Lack of conventional oxygen-linked proton and anion binding sites does not impair allosteric regulation of oxygen binding in dwarf caiman hemoglobin

Roy E. Weber,1 Angela Fago,1 Hans Malte,1 Jay F. Storz,2* and Thomas A. Gorr3,4*

1Zoophysiology, Department of Bioscience, Aarhus University, Aarhus, Denmark; 2School of Biological Sciences, University of Nebraska, Lincoln, Nebraska; 3Institute of Veterinary Physiology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland; and 4Center for Pediatrics and Adolescent Medicine, University Medical Center Freiburg, Freiburg, Germany

Submitted 24 January 2013; accepted in final form 22 May 2013

Weber RE, Fago A, Malte H, Storz JF, Gorr TA. Lack of conventional oxygen-linked proton and anion binding sites does not impair allosteric regulation of oxygen binding in dwarf caiman hemoglobin. Am J Physiol Regul Integr Comp Physiol 305: R300–R312, 2013. First published May 29, 2013; doi:10.1152/ajpregu.00014.2013.—In contrast to other vertebrate hemoglobins (Hbs) whose high intrinsic O2 affinities are reduced by red cell allosteric effectors (mainly protons, CO2, organic phosphates, and chloride ions), crocodilian Hbs exhibit low sensitivity to organic phosphates and high sensitivity to bicarbonate (HCO3−), which is believed to augment Hb-O2 unloading during diving and postprandial alkaline tides when blood HCO3− levels and metabolic rates increase. Examination of the effectors that modulate Hb-O2 affinity, levels of those effectors in the red blood cells, and the O2 tensions for loading the protein molecules from the low-affinity, tense (T) state to the high-affinity, relaxed (R) state, which is basic to cooperativity in O2 binding. Hb-O2 affinity is modulated by allosteric effectors, chiefly protons (low pH) and CO2 that decrease O2 affinity (increase O2 unloading in the tissues via the Bohr effect), and organic phosphate and chloride ions that commonly reduce Hb-O2 affinity by preferential binding at specific sites of the molecules in the T state (62, 91). Thus, whereas protons mainly bind at β146His (the COOH-terminal histidines of the β chains), the organic phosphates [typically 2,3-diphosphoglycerate (DPG) in mammals, and ATP in ectothermic vertebrates] interact with seven amino acid residues in the cavity between the β chains (β1Val of one chain; and β2His, β82Lys, and β143His of both β chains). CO2 binds at the unprotonated NH2-terminal residues of both chains, and Cl− ions at one α chain site (between α Val and α131Ser) and one β chain site (between β1Val and β82Lys) (42, 53, 62, 70, 78).

Crocodilian Hbs exhibit striking, distinguishing characteristics. In contrast to the vast majority of vertebrates, they show little or no response to organic phosphates, Cl−, or CO2 (8, 9, 39). The insensitivity to phosphates correlates with the replacement of three phosphate binding residues. Thus, compared to human Hb, β143His is replaced by Ala and β1Val-β2His by acetylated-Ala-Ser in the Nile crocodile (Crocodylus niloticus) and the American alligator (Alligator mississippiensis) and by Ser-Pro in the spectacled caiman (Caiman crocodilus) (51, 75). Perhaps the best known feature of crocodilian Hbs is that O2 affinity is drastically decreased by HCO3− ions (8), which is considered to play an important role in unloading O2 and maintaining aerobic metabolism when crocodilians dive and drown their prey (8, 95), compensating for their low myoglobin O2 stores (46). The HCO3− effect may also play a vital role in unloading O2 from the blood during postprandial alkaline tides (95) when increased blood HCO3− concentrations (resulting from HCl secretion into the stomach to digest bone) coincide neatly with the increased demand for O2 (the specific dynamic action of food) (19). This view aligns with the fact that crocodilians consume large amounts of bone (one alligator stomach contained remnants of up to 12 turtles) (20) and that the postfeeding metabolic peak in C. porosus was 70% higher when fed bone-rich chicken necks than when fed homogenized chicken (30).

The mechanisms basic to allosteric regulation of Hb-O2 affinity in crocodilians remain controversial. Although modeling indicates deoxygenation-linked binding of HCO3− at three residues in the central cavity between the two β chains; viz., β82Lys, β144Glu of one β chain, and the NH2-terminal residue of the partner β chain (Ser in C. crocodilus) (63), mutagenic replacement of β82Lys did not change the HCO3−

the protein molecules from the low-affinity, tense (T) state to the high-affinity, relaxed (R) state, which is basic to cooperativity in O2 binding. Hb-O2 affinity is modulated by allosteric effectors, chiefly protons (low pH) and CO2 that decrease O2 affinity (increase O2 unloading in the tissues via the Bohr effect), and organic phosphate and chloride ions that commonly reduce Hb-O2 affinity by preferential binding at specific sites of the molecules in the T state (62, 91). Thus, whereas protons mainly bind at β146His (the COOH-terminal histidines of the β chains), the organic phosphates [typically 2,3-diphosphoglycerate (DPG) in mammals, and ATP in ectothermic vertebrates] interact with seven amino acid residues in the cavity between the β chains (β1Val of one chain; and β2His, β82Lys, and β143His of both β chains). CO2 binds at the unprotonated NH2-terminal residues of both chains, and Cl− ions at one α chain site (between α Val and α131Ser) and one β chain site (between β1Val and β82Lys) (42, 53, 62, 70, 78).

Crocodilian Hbs exhibit striking, distinguishing characteristics. In contrast to the vast majority of vertebrates, they show little or no response to organic phosphates, Cl−, or CO2 (8, 9, 39). The insensitivity to phosphates correlates with the replacement of three phosphate binding residues. Thus, compared to human Hb, β143His is replaced by Ala and β1Val-β2His by acetylated-Ala-Ser in the Nile crocodile (Crocodylus niloticus) and the American alligator (Alligator mississippiensis) and by Ser-Pro in the spectacled caiman (Caiman crocodilus) (51, 75). Perhaps the best known feature of crocodilian Hbs is that O2 affinity is drastically decreased by HCO3− ions (8), which is considered to play an important role in unloading O2 and maintaining aerobic metabolism when crocodilians dive and drown their prey (8, 95), compensating for their low myoglobin O2 stores (46). The HCO3− effect may also play a vital role in unloading O2 from the blood during postprandial alkaline tides (95) when increased blood HCO3− concentrations (resulting from HCl secretion into the stomach to digest bone) coincide neatly with the increased demand for O2 (the specific dynamic action of food) (19). This view aligns with the fact that crocodilians consume large amounts of bone (one alligator stomach contained remnants of up to 12 turtles) (20) and that the postfeeding metabolic peak in C. porosus was 70% higher when fed bone-rich chicken necks than when fed homogenized chicken (30).

The mechanisms basic to allosteric regulation of Hb-O2 affinity in crocodilians remain controversial. Although modeling indicates deoxygenation-linked binding of HCO3− at three residues in the central cavity between the two β chains; viz., β82Lys, β144Glu of one β chain, and the NH2-terminal residue of the partner β chain (Ser in C. crocodilus) (63), mutagenic replacement of β82Lys did not change the HCO3−

143His compared with other vertebrate and crocodilian Hbs: (91) revealed a metabolic rates increase. Examination of different species and may also undergo reversible changes respectively (91). Each of these factors may vary among chains, O2 binding at the heme groups triggers a transition of tissues is governed by its intrinsic O2 affinity, its sensitivity to effectors in the red blood cells, and the O2 tensions for loading and unloading O2 at the respiratory surfaces and in tissues, respectively (91). Each of these factors may vary among different species and may also undergo reversible changes within the same individual animals in response to changes in environmental conditions, metabolic requirements, and mode of life.

