C. R. Biologies 336 (2013) 134–141

Contents lists available at SciVerse ScienceDirect

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Molecular biology and genetics/Biologie et génétique moléculaires

# Middle ferritin genes from the icefish *Chionodraco rastrospinosus*: Comparative analysis and evolution of fish ferritins

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ARTICLE INFO

*Article history:*
Received 8 February 2013
Accepted after revision 12 March 2013
Available online 13 April 2013

*Keywords:*
Antarctic fish
Ferritin
Gene expression
Phylogeny

ABSTRACT

Ferritin is a major intracellular iron storage protein in higher vertebrates and plays an important role in iron metabolism. This study reports the identification from the Antarctic icefish *Chionodraco rastrospinosus* of a complete mRNA sequence and four partial mRNA sequences, all encoding the ferritin M subunit and sharing a clear homology with the ferritin M-chain of other fish species. The open reading frame of the complete ferritin M transcript is of 528 base pairs and encodes a protein of 176 amino acids that retains the residues involved in the ferroxidase diiron center and in the ferrihydrite nucleation center. Despite the absence of hemoglobin and of any appreciable amount of iron in the icefish blood, RT-PCR analysis shows that H and M ferritin subunits are expressed both in blood and in other tissues, such as spleen, head kidney, liver and kidney. Phylogenetic analysis shows that the H and M subunits form two well separated clusters. Basal to these two clusters emerges a heterogeneous cluster, formed by two *Danio rerio* M, a *Salmo salar* M and an *Orechromis niloticus* H isoforms; these forms maybe represent the heritage of ancestral forms from which arose the two major H and M subunits of the fishes.



## 1. Introduction

Ferritin is the most common and ancient molecule of iron homeostasis, essentially ubiquitous and expressed in most eubacteria, archea, plants and animals. The ubiquitous ness and conservedness of ferritin are attributed to its essential role in the control of iron homeostasis and oxygen chemistry [1,2]. Iron (Fe) is an essential nutrient on account of its indispensable function in many fundamental cellular processes. However, in the presence of oxygen, iron can catalyze the production of reactive oxygen species (ROS), which is greatly dangerous for the cell [3].

The typical ferritin is a spherical protein composed of 24 subunits, which fold in a 4-helical bundle. The major property of the protein is the large cavity, designed to accommodate up to 4000 Fe atoms. This very high iron-binding capacity concentrates iron in a compact and safe form, which can be made readily available when needed; therefore, ferritin acts as an iron storage molecule to avoid loss of the precious metal [4].

Most eukaryotes have two major ferritin genes that encode subunits with different properties, generally named H (heavy) and L (light) that co-assemble to form heteropolymers. In mammals, the two subunit-types have little propensity to form homopolymers, and assemble in different proportions thus, originating a large number of isoferritins, with a tissue-specific distribution [1,5]. Thus, L-rich copolymers predominate in spleen and liver, whereas H-rich ferritins are found in other tissues, such as heart and kidney. In heteropolymeric ferritins, the H and L subunits have distinct and complementary functions. The H subunit contains the four-helix bundle a dinuclear ferroxidase center, which promotes the oxidation of Fe(II) in the presence of molecular oxygen [6]. The ferroxidase center sequesters Fe(II) from Fenton-like reactions in which the spontaneous oxidation to Fe(III) donates single electrons to transform innocuous reactive oxygen species,

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1631-0691/$ – see front matter 
http://dx.doi.org/10.1016/j.crvi.2013.03.003

like $H_2O_2$, into highly toxic radicals. The iron ligands are highly conserved and are provided by residues E27, E61, E62, H65, E107 and Q141 [7]. The L-chain lacks the catalytic center, but contains specific carboxylic groups on the cavity surface (E57, E60, and E64 using the H-chain numbering), that enable an efficient process of nucleation and mineralization of Fe(III), facilitating iron accumulation [8,9]. As a result, ferritin, through its ability to uptake, oxidize, and sequester iron, reduces free iron level and relieves oxidative stress [10]. The H-rich ferritins have a high ferroxidase activity, therefore, they oxidize and sequester actively the iron and have a more pronounced anti-oxidant activity, while the L-rich ferritins form molecules physically more stable which may contain a larger amount of iron in the cavity and have a more pronounced iron storage function [4,11].

