ELSEVIER

Journal of Experimental Marine Biology and Ecology 329 (2006) 135–143

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

www.elsevier.com/locate/jembe

Transition metal binding to cod otolith proteins

Matthew B. Miller, Alexis M. Clough, Jennifer N. Batson, Richard W. Vachet *

Department of Chemistry, University of Massachusetts-Amherst, 701 LGRT A, 710 N. Pleasant St., Amherst, MA 01003, United States

Received 12 March 2005; received in revised form 5 August 2005; accepted 25 August 2005

Abstract

Otolith microchemistry can be very useful in identifying fish populations and reconstructing fish movements. Recent attempts have been made to evaluate otoliths as proxies of ambient levels of transition metals, but findings have been inconsistent. Some of the difficulty with obtaining a definitive answer stems from an incomplete understanding of the biological control of transition metal speciation in otoliths. Metals may be incorporated as part of the calcium carbonate phase, trapped in interstitial spaces within the crystal, or associated with the protein matrix. Metal binding to the protein phase may be inferred from its structural and biochemical properties but has not been observed previously. Inherent difficulties with the extraction of metal-binding proteins in their native state from the calcium carbonate phase make them extraordinarily difficult to measure. We have developed a method that facilitates the extraction of otolith proteins without total disruption of transition metal binding. Chelating agents such as EDTA, used in the decalcification of otoliths, can demetallate the proteins if allowed to reach equilibrium; however, if the reaction is halted prior to equilibration, intact metal–protein complexes can be obtained. Using such an approach, we have confirmed the presence of copper and zinc in the soluble portion of the protein matrix of cod otoliths, and we have established that between 70% and 100% of copper and 40% to 60% of zinc found in whole otoliths are associated with the soluble part of the protein matrix. Manganese was not observed to be associated with the protein, indicating that it is either weakly bound or that no binding is present. Our results, combined with an understanding of the biological control of these metals, suggest that otoliths are not likely to be reliable indicators of copper and zinc exposure, but they may provide useful insight into fish growth and physiological development.

Keywords: Cod; Copper; Manganese; Otolith; Protein matrix; Zinc

1. Introduction

Otoliths are small, paired, calcified structures which form in the vestibular organs of teleost fish (Campana, 1999). They develop as calcium carbonate precipitates from the endolymphatic fluid onto a protein matrix, resulting in the formation of an aragonitic crystal. The protein scaffolding, which serves to regulate crystal growth, has an abundance of aspartic and glutamic acids in its sequence and is partly glycosylated (Degens et al., 1969; Dauphin and Dufour, 2003). It generally comprises 3–4% of the total mass of the otolith and is 40–50% water-soluble. Variations in growth rate affect the proportions in which aragonite and protein are added to the otolith, resulting in seasonal, and sometimes diurnal, growth rings. These annular rings are usually 20–50 μm in width and are useful in aging studies (Campana and Thorrold, 2001). Unlike other bone tissue, growth of the otolith continues throughout the life of the fish, even in advanced age or times of starvation. Furthermore, because the otolith is acellular in nature, it is not subject to the

* Corresponding author. Tel.: +1 413 545 2733; fax: +1 413 545 4490.

E-mail address: rwvachet@chem.umass.edu (R.W. Vachet).

0022-0981/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jembe.2005.08.016

same metabolic reworking or resorption as skeletal tissue.

The permanence of the otolith allows its microchemistry to function as a biological record of the chemical environment in the endolymph, which can serve as a useful tool for fisheries biologists. It can allow reconstruction of migration pathways based upon the ratio of strontium/barium substitutions into the aragonite (Campana, 1999). Additionally, the trace-element signature of the otolith can be used to pinpoint the river of origin of anadramous fish, as well as serve as a natural tag to identify stock mixing. Several studies have explored the use of otoliths as proxies of ambient concentrations of transition metals both as indicators of pollution and for additional dimensions in natural tagging, but results have been variable by metal and species (Swearer et al., 1999; Milton and Chenery, 2001; Saquet et al., 2002). Explanations for fluctuations in transition metal content have included differences in metabolic rates, diet, and ambient concentration (Halden et al., 2000). The latter two explanations favor environmental origin and passive inclusion of the metal, while the first implies a strong degree of biological control.

If otoliths are to serve as effective indicators of ambient transition metal levels, more must be known about the manner in which transition metals are incorporated into the otolith. The presence of transition metals in the otolith is partially dependent on the unique biological control of these metals, which allows discrimination either for or against the inclusion of a particular element at a given biological barrier. It has been suggested that transition metals incorporated into the otolith may be substituted for calcium in the aragonite crystal, co-precipitate as a metal carbonate, be occluded in the interstitial spaces within the crystal structure, and/or associated with the protein matrix, either specifically or non-specifically (Campana, 1999). Similarities in sequence (Murayama et al., 2000) and function (Mugiya et al., 1979; Morales-Nin, 2000; Borelli et al., 2003) between otolith proteins and known metal-binding proteins suggest metal–protein binding, but this association remains speculative, as no direct evidence is available. Confirming transition metal–protein interactions is complicated by difficulties in extracting otolith proteins in a way that avoids disruption of any metal–protein complexes. Calcium carbonate is most easily dissolved using acid solutions or chelating agents. The use of acids, however, can result in protein denaturation or hydrolysis, most likely resulting in metal loss. Even in relatively mild acid solutions (pH 6 or below), metals can be displaced through competitive binding of amino acid residues by more abundant protons. Chelators such as nitrilotriacetic acid (NTA) and ethylenediaminetetraacetic acid (EDTA) have higher binding constants for transition metals than for calcium (Martell and Smith, 1974), and these binding strengths likely exceed the strength of any metal–protein binding. Consequently, upon reaching equilibrium, solvent-accessible metals will be stripped from proteins, unless the proteins have higher metal affinity constants.

We have developed an extraction procedure that allows for the observation of metal–protein binding in otoliths that is both economical and relatively simple to perform. Using this procedure, we demonstrate that large portions of the Cu and Zn present in the otolith are associated with the protein phase, while Mn is either weakly bound or not associated with the protein matrix at all. This work provides the first direct observation of metalloproteins from an otolith and establishes a means by which the total quantity of protein-bound metal may be determined.

2. Experimental

2.1. Samples

Otoliths from Pacific-habitat Cod (*Gadus morhua*, Linne: 1758) weighing 200–500 mg were used for all studies. Following removal from the specimens, samples were cleaned with hydrogen peroxide and 1% HNO_3 and subsequently stored in an acid-cleaned HDPE jar until use.

Prior to analysis, samples for protein extraction were hand-ground for approximately 2 h with an agate mortar and pestle so that the majority of the mass was present in approximately 10 μm diameter particles, with no particles exceeding 20 μm. The size of the particles was verified by optical microscopy of a sample immersed in deionized water using an Olympus BH2 (Olympus America, Melville, NY) microscope with polarized transmitted light. Sample grinding was performed in a laminar flow hood, and all preparative steps were carried out in a clean room. All contact surfaces were cleaned with dilute HNO_3 (J.T. Baker, Phillipsburg, NJ).

2.2. Protein extraction

The protein extraction solution consisted of 0.125 M EDTA (J.T. Baker), adjusted to pH 6 with ammonium hydroxide (Fisher Scientific, Hampton, NH). Upon calcium carbonate dissolution, the pH of the sample solution rises to a pH slightly below 8, approximating that of the endolymph pH of 7.9 (Takagi, 2002), thus minimizing any pH-induced metal loss.