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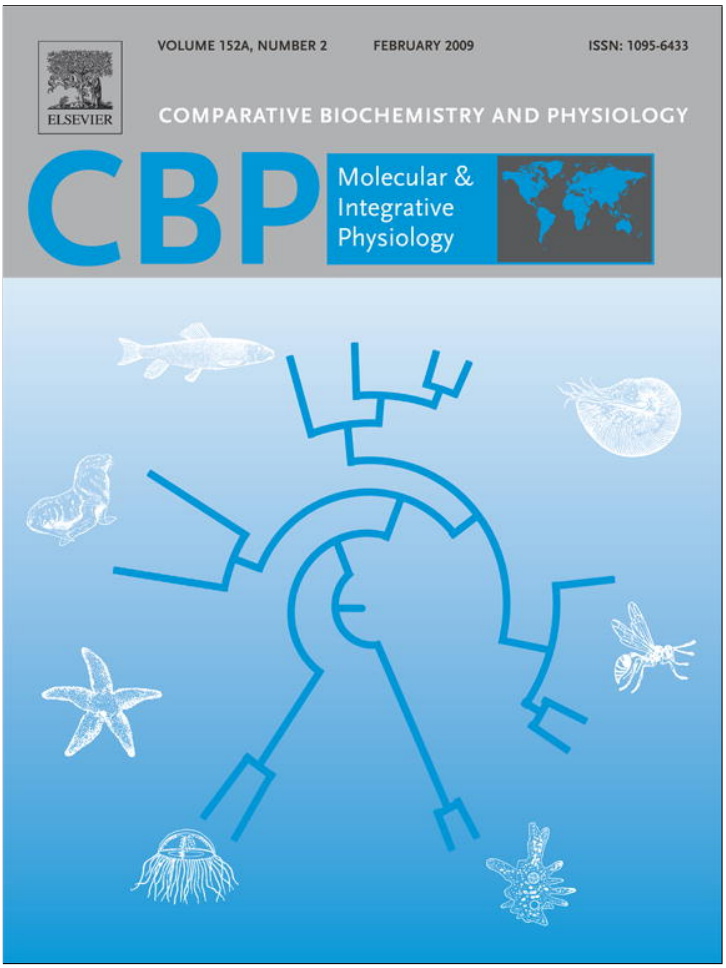

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Characterization of the hemoglobins of the Australian lungfish *Neoceratodus forsteri* (Krefft)

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ABSTRACT

We examined for the first time the hemoglobin components of the blood of the Australian lungfish, *Neoceratodus forsteri* and their functional responses to pH and the allosteric modulators adenosine triphosphate (ATP), guanosine triphosphate (GTP), 2,3-bisphosphoglyceric acid (BPG) and inositol hexaphosphate (IHP) at 25 °C. Lysates prepared from stripped, unfractionated hemolysate produced sigmoidal oxygen equilibrium curves with high oxygen affinity (oxygen partial pressure required for 50% hemoglobin saturation, p_{50}=5.3 mmHg) and a Hill coefficient of 1.9 at pH 7.5. p_{50} was 8.3 and 4.5 mmHg at pH 6 and 8, respectively, which corresponded to a modest Bohr coefficient ($\Delta\log p_{50}/\Delta pH$) of −0.13. GTP increased the pH sensitivity of oxygen binding more than ATP, such that the Bohr coefficient was −0.77 in the presence of 2 mmol L^{-1} GTP. GTP was the most potent regulator of hemoglobin affinity, with concentrations of 5 mmol L^{-1} causing an increase in p_{50} from 5 to 19 mm Hg at pH 7.5, while the order of potency of the other phosphates was IHP>ATP>BPG. Three hemoglobin isoforms were present and each contained both α and β chains with distinct molecular weights. Oxygen affinity and pH-dependence of isoforms I and II were essentially identical, while isoform III had a lower affinity and increased pH-dependence. The functional properties of the hemoglobin system of *Neoceratodus* appeared consistent with an active aquatic breather adapted for periodic hypoxic episodes.

