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Myoglobin oxygenation and autoxidation in three reptilian species

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ABSTRACT

Differences between species in the oxygen (O_2) affinity (P_{50}) of myoglobin (Mb) may serve to fine tune O_2 supply to cardiac and skeletal muscle in ectotherms. In support of this view, it has been shown that fish Mb O_2 affinities differ between species when measured at the same temperature, but are in fact similar when adjusted for in vivo muscle temperatures, most likely to maintain intracellular O_2 delivery in species adapted to different environments. It is unknown whether similar adaptations exist in the O_2 affinity of Mb from reptiles, despite this group of ectothermic vertebrates displaying great variation in the tolerance to both temperature and hypoxia. In this study, we have purified Mb from muscle tissues of three reptilian species (turtle, tortoise and alligator) with different lifestyles. We have measured O_2 binding characteristics and autoxidation rates of the three Mbs and measured the effects of temperature, lactate and blocking of reactive thiols on the O_2 affinity of turtle Mb. Our data show that, at a constant temperature, reptilian Mbs have similar O_2 affinities that are lower than those of mammalian Mbs, which may optimize intracellular O_2 transport at lower body temperatures. Reptilian Mbs have lower autoxidation rates than both mammalian and fish Mbs, which may be beneficial during oxidative stress. Furthermore, the O_2 affinity of turtle Mb is without allosteric control and independent of either lactate or thiol covalent modification. This study reveals some common adaptive patterns in the temperature-dependent regulation of Mb oxygenation in vertebrates.

1. Introduction

The main physiological role of the oxygen (O_2) binding heme-protein myoglobin (Mb) is to facilitate O_2 diffusion in heart and skeletal muscle during hypoxia (Wittenberg and Wittenberg, 2003; Gros et al., 2010) and to function as an O_2 store, a property which contributes to prolong aerobic diving in marine mammals and birds (Kooyman and Ponganis, 1998). Consistent with its dual role in intracellular O_2 delivery, Mb has an O_2 affinity (P_{50}, the oxygen tension (PO_2) at 50% saturation) that lies between that of hemoglobin (Hb) in the blood and cytochrome c oxidase in the mitochondria and that is close to the PO_2 within the muscle cell so that Mb exists in a partially deoxygenated state in vivo (Wittenberg and Wittenberg, 1989). Differences between species in the Mb P_{50} may thus serve to fine tune O_2 delivery according to tissue PO_2 and O_2 demand.

P_{50} values of vertebrate Mbs decrease at lower temperatures and the dependency on temperature (reflecting the heat of oxygenation, ΔH) is very similar in Mbs from mammals and fish. Mammalian Mb P_{50} values lie invariably close to ~1 Torr at 25 °C and ~2.3 Torr at 37 °C (Nichols and Weber, 1989; Helbo et al., 2013), most likely reflecting that mammals maintain a constant, high body temperature in order to sustain an active lifestyle. In contrast, greater diversity is found in fish Mbs with P_{50} values ranging between ~1 and ~5 Torr at 25 °C (Helbo et al., 2013), suggesting that Mb must function efficiently over the wider range of in vivo O_2 tensions and body temperatures of fish compared to mammals. In support of this hypothesis, Marcinek et al. (2001) showed that fish Mb O_2 affinities differ between species when measured at the same temperature, but that they are indeed similar when adjusted for in vivo muscle temperatures, most likely to maintain a constant intracellular O_2 delivery in species adapted to different environments.

There is general agreement that both fish and mammalian Mbs are insensitive to allosteric cofactors such as H^+ and lactate (see Helbo et al., 2013 for references) that bind non-covalently to the protein. However, we have recently shown that covalent S-nitrosation by the signaling molecule nitric oxide (NO) of a specific cysteine (Cys) residue (most likely in position 107) of salmonid Mbs (generating S-nitrosated Mb, Mb-SNO) allosterically increases the O_2 affinity, a mechanism that may aid in the release of protective NO from Mb to the hypoxic heart (Helbo and Fago, 2011; Howes et al., 2012; Helbo et al., 2014). Reptilian Mbs contain a conserved Cys in position 109 (Helbo et al., 2013 and references herein), but it is unknown whether allosteric regulation by S-nitrosation and in addition whether allosteric non-covalent regulation by lactate and H^+ is present in this group.