In vertebrate Hbs that comprise two α chains and two β chains, O2 binding at the heme groups triggers a transition of the role of hemoglobin (Hb) in transporting O2 to respiring tissues is governed by its intrinsic O2 affinity, its sensitivity to the effectors that modulate Hb-O2 affinity, levels of those effectors in the red blood cells, and the O2 tensions for loading and unloading O2 at the respiratory surfaces and in tissues, respectively (91). Each of these factors may vary among different species and may also undergo reversible changes within the same individual animals in response to changes in environmental conditions, metabolic requirements, and mode of life.

In vertebrate Hbs that comprise two α chains and two β chains, O2 binding at the heme groups triggers a transition of the role of hemoglobin (Hb) in transporting O2 to respiring tissues is governed by its intrinsic O2 affinity, its sensitivity to the effectors that modulate Hb-O2 affinity, levels of those effectors in the red blood cells, and the O2 tensions for loading and unloading O2 at the respiratory surfaces and in tissues, respectively (91). Each of these factors may vary among different species and may also undergo reversible changes within the same individual animals in response to changes in environmental conditions, metabolic requirements, and mode of life.
sensitivity of Nile crocodile (C. niloticus) Hb, and replacements of 12 other amino acid residues clustered at the αβ2 interface were required to transplant the bicarbonate sensitivity into human HbA (16, 46, 47). Also, in contrast to the reported absence of oxygenation-linked binding of CO2 in Cai. crocodilus Hb (8), carbamate (carbamino) formation in crocodilians is reflected by the much (twofold to fivefold) larger CO2 Bohr effect than fixed-acid Bohr effect (measured when pH is changed by adding CO2 and buffers, respectively) in Cai. crocodilus and A. mississippiensis blood (39, 95). Finally, contrary to previous reports [cf. (46)], crocodilian red cells may contain a wide spectrum of organic phosphate effectors, albeit at low concentrations. In fact Cai. crocodilus red cells contain ATP and DPG, as well as inositol pentaphosphate (IPP, found in avian red cells), inositol hexaphosphate (IHP), and guanosine triphosphate (GTP, which is commonly encountered in fish red cells) (86).

Aiming to elucidate molecular adaptations and structure-function relationships in crocodilian Hbs, we determined the amino acid sequences of the α and β chains of the Hb of the dwarf caiman, Paleosuchus palpebrosus. This species differs from larger alligators and crocodiles in that it frequents stony creeks with clean, fast-running and cooler water, and mainly feeds on small vertebrates (e.g., tadpoles, frogs, fish, small mammals) and a variety of insects and snails (18). Finding a unique combination of amino acid changes at highly conserved effector binding sites, we investigated the O2 binding properties of the Hb and its sensitivities to chloride ions, ATP, DPG, and IHP, and to CO2 and temperature, over a wide pH range to discern possible alternative allosteric regulatory mechanisms. On the basis of the aggregation of deoxygenated Hbs to octamers and larger polymers (that may occur in vivo) observed in a diverse array of saurupod taxa (birds and nonavian reptiles) (67, 71, 80), we also assessed polymerization in dwarf caiman Hb, its dependence on oxygenation state, and its possible effects on Hb-O2 affinity.

MATERIALS AND METHODS

Primary Structure

Blood from two young (2–3 yr old) specimens of dwarf caiman, P. palpebrosus, sampled in connection with routine diagnostic controls by the veterinarian at Cologne Zoological Gardens in accordance with existing regulations, was a generous gift from the zoo. Red cells separated by centrifugation were lysed in five times their volumes of 20 mM Tris-HCl, 40 mM DTE, pH 8.5 buffer. The hemolysate resolved into three electrophoretic bands by native alkaline disc electrophoresis, each of which was identified by NH2-terminal sequences as a mixture of the same αα and β chains without any hint of the presence of additional subunits (not shown). This indicates that P. palpebrosus Hb consists of a single component whose αα/β chain subunits show a strong tendency to form aggregates that involve disulfide bonds.

For a better yield of purified peptides we subjected a 1:1 mix (w/w) of lyophilized native and oxidized globin to a tryptic digest followed by size-exclusion chromatography (Sephadex G-25 fine) with acidic elution (0.1 N acetic acid), which resolved the digest into 10 fractions of various sized peptides. Next, we employed reverse-phase HPLC with a LiChrospher 60 RP select B column and different trifluoroacetic acid (TFA)/acetonitrile gradients to separate the 10 fractions into a total of 281 peptide-containing peaks, including all αα and β chain tryptic peptides (Tp). The complete primary sequences of the αα and β subunits were then determined by means of automated Edman degradation in conjunction with amino acid analyses of these tryptic and some additional chymotryptic peptides. NH2-terminal and peptide sequencing schemes of either subunit and tables of the amino acid analyses of αα and β peptides are available upon request.

For proper placement of tryptic peptides within the αα or β chain, comparison to the complete globins from the closely related Cai. crocodilus was crucial. In addition, enriched native subunits and pyridyl-ethylated globin, whose reactive cysteinyl SH-groups could no longer form disulfide-based aggregates, were NH2-terminally sequenced up to αα position 24 and β position 58. This approach allowed the unequivocal identification of alkylated cysteines at αα positions 18/19 and β position 23, respectively (see Fig. 1).

Recovery of β Tp9α from the HPLC column occurred in two peaks, 70% of which contained Gltu and 30% Gln at position 73. This Gltu/Gln ambiguity might be explained either by a true allelic difference (heterozygosity) at site 73 or by the spontaneous demediation of the original amide (Gln). Proof of the critically important replacement of the COOH-terminal His by Tyr in the β chain (i.e., of β146 Tyr) was obtained by: 1) amino acid analyses of Tp 14b+15I (position 136–141) and Tp 14b+15II (position 142–146), which provided evidence for a single His existing in Tp 14b+15I, whereas two Tyr residues and no His residues were recovered for Tp 14b+15II; and 2) the sequence of the entire Tp 14b+15 that demonstrated a sole His residue in position 1 (β136) along with Tyr phenylhydantoin (PTH)-derivative peaks of almost equal yields for β145 and β146.

For comparisons of Paleosuchus globins with other cytogastic chains of other species, percentage similarities and identities of amino acid residues (Table 1) were calculated using the EMBoss tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) based on the global alignment Needleman-Wunsch algorithm operating with the Blosum62 matrix.

O2 Binding Measurements

Hemoglobin preparation. Hb solutions obtained by lysing washed red blood cells were centrifuged for 10 min at 400 g to remove cellular debris, and were stripped of organic phosphates on a 38 × 2.1 cm (height × diameter) column of Sephadex G25 Fine gel and dialysed for 24 h against 0.01 mol/l HEPES buffer containing 5 × 10−4 mol/l EDTA, as earlier described (92). The Hb showed no oxygenation as judged from equal absorption values at 539 and 569 nm after brief equilibration with carbon monoxide, and was frozen at −80°C in 150-μl aliquots that were thawed individually for O2 equilibrium and other measurements.

