CHLORIDE-DEPENDENT ORGANIC PHOSPHATE SENSITIVITY OF THE OXYGENATION REACTION IN CROCODILIAN HEMOGLOBINS

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Summary

We show that crocodilian hemoglobins (Hbs), which previously have been considered to be model pigments lacking allosteric interaction with organic phosphate esters, do exhibit oxylabile ATP and 2,3-diphosphoglycerate (DPG) binding that decreases O_2 affinity and increases pH sensitivity (Bohr effect), in the absence of Cl^- and at the low Cl^- concentrations that may occur in crocodilian plasma during 'post-ingestive alkaline tides'. Hbs from different species vary in their phosphate sensitivities. In *Alligator mississippiensis* Hb, O_2 affinity shows greater ATP than DPG sensitivity at low [cofactor]/[Hb] ratios. In *Paleosuchus palpebrosus* Hb, where even a high Cl^- concentration $(0.1\,\mathrm{mol\,l^{-1}})$ does not completely suppress the phosphate effects, the opposite is true, whereas both ATP and DPG exert similar effects in *Caiman crocodilus* Hb. Lactate, at concentrations that may occur after intensive exercise, similarly depresses Hb O_2-affinity, indicating an O_2 demand/O_2 supply feedback regulation. Curiously, inositol hexaphosphate (IHP), a potent allosteric effector in other vertebrate Hbs, has no effect on *A. mississippiensis* and *P. palpebrosus* Hb and only small effects on *C. crocodilus* Hb, presumably because of steric hindrance at the binding site. The molecular mechanisms underlying the observations (particularly the implication of β82-Lys, i.e. the lysine residue at position 82 of the β-chains that binds phosphate and Cl^-) are considered. Their physiological significance requires further study.

Introduction

Vertebrate Hbs have high intrinsic O_2 affinities that are decreased by organic phosphates found in the red cells. These allosteric effectors, which are commonly ATP and/or GTP in fish, ATP and/or DPG in amphibians and reptiles, inositol pentaphosphate

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(IPP) in birds and DPG in mammals, bind in 1:1 stoichiometry to deoxygenated Hb tetramers, becoming displaced upon oxygenation as the Hb molecules shift into the oxygenated structure (Perutz, 1983; Weber and Jensen, 1988; Weber and Wells, 1989).

Crocodilians, however, have low organic phosphate concentrations and their Hb O_2-affinities have been found to be insensitive to ATP, DPG and IPP but to be strongly depressed by CO_2 (Bauer and Jelkmann, 1977; Jelkmann and Bauer, 1980; Grigg *et al.* 1993). This functional transformation is attributed to a few key amino acid substitutions that convert the phosphate binding site into two HCO_3^- binding sites and concomitantly depress the oxygenation-linked binding of chloride and CO_2 (Bauer *et al.* 1981; Leclercq *et al.* 1981; Perutz *et al.* 1981).

Previous studies on crocodilian Hbs were carried out at high ($100\,mmol\,l^{-1}$) Cl^- concentrations, whereas Cl^- levels in vertebrate red cells may be considerably lower (Nikinmaa, 1990), especially in reptiles (e.g. $32\,mmol\,l^{-1}$ in *Chrysemys picta belli*, Maginniss and Hitzig, 1987). In crocodilians, plasma Cl^- levels may fall drastically (e.g. from 95 to $7\,mmol\,l^{-1}$ while plasma HCO_3^- concentrations may increase to $105\,mmol\,l^{-1}$, in *Alligator mississippienses*, Coulson and Hernandez, 1983) during the 'alkaline tides' that follow feeding. Given, moreover, that red cell Cl^- levels are influenced by the same pathways that control H^+ and HCO_3^- concentrations and that the same amino acid residue, β82-Lys (which is preserved in the crocodilian Hbs, LeClercq *et al.* 1981), is implicated in binding of the phosphate cofactors, as well as Cl^-, we investigated the effects of phosphates in crocodilian Hbs at different pH conditions and divergent Cl^- concentrations.

Materials and methods

Blood was drawn by caudal or cardiac venepuncture from adult specimens of the American alligator *Alligator mississippiensis* (Daudin), the spectacled caiman *Caiman crocodilus* (Linnaeus) and the dwarf caiman *Paleosuchus palpebrosus* (Cuvier). The red cells were washed in 0.9 % NaCl and lysed by osmotic shock or freezing. After the cell ghosts had been separated by centrifugation, the supernatant Hb was stripped by column chromatography using Sephadex G25 Fine beads (Berman *et al.* 1971) or MB1 mixed-ion exchanger. The Hb was dialysed for 24 h against at least three changes of $0.01\,mol\,l^{-1}$ Hepes buffer containing $5\times10^{-4}\,mol\,l^{-1}$ EDTA. The Hb showed no oxidation, as judged from equal absorption values at 539 and 569 nm after brief equilibration with carbon monoxide, and was frozen at $-80\,°C$ in $150\,\mu l$ samples that were individually thawed for O_2 equilibrium measurements.