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1. Introduction

Lungfishes derive from an ancient lineage of air-breathing dipnoan fishes first appearing in the fossil record 380 million years ago (Campbell and Barwick, 1990). There are six extant species of lungfishes: four African species in the genus *Protopterus* (Family Protopteridae), one South American species, *Lepidosiren paradoxa* (Family Lepidosirenidae), and one Australian species, *Neoceratodus forsteri* (Family Ceratodontidae). They are all bimodal breathers and possess both gills and lungs, the lungs being homologous with the pulmonary gas exchange organs of higher vertebrates (Lomholt et al., 1975; Brainerd, 1994; Graham, 2006). During the alternation of water breathing and air breathing, the pattern of circulation oscillates between that of a fish and a tetrapod (Fishman et al., 1985). In addition, molecular studies point to a link between lungfishes and early tetrapod evolution and thus the critical transition from water- to air-breathing that led to a vertebrate conquest of terrestrial habitats (Meyer and Wilson, 1990; Brinkmann et al., 2004).

The impressive abilities of *Protopterus* and *Lepidosiren* to survive for long periods in hypoxic or anoxic water, or in mud-lined cocoons when pools dry out, are supported by diversion of the branchial circulation to the pulmonary gas exchange surfaces to enable air breathing (Johansen et al., 1976; Abe and Steffensen, 1996; Jucá-Chagas, 2004). In contrast to these obligate air-breathers with paired lungs, *Neoceratodus* is predominantly a water-breather, has a single lung, and is less well able to survive extended periods out of water (Grigg, 1965; Lenfant et al., 1966; Fritsche et al., 1993). *Neoceratodus* may therefore be considered the most primitive of the extant Dipnoi and represents an important stage in the transition from water to air-breathing. It occurs naturally in slow flowing waters of the Burnett and Mary River systems in South-Eastern Queensland. During dry periods when aquatic oxygen levels decline sharply, *Neoceratodus* is reported to supplement its oxygen uptake by breathing air (Gannon et al., 1983). Field observations by Grigg (1965) however, linked air-breathing in *Neoceratodus* to periods of physical activity, an idea only recently resurrected as an adaptation for air-breathing (Farmer and Jackson, 1998; Clark et al., 2007; Wells et al., 2007). The Queensland lungfish does not build a mud-lined

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cocoon like the African species, and cannot survive for more than a few days out of water (Kind, 2003).

Several studies have examined aspects of the blood oxygen transport system that support air-breathing in lungfishes. During estivation the African lungfish *Protopterus amphibious* increased hematocrit and hemoglobin concentration by 50%, reflecting a raised oxygen carrying capacity, while the concentration of erythrocyte organic phosphates decreased thus leading to a sharp increase in whole blood-oxygen affinity where p_{50} (oxygen partial pressure required for 50% hemoglobin saturation) changed from 33 to 9 mmHg (Johansen et al., 1976). The increase in oxygen affinity has been interpreted as providing improved oxygenation during estivation in a low oxygen environment. The red blood cells of the African lungfish *Protopterus aethiopicus*, and the South American lungfish *Lepidosiren paradoxa* contain high concentrations of adenosine and guanosine triphosphates (ATP and GTP) together with an inositol polyphosphate (IP2), a potent category of allosteric regulators of hemoglobin oxygen affinity occurring in birds (Bartlett 1978; Isaacks et al., 1978). Two major hemoglobin components, each with similar sensitivity to GTP, are present in *Protopterus amphibious*, and a single GTP-sensitive hemoglobin occurs in *Protopterus annectens* (Weber et al., 1977).

Although the erythrocytes of *Neoceratodus forsteri* contain both ATP and GTP, IP2 was not detected (Isaacks and Kim, 1984), which could reflect an earlier evolutionary stage of facultative air-breathing. To date, no detailed studies have appeared on the hemoglobin components of the blood of *Neoceratodus* or their functional responses to pH and organic phosphate modulators. In view of both the interesting phylogeny of this lungfish species, and its contrasting behaviour of facultative air-breathing, we have examined aspects of the hemoglobin system in *Neoceratodus*, and in particular, structure-function relationships of the highly adaptable hemoglobin protein.

2. Materials and methods

Six lungfish (body mass range 346–942 g; body length range 0.4–0.6 m) were obtained from Macquarie University, NSW, Australia, and held in aerated indoor tanks (0.5×1.1×0.5 m deep) at the University of Adelaide, SA, Australia. Water depth was kept at approximately 0.3 m, water temperature was maintained between 22 and 24 °C, photoperiod was 12:12 (lights on 07:00, lights off 19:00), and fish were fed once every 3–5 days on pellets (Classic SS 6 mm, Skretting, Cambridge, TAS, Australia). Animals were fasted for 6 days prior to taking blood samples.