O2 equilibria. Hb-O2 equilibria were measured in the presence of 0.05 mol/l HEPES buffer (89) unless otherwise specified, using a modified gas diffusion chamber coupled to two cascaded Wösthoff pumps (Bochum, Germany) for mixing of air and pure (>99.998%) N2 (88, 93). P02 and n50 (respectively, O2 tension and Hill’s cooperativity coefficients at half-saturation) values were interpolated from linear regressions (r2 > 0.99) of Hill plots (log Y/(1 − Y) vs. log P02, where Y is the fractional saturation) of four or more equilibration steps between 30% and 70% O2-Hb saturation. The effects of ATP (disodium salt), DPG (pentacyclohexyl-ammonium salt), IHP (inositol hexaphosphate, sodium salt), and Cl− (KCl) were investigated by adding accurate volumes of standard, ~100 mM solutions of these effectors to the stripped Hb solutions. ATP was assayed using Sigma test chemicals, and Cl− was assayed using a CMT10 chloride titrator (Radiometer, Copenhagen, Denmark). The pH values were measured against 0.01 mol/l HEPES buffer containing 5 × 10−4 mol/l EDTA, as earlier described (92). The pH values were measured against 0.01 mol/l HEPES buffer containing 5 × 10−4 mol/l EDTA, as earlier described (92).
fit to the data in the form \( \log \frac{[Y]}{[1 - Y]} \) vs. \( \log P_{O2} \) (end weighting) using the curve-fitting procedure previously described (94), and fitting the number of binding sites, \( q \), along with the other parameters to obtain the best possible fit. Additional fits were performed with \( q \) fixed at 4 (as applies to tetrameric Hb). Derived parameters, including the median oxygen tension, \( P_{o2} \); the maximum slope of the Hill plot, \( n_{max} \); and the free energy of hem-o-heme interaction, \( AG \); were evaluated as previously detailed (94). Heats of oxygenation were calculated from the van’t Hoff isochore \( [\Delta H = 2.303 \cdot R \cdot \Delta \log P_{O2}/\Delta(T)] \), where \( R \) is the gas constant and \( T \) is the absolute temperature. All quoted \( \Delta H \) values are exclusive of the heat of solvation of \( O_2 \) (~12.6 kJ/mol).

### Size Exclusion Chromatography

Hb quaternary structure and its oxygenation dependence were investigated by gel filtration of Hb preparations that had not been in contact with CO on a 59.3 × 2.6 cm (height × diameter) column of Sephacryl S-200 HR. The proteins were eluted with 0.025 M Tris buffer (pH 7.40 at 5°C) containing 0.025 M NaCl and 0.003 M NaN3. The partition coefficients, \( K_{el} \) \( (= V_t/V_s) \), of Hb fractions were compared with those of horse heart cytochrome c, oval albumin, and aldolase obtained from Boehringer-Mannheim; myoglobin and bovine serum albumin obtained from Sigma (A-0380 and M-4503, respectively); and catalase and ferritin from GE Health Care. Gel filtration of deoxygenated Hb was carried out by adding Na-dithionite (1 mg/ml) to the Hb sample applied to the column and to the elution buffer after both solutions had been equilibrated with gaseous \( N_2 \). Hb fractions retrieved for \( O_2 \) binding experiments were concentrated by ultrafiltration in Milipore ultrafree filter units (cut-off molecular mass 10,000 Daltons). Fractions showing slight oxidation were briefly equilibrated with CO and reduced by dialysis (30 min) against \( N_2 \)-equilibrated 0.01 M HEPES buffer (pH ~7.6) containing freshly added Na-dithionite (0.1%).

---

**Fig. 1.** Amino acid sequences of the \( \alpha \) and \( \beta \) globins of dwarf caiman (Paleosuchus palpebrosus) hemoglobin (Hb) compared with those for spectacle caiman (Caiman crocodilus), Nile crocodile (Crocodylus niloticus), the American alligator (Alligator mississippiensis) (51), chicken, and human. Residues in the other species are shown only where they differ from those in human Hbs. His residues highlighted in yellow and blue are titratable (surface) residues and nontitratable residues, respectively, in human Hb (11, 53); those highlighted in green represent potential gains of titratable His residues compared with human Hb.

<table>
<thead>
<tr>
<th>A</th>
<th>( \alpha )-globin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human HBA</td>
<td>A1</td>
</tr>
<tr>
<td>Chicken HBA</td>
<td>A16</td>
</tr>
<tr>
<td>P. palpebrosus HBA</td>
<td>B1</td>
</tr>
<tr>
<td>C. crocodilus HBA</td>
<td></td>
</tr>
<tr>
<td>C. niloticus HBA</td>
<td></td>
</tr>
<tr>
<td>A. mississippiensis HBA</td>
<td>C7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>( \beta )-globin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human HBB</td>
<td>A1</td>
</tr>
<tr>
<td>Chicken HBB</td>
<td>A16</td>
</tr>
<tr>
<td>P. palpebrosus HBB</td>
<td>B1</td>
</tr>
<tr>
<td>C. crocodilus HBB</td>
<td></td>
</tr>
<tr>
<td>C. niloticus HBB</td>
<td></td>
</tr>
<tr>
<td>A. mississippiensis HBB</td>
<td>C7</td>
</tr>
</tbody>
</table>

---

R302

ALLOSTERIC REGULATION OF DWARF CAIMAN HEMOGLOBIN

---

**AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00014.2013 • www.ajpregu.org**
ALLOSTERIC REGULATION OF DWARF CAIMAN HEMOGLOBIN

Table 1. Identities and similarities in α and β chain sequences of P. palpebrosus hemoglobin compared with those of other crocodilian, chicken and human hemoglobins

<table>
<thead>
<tr>
<th></th>
<th>α Chains</th>
<th>β Chains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Identity</td>
<td>Similarity</td>
</tr>
<tr>
<td>Cal. crocodilus</td>
<td>83.7</td>
<td>90.1</td>
</tr>
<tr>
<td>C. niloticus</td>
<td>79.4</td>
<td>87.2</td>
</tr>
<tr>
<td>A. mississippiiensis</td>
<td>79.4</td>
<td>87.2</td>
</tr>
<tr>
<td>Chicken</td>
<td>75.2</td>
<td>84.4</td>
</tr>
<tr>
<td>Human</td>
<td>67.4</td>
<td>75.9</td>
</tr>
</tbody>
</table>

All values are %.

followed by extensive dialysis against N2CO equilibrated HEPES buffer without Na-dithionite.

RESULTS

Structural Analyses

Figure 1 shows the amino acid sequences of P. palpebrosus Hb aligned with those for Cal. crocodilus, C. niloticus, A. mississippiiensis, chicken, and human. The entire collection of globin peptides isolated from the 10 size exclusion fractions (see MATERIALS AND METHODS) was free of peptides that matched αD subunits of birds and nonavian reptiles, revealing that P. palpebrosus, like other adult crocodilians, does not express αD globin (31). The α and β chain sequences of P. palpebrosus (Table 1) show higher identity with those of the other cai-molus member (Cal. crocodilus) than with those of C. niloticus and A. mississippiiensis and with chicken than with human α and β chains. Relative to the α chains, the β chains showed higher sequence divergence in comparisons among species, which is consistent with previous reports of unusually high substitution rates in the β chain subunits of crocodilian Hbs (31, 36).

