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High blood oxygen affinity in the air-breathing swamp eel *Monopterus albus*

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ABSTRACT

The Asian swamp eel (*Monopterus albus*, Zuiew 1793) is a facultative air-breathing fish with reduced gills. Previous studies have shown that gas exchange seems to occur across the epithelium of the buccopharyngeal cavity, the esophagus and the integument, resulting in substantial diffusion limitations that must be compensated by adaptations in others steps of the O_2 transport system to secure adequate O_2 delivery to the respiring tissues. We therefore investigated O_2 binding properties of whole blood, stripped hemoglobin (Hb), two major isoHb components and the myoglobin (Mb) from *M. albus*. Whole blood was sampled using indwelling catheters for blood gas analysis and determination of O_2 equilibrium curves. Hb was purified to assess the effects of endogenous allosteric effectors, and Mb was isolated from heart and skeletal muscle to determine its O_2 binding properties. The blood of *M. albus* has a high O_2 carrying capacity [hematocrit (Hct) of 42.4 ± 4.5%] and binds O_2 with an unusually high affinity (P_{50} = 2.8 ± 0.4 mmHg at 27 °C and pH 7.7), correlating with insensitivity of the Hb to the anionic allosteric effectors that normally decrease Hb-O_2 affinity. In addition, Mb is present at high concentrations in both heart and muscle (5.16 ± 0.99 and 1.08 ± 0.19 mg · g wet tissue^{-1}, respectively). We suggest that the high Hct and high blood O_2 affinity serve to overcome the low diffusion capacity in the relatively inefficient respiratory surfaces, while high Hct and Mb concentration aid in increasing the O_2 flux from the blood to the muscles.

1. Introduction

The Asian swamp eel (*Monopterus albus*) is an air-breathing member of the *Synbranchidae* that is widely distributed across South-east Asia (Rosen and Greenwood, 1976), where it inhabits slow flowing and often hypoxic waters. Air-breathing is believed to have evolved as an adaptation to aquatic hypoxia and/or seasonal water level fluctuations, and the vast majority of extant air-breathing fish are found in tropical hypoxic waters (Graham, 1997). Unlike most air-breathing fish, *M. albus* lacks a distinct air-breathing organ (ABO) and relies on extra-branchial gas exchange using a highly vascularised epithelium in the buccopharyngeal cavity as well as a vascularized esophagus and integument (Taylor, 1831; Liem, 1967; Lefevre et al., 2014). The buccal cavity of *M. albus* expands during air-breathing remaining initially inflated during submergence and exhalation occurs both under water and at the surface prior to the next inhalation (Wu and Kung, 1940). Given the reduced gills, *M. albus* was originally classified as an obligate air-breather, like its close relative *M. cuchia* (Carter, 1931; Wu and Lui, 1943; Lomholt and Johansen, 1976), but because *M. albus* maintains blood O_2 concentrations during forced submersion in normoxic water, it was argued to be a facultative rather than obligate air-breather (Iversen et al., 2013). The pharyngeal air-breathing structures are characterised by respiratory islets divided by non-respiratory section (Iversen et al., 2013), where only the former is perfused by intra-epithelial capillaries (Liem, 1967). This, in combination with a smaller respiratory surface area and presumably con-current gas exchange represent a considerably lower diffusion capacity and lower gas exchange efficiency than normal piscine gills (Hughes, 1972). Thus, to sustain O_2 uptake with a low diffusion capacity it would seem beneficial to maintain a large PO_2 (partial pressure of oxygen) gradient across this epithelium, which can be achieved by high O_2 affinity and O_2 carrying capacity of the blood (Hlastala and Berger, 2001).

The correlation between blood O_2 affinity across fish species and their natural environmental O_2 availability was first noted by Krogh

Abbreviations: $[CO_2]_{pl}$, Total concentration of carbon dioxide in blood plasma; Hb, Hemoglobin; [Hb-O_2], Concentration of oxygen bound to hemoglobin; Hct, Hematocrit; Mb, Myoglobin; n_{50}, Hill's cooperativity coefficient at half saturation; $[O_2]_{total}$, Total concentration of oxygen in blood; P_{50}, Partial pressure of oxygen at half saturation; PCO_2, Partial pressure of carbon dioxide; PO_2, Partial pressure of oxygen; RBC, Red blood cell; *Y*, Fractional saturation; α_{CO2}, Solubility coefficient of carbon dioxide in plasma; $\alpha_{O2,blood}$, Solubility coefficient of oxygen in blood; φ, Bohr factor.