Animals were individually netted from their holding tank and a blood sample (3–6 ml) was taken by caudal venipuncture into heparinised tubes. A sub-sample of blood was used immediately to measure hemoglobin concentration with an analyzer calibrated for fish blood (HemoCue AB, Ängelholm, Sweden; Clark et al., in press) and the rest of the sample was placed on ice. Another sub-sample was used to determine hematocrit by centrifugation in microhematocrit tubes at 12,000 ×*g* for 5 min (Red blood cell volume was not determined in this study and hence the reported hematocrit values have not been adjusted for any effects which may have arisen from red blood cell swelling induced by beta adrenergic activation). The remaining samples were then centrifuged at 1000 ×*g* to separate the cells from the plasma. The plasma was decanted and the packed cells were placed at −80 °C. After 4 days of storage, the packed cells were shipped on dry ice to The University of Auckland, New Zealand, for purification, isolation, and functional studies. Red blood cells collected from either individuals or pooled samples were treated by the addition of two volumes of distilled water, and cell debris was removed by centrifugation at 12,000 ×*g* for 30 min at 4 °C.

Hemoglobin solutions were depleted of endogenous small molecules, particularly red cell organic phosphates, by passage down a Sephadex G25 column (8×270 mm) equilibrated with 10 mmol L^{-1} Tris–HCl buffer containing 300 mmol L^{-1} NaCl at pH 8.2, and reduced by the addition of ascorbic acid and tetramethylene phenylene diamine to final concentrations of 6 mmol L^{-1} and 1 μmol L^{-1}, respectively. Functional studies were carried out using 10 mmol L^{-1} HEPES buffers with final chloride concentration in the range 0–1000 mmol L^{-1} NaCl in order to determine the effect of chloride ion concentration on the oxygen binding equilibrium (These results have not been normalized to account for associated water concentration effects, Hundahl et al. 2003; Colombo et al 1994). The effects of the exogenous allosteric effectors ATP, GTP, 2,3-bisphosphoglyceric acid (BPG), and inositol hexaphosphate (IHP) (range 0 to 2 mmol L^{-1} final concentration) were evaluated by addition of pH-adjusted cofactors to the buffer. The binding constants for organic phosphates were determined by non-linear-least-squares fitting of the experimental data to the function:

$$\log\ p_{50} = A + B^{*}([X\}/(K + [X\})$$

using Tablecurve 2D (Jandel Scientific, San Rafael, CA, USA)
where A is the log p_{50} in the absence of organic phosphate, B is the change in log p_{50} produced on saturation with organic phosphate, [X] is the concentration of organic phosphate and K is the apparent binding constant. In all cases the fitting yielded $r^2 > 0.99$.

Oxygen binding curves were obtained at 25 °C using a Hemox Analyzer (TCS Scientific, PA., USA). Particular care was taken to determine full saturation in order to avoid reporting spurious co-operativity data (see Riggs, 1998). Curves obtained up to oxygen partial pressures of 160 mmHg were used to determine full saturation by fitting a hyperbolic function to the final 15% of the binding curve. The validity of this method was investigated by running a number of binding curves up to one atmosphere pressure of pure oxygen (760 mmHg). The extrapolation method in this instance was found to predict end points for the binding curves which were within 0.2% of the measurement made using pure oxygen and avoided misinterpretation of p_{50} and Hill coefficient values (Lapennas et al., 1981). The p_{50} values varied by <0.5 mmHg (n=4) for a given sample (Guarnone et al., 1995).

Samples employed in isoelectric focusing experiments were pre-treated by the addition of a small quantity of sodium dithionite in order to reduce any met-hemoglobin present, and then equilibrated with one atmosphere pressure of pure carbon monoxide. Excess reductant was then removed by passage down an 8×270 mm column of Sephadex G25 equilibrated with water. Isoelectric focusing was performed using Phast gels (GE Healthcare, Amersham, U.K.) at pH 5–8, pre-cooled to 10 °C, and pre-focused for 10 min at 2000 V. The hemoglobin samples were loaded and run at 2000 V. Equilibrated gels were immersed in 0.2 mol L^{-1} trichloroacetic acid for 5 min, then placed in Coommassie Blue until sufficiently stained, and subsequently destained with a 3:1:6 ratio by volume of methanol: acetic acid: water until clarified. The scanned image of the gel and the relative densities of each band from each sample were recorded. Isoelectric points were determined by reference to a commercial pI-calibration ladder (GE Healthcare).

