, was designed corresponding to the sense strand predicted by the peptide...
fragments WGIKHD (Fig. 10). Total RNA was isolated from intact erythrocytes with a micro RNA isolation kit (Stratagene). First strand cDNA was synthesized with MMLV-RT (Promega) using an oligo(dT) primer. PCR reactions were carried out using HOPLO F1 and oligo(dT). The PCRs were carried out for 30 cycles of 94 °C for 30 s, 50 °C for 1.0 min, and 72 °C for 1.5 min with Taq polymerase on a GeneAmp PCR system 9600 (Perkin-Elmer). Sequencing was then performed with HOPLO F1 as primer on an ABI 377 automatic sequencer (Applied Biosystems, Inc.) according to the manufacturer’s recommendations.

Electrospray Ionization Mass Spectrometry—Electrospray data were acquired on a Quattro II triple quadrupole mass spectrometer (Micromass Ltd.) as described elsewhere (30).

RESULTS

Oxygenation Studies—Anion exchange chromatography resolves the Hb into two distinct fractions, HbCa and HbAn, occurring in a ratio of approximately 38:62 (Fig. 1). The oxygenation characteristics of HbAn and HbCa are radically different. At pH 7.2, the approximate intracellular value, the affinity of stripped HbCa markedly exceeds that of HbAn (#P_50$ = 2.4 and 8.7 mm Hg, respectively, at 25 °C) (Figs. 2 and 3). In contrast to the pronounced normal Bohr effect in HbAn ($\varphi = \Delta \log P_50/\Delta pH = -0.56$ at pH 7.2), HbCa exhibits a marked, reverse Bohr effect ($\varphi = +0.38$). Due to opposite pH effects the functional differentiation between the two isoHbs increases with falling pH.

HbAn exhibits much greater sensitivity to ATP than HbCa. The phosphate effects increase with falling pH, whereby the presence of ATP induces a slight normal Bohr effect in HbCa ($\varphi = -0.14$ at pH 7.2) and almost obliterates the affinity difference between the two Hb components (Fig. 3). Significantly, ATP alone decreases O₂ affinity of both components more than ATP in the presence of 100 mM Cl⁻ (as illustrated for pH 7.2 by the $\Delta \log P_50$ columns in Fig. 3). The Hill coefficient $n_{50}$ approximates 2.0 in both Hbs at pH 6.5–8.0, decreases at low and high pH to 1.5 in HbAn, and at low pH to 1.7 in HbCa (Fig. 3) but increases to 2.4 in HbCa in the presence of ATP.

The difference between the Bohr effect curves at 10 and 25 °C (Fig. 4) illustrates a large overall temperature effect ($\Delta H^*$ about $-85$ kJ mol$^{-1}$) in HbAn at high pH (8.7) where the Bohr effect and phosphate binding disappear (cf. Fig. 3). At lower pH, where the Bohr effect is operative, the enthalpy of oxygenation decreases to approximately $-45$ kJ mol$^{-1}$ at pH 6.8 reflecting endothermic proton release. Given that the Bohr factor (0.65) gives the moles of protons dissociated per mol of O₂
bound, the enthalpy difference (+40 kJ mol⁻¹ of heme) indicates an apparent heat of proton dissociation of 62 kJ mol⁻¹. Analogously the increase in enthalpy for HbCa (by approximately 18 kJ mol⁻¹ as pH decreases from pH 9, Fig. 4) reflects proton association upon O₂ binding, in accordance with the reverse Bohr effect. Related to the Bohr factor (+0.38) this increase indicates an apparent ionization enthalpy of approximately 47 kJ per mol of protons bound. These values may, however, be biased by thermodynamic contributions from other oxygenation linked processes, such as Cl⁻ binding, that may account for the lower ΔH value found in HbCa at pH 6.0 than at pH 8.5 (where oxygen-linked proton binding approximates zero).

Chloride ions reduce O₂ affinity of both HbAn and HbCa, except for HbA at pH >7.7 where 0.1 M chloride increased affinity (Fig. 3). Below pH 7.7 chloride and saturating ATP concentration raise the Bohr effect of HbAn to affinity (Fig. 3). Below pH 7.7 where 0.1 M chloride increased affinity (Fig. 3). Below pH 7.7 chloride and saturating ATP concentration raise the Bohr effect of HbAn to affinity (Fig. 3). Below pH 7.7 where 0.1 M chloride increased affinity (Fig. 3). Below pH 7.7 chloride and saturating ATP concentration raise the Bohr effect of HbAn to affinity (Fig. 3). Below pH 7.7 where 0.1 M chloride increased affinity (Fig. 3). Below pH 7.7 chloride and saturating ATP concentration raise the Bohr effect of HbAn to affinity (Fig. 3). Below pH 7.7 where 0.1 M chloride increased affinity (Fig. 3). Below pH 7.7 chloride and saturating ATP concentration raise the Bohr effect of HbAn to affinity (Fig. 3).