The primary structures of P. palpebrosus Hb chains reveal a unique combination of amino acid substitutions, including β82Lys→Gln, β143His→Val, and β146His→Tyr (Fig. 1) that delete positively charged (anion-binding and proton-dissociating) residues and thus may be expected to drastically impact the sensitivities of the Hb to allosteric effectors. Another impinging substitution of P. palpebrosus Hb is β93Cys→Tyr, which removes a solvent-exposed cysteine residue that is highly conserved among mammalian, avian, and reptilian Hbs.

Charge, Polarity, and Histidine Content

The sequence of P. palpebrosus Hb differs markedly from that of other crocodilian, avian, and human Hbs in charge, polarity, and hydrophathy (hydrophobicity) indices of amino acid residues, as well as the number and position of histidine residues that may function as buffer groups and source of Bohr protons and in stabilizing the Hb’s T-structure through the formation of internal salt bridges (11). P. palpebrosus Hb contains 24 (11 α chain and 13 β chain) His residues in each dimeric half-molecule (Fig. 1), which is considerably more than in other vertebrates, including humans, who have 19 (10 + 9) residues, but they align with the high numbers of physiological buffer groups in crocodilian Hbs (11).

Table 2. Amino acid residues specifically encountered in α and β chains of P. palpebrosus hemoglobin compared with those of other species

<table>
<thead>
<tr>
<th>Human</th>
<th>P. palpebrosus</th>
<th>Cal. crocodilus</th>
<th>C. niloticus</th>
<th>A. mississippiiensis</th>
<th>Comparison*</th>
</tr>
</thead>
<tbody>
<tr>
<td>α12</td>
<td>Ala</td>
<td>Gly</td>
<td>Ala</td>
<td>Ala</td>
<td>=</td>
</tr>
<tr>
<td>α18</td>
<td>Gly</td>
<td>Cys</td>
<td>Ala</td>
<td>Ser</td>
<td>=</td>
</tr>
<tr>
<td>α19</td>
<td>Ala</td>
<td>Cys</td>
<td>Gly</td>
<td>Gly</td>
<td>=</td>
</tr>
<tr>
<td>α23</td>
<td>Glu−</td>
<td>Asp−</td>
<td>Glu−</td>
<td>Glu−</td>
<td>=</td>
</tr>
<tr>
<td>α28</td>
<td>Ala</td>
<td>Tyr</td>
<td>Ala</td>
<td>Ala</td>
<td>=</td>
</tr>
<tr>
<td>α32</td>
<td>Met</td>
<td>Leu</td>
<td>Met</td>
<td>Met</td>
<td>=</td>
</tr>
<tr>
<td>α34</td>
<td>Leu</td>
<td>Phe</td>
<td>Cys</td>
<td>Cys</td>
<td>=</td>
</tr>
<tr>
<td>α35</td>
<td>Ser</td>
<td>Val</td>
<td>Ala</td>
<td>Ala</td>
<td>p (npp)</td>
</tr>
<tr>
<td>α49</td>
<td>Ser</td>
<td>Tyr</td>
<td>Ser</td>
<td>Ser</td>
<td>=</td>
</tr>
<tr>
<td>α64</td>
<td>Asp−</td>
<td>Leu</td>
<td>Ala</td>
<td>Ser</td>
<td>= o (o o o)</td>
</tr>
<tr>
<td>α100</td>
<td>Leu</td>
<td>Leu</td>
<td>Phe</td>
<td>Phe</td>
<td>=</td>
</tr>
<tr>
<td>α134</td>
<td>Tyr</td>
<td>Tyr</td>
<td>Ser</td>
<td>Ser</td>
<td>=</td>
</tr>
<tr>
<td>β12</td>
<td>Tyr</td>
<td>Tyr</td>
<td>Val</td>
<td>Val</td>
<td>=</td>
</tr>
<tr>
<td>β19</td>
<td>Asp−</td>
<td>Asp−</td>
<td>Asp−</td>
<td>Asp−</td>
<td>o o (-)</td>
</tr>
<tr>
<td>β22</td>
<td>Asp−</td>
<td>Ala</td>
<td>Ser</td>
<td>His+</td>
<td>=</td>
</tr>
<tr>
<td>β31</td>
<td>Leu</td>
<td>Leu</td>
<td>Met</td>
<td>Met</td>
<td>=</td>
</tr>
<tr>
<td>β49</td>
<td>Ser</td>
<td>Ala</td>
<td>Ser</td>
<td>Cys</td>
<td>= p (npp)</td>
</tr>
<tr>
<td>β52</td>
<td>Asp−</td>
<td>Glu−</td>
<td>Glu−</td>
<td>His+</td>
<td>=</td>
</tr>
<tr>
<td>β82</td>
<td>Lys+</td>
<td>Gln</td>
<td>Arg+</td>
<td>Lys+</td>
<td>+ o (+++ )</td>
</tr>
<tr>
<td>β93</td>
<td>Cys</td>
<td>Tyr</td>
<td>Phe</td>
<td>Phe</td>
<td>=</td>
</tr>
<tr>
<td>β108</td>
<td>Asp−</td>
<td>Glu−</td>
<td>Asp−</td>
<td>Asp−</td>
<td>o o (-)</td>
</tr>
<tr>
<td>β121</td>
<td>Glu−</td>
<td>Asp−</td>
<td>Asp−</td>
<td>Asp−</td>
<td>=</td>
</tr>
<tr>
<td>β124</td>
<td>Pro</td>
<td>Met</td>
<td>Leu</td>
<td>Val</td>
<td>=</td>
</tr>
<tr>
<td>β134</td>
<td>Val</td>
<td>Ala</td>
<td>Ala</td>
<td>Ala</td>
<td>=</td>
</tr>
<tr>
<td>β143</td>
<td>His+</td>
<td>Val</td>
<td>Ala</td>
<td>Ala</td>
<td>+ o (o o o)</td>
</tr>
<tr>
<td>β146</td>
<td>His+</td>
<td>Tyr</td>
<td>His+</td>
<td>His+</td>
<td>+ o (+ + )</td>
</tr>
</tbody>
</table>

Underlined residues have polar side chains; + and − denote residues whose side chains are positively and negatively charged, respectively, at pH 7.4.

*Comparison between hemoglobins of, respectively, humans, P. palpebrosus, and (in brackets) Cal. crocodilus, C. niloticus, and A. mississippiensis: =, same charge/polarity in all species; +, positively charged side chains; −, negatively charged side chains, o, neutral side-chains, p, Polar side chain; n, nonpolar side chain.
Palpebrosus\(Hb\) exhibits a high \(O_2\) affinity that is substantially reduced by \(Cl^-\) ions, decreased pH, and increased temperature (Fig. 2; Table 3); at pH 7.4, \(P_{50}\) values at 10°C and 25°C are 0.65 and 5.43 mmHg, respectively, in the presence of 0.1 M Cl\(^-\) and 2.69 mmHg, respectively, in the stripped \(P.\) palpebrosus\(Hb\); and 1.53 and 0.089, respectively, at 25°C and pH 7.4 (Fig. 3B). Also, whereas the effects of IHP and chloride are additive (i.e., chloride plus IHP depress \(O_2\) affinity more than IHP alone), addition of chloride increases \(Hb\)-\(O_2\) affinity in the presence of ATP or DPG (Fig. 3B). Similar relative sensitivities to these effectors were observed in duplicate measurements at pH 7.0 (data not shown).