The hemoglobin isoforms in the stripped lysate were separated using ion exchange on a column of Q-Sepharose equilibrated with 10 mmol L^{-1} HEPES pH 8.0. The proteins were eluted in a salt gradient from 0 to 300 mmol L^{-1} sodium chloride in the same buffer over 70 min. After collection, the isoforms were concentrated and buffer-

Table 1
Hematological parameters from whole blood of lungfishes (mean±S.E.M.)

	Hemoglobin g L^{-1}	Hematocrit %	MCHC g L^{-1}
Neoceratodus forsteri	44.6±4.7	23.0±1.9	192.1±6.4
Lepidosiren paradoxa[a]	45.3±7.8	15.7±2.9	289.9±15.1
Protopterus aethiopicus[b]	62	25	248

[a]Johansen and Lenfant (1967), [b]Lenfant and Johansen (1968).

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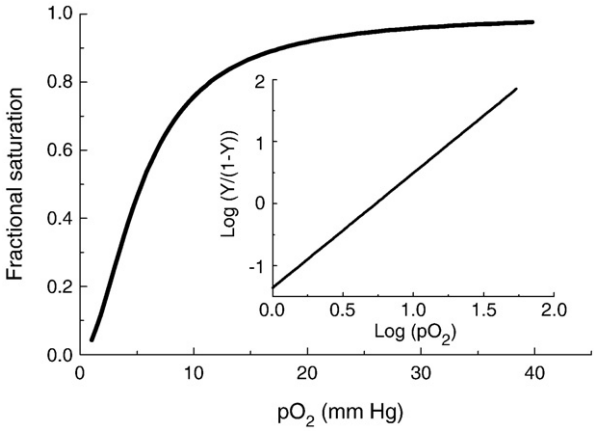

Fig. 1. Oxygen equilibrium binding curve and Hill plot inset for stripped red blood cell lysate at 25 °C in 10 mmol L^{-1} HEPES buffer at pH 7.5.

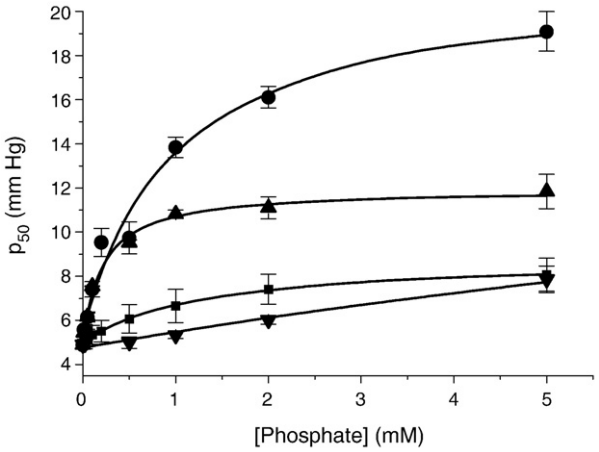

Fig. 3. Concentration effect of added organic phosphates GTP (●), IHP (▲), ATP (■) and BPG (▼) on p_{50} values for oxygen binding to red cell lysate at pH 7.5 in 10 mmol L^{-1} HEPES buffer and 25 °C. (Mean values ± S.D.).

exchanged to 10 mmol L^{-1} HEPES pH 7.5 using Vivaspin 5000 MWCO PES concentrators.

Precise molecular masses for the α- and β-globin chains of each hemoglobin isoform were determined by ESI mass spectrometry using a Qstar ESI-TOF. Hemoglobin samples were exchanged into water prior to mass spectrometric measurements.

3. Results

Hematological measurements from fresh, whole blood of *Neoceratodus* are summarized and compared with values from obligate air-breathing lungfishes in Table 1. Lysates prepared from whole blood from each individual were checked for autoxidisability. At pH>6.0, no detectable autoxidation occurred over a 2 h period at room temperature (20 °C), or overnight at −20 °C. Addition of saturating amounts of allosteric effectors did not promote t-state autoxidation. At room temperature and pH 6.0, however, the lysate was found to oxidize to a level of 3% after 30 min, a time well in excess of the 10–15 min required to determine oxygen equilibrium curves. Preliminary isoelectric focusing of individual fish did not reveal polymorphic differences, and thereafter pooled lysate was used as the basis of experimental observations. At pH 7.5 and 25 °C, the lysates showed typical high affinity (p_{50}=5.3±0.3 mmHg; S.E., n=6, 1 mmHg=0.133 kPa) and sigmoidal oxygen binding curves with associated Hill coefficients of 1.9±0.1) (Fig. 1).

