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Interspecific variation and plasticity in hemoglobin nitrite reductase activity and its correlation with oxygen affinity in vertebrates

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ABSTRACT

Deoxygenated hemoglobin (Hb) is a nitrite reductase that reduces naturally occurring nitrite to nitric oxide (NO), supplying physiological relevant NO under hypoxic conditions. The nitrite reductase activity is modulated by the allosteric equilibrium between the R and T structures of Hb that also determines oxygen affinity. In the present study we investigated nitrite reductase activity and O_2 affinity in Hbs from ten different vertebrate species under identical conditions to disclose interspecific variations and allow an extended test for a correlation between the rate constant for nitrite reduction and O_2 affinity. We also tested plastic changes in Hb properties via addition of T-structure-stabilizing organic phosphates (ATP and GTP). The decay in deoxyHb during its reaction with nitrite was exponential-like in ectotherms (Atlantic hagfish, carp, crucian carp, brown trout, rainbow trout, cane toad, Indian python and red-eared slider turtle), while it was sigmoid in mammals (harbor porpoise and rabbit). Typically, hypoxia-tolerant species showed a faster reaction than intolerant species. Addition of ATP and GTP decreased O_2 affinity and slowed the rate of nitrite reduction in a concentration-dependent manner. The initial second order rate constant of the deoxyHb-mediated nitrite reduction showed a strong curvilinear correlation with oxygen affinity among all ectothermic vertebrates, and the relationship also applied to plastic variations of Hb properties via organic phosphates. The relationship predicts high nitrite reductase activity in hypoxic tolerant species with high Hb-O_2 affinity and reveals that the decrease in erythrocyte ATP and/or GTP during acclimation to hypoxia in ectotherms increases the erythrocyte NO generating capacity.

1. Introduction

Hemoglobin (Hb) is essential for blood O_2 and CO_2 transport in vertebrates via its reversible binding of O_2, CO_2 and H^+ and appropriate allosteric interactions between the different ligand binding sites. In addition to this fundamental function, Hb is now recognized as an important player in nitric oxide (NO) homeostasis. Hemoglobin can scavenge surplus NO entering the blood (e.g. from the endothelium) by converting NO to nitrate (reaction with oxygenated Hb) or by binding it tightly to deoxygenated heme (forming nitrosyl-Hb), but Hb also functions as a NO synthase. Thus, deoxygenated Hb (deoxyHb) is a nitrite reductase that reduces naturally occurring nitrite (produced by NO autoxidation) to NO (Doyle et al., 1981; Cosby et al., 2003; Nagababu et al., 2003; Gladwin and Kim-Shapiro, 2008). This reaction has attracted much attention, because it represents one of the pathways by which nitrite can be bioactivated and recycled into NO under hypoxic conditions, where the production of NO by nitric oxide synthases is compromised by lack of O_2 (Lundberg et al., 2008; Kim-Shapiro and Gladwin, 2014; Fago and Jensen, 2015). Indeed, the escape of some of the NO produced inside red blood cells (RBCs) contributes to hypoxic vasodilation and regulation of hemostasis (Cosby et al., 2003; Crawford et al., 2006; Srihirun et al., 2012).

The reduction of nitrite to NO by deoxyHb is influenced by the allosteric equilibrium between the T structure (with low O_2 affinity) and R structure (high affinity) of the Hb. Deoxygenated heme shows better nitrite reductase capability in the R structure than the T structure (Huang et al., 2005), which may relate to a lower heme redox potential and/or an improved nitrite accessibility to the heme cavity in the R structure. This means that Hb O_2 affinity should influence the nitrite reductase reaction, so that Hbs with high O_2 affinity (i.e. allosteric equilibrium shifted towards the R state) are better nitrite reductases than Hbs with low O_2 affinity (with high T state character), which subsequently suggests that Hbs of hypoxia-tolerant species that generally have high O_2 affinity (Jensen, 2004) should be better nitrite reductases than Hbs from hypoxia-intolerant species. Indeed, the high affinity Hb from hypoxia-tolerant carp is a faster nitrite reductase than the low affinity Hb from hypoxia-intolerant rainbow trout, and a similar trend may exist in other vertebrate groups (Jensen, 2009), but so far only limited comparative data are available. We have previously studied the Hb nitrite reductase activity in carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), red-eared slider turtle (*Trachemys scripta*), harbor