Dose-response curves (Fig. 4) for the effects of increasing concentrations of free phosphates calculated as [total phosphate] – 0.5[Hb\(^+\)], on the assumption that at \(P_{50}\) half of the tetrameric \(Hb\) molecules are phosphate-liganded (97), demonstrate virtual annihilation of the phosphate effects in the presence of 0.1 M chloride. Strikingly, the double logarithmic plots reveal slopes that markedly exceed 0.25 (i.e., the value expected for one-to-one stoichiometry between phosphate and tetrameric \(Hb\) molecules if phosphate binding is limited to the T state). For DPG binding at pH 7.4 the slope of 0.37 (Fig. 4) indicates a stoichiometry of \(~1.5\) DPG molecules bound per \(Hb\) tetramer.

\(CO_2\) sensitivity. Admixture of \(CO_2\) in the equilibration gases conspicuously lowers \(O_2\) affinity of the stripped \(P.\) palpebrosus Hb at constant pH. This specific \(CO_2\) effect increases with increasing pH and is markedly reduced by \(Cl^-\) (Fig. 5). Thus at pH 7.0 and 7.4, addition of 29.7 mmHg \(CO_2\) raises \(log\ \(P_{50}\) by 0.85 and 0.98 units, respectively, in the absence of chloride, compared with 0.49 and 0.64, respectively, in 0.1 M chloride.

This decrease in Bohr effect with increasing temperature accords with the temperature dependence of ionization of Bohr groups. Unlike in human and most vertebrate \(Hbs\) (37, 73, 92) the Bohr effect is not increased by 0.1 M chloride, suggesting that allosteric proton binding to this \(Hb\) is not enhanced by chloride. \(O_2\) equilibria of stripped \(P.\) palpebrosus\(Hb\) in MES buffer (Fig. 2) reveal the absence of a reverse (acid) Bohr effect (increasing \(O_2\) affinity with decreasing pH) as found in human \(Hb\) below \(~6.5\).

**Oxygen Binding**

\(O_2\) affinity and its pH and chloride sensitivities. Stripped \(P.\) palpebrosus Hb exhibits a high \(O_2\) affinity that is substantially reduced by \(Cl^-\) ions, decreased pH, and increased temperature (Table 3); at pH 7.4, \(P_{50}\) values at 10°C and 25°C are 0.65 and 2.69 mmHg, respectively, in the stripped Hb; and 1.53 and 5.43 mmHg, respectively, in the presence of 0.1 M Cl\(^-\). Expressed as \(log\ \(P_{50,Cl}\) \(-\) \(log\ \(P_{50,stra}\), the chloride effect decreases markedly at high pH where cationic binding sites are neutralized (Table 3). Cooperativity of \(O_2\) binding was low (\(n_{50} = 1.2\--1.5\) under all conditions investigated (Fig. 2).

Significantly, \(P.\) palpebrosus Hb exhibits a pronounced Bohr effect. At pH 7.0–7.4, which characterizes physiological values in crocodilians (77), the Bohr factors (\(\varphi = \Delta log\(P_{50}\)/\(\Delta\)pH) in the absence and presence of 0.1 M chloride were −0.67 and −0.61, respectively, at 10°C; and −0.45 and −0.50, respectively, at 25°C (Fig. 2; Table 3). The decrease in Bohr effects with increasing temperature accords with the temperature dependence of ionization of Bohr groups. Unlike in human and most vertebrate \(Hbs\) (37, 73, 92) the Bohr effect is not increased by 0.1 M chloride, suggesting that allosteric proton binding to this \(Hb\) is not enhanced by chloride. \(O_2\) equilibria of stripped \(P.\) palpebrosus\(Hb\) in MES buffer (Fig. 2) reveal the absence of a reverse (acid) Bohr effect (increasing \(O_2\) affinity with decreasing pH) as found in human \(Hb\) below \(~6.5\).

**Phosphate sensitivities.** Measurements of the interactive effects of organic phosphates and chloride at physiological pH (Fig. 3) show insensitivity of \(P.\) palpebrosus Hb to organic phosphates in the presence of chloride ions, as observed in other crocodilians (8, 9, 63). In contrast, pronounced sensitivities to ATP and DPG are unmasked in the absence of chloride, as is also observed in \(A.\) mississippiensis and \(Cai.\) crocodilus Hbs (96). Strikingly, however, the \(Hb\)-\(O_2\) affinity is virtually insensitive to polyanionic IHP. Thus in the absence of chloride, addition of ATP, DPG, and IHP increase \(P_{50}\) by 0.49, 0.52, and 0.089, respectively, at 25°C and pH 7.4 (Fig. 3B). Also, whereas the effects of IHP and chloride are additive (i.e., chloride plus IHP depress \(O_2\) affinity more than IHP alone), addition of chloride increases \(Hb\)-\(O_2\) affinity in the presence of ATP or DPG (Fig. 3B). Similar relative sensitivities to these effectors were observed in duplicate measurements at pH 7.0 (data not shown).

**Table 3.** \(P_{50}\) values and Bohr factors of stripped \(P.\) palpebrosus hemoglobin and their dependence on pH, [\(Cl^-\)] and temperature

<table>
<thead>
<tr>
<th>C</th>
<th>Property</th>
<th>pH 8.0</th>
<th>pH 7.4</th>
<th>pH 7.0</th>
<th>(\varphi) (7.0–7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>(P_{50,stra}) (mmHg)</td>
<td>0.45</td>
<td>0.65</td>
<td>1.20</td>
<td>−0.67</td>
</tr>
<tr>
<td></td>
<td>(P_{50,stra} + 0.1) M Cl (mmHg)</td>
<td>0.78</td>
<td>1.53</td>
<td>2.66</td>
<td>−0.61</td>
</tr>
<tr>
<td></td>
<td>(log\ (P_{50,Cl}) (-) (log\ (P_{50,stra}))</td>
<td>0.23</td>
<td>0.37</td>
<td>0.35</td>
<td>−0.61</td>
</tr>
<tr>
<td>25</td>
<td>(P_{50,stra}) (mmHg)</td>
<td>2.02</td>
<td>2.69</td>
<td>4.26</td>
<td>−0.45</td>
</tr>
<tr>
<td></td>
<td>(P_{50,stra} + 0.1) M Cl (mmHg)</td>
<td>3.09</td>
<td>5.43</td>
<td>8.61</td>
<td>−0.50</td>
</tr>
<tr>
<td></td>
<td>(log\ (P_{50,Cl}) (-) (log\ (P_{50,stra}))</td>
<td>0.18</td>
<td>0.31</td>
<td>0.32</td>
<td>−0.45</td>
</tr>
</tbody>
</table>

\(\varphi\), Bohr factor; str., stripped.
the 7–30 mmHg PCO2 range) of ~0.5 in the absence, and ~0.4 in the presence of Cl, indicate binding of one CO2 molecule per two O2 molecules released.

Allosteric control. Extended Hill plots (Fig. 6) reveal slopes of unity at extremely low and high O2 saturations consistent with the Hb, and good correspondence between Pm and P50 values (O2 tensions at, respectively, median and half-saturations), permitting assessment of allosteric effects from alterations in P50 values. As shown (Table 4), the n50 values are low, which hampered fits of the MWC two-state model to the data. Thus, at high pH and in the presence of chloride, it was not possible to fit the model to the data with q (the number of interacting binding sites) floating and with the constraint that the allosteric constant (L) > 1. For the other curves, the fitted values of q were close to 4, indicating that the functional units are tetramers, despite Hb polymerization (see below). As illustrated (Fig. 6), lowered pH decreases O2 affinity by reducing KT and KR, whereas chloride ions decrease O2 affinity mainly by lowering KT. Fits with q fixed at 4 (Table 4) show that the interaction energy is increased in the presence of 0.1 M chloride and that this was due in part to a stabilizing effect of chloride on the T state (lowered KT). At pH 7.6 this was augmented by a destabilizing effect of chloride on the R state (increased KR), whereas this was not the case at pH 6.9. As evident from the standard error on the fits, however, it is not safe to draw firm conclusions about KR at the low pH.