3.1. Bohr effect

When oxygen binding curves were measured over a range of pH values from 6.0–8.0 it was found that p_{50} decreased from 8.3±1.0 mmHg at pH 6.0, to 4.5±0.2 mmHg at pH 8.0 (Fig. 2). Over this pH range it was also found that the Hill coefficient associated with oxygen binding rose from 1.50±0.03 at pH 6.0, to 1.90±0.10 mmHg at pH 8.0. The Bohr coefficient, Ø,=Δlog p_{50}/ΔpH for these data was −0.13. On addition of saturating amounts (2 mmol L^{-1}) of either ATP or GTP, the pH sensitivity of oxygen binding was found to increase markedly. In the presence of ATP the Bohr coefficient rose to −0.49, and in the presence of GTP to −0.77 (Fig. 2).

3.2. Concentration effect of organic phosphates

Titration of the lysate with increasing concentrations of organic phosphate at a constant pH of 7.5 showed that both ATP and GTP raised the p_{50} value for oxygen binding, but to differing extents (Fig. 4). Addition of ATP increased the p_{50} value from 5 (SD 0.1) to 8 (SD 0.7) mmHg at a concentration of 5 mmol L^{-1} with an associated increase in the Hill coefficient from 1.7 to 2.1. A similar addition of GTP shifted the p_{50} value from 5 (SD 0.2) to 19 (SD 0.9) mmHg, and increased the Hill coefficient to a value of 2.0. BPG and IHP shifted the p_{50} value from 5 (SD 0.1) mmHg to 8 (SD 0.6) and 12 (SD 0.7) mmHg respectively, with

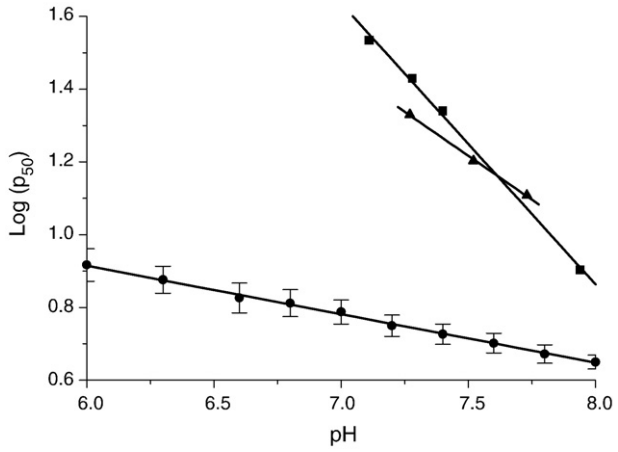

Fig. 2. Bohr plot showing the effect of pH on the oxygen affinity (p_{50}) of red cell lysate at 25 °C in 10 mmol L^{-1} HEPES buffers (●) and in the presence of 2 mmol L^{-1} phosphate cofactors ATP (▲) and GTP(■) for a single sample. (Mean values ± S.D.).

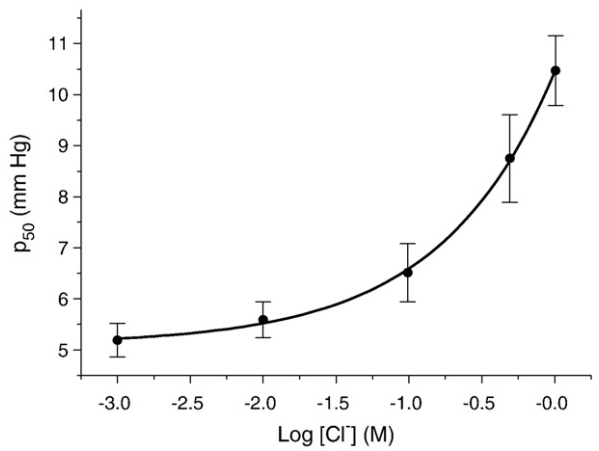

Fig. 4. Allosteric effect of chloride ion concentration on p_{50} values for oxygen binding to red cell lysate at 25 °C in 10 mmol L^{-1} HEPES buffer. (Mean values ± S.D.).