Interpolated on the basis of the P₅₀ maximum induced by IHP (Fig. 6A), the data indicate apparent dissociation equilibrium constants, Kₑ (estimated as the effector concentration that induces half of the maximum change in log P₅₀) for the reactions of HbCa with ATP, GTP, and IHP at pH 7.0 of approximately 11 × 10⁻⁴, 5.4 × 10⁻⁴, and 2.2 × 10⁻⁴ m, respectively. Interpolated in terms of the P₅₀ maximum induced by DPG, the constant for DPG approximates 13.2 × 10⁻⁴ m. Compared with values for the reaction of DPG with human and Eskimo dog Hbs (3.2 × 10⁻⁴ m at pH 7.5 and ~1 × 10⁻⁴ m at pH 7.2, respectively, at 20 °C and in the presence of 100 mM Cl⁻) (33, 34), this illustrates relatively low DPG sensitivity in Hoplosternum HbCa.

In contrast to the pronounced effects of the 10- and 20-mer synthetic trout band 3 peptides on the O₂ affinity of human Hb (Fig. 7; see also Ref. 17), the peptides had no effect on Hoplosternum HbAn at pH 7.2 and only marginally decreased the O₂ affinity at lower pH (6.4) (Fig. 7). This aligns with the absence of effects in trout Hbs I-IV (17),² despite the large effects of these peptides in human Hb (17). Significantly, the peptide exerts a distinct effect on Hoplosternum HbCa at pH 7.2 and an even greater effect at lower pH (6.58) (Fig. 7). The effect on human Hb (17) and the marked pH-dependent effects in Hoplosternum HbCa (Fig. 7) attest to the functionality of the peptides and the presence of a putative band 3-binding site in both Hbs.

The allosteric and derived MWC model parameters are given in Table I. The agreement between n₅₀ and nₘₐₓ and between P₅₀ and Pₘ values reflects highly symmetrical O₂ equilibrium curves that permit rigorous analysis of P₅₀ plots. Moreover, the mean value for the number of interacting O₂-binding sites per molecule (q = 4.03 ± 0.74), obtained when q was fit along with the other parameters to obtain the best possible fit in the 13 condition sets described in Table I, tallies neatly with a tetrameric structure. The derived parameters summarized in Table I were thus obtained with q fixed at 4.

Extended Hill plots for the effects of pH and organic phosphates in HbCa are shown (Figs. 8 and 9). In contrast to anionic vertebrate Hbs where the normal alkaline Bohr effect primarily results from a decrease in Kᵣ with increasing proton concentrations (23, 35, 36), the control mechanism of the reverse Bohr effect of Hoplosternum HbCa is an increase in Kᵣ with falling pH (Fig. 8, Table I), indicating a more constrained T

² R. E. Weber, unpublished observations.
state with increasing pH. The Bohr factor of the deoxygennated (T state) Hb markedly exceeds that at median saturation (φ = +0.35 versus +0.25, respectively, at pH 7–8; Fig. 8, inset). Increased pH accordingly raises the free energy of cooperativity (∆G increases from approximately 6.5 to 8.5 kJ·mol between pH 7 and 8, see Table I) as illustrated by the greater distance
 IsoHb Differentiation in Hoplosternum

The marked functional differentiation between *Hoplosternum* HbAn and HbCa agrees with earlier findings of Garlick et al. (21). In contrast to their study carried out in the presence of ionic (Tris/BisTris) buffers that may perturb the Bohr and phosphate effects due to higher chloride levels at low pH values (24), the present work carried out using zwitterionic HEPES buffer shows much lower Bohr factors \( \varphi = -0.56 \) compared with \(-0.98\) for HbAn.

**The Reverse Bohr Effect**—What may be the significance of a reverse Bohr effect in HbC that is obliterated by ATP? In view of the greater reduction of \( \text{O}_2 \) affinity by phosphates at low pH, we propose that a reverse Bohr effect in phosphate-free solution is a precondition for small in vivo pH effects associated with pronounced phosphate sensitivity.

Apart from *Hoplosternum*, Hbs with pronounced reverse Bohr effects occur in the facultative air-breathing teleost *Pterygoplichthys pardalis* (21, 40), the surface skimmer *Mylossoma sp.* (41), frog tadpoles, and aquatic salamanders (cf. Ref. 13) suggesting implication in the utilization of alternative sources of \( \text{O}_2 \). The reverse Bohr effect and strong phosphate sensitivity in *Hoplosternum* HbCa contrast with lack of Bohr and NTP effects in cathodic trout HbI but accord with data for eel *Anguilla* (12–14), *Mylossoma* (41), and *Pterygoplichthys* (40), indicating that the intensively studied trout HbI is an exceptional rather than prototype cathodic Hb.