O_2 equilibria were determined at $25\,°C$ using a modified gas diffusion chamber, fed by cascaded Wösthoff pumps (Bochum, Germany) for mixing air and pure N_2 (>99.998 %) (Weber, 1981; Weber *et al.* 1987). The effects of ATP (disodium salt), DPG (pentacyclohexylammonium salt), IHP (sodium salt) and lactate [L(+) lithium salt] were investigated by addition of accurate volumes of approximately $100\,mmol\,l^{-1}$, Cl^--free standard solutions. Concentrations of the nucleoside triphosphate and lactate in these solutions were assayed (to $2\,mmol\,l^{-1}$ accuracy) using, respectively, Sigma and Boehringer test chemicals. Cl^- was added as KCl and assayed (to $1\,mmol\,l^{-1}$ accuracy)
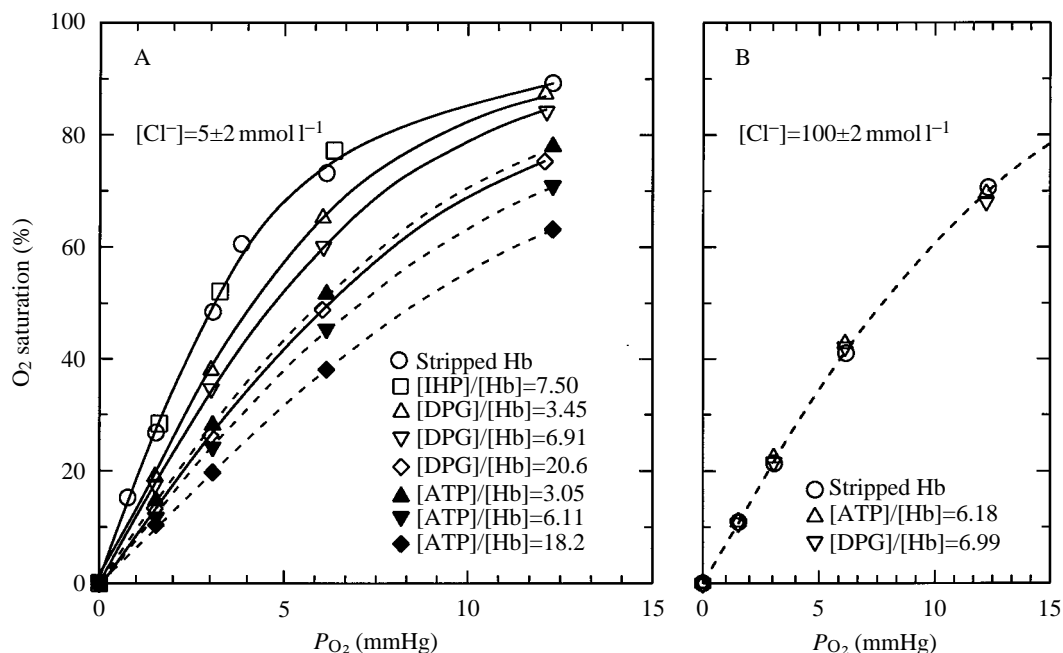
Fig. 1. Effects of [DPG]/[Hb], [ATP]/[Hb] and [IHP]/[Hb] ratios on the O_2 equilibria of *Alligator mississippiensis* Hb in 0.1 mol l^{-1} Hepes buffer, pH 7.1, at 25 °C. Hb concentration (tetramer basis), 0.17 mmol l^{-1}. (A) At 5±2 mmol l^{-1} Cl$^-$. (B) Overlapping O_2 equilibrium curves at 100 mmol l^{-1} Cl$^-$ in the absence and presence of ATP and DPG.

using a Radiometer (Copenhagen) CMT10 chloride titrator. O_2 equilibria were measured in 0.1 mol l^{-1} Hepes buffer, which does not perturb the free Cl$^-$ concentration, as may occur with (Bis)Tris buffers (Weber, 1992). The pH was measured using a Radiometer BMS 2 Mk thermostatted microelectrode. Values of P_{50} (oxygen tensions at half-saturation of the Hb) obtained at slightly different values from the target pH values were corrected using the appropriate Bohr factors ($\phi=\Delta \log P_{50}/\Delta \mathrm{pH}$), obtained by P_{50} measurement at other pH values.

Results

The effect of organic phosphates and of Cl$^-$ on *A. mississippiensis* Hb at pH 7.1 are shown in Figs 1–4. While ATP and DPG had no effect on the O_2 affinity of the Hb in the presence of 100 mmol l^{-1} Cl$^-$ (Fig. 1B), they markedly reduced Hb O_2-affinity at low Cl$^-$ concentration (5±2 mmol l^{-1}), the effect of ATP being more pronounced than that of DPG. Significantly, IHP did not influence oxygen binding (Fig. 1A).