Temperature sensitivity and enthalpic effects. At pH 7.5–8.0, at which stripped P. palpebrosus Hb virtually lacks a Bohr effect (cf. Fig. 2), the overall oxygenation enthalpy (~56.3 kJ/mol) corresponds closely with the intrinsic heat of oxygenation of human Hb (~59 kJ/mol) (4). The numerically lower value (~48.8 kJ/mol) found at pH 7.0 where the Bohr effect is operative, is consistent with endothermic contributions from Bohr proton dissociation (~26 kJ/mol for imidazole groups of histidines (3, 4)). Analogously, the reduced enthalpy values observed in the presence of 0.1 M Cl<sup>-</sup> (~47.7, ~46.6, and ~42.3 kJ/mol at pH 8.0, 7.5, and 7.0, respectively) reflect endothermic contributions (~8.6, ~7.7, and ~6.5 kJ/mol, respectively) from oxygenation-linked chloride ion dissociation. The semblance of these enthalpic effects with those in other (nonendothermic) vertebrates (90) indicates the absence of distinguishing adaptive traits related to body temperature variation in P. palpebrosus Hb.

Quaternary Structure and Effects

Unexpectedly, the size exclusion gel-filtration chromatography experiments showed that oxygenated P. palpebrosus Hb elutes with the void volume of the Sephacryl S-200 HR medium, revealing an exceptionally high degree of polymerization (Fig. 7). In contrast, the elution pattern of Hb deoxygenated with dithionite reveals the presence of three fractions.
of smaller molecules, whose partition coefficients ($K_{av}$) indicate molecular masses of 31.6, 81.3, and 263 kDa. The value of 38 kDa obtained for human Hb (Fig. 7, inset) is consistent with observations that molecular mass estimates for tetrameric vertebrate Hbs on the basis of gel filtration are lower than established values (64–68 kDa) due to reversible dissociation to dimers (17) and to elution volumes being proportional to molecular Stokes radii of proteins rather than their molecular weights (1). In this light, the three fractions observed in deoxygenated Hb (labeled 4, 3, and 2 in Fig. 7) likely comprise tetramers, octamers (dimers of tetramers), and high-order polymers, respectively.

O$_2$ equilibrium measurements of three major molecular mass fractions obtained from gel filtration of deoxygenated Hb show similar O$_2$ affinities and Bohr effects (Fig. 8) indicating that aggregation state does not affect oxygenation properties of $P$. palpebrosus Hb.

**DISCUSSION**

**O$_2$ Affinity and its Sensitivity to Allosteric Effectors**

$P$. palpebrosus Hb exhibits a high intrinsic O$_2$ affinity combined with low cooperativity, suggesting that the allosteric T-R equilibrium in this Hb is markedly shifted to the high-affinity R state. Its O$_2$ affinity is reduced by allosteric effectors (protons and CO$_2$, and organic phosphate and chloride anions) despite replacements of functionally important amino acid residues compared with other Hbs. $P$. palpebrosus Hb provides a unique opportunity to reevaluate the roles of specific amino acid residues implicated in the allosteric regulation of O$_2$ affinity, and the possible compensatory mechanisms in naturally occurring Hbs that lack specific effector binding sites.

**CO$_2$ Effects**

$P$. palpebrosus Hb binds molecular CO$_2$. The distinct, specific effect of CO$_2$ on O$_2$ affinity (Fig. 5) is at variance with the reported absence of carbamate formation in crocodilian Hbs...
The reduction in the CO₂ effect observed in the presence of chloride and increased proton activity (pH decrease) (cf. Fig. 5) is consistent with competition between these effectors for binding at the NH₂-terminal amino acid residues, and thus is analogous to the antagonism between binding of CO₂ on the one hand, and of lactate (58) and DPG (7) on the other, in human HbA.

The slope of ~0.5 for the log P₅₀/log P₅₇₉ relationship (Fig. 5) signifies a stoichiometry of two CO₂ molecules bound per Hb tetramer under physiological conditions, which indicates that CO₂ binds to either the α or the β chains of P. palpebrosus Hb as in human Hb, where O₂-linked carbamate formation is confined to the NH₂-terminal residues (75) and are therefore not available for carbamate formation.

In A. mississippiensis Hb carbamate, free HCO₃⁻ and Hb-bound HCO₃⁻ contribute approximately equally to the high CO₂ carrying capacity of deoxygenated red cells (41). Two observations indicate that HCO₃⁻ binding to P. palpebrosus Hb is markedly reduced compared with that of other crocodilians. First, the CO₂ effect increases strongly with increasing pH (Fig. 5A), whereas the association constants for the reaction of Cai. crocodilus Hb with HCO₃⁻ are essentially pH independent below pH 7.3 (8). Second, compared with other crocodilian Hbs, P. palpebrosus Hb exhibits substitutions (viz., H103Phe→Val, H100Phe→Leu, and B31Met→Leu) (Fig. 1) that replace 4 of the 12 amino acid residues (viz., 34Cys, 35Ala, 36Tyr, 38Gln, 38Lys, 39Arg, and 41Tyr of the β chain) that are present in other crocodilian Hbs, and confer a HCO₃⁻ effect when engineered into human Hb (47). Among these the loss of polar α34Cys in P. palpebrosus Hb may be significant. If β82-Lys is a bicarbonate-binding site as proposed by Perutz and his colleagues (63), its substitution by Gln would similarly contribute to loss of bicarbonate binding in P. palpebrosus Hb, but not in Cai. crocodilus and A. mississippiensis Hbs that have retained this residue, nor in C. niloticus Hb that shows a polar- and charge-neutral substitution (β82Lys→Arg).(1)

The Bohr Effect

β146His→Tyr substitution. The pronounced Bohr effect in stripped Hb (φ = −0.50 at 25°C) (Fig. 2, Table 3) is remark-

---

Table 4. MWC and derived parameters obtained by fitting the MWC two-state model to the data with four fixed interacting O₂-binding sites

<table>
<thead>
<tr>
<th>pH</th>
<th>Cl mM</th>
<th>P₅₀ mmHg</th>
<th>P₅₇₉ mmHg</th>
<th>n₅₀</th>
<th>log L</th>
<th>SE*</th>
<th>log K₇</th>
<th>SE*</th>
<th>log K₉</th>
<th>SE*</th>
<th>ΔG kJ·(M heme)⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.608</td>
<td>0.001</td>
<td>3.58</td>
<td>3.53</td>
<td>1.29</td>
<td>1.66</td>
<td>0.033</td>
<td>−0.758</td>
<td>0.040</td>
<td>−0.146</td>
<td>0.075</td>
<td>2.44</td>
</tr>
<tr>
<td>6.964</td>
<td>0.001</td>
<td>8.02</td>
<td>7.61</td>
<td>1.21</td>
<td>3.49</td>
<td>1.593</td>
<td>−0.993</td>
<td>0.025</td>
<td>−0.0562</td>
<td>0.386</td>
<td>2.07</td>
</tr>
<tr>
<td>7.595</td>
<td>0.010</td>
<td>9.38</td>
<td>8.31</td>
<td>1.43</td>
<td>65.61</td>
<td>0.0026</td>
<td>−1.102</td>
<td>0.019</td>
<td>15.46</td>
<td>0.011</td>
<td>4.16</td>
</tr>
<tr>
<td>6.961</td>
<td>0.010</td>
<td>14.00</td>
<td>12.84</td>
<td>1.37</td>
<td>4.07</td>
<td>4.085</td>
<td>−1.279</td>
<td>0.063</td>
<td>−0.117</td>
<td>1.012</td>
<td>3.31</td>
</tr>
</tbody>
</table>