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and Leitch (1919). In addition, given the much greater O_2 availability in air than in water, it has been postulated that the evolutionary transition from water to air-breathing would be associated with a decrease in blood O_2 affinity (McCutcheon and Hall, 1937; Johansen et al., 1978b), as documented in at least two pairs of closely related species with different breathing mode (Powers et al., 1979). Although there are large variations in blood O_2 binding affinities amongst air-breathing fish (Lenfant and Johansen, 1968; Lomholt and Johansen, 1976; Heisler, 1982) an analysis of the O_2 affinity of whole blood from 40 genera of water and air-breathers from the Amazon basin (Powers et al., 1979) revealed no evidence of any systematic difference associated to breathing mode, while noting a trend that O_2 affinity in species inhabiting slow-flowing hypoxic water was higher compared to species living in fast flowing waters. While the evolution of blood with a high O_2 affinity would benefit branchial O_2 uptake, the unloading of O_2 in the tissues becomes more difficult (Brauner and Wang, 1997; Wang and Malte, 2011). This can be countered in part by the Bohr effect, where proton binding to Hb and stabilizes its low affinity T(ense)-state conformation, increases O_2 unloading and causes blood PO_2 to increase. Unloading of O_2 in the tissues can further be enhanced by increasing the O_2 flux from the blood to the tissues. Myoglobin (Mb) functions as an intracellular O_2 carrier in the skeletal and heart muscles of most vertebrates (Wittenberg and Wittenberg, 2003) and when expressed at high concentrations it would increase the flux of O_2 from blood to the mitochondria.

Teleost fishes display the most extensive heterogeneity in adult Hb structure and function amongst vertebrates and can accordingly be categorized (Weber, 2000) as class I species like plaice and carp (Weber and de Wilde, 1976) that have multiple (electrophoretically-) anodal Hbs with almost identical O_2 affinities and Bohr effects, and Class II species (anquillid eels, salmonids and catfish) (Weber et al., 1976; Weber and Lykkeboe, 1978) that additionally express electrophoretically-cathodal isoHbs that have high isoelectric points (pI > 8.2), commonly exhibit high O_2 affinities, and show reverse or no Bohr effects in the absence of anionic effectors. The latter isoHbs have variously been postulated to function as a blood O_2 reserve that can be drawn upon during hypoxia or when blood pH decreases (e.g. due to increased physical activity in fast-flowing water) (Powers, 1972; Weber, 1990). No information appears to be available on the functional consequences of Hb multiplicity in *Monopterus albus*.

We hypothesized that *M. albus* would exhibit high blood O_2 carrying capacity, a high blood O_2 affinity, and high Mb concentrations in the O_2 consuming muscles. To examine these hypotheses, we measured hematocrit (Hct) and whole blood O_2 equilibria at two CO_2 levels to determine blood O_2 affinity and Bohr effect. Because the O_2 affinity of the Hb in blood depends on its intrinsic O_2 affinity and its interaction with protons and red blood cell (RBC) anionic effectors (Weber and Fago, 2004), we also measured O_2 equilibrium curves of the stripped (cofactor-free) Hb and the major isoHb components, variously in the absence and presence of chloride, and of physiological levels of RBC co-factors (unstripped Hb solutions). Finally, we measured the concentration of Mb in the heart and skeletal muscle and determined O_2 equilibrium curves of purified Mb.

2. Materials and Methods

2.1. Fish

Specimens of *Monopterus albus* (Zuiew, 1793) were obtained from a local aquaculture facility in Can Tho City (Vietnam) and transported to Aarhus University (Denmark). They were kept in large aquaria at 27 ± 0.5 °C, fed to satiation with mussels every third day and were acclimated for three months prior to the experiments.