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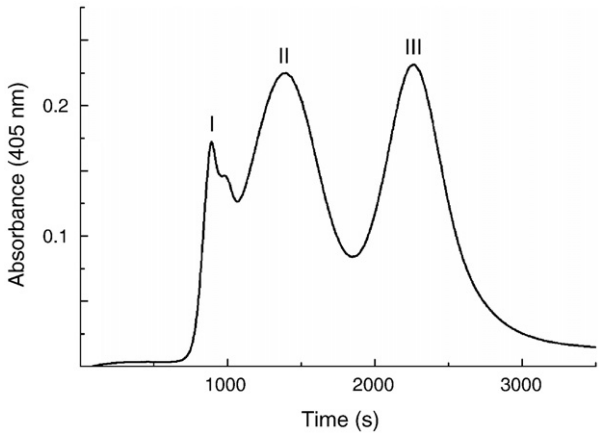

Fig. 5. Elution profile of hemolysate absorbed on Sepharose equilibrated with 10 mmol L^{-1} HEPES, pH 8.0. The proteins were eluted by a linear salt gradient from 0 to 300 mmol L^{-1} NaCl over 70 min. Fractions were labeled from I to III in order of elution.

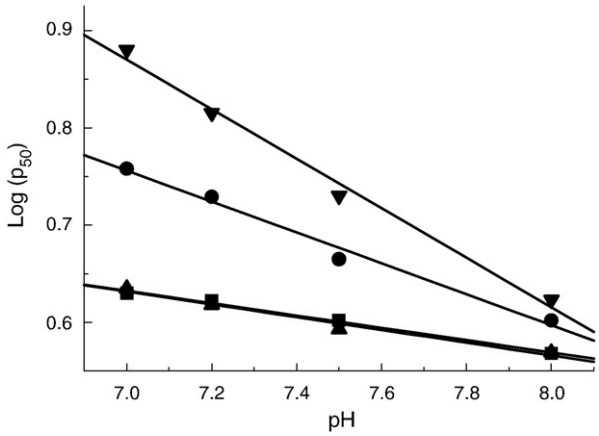

Fig. 6. Bohr plots showing the effect of pH on p_{50} of isolated, purified fractions I (▲), II (■), and III (▼) in 10 mmol L^{-1} HEPES buffer at 25 °C, compared with red cell lysate (●).

BPG increasing the Hill coefficient to 2.0, whereas IHP had no discernable effect. Fitting the curves of p_{50} *vs* the concentration of organic phosphate to a hyperbolic function yielded equilibrium constants (K) for the binding of the organic phosphate to hemoglobin with values of 1.34 (SE 0.046, r^2 0.999), 0.97 (SE 0.16, r^2 0.998), 28.32 (SE 0.02, r^2 0.999) and 0.20 (SE 0.18, r^2 0.992) mmol L^{-1} for ATP, GTP, BPG, and IHP respectively. The smaller the value of K, the tighter the cofactor binding required to produce 50% of the saturating effect (cf. maximal effects in Fig. 3). Thus, GTP was bound more tightly than ATP and also had the larger allosteric effect.

3.3. Chloride effect

Addition of NaCl to the lysate increased the p_{50} value for oxygen binding at pH 7.5 from 5(SD 0.1) to 11 (SD 0.6) mmHg as the salt concentration was raised to 1000 mmol L^{-1} (Fig. 4). The addition of salt was also associated with an increase in the Hill coefficient from 1.7 to 2.0.

3.4. Molecular characterization of hemoglobin isoforms

Isoelectric focusing of the red cell lysate in the pH range 5 to 8 indicated the presence of three hemoglobin isoforms in each specimen with isoelectric points of 6.86, 6.94 and 6.97. The proportion of each isoform was essentially constant in each fish studied. Chromatography of the red cell lysate yielded three components: I, II, and III (Fig. 5). Electro-spray mass spectrometry of each of the components showed that each hemoglobin isoform contained both α and β chains with distinct molecular weights (Table 2).

3.5. Functional properties of purified isoforms

When exposed to pH values between 7.0 and 8.0 the three hemoglobin isoforms showed pH-dependent oxygen binding characteristics (Fig. 6). Isoforms I and II exhibited almost identical oxygen affinities which were slightly higher than that of the whole lysate with Bohr coefficients both approximately −0.06. Isoform III showed a greater sensitivity towards solution pH with a higher p_{50} (lower oxygen affinity) than the whole lysate, and a Bohr coefficient of −0.25. Addition of GTP to solutions of the hemoglobin isoforms produced a rise in p_{50} (decrease in oxygen binding affinity) in all cases (Fig. 7).