Measurements at different pH values (Fig. 2) confirm that ATP has a greater effect than DPG and show that effector action and the difference between the ATP and DPG effects increase with decreasing pH, where the cationic binding sites become increasingly charged. The ϕ value of -0.20 observed at 7 mmol l^{-1} Cl$^-$ (interpolated for pH 7.4–7.6; see Fig. 2) equals the fixed-acid Bohr factor observed in *A. mississippiensis* blood (Weber
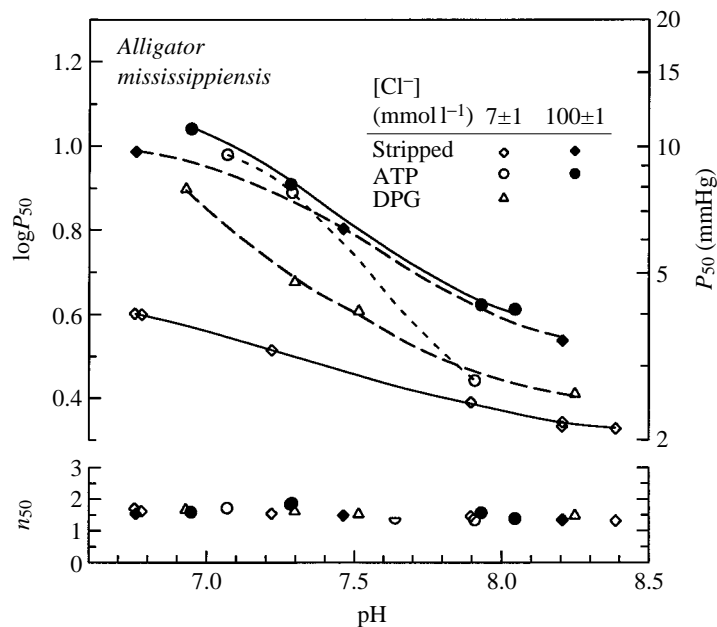
Fig. 2. Effects of saturating concentrations (molar excess over Hb tetramers >25) of ATP (circles) and DPG (triangles) on P_{50} and n_{50} of stripped *Alligator mississippiensis* Hb in $0.1 \, mol \, l^{-1}$ Hepes buffer at different pH values. Open symbols, $7\pm1 \, mmol \, l^{-1}$ Cl^-; filled symbols, $100\pm1 \, mmol \, l^{-1}$ Cl^-. Hb concentration (tetramer basis), $0.21 \, mmol \, l^{-1}$.

and White, 1986). This value is raised to -0.33 in the presence of $100 \, mmol \, l^{-1}$ Cl^- and to -0.36 and -0.75 in the presence of saturating concentrations of, respectively, DPG and ATP. At pH values below 7.3, the effectiveness of ATP approximates that of $100 \, mmol \, l^{-1}$ Cl^-. In contrast to the phosphate effects, the Cl^- effect remains pronounced at high pH (Fig. 2). Lactate decreased Hb O_2-affinity at high as well as at low $[Cl^-]$ (Fig. 3). The tested effectors had no significant effects on the Hill's cooperativity coefficients at half-saturation (n_{50}), which varied between 1.5 and 1.7 (Figs 2 and 3). These values are low compared with those of mammalian Hbs, but may be raised by the presence of HCO_3^- (as in *Crocodylus porosus* Hb, Brittain and Wells, 1991).

While confirming the lack of an IHP effect, dose–response curves (Fig. 4) show greatest ATP and DPG sensitivities at low [phosphate]/[Hb] ratios, greater oxygenation-linked binding of ATP than of DPG at all ratios tested and that all these effects are obliterated in the presence of added Cl^-.

In *C. crocodilus* Hb, the phosphate effects expressed at low Cl^- levels were similarly abolished in the presence of $100 \, mmol \, l^{-1}$ Cl^- (Fig. 5). However, in contrast to the greater effect of ATP in *A. mississippiensis*, the ATP and DPG effects were similar. Also, *C. crocodilus* Hb showed distinct oxygenation-linked IHP binding at low Cl^- concentration.

P. palpebrosus Hb measured at pH 7.4 and 7.0 (Fig. 6A,B) showed pronounced ATP and DPG sensitivity in the absence of Cl^-. In contrast to *A. mississippiensis* Hb, however,
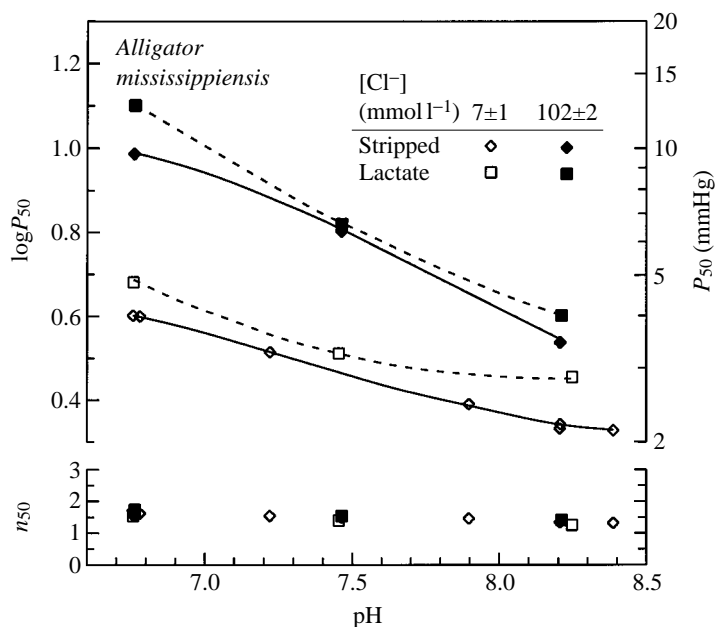
Fig. 3. Effect of $0.23\,\mathrm{mmol\,l^{-1}}$ lactate (squares) on oxygenation properties of stripped *Alligator mississippiensis* Hb measured at 7 ± 1 and $102\pm2\,\mathrm{mmol\,l^{-1}}$ Cl$^-$ (open and filled symbols, respectively) in $0.1\,\mathrm{mol\,l^{-1}}$ Hepes buffer. Hb concentration (tetramer basis), $0.21\,\mathrm{mmol\,l^{-1}}$.

