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Energetic costs of protein synthesis do not differ between red- and white-blooded Antarctic notothenioid fishes

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ABSTRACT

Antarctic icefishes (Family Channichthyidae) within the suborder Notothenioidei lack the oxygen-binding protein hemoglobin (Hb), and six of the 16 species of icefishes lack myoglobin (Mb) in heart ventricle. As iron-centered proteins, Hb and Mb can promote the formation of reactive oxygen species (ROS) that damage biological macromolecules. Consistent with this, our previous studies have shown that icefishes have lower levels of oxidized proteins and lipids in oxidative muscle compared to red-blooded notothenioids. Because oxidized proteins are usually degraded by the 20S proteasome and must be resynthesized, we hypothesized that rates of protein synthesis would be lower in icefishes compared to red-blooded notothenioids, thereby reducing the energetic costs of protein synthesis and conferring a benefit to the loss of Hb and Mb. Rates of protein synthesis were quantified in hearts, and the fraction of oxygen consumption devoted to protein synthesis was measured in isolated hepatocytes and cardiomyocytes of notothenioids differing in the expression of Hb and cardiac Mb. Neither rates of protein synthesis nor the energetic costs of protein synthesis differed among species, suggesting that red-blooded species do not degrade and replace oxidatively modified proteins at a higher rate compared to icefishes but rather, persist with higher levels of oxidized proteins.

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1. Introduction

Antarctic icefishes of the notothenioid family Channichthyidae are a remarkable example of evolutionary innovation at cold temperature and singular among vertebrates for their lack of the oxygen-binding protein, hemoglobin (Hb) (Ruud, 1954). The ancestral notothenioid incurring the mutation resulting in the loss of Hb approximately 8.5 MYA (Near, 2004) survived because of the prevailing conditions in the Southern Ocean at that time. Foremost, the Southern Ocean had cooled to less than 5 °C by 12 MYA, enhancing oxygen solubility in blood plasma, which is inversely proportional to temperature (Eastman, 1993). Today, water temperatures are near −1.9 °C in many regions of the Antarctic, although the Western Antarctic Peninsula region is rapidly warming (Littlepage, 1965; Meredith and King, 2005). Secondly, the diversity and biomass of the Antarctic fish fauna declined precipitously during the mid-tertiary period, likely due to a combination of cooling waters and loss of near-shore-habitats as ice sheets expanded and scoured the continental shelf (Eastman, 1993; Near et al., 2012). This reduced competition for the ancestral icefish that inhabited an expansive ocean, comprising 10% of the world's oceans. Thirdly, prior or subsequent to the loss of Hb, the cardiovascular system of icefishes was extensively remodeled to maximize oxygen delivery. Blood volumes of icefishes are 2–4-fold greater than red-blooded fishes, and vascular diameters are 2–3 times greater, minimizing the work of the heart (Fitch et al., 1984; Hemmingsen and Douglas, 1970). Cardiac output is high, and blood is circulated at a high velocity, maximizing the partial pressure gradient of oxygen between capillaries and tissues (Holeton, 1970). Additionally, vascular density is higher in some tissues, such as retina, compared to red-blooded species (Wujcik et al., 2007). These dramatic alterations in the icefish cardiovascular system suggest that the loss of Hb is a disaptation, with the ancestral Hb-expressing state superior to the hemoglobinless one, or, at best, a neutral mutation neither enhancing nor diminishing fitness (Montgomery and Clements, 2000).

More puzzling than the loss of Hb is the pattern of loss of expression of the intracellular oxygen-binding protein myoglobin (Mb) in hearts of icefishes. Six of the 16 species of icefishes lack Mb in their heart ventricle, and all notothenioids lack Mb in oxidative skeletal muscle (Grove et al., 2004; Moylan and Sidell, 2000; Sidell et al., 1997). Unlike the loss of Hb, which occurred only once prior to the radiation of the Channichthyidae family, the loss of Mb expression occurred four times during the radiation of this family, suggesting weak selective pressure maintaining the Mb gene (Grove et al., 2004; Moylan and Sidell, 2000). Yet, Mb is functional at the cold body temperature of notothenioids and enhances cardiac performance when present. Oxygen dissociation rates of notothenioid Mb are only 3–4-fold lower

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at 0 °C compared to mammalian Mb at 37 °C (Cashon et al., 1997). Additionally, studies with isolated perfused hearts show that the loss of Mb reduces cardiac performance at high afterload pressures compared to hearts that express the protein (Acierno et al., 1997). It is difficult to reconcile the multiple losses of Mb expression with functional data unless one considers a potential disadvantage to the presence of Mb, offsetting its benefits.