Table 2
Mass spectral data for the three isoforms of *Neoceratodus* hemoglobin

	α-chain MW	β-chain MW
Isoform 1	15328.2±0.0	16176.4±0.5
Isoform 2	15348.8±0.0	16291.6±0.5
Isoform 3	15387.8±0.5	16754.8±0.5

4. Discussion

The hemoglobin system of the Australian lungfish *Neoceratodus forsteri* showed typical vertebrate characteristics with tetrameric proteins which bind oxygen co-operatively, have a relatively high intrinsic oxygen affinity, and are sensitive to organic phosphates. Neither *Neoceratodus*, nor the obligate air-breathing lungfishes have an appreciable Root effect (Lenfant et al., 1966; Berenbrink et al., 2005). Despite an effective mechanism for allosteric regulation of hemoglobin-oxygen affinity through GTP and, to a lesser extent, ATP, prolonged exposure of *N. forsteri* to hypoxia (pO_2 = 44 mm Hg) did not appreciably alter blood oxygen affinity or red cell organic phosphate composition (Kind et al., 2002). In contrast, the African lungfish, *Protopterus amphibius*, showed a GTP-mediated increase in whole blood oxygen affinity when exposed to the low oxygen environment in the cocoon during estivation (Johansen et al., 1976). Interestingly, the obligate air-breathing *Protopterus* and *Lepidosiren* possess the additional allosteric cofactor IP2, a phosphate not found in *Neoceratodus* (Isaacks and Kim, 1984). The significance of IP2 and its potential role in hypoxia

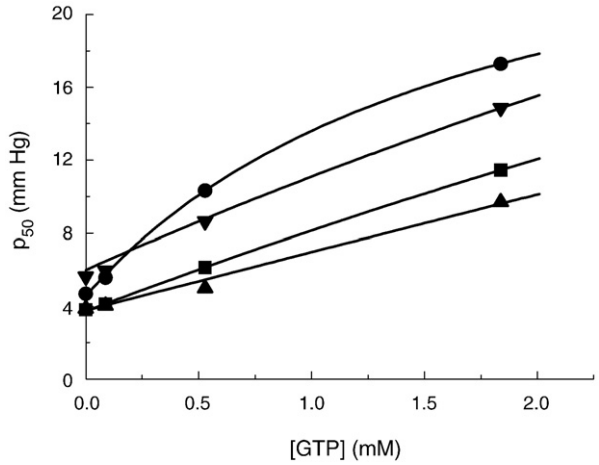

Fig. 7. Concentration effect of added GTP on the p_{50} values for isolated, purified fractions I (▲), II (■), and III (▼) compared with red cell lysate (●), at pH 7.5 in 10 mmol L^{-1} HEPES buffer at 25 °C.

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regulation is unknown. The oxygen binding characteristics of the hemoglobins of *Neoceratodus* are therefore consistent with a first line of hypoxic defense in securing saturation during gill breathing. This marks *Neoceratodus* apart from the obligate air-breathing lungfishes and most tetrapods (cf. Weber and Wells 1989; Nikinmaa, 2001).

The greater potency of GTP compared to IHP and BPG in modulating *Neoceratodus* hemoglobin was unexpected. The stronger allosteric effect of IHP over GTP in the air-breathing fish, *Arapaima gigas* reflects the generally higher potency of this cofactor in vertebrate hemoglobins. However, among several other Amazonian fishes regularly subjected to hypoxia, GTP and ATP exert similar effects, and in the armoured catfish, *Hoplosternum littorale*, BPG is present, yet exerts little of the expected allosteric effect (Weber, 2000). The explanation for modest IHP and BPG effects upon *Neoceratodus* hemoglobin is unknown, but might relate to the molecular architecture of the central binding cavity whereby their binding is influenced by steric hindrance. The physiological significance of such a diverse range of red cell organic phosphates among air-breathing fishes in general, and in the lungfishes particularly, is uncertain and not clearly related to hypoxic tolerance (Weber, 1982).

In the presence of increasing concentration of chloride ions, the red blood cell lysate of *Neoceratodus* showed a decrease of oxygen affinity, reminiscent of that seen in the human (Perutz et al., 1994). Interestingly, addition of increasing concentrations of chloride ions to the red cell lysate was also associated with a slight increase in the Hill coefficient, in contrast to the case of the hagfish *Myxine glutinosa* in which addition of chloride at modest levels (100 mmol L^{-1}) leads to dissociation of the hemoglobin (Fago and Weber, 1995). It would thus appear that in the case of the hemoglobins of the lungfish, chloride ions exert their allosteric role by affecting the differential binding of water to the oxy and deoxy forms of the protein as has been shown to be the case in the human system (Colombo et al., 1994).