P₅₀, O₂ tension at half-saturation; P₅₇₉, median O₂ tension; n₅₀, Hill’s cooperativity coefficient at half-saturation; L, allosteric constant; K₇ and K₉, association equilibrium constants of the T and R structures, respectively; ΔG, free energy of heme-heme interaction. *Standard errors as estimated from the covariance matrix associated with the fit.
able given the substitution of \(\beta 146\)His by neutral Tyr in \(P.\ palpebrosus\), and the prodigious evidence for the importance of \(\beta 146\)His for expression of the Bohr effect in vertebrate Hbs. In humans where His residues account for almost 90% of the total alkaline Bohr effect (11, 27, 53), the majority of this effect (63% at pH 7.4) (27) originates from proton release upon oxygenation-linked breakage of the T-state salt bridge between \(\beta 146\)His and \(\beta 94\)-Asp (61). The overriding role of \(\beta 146\)His in expression of the alkaline Bohr effect is supported by a host of investigations. Enzymatic removal of the \(\beta\) chain COOH-terminal residues (145-Tyr and 146-His) from human HbA drastically reduces the Bohr effect and cooperativity but increases \(O_2\) affinity, confirming its role in stabilizing the T state relative to the R state (12, 43, 49, 56). Moreover, abnormal human Hb with \(\beta 146\)His replaced by a wide range of other residues [viz., by Asp in Hb Hiroshima (38, 64), Pro in Hb York (48, 55), Tyr in Hb Bologna-St. Orsola (also called Hb Halamshire) (50, 52), Gln in Hb Kodaira (33), Leu in Hb Cowtown (76), and Arg in Hb Cochin-Port Royal (87)] show a similar reduction in the Bohr effect, and generally decreased heme-heme cooperativity and increased \(O_2\) affinities.

Available data indicate a similarly important role for \(\beta 146\)His in ectothermic vertebrates. The Bohr effect of carp Hb is halved following enzymatic cleavage of the \(\beta\) chain COOH-terminal His residues (60), and that of \(Aethotaxis\) mitopteryx Hb (\(\beta 146\)His→Val) is about half of that in other Antarctic fishes (21). In conjunction with the pervasive correlation between \(\beta 146\)His and Bohr effects in widely different vertebrates, the strong Bohr effect in \(P.\ palpebrosus\) Hb indicates that other titratable residues take over the role of \(\beta 146\)His. Which residues might these be?

The high His content of \(P.\ palpebrosus\) Hb (24 per \(\alpha\) dimer, Fig. 1) confirms the generality of this trait in crocodilians (11, 40), predicting high nonbicarbonate buffer values in \(P.\ palpebrosus\) blood that reduces arteriovenous pH changes and thus the in vivo contribution of the fixed-acid Bohr effect to tissue \(O_2\) release. In bicarbonate-sensitive Hbs, pH changes are further dampened because proton binding by Hb is balanced by protons liberated upon \(O_2\)-linked HCO\(_3\) binding (41).

Apart from increasing the buffer capacity, the additional His residues in crocodilean Hbs may take over the role of \(\beta 146\)His in accounting for a strong fixed-acid Bohr effect expressed in \(P.\ palpebrosus\) Hb (Fig. 2). Although four of the His residues found in human Hb are substituted for other residues (i.e., \(\beta 2\)Pro, \(\beta 116\)Met, \(\beta 143\)Val, and \(\beta 146\)Tyr), \(P.\ palpebrosus\) has 10 His residues (at positions \(\alpha 67\), \(\alpha 113\), \(\beta 6\), \(\beta 44\), \(\beta 56\), \(\beta 84\), \(\beta 87\), \(\beta 118\), \(\beta 127\), and \(\beta 136\)), which are not found in human Hb and may be sources of Hb protons and may be found in cytoplasm of fetal blood via lesser DPG interaction. Analogously, abnormal human Hbs in which \(\beta 143\)His is substituted by neutral residues, including Hb Little Rock (\(\beta 143\)His→Gln), Hb Syracuse (\(\beta 143\)His→Pro), and Hb Old Dominion (\(\beta 143\)His→Tyr) all exhibit increased affinities in the presence of DPG [cf. (22)].

**Phosphate Effects**

Contrary to the reported insensitivity of crocodilian Hbs to organic phosphates (8, 46, 63), ATP and DPG decrease \(O_2\) affinities of crocodilian Hb at low chloride levels (96) as may occur during the postprandial alkaline tide (20).

The observation that slopes of double logarithmic plots of \(P_50\) against the free DPG and ATP concentrations (Fig. 4) clearly exceed the maximum value (0.25) expected for one-to-one stoichiometry between bound phosphate and Hb molecules provides evidence for an additional phosphate binding site (compared with that between the \(\beta\) chains), as observed for Hbs of several other vertebrates including eel, billfish, and dromedary (2, 59, 97). Additional evidence for a second binding site derives from association/disassociation kinetics of the reaction of IHP with deoxygenated and carboxy human Hb (99) and molecular dynamic simulations of skua (seabird \(Catharacta\) maccormicki) and pheasant Hbs (32, 68, 84). This second site that comprises a cluster of positive charges between the \(\alpha\) chains may serve as an entry-leaving site, implying the existence of a migration pathway for phosphates along the central cavity between the two phosphate binding sites (68). In conjunction with the studies of fish, bird, and mammalian Hbs, our findings in \(P.\ palpebrosus\) provide further evidence of a wide occurrence of an \(\alpha\) chain phosphate binding site among vertebrates.

Given the distinct effects of ATP and DPG, the insensitivity of \(P.\ palpebrosus\) Hb to IHP molecules (which carry six negative charges compared with four for ATP at physiological pH) indicates that binding of IHP molecules in the cavity between the \(\beta\) chains is impeded by steric hindrance or stereo-chemical mismatch with the positive charges lining the cavity. The observation that 0.1 M chloride decreases Hb-\(O_2\) affinity in the presence of IHP, but increases it in the presence of DPG and ATP indicates that chloride is a less potent effector than ATP or DPG but obstructs binding of these phosphates at shared cationic binding sites.

Collectively the \(\beta 143\)His→Val and \(\beta 82\)Lys→Gln substitutions in \(P.\ palpebrosus\) Hb delete four of seven phosphate binding sites commonly found in vertebrates, which accords with the loss of phosphate sensitivity in the presence of 0.1 M \(Cl^-\) (Fig. 4). Given that \(\beta 82\)-Lys also is a \(Cl^-\) binding site, its replacement by neutral Gln predictably reduces the chloride effect in \(P.\ palpebrosus\), but not in \(C.\ niloticus\), where it is replaced by another positively charged residue (Arg) (Fig. 1).