the DPG effect exceeded that of ATP at low phosphate levels. While both effects were reduced, they remained distinct in the presence of $100\,\mathrm{mmol\,l^{-1}}$ Cl$^-$, particularly at the lower pH (Fig. 6B), where ATP and DPG compete more effectively with Cl$^-$ for binding sites. No oxylabile IHP binding was evident at either pH tested. Surprisingly, high levels of DPG decreased O_2 affinity more in the absence than in the presence of Cl$^-$.

Discussion

Our data show that the vertebrate allosteric cofactors ATP and DPG do modulate O_2 affinity of crocodilian Hbs at low Cl$^-$ concentrations; claims of phosphate insensitivity of these Hbs thus require qualification. The finding is interesting with respect to structure–function relationships in heme proteins and also has possible physiological implications, given the low plasma Cl$^-$ values associated with the post-feeding 'alkaline tides' that follow massive secretion of HCl into the stomach to dissolve consumed bones (Coulson and Hernandez, 1983).

ATP is the main potential organic modulator encountered in adult crocodilian erythrocytes. In *Crocodylus porosus*, *C. johnstoni* and *C. novaeguinea*, cellular ATP concentrations vary between 0.54 and $0.70\,\mathrm{mmol\,l^{-1}}$ (0.14–$0.15\,\mathrm{mol\,mol^{-1}}$ Hb) and are 2–3 times greater than the corresponding DPG levels of 0.23–$0.27\,\mathrm{mmol\,l^{-1}}$ (Grigg and Gruca, 1979). Cofactor concentrations may be much higher in the embryonic stages. In
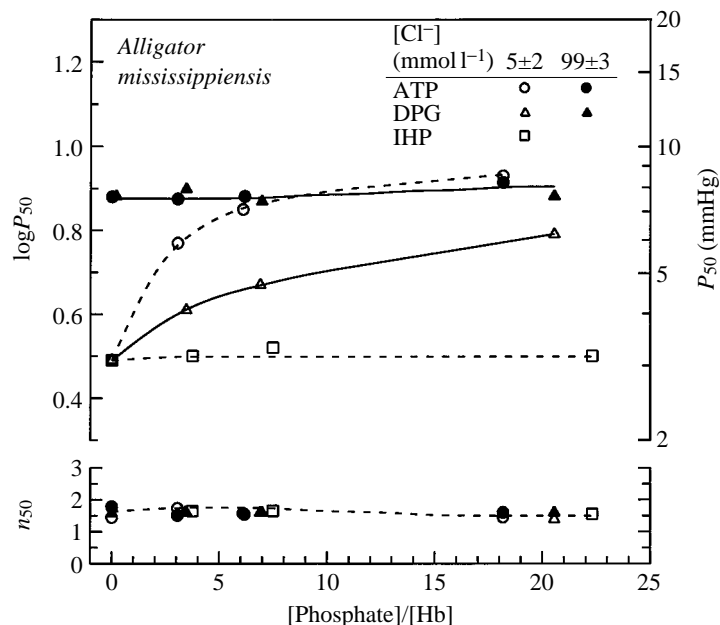
Fig. 4. Dose–response curves for the effects of ATP (circles), DPG (triangles) and IHP (squares) on P_{50} and n_{50} values of *Alligator mississippiensis* Hb in 0.1 mol l^{-1} Hepes buffer, pH 7.1, at 5 mmol l^{-1} (open symbols) and 99 mmol l^{-1} (filled symbols) Cl$^-$. Hb concentration (tetramer basis), 0.17 mmol l^{-1}.