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porpoise (*Phocoena phocoena*) and rabbit (*Oryctolagus cuniculus*) under identical experimental conditions (Jensen, 2008; Jensen, 2009; Soegaard et al., 2012; Jacobsen et al., 2012). We now compare and integrate these findings with new results on Hbs from Atlantic hagfish (*Myxine glutinosa*), crucian carp (*Carassius carassius*), brown trout (*Salmo trutta*), cane toad (*Rhinella marina*) and Indian python (*Python molorus*). The species were carefully selected to cover a broad range of O_2 affinities and hypoxia tolerances with the aim to obtain detailed insight into variations in Hb nitrite reduction activity among vertebrates and to enable an extended test for an interspecific correlation between the rate constant of the reaction and Hb oxygen affinity. We hypothesized that there would be a strong interspecific correlation, and that this would apply also to plastic changes of Hb properties. Modulation of Hb O_2 affinity often involves changes in the concentration of erythrocyte organic phosphates (Weber and Jensen, 1988). We therefore tested changes in Hb-mediated nitrite reduction by adding T structure-stabilizing organic phosphates (ATP and GTP) at different concentrations to crucian carp Hb. Our results demonstrate a very strong correlation between the second order rate constant for nitrite reduction and O_2 affinity, which applies to both interspecific variation and plastic changes of Hb properties.

2. Materials and methods

Blood was drawn from anesthetized animals, using procedures in accordance with Danish laws of animal experimentation. Freshly drawn blood was centrifuged and both plasma and buffy coat were removed. The RBCs were washed twice in cold 0.9% NaCl and hemolyzed in cold distilled water. The hemolysates were purified by passage through a Sephadex G25 superfine (Amersham, Uppsala, Sweden) gel filtration column that was equilibrated and eluted with a stripping buffer (0.05 M Tris buffer, pH 7.3, 0.1 M KCl) that simulated natural RBC pH and ionic strength. For each species, the Hb solutions were divided among a series of Eppendorf tubes and stored at −80 °C.

Experiments were performed as previously described (Jensen, 2008). In brief, individual tubes were thawed and the Hb was diluted with stripping buffer to a heme concentration of 150–160 μM. Three milliliter of Hb solution was placed in a glass tonometer with a built-in 1-cm light path cuvette. In experiments testing the influence of ATP (adenosine 5′-triphosphate disodium salt hydrate, Sigma-Aldrich A2383) and GTP (guanosine 5′-triphosphate sodium salt hydrate, Sigma-Aldrich G8877), these nucleoside triphosphates (NTP) were added from freshly prepared stock solutions to obtain a [NTP]/[heme] ratio of either 0.5 or 5 (equivalent to [NTP]/[Hb tetramer] ratios of 2 and 20, respectively). The Hb was equilibrated for 1 h at 25 °C to humidified gas delivered from a Wösthoff (Bochum, Germany) Digamix gas mixing pump. The gas was either pure N_2 (to obtain fully deoxygenated Hb) or a mixture of air and N_2 with known oxygen tension, Po_2 (to obtain intermediate oxygen saturations). The tonometer cuvette was then placed in the thermostatted (25 °C) cuvette holder of a Cecil CE2041 (Cambridge, UK) spectrophotometer, and gas flow was maintained to ensure constant Po_2. A spectral scan from 480 to 700 nm was recorded. The reaction between nitrite and Hb was initiated by adding nitrite (from a 140 mM $NaNO_2$ stock solution) to a [nitrite]/[heme] ratio of 2.7 (time zero). Subsequent spectral scans were run at specified time points during the reaction. Reproducibility was assured by performing 2–3 experiments with the fully deoxygenated Hb for each species.

The concentrations of deoxygenated Hb (deoxyHb), oxygenated Hb (oxyHb), nitrosyl-hemoglobin (HbNO) and methemoglobin (metHb) during the reaction were assessed by spectral deconvolution, using a least-square curve fitting procedure and species-specific reference spectra of deoxyHb, oxyHb, metHb and HbNO (Jensen, 2007; Jensen, 2008). Reaction kinetics were evaluated from plots of [deoxyHb] versus time. Oxygen equilibria were assessed by equilibrating Hb solutions to different Po_2 values. Oxygen saturation (So_2, %) was calculated as 100[oxyHb]/([oxyHb] + [deoxyHb]). Connected values of So_2 and Po_2 were used to make Hill plots [$\log(So_2/(100\text{-}So_2))$ versus $\log Po_2$], from which P_{50} (O_2 tension at 50% So_2) and Hill's n (index of cooperativity) were calculated.