**Polymerization and Its Effect on Hb-\(O_2\) Binding**

Polymerization of Hbs may begin at hemolysis (69), but may also occur in vivo, as observed in the turtle \(Pseudemys\) scripta (81) and witnessed by inclusions resembling Hb crystals in erythrocytes of healthy iguanas (79). In vivo polymerization moreover may be associated with red cell sickling found in iguanas and several teleosts (45). In polymerizing to large aggregates (Fig. 7), oxygenated \(P.\ palpebrosus\) Hb differs from Hbs of other reptiles, including \(Cai.\ crocodilus\) (9) and a large

---

2 The His→Asp replacement in Hb Hiroshima originally reported at \(\beta 143\) was later shown to be at \(\beta 146\).
majority of turtles (69, 82) in which polymerization is deoxygenation-linked and does not proceed beyond octamers (dimers of tetramers).

Hb polymerization commonly involves Cys residues, as evident from the correlation between disulfide bridge formation and polymerization in ectotherm Hbs (13, 25, 67, 69, 85). In human Hb mutants Mississippi (β9Ser→Cys) and Hb Ta-li (β83Gly→Cys), polymerization is induced by the incorporation of a single Cys residue (6).

The dissociation of the large complexes of oxygenated P. palpebrosus Hb into smaller molecules upon deoxygenation contrasts starkly with the deoxygenation-linked self-association encountered in Hbs of other ectothermic vertebrates (65), including hagfish and lampreys (monomer to dimer (14, 26), snakes (dimer to tetramer) (29, 54), frogs Rana esculenta and R. temporaria (tetramer to dimer of tetramers) (5, 23), and bullfrog R. catesbiana (tetramer to heterotrimer of tetramers) (83).

Given that dithionite (used to deoxygenate Hb) may reduce disulfide bonds (44), the possibility cannot be excluded that such interaction contributed to the dissociation of the large Hb complexes upon deoxygenation. Nevertheless P. palpebrosus Hb appears to be unique in polymerizing to giant complexes in the oxygenated state (in the absence of dithionite).

The residues mediating the distinctive self-association remain unknown. P. palpebrosus Hb lacks the only surface Cys residue known in humans (β93Cys) (98), and α49Cys, which can polymerize through intermolecular disulfide bridges in a teleost fish (25), but has six Cys residues per dimer (at α18, α19, α104, α130, β23, and β100). Available data (36) show that apart from the highly conserved α104Cys, all crocodilian Hbs (as with chicken Hb) have Cys at α130 and β23 (Table 5). Strikingly, P. palpebrosus differs from other crocodilians in lacking α34Cys and α81Cys and uniquely possessing two adjacent Cys residues at α18 and α19 (surface helical positions A16 and AB1) (28) (Fig. 1), implying that these residues may be involved in its unusual, oxygenation-linked polymerization.

The similar O2 affinities and Bohr effects in the three major molecular mass fractions isolated in the deoxygenation state (cf. Figs. 7 and 8) indicates that polymerization does not affect the O2 binding properties of Hb. This inference is supported by q values (the number of interacting O2 binding sites) near 4 obtained in fitting the two-state allosteric model to the data. It also aligns with observations that different quaternary assemblies of C. porosus Hb exhibit the same CO2 effect (9) and that the O2 binding properties of turtle (Dermatemys mawii) Hb are not altered by mercaptoethanol-induced dissociation (80). Similarly, polymerization of teleost fish (25) and mouse (72) Hbs has no significant effect on O2 affinity, and recombinant human Hb β83Gly→Cys, which oligomerizes through disulfide bonds, has similar CO binding properties as native human HbA (24).

The lack of polymerization effects on O2 binding properties indicates that the highly variable number of Cys residues in vertebrate Hbs has no consequence for O2 transport. Given that Cys (-SH) residues contribute to redox buffering during oxidative stress in periodic hypoxia and reoxygenation (66), it would seem appropriate to compare Hb polymerization and red cell antioxidant defenses in crocodilians (in which Hbs have high Cys contents) and sauropsids such as the side-neck turtle, Podocnemis unifilis, whose Hbs completely lack Cys residues (34).

### Table 5. Positions of cysteine residues in the α and β chains of crocodilian, human, and chicken hemoglobins

<table>
<thead>
<tr>
<th></th>
<th>α Chain</th>
<th>β Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human HbA</td>
<td>104</td>
<td>93, 112</td>
</tr>
<tr>
<td>Chicken HbA</td>
<td>104, 130</td>
<td>23, 93, 126</td>
</tr>
<tr>
<td>P. palpebrosus</td>
<td>18, 19, 104, 130</td>
<td>23, 100</td>
</tr>
<tr>
<td>Cat. crocodilus</td>
<td>34, 81, 104, 115, 130</td>
<td>23, 100</td>
</tr>
<tr>
<td>C. niloticus</td>
<td>8, 34, 81, 104, 130</td>
<td>23, 76, 93, 126</td>
</tr>
<tr>
<td>A. mississippiensis</td>
<td>34, 81, 104, 130</td>
<td>23, 49, 93, 126</td>
</tr>
</tbody>
</table>

### Perspectives and Significance

P. palpebrosus Hb exhibits a unique combination of distinctive amino acid exchanges compared with other vertebrate and
crocodilian Hbs that could potentially disrupt the allosteric regulation of Hb function. The finding that Hb sustains marked sensitivities to allosteric effectors demonstrates the existence of alternative, compensatory molecular mechanisms to maintain O2 delivery to the tissues in this species. The functional properties of *P. palpebrosus* Hb disprove several published assumptions and tenets: notably, 1) that β146His is essential for expression of pronounced Bohr effects (*P. palpebrosus* Hb lacks this residue but expresses a strong Bohr effect); 2) that crocodilian Hbs lack phosphate effects, that β82Lys is essential for phosphate binding, and that IHP invariably is a more potent effector than ATP and DPG (*P. palpebrosus* Hb lacks β82Lys and is sensitive to ATP and DPG but not to IHP); 3) that crocodilian Hbs bind bicarbonate and do not form carbamino compounds (*P. palpebrosus* Hb binds CO2); and 4) that oxygenated reptilian Hbs do not polymerize (the oxygenated Hb aggregates to large complexes). *P. palpebrosus* Hb thus provides unique opportunity for further studies on structure-function coupling and the evolution of effector sensitivities in vertebrate Hbs.

ACKNOWLEDGMENTS

We thank Anny Bang (Aarhus) for technical assistance, and Prof. W. Böhme, Dr. O. Behlert, Dr. T. Ziegler, and U. Bott (Zoological Gardens of Cologne, Germany) for the blood samples. Primary structure determinations were carried out by T.A. Gorr under expert supervision of the late Prof. G. Braunitzer and Dr. T. Kleinschmidt, Max Planck Institute of Biochemistry, Munich-Münchsmried, Germany.

GRANTS

Support for this study was provided by a Faculty of Science and Technology, Aarhus University grant to R. E. Weber, by Danish Council for Independent Research Natural Sciences Grant 10-084565 to A. Fago, by National Heart, Lung, and Blood Institute Grants R01 HL087216 and HL087216-S1 and National Science Foundation Grant IOS-0949391 to J.F. Storz, and by personal fellowships from the Studienstiftung des Deutschen Volkes and Max Planck Society to T.A. Gorr.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