For *Neoceratodus*, the primary response to low aquatic oxygen concentration appears to be an increase in both air-breathing frequency and cardiac output (Johansen et al., 1967; Fritsche et al., 1993). The single lung of *Neoceratodus* appears less efficient in CO_2 exchange than in the species with paired lungs (Graham, 1997; Johansen et al., 1967) and when removed from water, *Neoceratodus* experiences a build up of CO_2 in the blood and an associated drop in blood pH. By removing *Neoceratodus* from water for 39 min, Lenfant et al. (1966) observed a drop in pH from 7.6 to 7.3 which led to a reduced ability of the hemoglobin to bind and transport oxygen. When returned to water the CO_2 was rapidly released via the branchial apparatus. Comparison of the obligate air-breathing lungfishes *Protopterus* and *Lepidosiren* with the facultative *Neoceratodus* reveals that, while all have high arterial pO_2, and complete hemoglobin saturation at rest in water, the former have very high arterial pCO_2 whereas *Neoceratodus* has values only one tenth that of the obligate air breathers (Grigg, 1974). *Neoceratodus* ventilates its single lung when exposed to hypoxic water and to a lesser degree while active in normoxic water (Grigg, 1965; Fritsche et al., 1993). Using aerial respiration, *Neoceratodus* of the size used in this study can withstand aquatic hypoxia of pO_2=55 mmHg for at least one week at 20 °C, and they can tolerate extreme hypoxia of pO_2=8 mmHg for at least 5 h at 19–27 °C (T.D. Clark, pers. obs.). Aquatic hypercapnia significantly increased lung ventilation in *Lepidosiren* (Sanchez and Glass, 2001) and ventilation frequency increased in *Protopterus* as pulmonary CO_2 increased (Babiker, 1979). The cardio-respiratory responses to low oxygen in *Lepidosiren* are similar to tetrapods (Sanchez and Glass, 2001) and thus differ from those in *Neoceratodus* (Fritsche et al., 1993).

The large Bohr effect of *Neoceratodus* hemoglobins in the presence of saturating amounts of GTP (Ø=−0.77) matches the relatively large Bohr effect seen in whole blood (Ø=−0.62; Lenfant et al., 1966) and contrasts with smaller Bohr effects in the obligate air-breathing lungfishes (Ø=−0.25 to −0.47; Lenfant and Johansen, 1968; Johansen et al., 1976). The substantial Bohr effect in *Neoceratodus* is typical of that seen in more active, athletic fishes, rather than in sedentary species coping with hypoxia (Wells, in press). This observation fits well with the detailed field observations on *Neoceratodus* by Grigg (1965) in which the author observed that air-breathing in the species was associated with activity when swimming in reasonably well-aerated rivers, and thus it does not accord with the air-breathing behavior of the African and South American species under extreme hypoxia in stagnant and dehydrating conditions. The smaller Bohr effects of the African and South American lungfishes are consistent with the general problem air-breathers have in eliminating respiratory carbon dioxide (Graham 1997).

Unlike the other species of lungfish, *Neoceratodus* does not have the capacity to estivate. During estivation, *Protopterus* increased its hematocrit by 50% while lowering the red cell concentration of GTP from 1.2 to 0.2 mmol L^{-1} which consequently lowered the p_{50} of its hemoglobin from 33 to 9 mm Hg (Johansen et al., 1976). These concurrent changes in hematocrit and oxygen affinity do not appear to correspond with any alteration in the hemoglobin isoform composition. *Protopterus* red blood cells contain three hemoglobin isoforms, as do those *of Neoceratodus.*

Thus *Neoceratodus* possesses a multiple hemoglobin system with marked sensitivity to pH (large Bohr effect) and allosteric effectors, with oxygen binding characteristics suited to a periodically active life in the aquatic habitat. Unlike *Protopterus*, *Neoceratodus* is unable to estivate in a mud cocoon or cope with prolonged air exposure. This inability appears to result not only from a less developed pulmonary system that is less efficient in venting carbon dioxide compared to paired lung species, but also from differences in the expression (e.g. IP2) and modulation (e.g. decrease in GTP) of allosteric regulators of haemoglobin oxygen affinity during hypoxic exposure. It is unlikely that these adaptations were prerequisites leading to the establishment of tetrapods in the terrestrial environment.

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