2.2. Surgical procedure and experimental protocol

Seven eels with a mean body mass of 145 ± 25 g (mean ± s.e.m) were anesthetized with benzocaine (0.3 g L^{-1}, dissolved in a small volume of acetone) and transferred to an operating table when reflexes to pinching subsided. The dorsal aorta was accessed ventrally through a 5 cm incision anterior to the anus and cannulated with a PE50 catheter containing heparinized saline (50 IU mL^{-1}). The fish were allowed to recover undisturbed for 24 h at 27 °C in normoxic water to allow blood gases to return to normal values before blood sampling. On the following day, the arterial catheter was extended and after having left the fish undisturbed for another 2 h, a 0.4 mL blood was drawn for *in vivo* measures of concentration of oxygen bound to Hb ($[Hb\text{-}O_2]$), Hct, pH and total concentration of carbon dioxide in plasma ($[CO_2]_{pl}$) as described below (Sections 2.5 and 2.6). Thereafter, an additional 1.5 mL blood was sampled for measurement of *in vitro* blood O_2 equilibrium curves.

2.3. Hemoglobin purification

RBC were separated from plasma by centrifugation, washed 5 times in 0.9% NaCl and lysed in 4-fold volume of ice-cold distilled water. 1 mol L^{-1} Hepes (pH 7.4) was added to reach a final buffer concentration of 10 mM and the mixture was left on ice for 15 min. The hemolysate was centrifuged at 8,100 *g* for 10 min. The supernatant containing the Hb was collected and Hb heterogeneity was evaluated on isoelectrofocusing (IEF) polyacrylamide gels (GE-Healthcare, pH gradient 3-10). To strip Hb from organic allosteric effectors, the hemolysate was mixed with AG 501-X8 mixed bed resin, centrifuged for 10 min at 10,000 rpm and the supernatant dialyzed against 10 mmol L^{-1} Hepes pH 7.4 for 24 h. IsoHb composition was moreover investigated by preparative IEF on a 110 mL (Amersham Biosciences, type 8102) column as previously described (Larsen et al., 2003), using Amersham ampholytes in pH ranges of 3-10.5, 5-8 and 6.7 – 7.7 (10, 30 and 60%, respectively).

2.4. Myoglobin purification

Heart and muscle tissue were dissected out of euthanized fish, washed in ice-cold saline, snap-frozen in liquid nitrogen and stored at -80 °C until further use.

Mb concentrations were measured using a modified version of the method developed by Reynafarje (1963). Heart ventricle and muscle tissues were homogenized for 1 min in 40 mmol L^{-1} phosphate, pH 6.6 buffer (19.25 mL buffer/g wet tissue) on ice using an Ultra-Turrax T25 homogenizer (IKA, Staufen, Germany). Samples were centrifuged (50 min, 15,000 g) and the supernatant collected and equilibrated with CO gas for 2 min. A pinch of sodium dithionite was added and the sample was equilibrated with CO for 2 min. Finally, absorbance at 538 and 568 nm was measured in quartz cuvettes using a HP 8453 UV-visible spectrophotometer and the Mb concentrations (mg protein/g wet tissue) were calculated as:

$$C(\mathrm{Mb}) = (A_{538\mathrm{nm}} - A_{568\mathrm{nm}}) \cdot 117.3 \mathrm{mg\ g\ wet\ tissue}^{-1} \quad (1)$$

To purify Mb heart ventricles and muscle tissue were homogenized on ice in buffer (~5 mL/g tissue, 50 mmol L^{-1} Tris, 0.5 mmol L^{-1} EDTA, 0.5 mg mL^{-1} DTT, pH 8.3) and centrifuged (Helbo and Fago, 2011). The supernatant was collected and submitted to two rounds of ammonium sulphate precipitation (40 and 80%) followed by desalting through a PD-10 column equilibrated with 50 mmol L^{-1} Tris, 0.5 mmol L^{-1} EDTA, 0.5 mg mL^{-1} DTT, pH 8.3. Finally the sample was passed through a Tricorn Superdex 75 10/300 GL fast protein liquid chromatography (FPLC) gel filtration column (Amersham Biosciences) equilibrated with 50 mmol L^{-1} Tris, 0.5 mmol L^{-1} EDTA, 0.5 mg mL^{-1} DTT, 150 mmol L^{-1} NaCl, pH 8.3 at a flow rate of 0.7 mL min^{-1}, to separate