In human Hb, the main Bohr groups are N-terminal Val-NA1(α1) and the C-terminal His-HC3(β146) that account for about 30 and 50–65%, respectively, of the normal Bohr effect, whereas His-H21(β143) is considered to be involved in the expression of the reverse ("acid") Bohr effect that reflects the uptake of protons upon oxygenation at low pH (<6.5) (42–44). With Val-NA1 acetylated in fish Hbs, the absence of a normal Bohr effect in stripped *Hoplosternum* HbCa correlates with the His→Phe-HC3(β146) replacement, as found in cathodic Hbs of trout, eel, and catfish (7, 13, 38). The reverse Bohr effect becomes apparent only when the major alkaline Bohr groups are replaced (as in cathodic Hbs) or inoperative (as in anodic Hbs that exhibit reverse Bohr effects at high pH) (13, 45). Apart from the HC3(β146) substitution, *Hoplosternum* HbCa shows a His→Asn-FG4(994) replacement that also is encountered in eel HbCa and other reverse Bohr effect Hbs, providing further evidence for involvement of His-FG4(994) in the alkaline Bohr effect of fish Hbs (45). Interestingly, Ser-F9(983), which typically is conserved in fish Hbs with normal Bohr and Root effects and which has been considered to donate a hydrogen bond to His-HC3(β146) (46), is substituted by Cys in *Hoplosternum* HbCa and by Asn in eel HbCa. Cys at F9(983) is another mammalian trait and highly exceptional in fish Hbs.

The molecular mechanism proposed for the reverse Bohr effect in eel HbC (13) visualizes the implication of the residues
at the phosphate-binding site that in fish Hbs include Val-NA1(b1), Glu-NA2(b2), Lys-EF6(b82), and Arg-H21(b143). In the T state the proximity of positively charged amino acid residues in the central cavity reduces their affinity for protons (whereas their $pK_a$ values are normal in the R state), whereby the groups implicated in organic phosphate binding become reverse Bohr groups in the absence of phosphates. In other words, protons destabilize the T state, as is evident from the increase of $K_T$ with pH decrease, whereas the O2 affinity of the R state is practically unaffected (Fig. 8; Ref. 14). Accordingly, the reverse Bohr effect in Hoplosternum HbCa having His at NA2(b2) is almost twice as large as that in eel Hb Ca having Glu-NA2(b2) (0.38 and 0.2, respectively), indicating that more positively charged groups in the central cavity contribute to this effect in Hoplosternum.

To our knowledge the increase in overall oxygenation enthalpy of HbCa (increased temperature sensitivity) with falling pH (Fig. 4) provides the first demonstration of the thermodynamic consequences of O2-linked proton binding associated with a reverse Bohr effect. The opposite pH dependence of the temperature effects in HbAn and HbCa (Fig. 4) would tend to keep a constant and pH-independent in vivo heat of oxygenation.

**Organic Phosphate Interaction**—Most fish Hbs have Glu at NA2(b2), which accepts hydrogen bonds from strain-free ATP and GTP molecules (47). The presence of His at NA2(b2) in Hoplosternum HbCa is exceptional for teleosts and other ectothermic vertebrates, where its distribution suggests a correlation with air breathing or the presence of alternative red cell phosphates. As listed (48) it occurs in the Hbs of the lungfish Lepidosiren paradoxa, where 6–8% of its erythrocytic phosphates is inositol diphosphate (49), the sharks Squalus acanthias and Heterodontus portusjacksoni, where high erythrocytic urea levels antagonize the modulator effectivity of ATP (50), and tadpoles of the frog Rana catesbeiana and the toad Xenopus laevis. Alternatively, the episodic occurrence of His-NA2 in elasmobranchs, lungfish, and developmental stages of higher vertebrates suggests that it may be a phylogenetically primitive character that was deleted in most non-mammalian vertebrates.

The occurrence of high levels of DPG in Hoplosternum erythrocytes together with the "mammalian DPG-binding" residue...
His-NA2(b2) in HbCa appears to impart no selective advantage for DPG binding, given that HbCa, as does HbAn, exhibits markedly lower sensitivities to DPG than to ATP and GTP (Fig. 6).