3. Results

We have studied the reaction of nitrite with deoxygenated Hb from 10 different vertebrate species under the same experimental conditions (i.e. [heme] = 150–160 μM, [nitrite]/[heme] = 2.7, 25 °C, pH = 7.3, 0.05 M Tris buffer, and [KCl] = 0.1 M). The velocity of the reaction was illustrated by plotting the decay in [deoxyHb] during the reaction as function of time (Fig. 1). It is evident that there were major species differences. The reaction proceeded much faster in the two hypoxia-tolerant teleost fish carp and crucian carp than in the more intolerant species rainbow trout and brown trout (Fig. 1B). The hypoxia-tolerant Atlantic hagfish also showed a fast reaction over the first half or the reaction, but then the decay in [deoxyHb] slowed somewhat (Fig. 1A). Both reptile species, Indian python and red-eared slider turtle, exhibited rapid reactions (Fig. 1C), with Indian python Hb coming out as the fastest nitrite reductase of all the examined species. The Hb of the

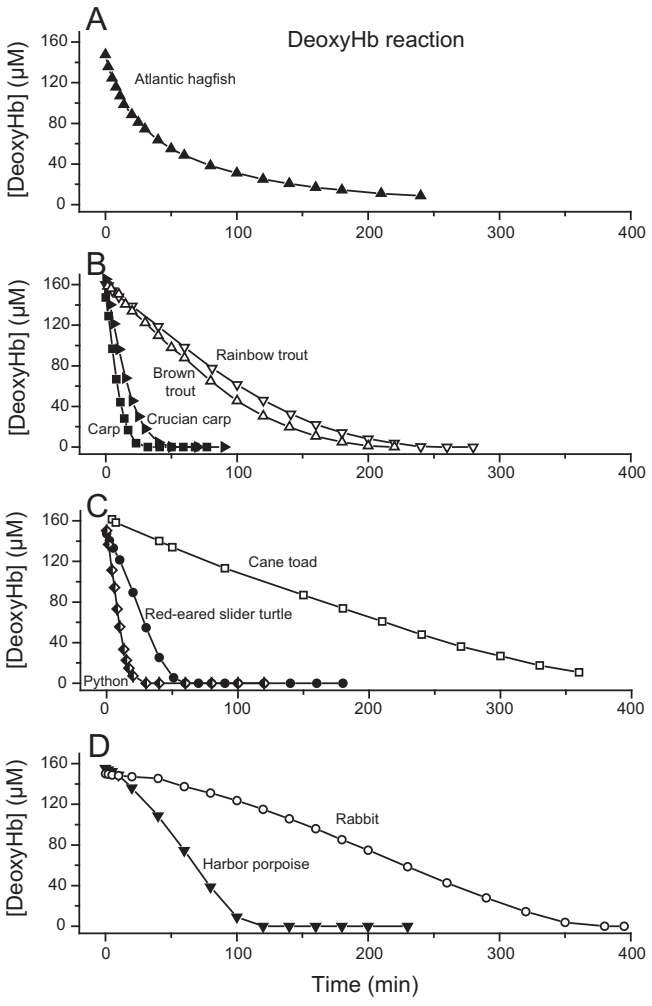

Fig. 1. Time-dependent decline in [deoxyHb] during the reaction of nitrite with deoxygenated Hb from 10 different chordate/vertebrate species: (A) Atlantic hagfish (*Myxine glutinosa*); (B) common carp (*Cyprinus carpio*), crucian carp (*Carassius carassius*), brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*); (C) Indian python (*Python molorus*), red-eared slider turtle (*Trachemys scripta*), cane toad (*Rhinella marina*); (D) harbor porpoise (*Phocoena phocoena*), rabbit (*Oryctolagus cuniculus*). All experiments were conducted under the same conditions: [heme] = 150–160 μM, [nitrite]/[heme] = 2.7, temperature = 25 °C, pH = 7.3 in 0.05 M Tris buffer and [KCl] = 0.1 M.