C. porosus (Grigg *et al.* 1993), ATP levels approach 100 μmol g^{-1} Hb (approximately 6.5 mol mol^{-1} Hb) at 15 days of embryonic development and DPG levels peak at 18 μmol g^{-1} Hb (approximately 1.2 mol mol^{-1} Hb) at about 80 days of development. Both effectors exert pronounced effects on the O$_2$ affinity of the embryonic Hb, that of ATP being greater (as here recorded for adult *A. mississippiensis* Hb). Grigg *et al.* (1993) observed small ATP and DPG effects in adult *C. porosus* Hb in the presence of 100 mmol l^{-1} Cl$^-$ (similar to those reported here for *P. palpebrosus* Hb), but decreased phosphate interaction as pH fell below 7.8.

In human Hb (which has served as a model for probing allosteric interactions in hemoglobins and other proteins), organic phosphates bind at four pairs of cationic amino acid residues facing the cavity between the two β-chains. These are β1-Val, β2-His, β82-Lys and β143-His (i.e. valine, histidine, lysine and histidine found at positions 1, 2, 82 and 143, respectively; Perutz, 1983). The phosphate-insensitivity of crocodilian Hbs (established in the presence of Cl$^-$) correlates with the substitution of β1-Val and β2-His by acetylated Ala and Ser, respectively, in *A. mississippiensis* and *Crocodylus niloticus*, and by Ser and Pro in *C. crocodilus*, and the replacement of β143-His by Ala in all these species (Leclercq *et al.* 1981). In crocodilian Hbs, HCO$_3^-$ binds at the β1 residue and at β82-Lys and β144-Glu (Perutz *et al.* 1981). Allosteric interaction of phosphates with the N-terminal residues is excluded, either by their acetylation or by the adjacent β2-His→Pro substitution that bends the N termini away from the 'phosphate site' (Perutz
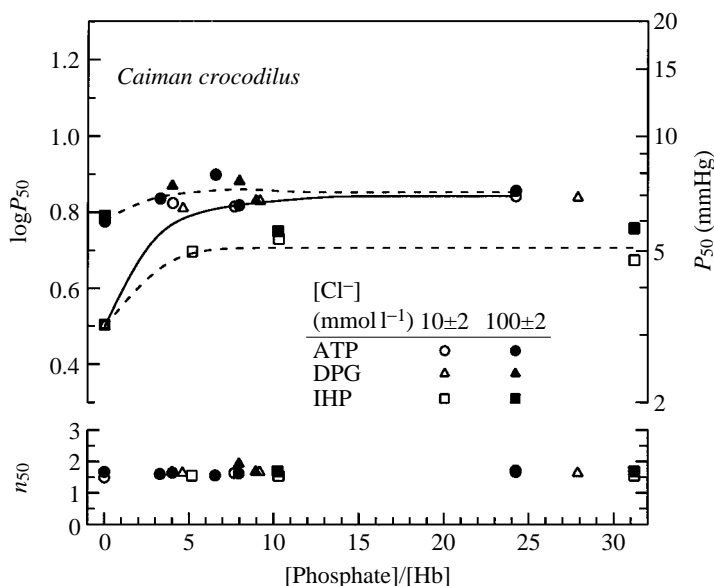
Fig. 5. Dose–response curves for the effects of ATP (circles), DPG (triangles) and IHP (squares) on P_{50} and n_{50} values of *Caiman crocodilus* Hb in $0.1\,\mathrm{mol\,l^{-1}}$ Hepes buffer, pH 7.1, and at $10\,\mathrm{mmol\,l^{-1}}$ (open symbols) and $100\,\mathrm{mmol\,l^{-1}}$ (filled symbols) Cl^-. Hb concentration (tetramer basis), $0.17\,\mathrm{mmol\,l^{-1}}$.

et al. 1981). This leaves only β82-Lys as a phosphate binding residue, which has been considered an insufficient basis for affinity modulation, since it is present in human fetal Hb that shows a reduced DPG sensitivity although it still has β2-His (cf. Bunn and Briehl, 1970; Perutz *et al.* 1981). The present data, however, indicate an important role for β82-Lys in phosphate modulation of tetrameric Hbs.

In human Hb, chloride ions bind mainly at α1-Val (also interacting with α131-Ser) and at β82-Lys (also interacting with β1-Val; cf. Riggs, 1988). Both these groups contribute to the Bohr effect in the absence of phosphates, and β82-Lys is considered to be important for the expression of the Bohr effect in Hbs that have no or reduced phosphate effects (Riggs, 1988; Perutz *et al.* 1980). In the crocodilian Hbs that have been sequenced (caiman, alligator and Nile crocodile), α131-Ser is replaced by Ala, which may inactivate Cl^- binding at α1-Val (Perutz *et al.* 1981), leaving β82-Lys as the predominant Cl^- binding site. This is consistent with the lesser augmentation of the Bohr factor by chloride in alligator Hb (where $100\,\mathrm{mmol\,l^{-1}}$ Cl^- raises ϕ from -0.20 to -0.33, see Fig. 2) than in deionized human Hb, where addition of Cl^- to $100\,\mathrm{mmol\,l^{-1}}$ increases ϕ from -0.2 to -0.5 by raising the pKa values of both α1-Val and β82-Lys (Perutz, 1989). The greater effect of ATP and DPG than of $0.1\,\mathrm{mol\,l^{-1}}$ Cl^- in increasing the Bohr factor of stripped Hb (see Fig. 2) is a manifestation of their pH-dependent oxylabile binding to crocodilian Hb.