In view of the large phosphate effects in Hoplosternum HbCa, the presence of uncharged Ser at H21(b143), compared with His in human Hb and Arg or Lys in other fish Hbs, is unexpected and calls for reconsideration of the importance of individual phosphate-binding sites. Moreover, Ser-H21(b143) also occurs in trout HbI and human fetal Hb that have no and small phosphate effects, respectively. These findings suggest minor significance of H21(b143) for phosphate interaction or that the Glu3His-NA2(b2) exchange in HbCa compensates for absence of phosphate binding at this site. A recent NMR study of mutant recombinant Hbs (44) similarly indicates that H21(b143) is not essential for DPG binding in the neutral pH range.

The progressively increasing effects of DPG, ATP, and GTP on O2 affinity of Hoplosternum Hbs (Fig. 6) contrast with human Hb that exhibits similar sensitivities to these effectors (51) and similar binding constants for ATP and DPG (35). In life, however, NTP effects may be drastically reduced as a result of complex formation with divalent cations, since the ATP-Mg2+ stability constant exceeds the DPG-Mg2+ constants by an order of magnitude (52, 53).

The maximal slope of log P50 versus log[DPG] curve (Fig. 6) is consistent with a 1:1 (DPG/Hb tetramer) stoichiometry found in human and other mammalian Hbs (34). The t values exceeding 0.25 observed with IHP and NTP (Fig. 6) could result from binding of these effectors at additional sites. In dromedary Hb, the pattern of Cl− and phosphate binding similarly indicates the presence of two polyanion sites per tetramer in deoxy and oxygenated Hb, one of which becomes stronger and the other weaker, in terms of affinity, as a result of oxygenation of the molecule (31).

The greater effects of phosphates on O2 affinity of HbCa than HbAn in Hoplosternum indicate a dominant role of Hb Ca in adapting blood O2 affinity to variations under the environmental conditions. In the armored catfish Hypostomus and Pterygoplichthys hypoxic exposure induces (gut) air breathing and lowers ATP and GTP levels that may increase blood O2 affinity and exploitation of the O2 reserves during submersion (54, 55).

Chloride Effects—In human Hb, Cl− may act either by neutralizing the positive charges in the central cavity without binding to specific residues (56) or bind at specific sites (57). Two major sites generally considered to be implicated in chloride binding in human Hb are Val-NA1(a1) that interacts with Ser-H14(a131) and Lys-EF6(b82) that interacts with Val-NA1(b1) (cf. Ref. 43). The t values for Hoplosternum Hbs A and C (0.22) and human Hb (0.48) (Fig. 5) indicate oxygenation-linked binding of 1 and 2 chloride ions, respectively, per tetramer, which accords with acetylation of Val-NA1(a) in Hoplosternum HbCa (Fig. 11).

The unexpected increase in O2 affinity induced in Hoplosternum HbAn by 0.1 M Cl− at pH 7.5 (Fig. 3) may result from Cl− binding to the R state. It agrees with the observation that in the presence of Cl−, low DPG and ATP levels raise O2 affinity of HbAn at pH 7.5 (Fig. 6D). Human Hb similarly provides...
evidence for Cl− binding in the oxygenated state (58). The lesser effects of ATP + Cl− than of ATP in both Hb components (Fig. 3) suggest that Cl− ions block binding of the phosphate effector at common binding sites.

**Band 3 Peptide Effects**—The effect of the synthetic trout cd-B3-peptide on the O2 affinity of *Hoplosternum* HbCa provides the first evidence for functionally significant interaction between fish Hb and band 3 proteins, suggesting a possible transducer role for *Hoplosternum* HbCa in regulating cellular processes in an oxygen-dependent manner. Band 3 proteins are responsible for HCO3−/Cl− exchange across the red cell membranes, and Hb and glycolytic enzymes (such as aldolase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase) compete for binding to their cytoplasmic domains (16, 59, 60). Thus high O2 availability releases Hb from the cd-B3 protein that thus become available for inhibiting glycolytic activity and controlling red cell volume via cAMP-dependent NaCl uptake (61).

Why does trout cd-B3 undergo oxygenation-linked binding with *Hoplosternum* HbC and human Hb but not with trout Hbs? We suggest that this is due to the presence of positively charged His at NA2(β2) in *Hoplosternum* HbCa and human Hb, given that the lack of effect on trout HbIV may result from lesser effects of ATP

\[ K_{ATP} = 1.9 \text{ kJ mol}^{-1} \]

respectively, at phosphate/Hb 2.5, 2.19, 3.16, 3.98, and 4.19 kJ mol−1, respectively, at phosphate/Hb ~28. These values are low compared with the stabilization energy of internal hydrogen bonds (12 kJ mol−1) (64) illustrating that small bond energy differences may account for large differences in the effects of individual heterotrophic phosphate effectors.

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