In general, knowledge about the functional properties, stability towards oxidation, temperature sensitivity and allosteric regulation of

Abbreviations: O_2, oxygen; Mb, myoglobin; P_{50}, O_2 tension at 50% saturation; PO_2, oxygen tension; Hb, hemoglobin; ΔH, heat of oxygenation; NO, nitric oxide; Cys, cysteine; Mb-SNO, S-nitrosated Mb; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; IEF, isoelectric focusing; n_{50}, cooperativity value; NEM, N-ethylmaleimide.

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reptilian Mbs is to our knowledge restricted to very few studies (Okotore and Aboderin, 1979; Weber et al., 1981; Okotore and Brown, 1983; Livingston et al., 1986) despite the fact that this large group of vertebrates shows great variation in lifestyle and adaptations to surviving in extreme environments. In fact, the most hypoxia-tolerant species known are found among the reptiles and within this group, the semi-aquatic turtles of the subfamily *Deirochelyinae*, including the painted turtle (*Chrysemys picta*) and the closely related red-eared slider (*Trachemys scripta*), are by far the most tolerant of severe and prolonged hypoxia or even anoxia among all vertebrates (Storey, 1996; Hermes-Lima and Zenteno-Savin, 2002; Bickler and Buck, 2007).

To expand our knowledge on Mb functional characteristics in reptiles, we have selected three species with different lifestyles and tolerances to hypoxia. The red-eared slider (*T. scripta elegans*) is a semi-aquatic turtle that is among the most hypoxia-tolerant vertebrates known as it can survive several months in total anoxia at freezing temperatures during winter hibernation (Milton and Prentice, 2007). In contrast, the steppe tortoise (*Agrionemys (Testudo) horsfieldii*) from Central Asia, is a land-living species that may rarely encounter hypoxia, but can withstand great temperature variations (Lagarde et al., 2002). The American alligator (*Alligator mississippiensis*) is a semi-aquatic species that inhabits more constantly warm climates and performs dives regularly (Andersen, 1961). In this study, we report O_2 binding characteristics and autoxidation rates of Mb purified from muscle tissues of the three reptilian species. Furthermore we show the effects of temperature, lactate and blocking of reactive thiols on the O_2 affinity of turtle Mb.

2. Materials and methods

7 hearts from juvenile American alligators (*A. mississippiensis*) (designated alligator onwards) kept at −80 °C were kindly donated by Tobias Wang, Aarhus University, in relation with another investigation (Skovgaard et al., 2008). 2 adult red-eared sliders (*T. scripta elegans*) (designated turtle onwards) were obtained from Lemberger (Oshkosh, WI, USA) and sent by air-freight to Aarhus University (Denmark). 6 steppe tortoises (*A. horsfieldii*) (designated tortoise onwards) were kindly donated by Copenhagen Zoo where they were predestined for euthanization due to chronic mycoplasmosis. Turtles and tortoises were kept at 21 °C in large aquaria with free access to dry platforms under infrared lamps and with access to food and water ad libitum at the department of Bioscience, Aarhus University. For functional comparisons, commercially purified horse heart Mb (Sigma-Aldrich) was used as representative for a mammalian Mb.

2.1. Purification of myoglobin

Turtles and tortoises were euthanized by injection of 200 mg/kg of sodium pentobarbital into the bloodstream where after hearts (tortoise) and skeletal muscle (turtle) were quickly dissected out, frozen in liquid nitrogen and stored at −80 °C. Animal care and sampling protocol were performed in compliance with the EU legislation directive for the treatment of laboratory animals (2010/63/EU).