That a common site (probably β82-Lys) is implicated in phosphate and chloride ion binding follows from the strong suppression of the phosphate effect caused by Cl^- addition. This effect is mirrored by the progressive decrease in the Cl^- effect (indicated
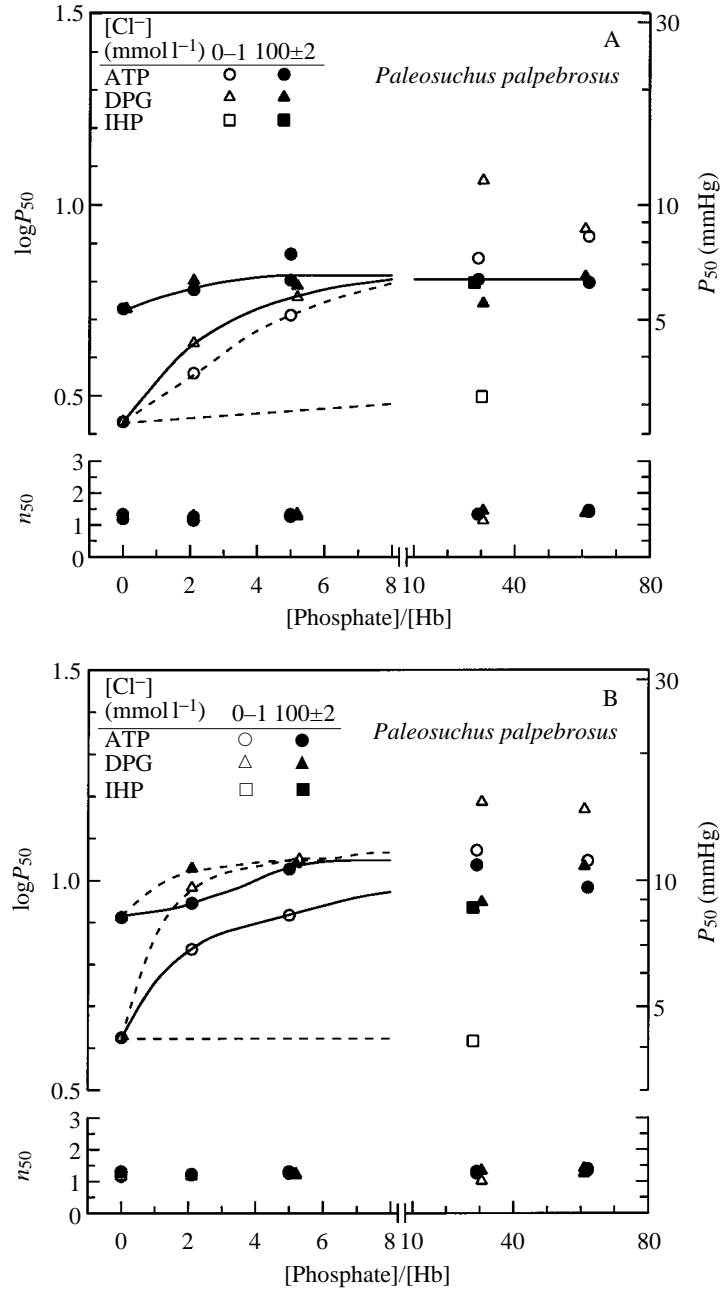
Fig. 6. (A) Dose–response curves for the effects of ATP (circles), DPG (triangles) and IHP (squares) on P_{50} and n_{50} values of *Paleosuchus palpebrosus* Hb in $0.1 \, mol \, l^{-1}$ Hepes buffer, pH 7.4, in the absence of Cl^- (open symbols) and the presence of $100 \, mmol \, l^{-1}$ Cl^- (filled symbols). Hb concentration (tetramer basis), $0.09 \, mmol \, l^{-1}$. (B) Effects of ATP, DPG and IHP on P_{50} and n_{50} values of *P. palpebrosus* Hb at pH 7.0. Other conditions as in A.

by the distances between similarly shaped open and filled symbols in Figs 4–6) with increasing phosphate concentration. Thus, at low [phosphate]/[Hb] ratios, ATP depresses Cl^- binding more than DPG does in *Alligator mississippiensis* Hb, whereas the opposite applies to *P. palpebrosus* Hb.

   The Hbs studied here illustrate a series of varying phosphate interaction. *A. mississippiensis* Hb distinguishes between ATP and DPG, showing greater oxygenation-linked binding of the former (Fig. 4). Given that ATP and DPG carry similar charges at physiological pH, this specificity presumably reflects stereochemical differences in the protein moiety, whereby ATP provides a better fit and thus is more effective in stabilizing the deoxygenated structure and in decreasing O_2 affinity. The lack of an IHP effect is opposite to expectations based on its greater negativity and suggests that allosteric interaction between crocodilian Hbs and large polyanions is impeded by steric hindrance at the phosphate binding sites.