The majority of reactive oxygen species (ROS) are generated by mitochondria as a byproduct of oxidative metabolism, but as iron-centered proteins both Hb and Mb can also propagate the formation of ROS (Halliwell and Gutteridge, 1989). The ferrous form of iron (Fe^{2+}) within the heme prosthetic group of Mb and Hb reacts with oxygen, forming the superoxide radical. Heme in the ferric state (Fe^{3+}) reacts with peroxide, forming the potent oxidant ferryl iron (Reeder and Wilson, 2005). Additionally, both ferrous and ferric iron promote the formation of the highly toxic hydroxyl radical via the Fenton reaction (Welch et al., 2002). While low levels of ROS serve as signaling molecules, high levels damage proteins, DNA and lipids unless mitigated by antioxidants (Valentine et al., 1998).

Free radicals react with both the protein backbone and amino acid side chains, causing hydrogen abstraction, oxidation and reduction, fragmentation, dimerization, disproportionation and substitution (Hawkins and Davies, 2001). Amino acids differ in their sensitivity to oxidation; histidine, leucine, methionine, cysteine and aromatic amino acids (phenylalanine, tyrosine, and tryptophan) are most sensitive to oxidation (Hohn et al., 2013). Oxidation of lysine, arginine, proline and threonine forms carbonyl derivatives that are easily detectable and therefore frequently used as a marker for oxidative stress (Levine et al., 2000). Consistent with iron-catalyzed ROS formation, we find the highest levels of protein carbonyls in hearts of red-blooded notothenioids, intermediate levels in hearts of icefishes expressing Mb and the lowest levels of carbonylated proteins in hearts of icefishes lacking Mb. (Mueller et al., 2012).

The fate of oxidized proteins depends on the extent of oxidation. Oxidation of methionine and cysteine is reversible, whereas oxidation of other amino acids may lead to protein denaturation, aggregation and loss of function (Hohn et al., 2013). Denatured proteins may be rescued and refolded by heat shock proteins, but the majority of damaged proteins are degraded by the 20S proteasome system and must be resynthesized (Davies, 2001). Protein synthesis represents a significant proportion of an organism's energy budget. An estimated 42% of oxygen consumed is devoted to protein synthesis in the Atlantic cod, *Gadus morhua*, with rates of protein synthesis highest in liver and gill and lowest in white muscle (Houlihan et al., 1988a).

If high levels of oxidized proteins in hearts of red-blooded notothenioids warrant greater rates of protein degradation and synthesis, then we anticipated that the rates and costs of protein synthesis would be greater in red-blooded fishes, conferring an energetic benefit to icefishes lacking Hb and Mb. To test this hypothesis, we measured rates of protein synthesis in hearts of notothenioid fishes differing in the expression of Hb and Mb and determined the metabolic cost of protein synthesis by measuring rates of oxygen consumption in isolated cardiac myocytes and hepatocytes in the presence and absence of the protein synthesis inhibitor cycloheximide.

2. Methods

2.1. Fish collection

Chaenocephalus aceratus (−Hb/−Mb) (Lönnberg 1906), *Gobionotothen gibberifrons* (+Hb/+Mb) (Lönnberg 1906), *Pseudochaenichthys georgianus* (−Hb/+Mb) (Norman 1937) and *Notothenia coriiceps* (+Hb/+Mb) (Richardson 1844) were collected between April and July 2013 in Dallmann Bay (64°S, 62°W) and off the southwestern shore of Low Island (63°S, 62°W), Antarctica. Fish were collected from the *ARSV Laurence M. Gould* by otter trawl or baited fish traps and held in aerated, circulating seawater tanks aboard the vessel at 0 ± 0.5 °C until transferred to the US Antarctic research station, Palmer Station. At Palmer Station, fish were maintained in circulating seawater tanks at 0 ± 0.5 °C. All experiments were conducted in accordance with IACUC regulations (University of Alaska, Fairbanks 247598-12).