Mb from each of the three reptilian species was purified as described in detail for trout Mb (Helbo and Fago, 2011). In brief, Mbs were purified from heart (alligator and tortoise) and skeletal muscle homogenates (turtle) by ammonium sulfate fractionation (40 and 80%), desalted on a PD10 column (GE-Healthcare) equilibrated with gel filtration buffer (50 mM Tris, 0.5 mM EDTA, 5 mg/mL dithiothreitol (DTT), 0.15 M NaCl, pH 8.2) and finally separated from contaminating Hb by fast protein liquid chromatography (FPLC) gel filtration using a Tricorn Superdex 75 10/300 GL column (GE-Healthcare) equilibrated with gel filtration buffer. All Mbs were then desalted on a PD10 column (GE-Healthcare) equilibrated with 50 mM Hepes, 0.5 mM EDTA pH 7.4 and stored at −80 °C for use in further experiments. Conversion of partly ferric (met) alligator Mb to the ferrous (oxy) form was obtained by standard procedures after adding solid dithionite and immediate desalting at 4 °C on a PD10 column (GE-Healthcare) equilibrated with 50 mM Hepes, 0.5 mM EDTA, pH 7.4. The same procedure was used to convert commercially available ferric horse Mb to the oxy form (oxyMb). Turtle and tortoise Mbs were already present as oxyMb after purification. Mb purity was assessed by SDS and isoelectric focusing (IEF) on polyacrylamide gels (Phast System, GE Healthcare) stained with Coomassie blue. Heme oxygenation/oxidation state was assessed by UV–vis absorption spectroscopy in the range of 400–700 nm by using known absorption peaks for mammalian Mbs (Antonini and Brunori, 1971).

2.2. Oxygen equilibria

O_2 binding curves were determined using a modified diffusion chamber technique previously described (Sick and Gersonde, 1969; Weber et al., 2000; Helbo and Fago, 2011). Briefly, water-saturated gas mixtures of O_2 or air and ultrapure (>99.998%) N_2 created by Wösthoff gas mixing pumps were used to equilibrate a thin smear (4 μL, ~100 μM heme, extinction coefficient $\varepsilon_{543} = 13.6\ mM^{-1}\ cm^{-1}$ (Antonini and Brunori, 1971)) of oxyMb solution in 50 mM Hepes, 0.5 mM EDTA pH 7.4 with stepwise (4–5 steps) increases in PO_2. Changes in absorbance upon oxygenation were recorded continuously at 436 nm by a photomultiplier (model RCA 931-A) and an Eppendorf model 1100 M photometer. The absorbance signal was measured using a laptop computer with the in-house made data acquisition software, Spectrosampler. P_{50} (PO_2 at half-saturation) and n_{50} (cooperativity) values were calculated from the zero intercept and slope, respectively, of Hill plots: log(Y) / (1 − Y) vs. logPO_2, where Y is the fractional saturation of Mb. Experiments were carried out in triplicate at 15, 20 and 25 °C. The effect of temperature on the P_{50} values was assessed by calculating the heats of oxygenation (ΔH) from the slope of the van't Hoff plots of logP_{50} as a function of 1/T as described previously (Helbo et al., 2012). Effects of sodium lactate (100 mM, 20 °C), low pH (6.5, 20 °C) and blocking of reactive thiols (see below) on the O_2 affinity of turtle Mb were also determined.

2.3. Blocking reactive thiols in tortoise Mb

To measure a possible effect of blocking reactive Cys on the O_2 equilibria and kinetics, turtle oxyMb was reacted with N-ethylmaleimide (NEM) at a 3:1 NEM/heme molar ratio for 1 h at room temperature (in 50 mM Hepes, 0.4 mM EDTA pH 7.4) to generate Mb-NEM, which is functionally equivalent to Mb-SNO but more stable and not photolabile (Helbo et al., 2014). Excess NEM was removed using a PD-10 desalting column (GE-Healthcare) equilibrated with 50 mM Hepes, 0.5 mM EDTA, pH 7.4 and O_2 binding curves were determined at 20 °C (n = 3).

2.4. Autoxidation rates

To determine oxyMb (Fe^{2+}) stability, the rate of spontaneous oxidation of MbO_2 to metMb (Fe^{3+}) was measured for all Mbs in air at 22 °C in 50 mM Hepes, 0.5 mM EDTA pH 7.4 (n = 2). The decrease in absorbance over time was monitored in quartz cuvettes at 543, 562 and 580 nm every hour for ~48 h using a HP 8543 UV–visible diode array spectrophotometer. Mb concentration in the cuvette was ~10 μM. Due to the long incubation time, to correct autoxidation rates for baseline changes, the autoxidation rate was calculated by plotting (A543 + A580) − 2 × A562, where A = absorbance at the given wavelength, as a function of time (t) and fitting according to a first-order exponential decay function.

3. Results

Tortoise and alligator Mbs were successfully purified from heart muscle as judged by IEF and SDS PAGE, with strong bands