   P. palpebrosus Hb similarly differentiates between ATP and DPG (Fig. 6A,B) but, in contrast to *A. mississippiensis* Hb, shows a greater response to the smaller DPG molecules. The greater ATP and DPG effects at pH 7.0 than at pH 7.4 accord with the pH-dependence of anion binding. The intriguing finding that O_2 affinities at high [DPG]/[Hb] ratios were lower in the absence, than in the presence, of Cl^- (Fig. 6A,B) may reflect a high stereochemical complementarity between DPG and the binding site, resulting in greater stabilization of the deoxygenated structure than that obtained in the presence of the smaller chloride ions. As with *A. mississippiensis*, the anion binding pocket of *P. palpebrosus* Hb appears to be unable to accommodate the relatively large IHP molecules (Fig. 6A,B). *C. crocodilus* Hb, displaying similar ATP and GTP effects and distinct, albeit reduced, IHP effects appears to be the least discriminating as regards phosphate binding.

   'The languid existence of crocodiles can be interrupted by fierce activity during escape, defense and predation' (Seymour *et al.* 1985, p. 144). In *C. porosus*, where the susceptibility to resultant acid–base disturbances increases with body size (Bennett *et al.* 1985), this may cause blood lactate concentrations to rise to $70\,mmol\,l^{-1}$ and pH to fall to 6.4 for long periods ($>14\,h$). As with ATP or HCO_3^-, lactate may influence Hb O_2-transporting properties and thus form a direct, regulatory feedback for adjusting tissue O_2 supply to demand. In human Hb, lactate has similar effects to Cl^- at concentrations below $0.1\,mmol\,l^{-1}$, but an approximately 40 % greater effect at higher concentrations (Guesnon *et al.* 1979). Lactate, at a concentration that approximates the plasma value in exercised *A. mississippiensis* (Coulson and Hernandez, 1979), decreases Hb O_2-affinity over a wide pH range (Fig. 3). Its seemingly greater effect at low Cl^- concentration suggests competition with chloride ions for the same binding sites. The lactate effect, which may improve O_2 unloading in the tissues, could be due either to greater lactate binding to deoxygenated Hb or to lesser binding to oxygenated Hb molecules compared with Cl^- binding. The binding constants obtained for human Hb (Guesnon *et al.* 1979) suggest the latter alternative to be the case.

   The data describe the functional consequences of the evolution of HCO_3^--sensitivity in crocodilian Hbs. Parallel observations on Hbs from crocodiles or gharials (which represent separate evolutionary lineages compared with the caimans and alligators

studied here, Desmore and Owen, 1989) would give further insight into the mechanisms of allosteric interaction in Hbs of the order Crocodilia. Evaluation of the physiological implications requires further information, e.g. on the Cl^- concentration variations 'seen' by the Hb inside the red cells and the interactive effects of CO_2/HCO_3^-.

Lastly, it may be recalled that organic phosphates may have significance other than for altering Hb O_2-affinity by allosteric regulation, such as by modifying intracellular pH (and thus O_2 affinity) *via* changes in the Donnan equilibrium of protons across the red cell membranes (Duhm, 1971; Wood and Johansen, 1973). Grigg and Gruca (1979) postulated that the low phosphate levels in crocodile red cells may be adaptive in decreasing the fixed-acid Bohr effect and thus in maintaining O_2 loading in the face of a lactate flush following dives.

References

Bauer, C., Forster, M., Gros, G., Mosca, A., Perrella, M., Rollema, H. S. and Vogel, D. (1981). Analysis of bicarbonate binding to crocodilian hemoglobin. *J. biol. Chem.* **256**, 8429–8435.

Bauer, C. and Jelkmann, W. (1977). Carbon dioxide governs the oxygen affinity of crocodile blood. *Nature* **269**, 825–827.

Bennett, A. F., Seymour, R. S., Bradford, D. F. and Webb, G. T. W. (1985). Mass-dependence of anaerobic metabolism and acid–base balance disturbance during activity in the salt-water crocodile, *Crocodylus porosus*. *J. exp. Biol.* **118**, 161–171.

Berman, M., Benesch, R. and Benesch, R. E. (1971). The removal of organic phosphates from hemoglobin. *Archs Biochem. Biophys.* **145**, 236–239.

Brittain, T. and Wells, R. M. (1991). An investigation of the co-operative function of the haemoglobin of the crocodile, *Crocodylus porosus*. *Comp. Biochem. Physiol.* **98**B, 641–646.

Bunn, H. F. and Briehl, R. W. (1970). The interaction of 2,3-diphosphoglycerate with various human hemoglobins. *J. clin. Invest.* **49**, 1088–1095.

Coulson, R. A. and Hernandez, T. (1979). Factors controlling glycogen breakdown in the alligator. *Comp. Biochem. Physiol.* **64**C, 115–121.

Coulson, R. A. and Hernandez, T. (1983). Alligator metabolism. Studies on chemical reactions *in vivo*. *Comp. Biochem. Physiol.* **74**B, 1–182.