2.2. Determination of rates of in vivo protein synthesis

Protein synthesis rates were measured using a flooding dose of radiolabeled phenylalanine based on methods first described by Garlick et al. (1980) and further modified for use in cold-water teleosts to compensate for low metabolic rates and low rates of amino acid incorporation into protein (Lewis and Driedzic, 2007; Treberg et al., 2005). The flooding dose methodology for measuring rates of protein synthesis requires three criteria to be met (Garlick et al., 1980). First, intracellular free phenylalanine pools must be flooded with radiolabeled phenylalanine. Secondly, the specific activity of radiolabeled phenylalanine must increase rapidly and remain stable throughout the time course that protein synthesis is measured. Thirdly, the incorporation of radiolabeled phenylalanine into protein must be linear over the time course used for measuring rates of protein synthesis. *Chaenocephalus aceratus* (671 ± 57 g), *G. gibberifrons* (864 ± 57 g), *P. georgianus* (730 ± 69 g) and *N. coriiceps* (1103 ± 44 g) were randomly selected from the holding tank, tagged for individual recognition and placed in a separate experimental tank with circulating seawater 0 ± 0.5 °C at least 24 h before injecting with [2,3-^{3}H] phenylalanine. Food was withheld from *N. coriiceps* for 4 days prior to injections. All other species rejected food but were used within 1 week to 10 days of capture. *N. coriiceps* held longer than 1 week were fed chopped fish once every 2–3 days. Fish were injected intraperitoneally with 1.0 mL 100 g^{-1} of [2,3-^{3}H] phenylalanine solution containing 135 mM phenylalanine and 100 μCi mL^{-1} [2,3-^{3}H] phenylalanine in notothenioid Ringer's solution (260 mmol L^{-1} NaCl, 2.5 mmol L^{-1} $MgCl_2$, 5.0 mmol L^{-1} KCl, 2.5 mmol L^{-1} $NaHCO_3$, 5.0 mmol L^{-1} NaH_2PO_4, pH 8.0 at 4 °C). Following the incubation period (3–24 h), fish were euthanized with a lethal dose of MS-222 (1:7500). Heart ventricles were quickly excised, blotted dry, frozen in liquid nitrogen and stored at −80 °C.

Hearts were homogenized with a Tekmar Tissuemizer (Teledyne Tekmar, Cincinnati, OH, USA) in 9 vol. of 6% trichloroacetic acid (TCA). After incubating 10 min on ice, homogenates were vortexed, and a 1.0 mL aliquot was transferred to a 1.5 mL microcentrifuge tube and centrifuged for 5 min at 15,600 *g*. The supernatant was removed and frozen at −20 °C for measuring the free pool of phenylalanine and specific radioactivity. The protein pellet was washed three times by resuspending the pellet in 1.0 mL of 6% TCA, vortexing and then centrifuging as described above. After the third wash, radioactivity of the discarded supernatant was at or below background levels ensuring only protein-bound [^{3}H] phenylalanine was measured in the protein pellet (data not shown). After washing, the protein pellet was dissolved in 1.0 mL of 0.3 mol L^{-1} NaOH in a 37 °C water bath (1–2 h). The dissolved protein pellet was stored at −20 °C until analyzed for protein concentration and protein-bound radioactivity.

Aliquots of supernatants and dissolved protein fractions were mixed with 10 mL of Ultima Gold scintillation cocktail (PerkinElmer, Waltham, MA, USA) and counted on an A2900 liquid scintillation counter (PerkinElmer) to determine the [2,3-^{3}H] phenylalanine content of the free and protein-bound pools. Free pool phenylalanine content was measured in the supernatant along with phenylalanine standards in 6% TCA using a fluorometric assay following the protocol described in McCaman and Robins (1962). Protein content was determined using the Pierce bicinchoninic acid protein assay kit as per manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA) using bovine serum albumin (BSA) as a standard. Rates of protein synthesis were expressed as nmol phenylalanine mg^{-1} protein h^{-1}.

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