Desmore, L. D. and Owen, R. D. (1989). Molecular systematics of the order Crocodilia. *Am. Zool.* **29**, 831–841.

Duhm, J. (1971). Effects of 2,3-diphosphoglycerate and other organic phosphate compounds on oxygen affinity and intracellular pH of human erythrocytes. *Pflügers Arch.* **326**, 341–356.

Grigg, G. C. and Gruca, M. (1979). Possible adaptive significance of low red cell organic phosphates in crocodiles. *J. exp. Zool.* **209**, 161–168.

Grigg, G. C., Wells, R. M. G. and Beard, L. A. (1993). Allosteric control of oxygen binding by haemoglobin during embryonic development in the crocodile *Crocodylus porosus*: the role of red cell organic phosphates and carbon dioxide. *J. exp. Biol.* **175**, 15–32.

Guesnon, P., Poyart, C., Bursaux, E. and Bohn, B. (1979). The binding of lactate and chloride ions to human adult hemoglobin. *Respir. Physiol.* **38**, 115–129.

Jelkmann, W. and Bauer, C. (1980). Oxygen binding properties of caiman blood in the absence and presence of carbon dioxide. *Comp. Biochem. Physiol.* **65**A, 331–336.

LECLERCQ, F., SCHNEK, A. G., BRAUNITZER, G., STANGL, A. AND SCHRANK, B. (1981). Direct reciprocal allosteric interaction of oxygen and hydrogen carbonate. Sequence of the haemoglobins of the caiman (*Caiman crocodilus*), the Nile crocodile (*Crocodylus niloticus*) and the Mississippi crocodile (*Alligator mississippiensis*). *Hoppe-Seyler's Z. physiol. Chem.* **362**, 1151–1158.

MAGINNISS, L. A. AND HITZIG, B. M. (1987). Acid–base status and electrolytes in red blood cells and plasma of turtles submerged at 3 ˚C. *Am. J. Physiol.* **253**, R64–R70.

NIKINMAA, M. (1990). *Vertebrate Red Blood Cells*. Berlin: Springer-Verlag.

PERUTZ, M. F. (1983). Species adaptation in a protein molecule. *Molec. Biol. Evol.* **1**, 1–28.

PERUTZ, M. F. (1989). Mechanisms of cooperativity and allosteric regulation in proteins. *Q. Rev. Biophys.* **22**, 139–236.

PERUTZ, M., BAUER, C., GROS, G., LECLERCQ, F., VANDECASSERIE, C., SCHNEK, A. G., BRAUNITZER, G., FRIDAY, A. E. AND JOYSEY, K. A. (1981). Allosteric regulation of crocodilian haemoglobin. *Nature* **291**, 682–684.

PERUTZ, M. F., KILMARTIN, J. V., NISHIKURA, K., FOGG, J. H., BUTLER, P. J. G. AND ROLLEMA, H. S. (1980). Identification of residues contributing to the Bohr effect of human haemoglobin. *J. molec. Biol.* **138**, 649–670.

RIGGS, A. F. (1988). The Bohr effect. *A. Rev. Physiol.* **50**, 181–204.

SEYMOUR, R. S., BENNETT, A. F. AND BRADFORD, D. F. (1985). Blood gas tensions and acid–base balance in the salt-water crocodile, *Crocodylus porosus*, at rest and after exhaustive exercise. *J. exp. Biol.* **118**, 143–159.

WEBER, R. E. (1981). Cationic control of O_2 affinity in lugworm erythrocruorin. *Nature* **292**, 386–387.

WEBER, R. E. (1992). Use of ionic and zwitterionic (Tris/BisTris and Hepes) buffers in studies on hemoglobin function. *J. appl. Physiol.* **72**, 1611–1615.

WEBER, R. E. AND JENSEN, F. B. (1988). Functional adaptations in hemoglobins from ectothermic vertebrates. *A. Rev. Physiol.* **50**, 161–179.

WEBER, R. E., JENSEN, F. B. AND COX, R. P. (1987). Analysis of teleost hemoglobin by Adair and Monod–Wyman–Changeux models. Effects of nucleoside triphosphates and pH on oxygenation of tench hemoglobin. *J. comp. Physiol.* **157**B, 145–152.

WEBER, R. E. AND WELLS, R. M. G. (1989). Hemoglobin structure and function. In *Lung Biology in Health and Disease; Comparative Pulmonary Physiology: Current Concepts* (ed. S. C. Wood and C. Lenfant), chapter 10, pp. 279–310. New York: Marcel Dekker, Inc.

WEBER, R. E. AND WHITE, F. N. (1986). Oxygen binding in alligator blood related to temperature, diving and 'alkaline tide'. *Am. J. Physiol.* **251**, R901–R908.

WOOD, S. C. AND JOHANSEN, K. (1973). Organic phosphate metabolism in nucleated red cells: influence of hypoxia on eel HbO_2 affinity. *Neth. J. Sea Res.* **7**, 328–338.