Ferritins from lower vertebrates received relatively little attention. Three distinct ferritin cDNAs, named H (heavy), M (middle) and L (light) on the basis of their mobilities in denaturing gels, have been described in bullfrog tadpole erythrocytes [12]. With respect to the sequence elements of functional importance, the L-chain contains the negatively charged residues responsible for iron nucleation and mineralization in the mammalian protein, the H-chain contains the ligands of the ferroxidase center, and the M subunits possess both the ferroxidase center of H subunits and the iron nucleation site of L subunits [4].

In fish ferritins, evidence for two or three subunits was obtained. The H and M subunits contain both the ligands typical of the mammalian H-chain ferroxidase center and the L-chain carboxylate residues in positions 60 and 64. The canonical L-chain glutamate residue in position 57 is present only in the M-chains and is substituted by an asparagine in the H-chains [13–17]. Evidence for the existence of ferritin L-chains in fish is scanty, since the only data available refer to the amino acid composition of the protein isolated from rainbow trout [18].

Recently, many nucleotidic sequences of H-chain ferritins were characterized from Antarctic teleosts [19–21]; on the contrary, sequences of the M-chain ferritins from Antarctic teleosts were only from the direct amino acid sequencing of the native proteins [15,22]. No L subunits were found in Antarctic fish. The structural and functional properties of the protein extracted from Antarctic fish tissues demonstrated that the presence of a ferritin homopolymer is a typical feature of Antarctic ferritin molecules, since it has been observed that a ferritin homopolymer, made of only M-chains, is present in the spleen of the notothenioids *Trematomus newnesi* and *Trematomus bernacchii* [15,22] and a homopolymer made only of H-chains in the spleen of the bathydraconid *Gymonodraco acuticeps* [23]. However, in both notothenioids, liver ferritin is a heteropolymer composed of M and H subunits [15].

The detection by northern blot analysis of a small but appreciable amount of H-chain mRNA in *T. bernacchii* spleen suggests the existence in *Trematomus* spleen of a minor ferritin isoform containing also the H-chain subunit [19]. Indeed, it has been demonstrated that the two subunit-types together improve the flexibility and functionality of the molecule [1].

The main objectives of this work were to increase the scarce molecular data on M-chain ferritins in Antarctic fish, and to provide new insights about the evolution of ferritin subunits in fish. To this end, a molecular characterization study has been made of the ferritin M-chains in the Channychtydae species *Chionodraco rastrospinosus*. Results report the identification of five different ferritin M-chain cDNA sequences. Gene expression analysis demonstrates that transcripts encoding both H and M subunits are present in all the tissues examined, whereas no L-chains have been found. Finally, a phylogenetic analysis was inferred to explore the evolutionary mechanisms of ferritin in fish.

## 2. Materials and methods

### 2.1. Animals

Adult *Chionodraco rastrospinosus* specimens were collected in the proximity of the Antarctica Peninsula and kept in aquaria supplied with aerated seawater at approximately −1.5 °C. Animals were anaesthetized in 0.50 g/L MS-222 (tricaine methane sulphonate) and then killed by severing their spinal cord behind the head plates. Tissues were quickly removed, frozen in liquid nitrogen, and stored at −70 °C until use.

### 2.2. Total RNA extraction

Total RNA was isolated from tissue samples using the TRI Reagent RNA Isolation Reagent (Sigma–Aldrich) according to the manufacturer's instructions, and resuspended in diethyl-pyrocarbonate-treated water. After removal of contaminating DNA using the TURBO DNA-free kit (Ambion), the concentration and integrity of the RNA samples were determined by absorbance at 260 nm and by formaldehyde–agarose gel electrophoresis.

### 2.3. First-strand cDNA synthesis

Total RNA (5 μg) isolated from fish tissues was reverse-transcribed using an anchored oligo-dT primer (PK71) and the Superscript-II reverse transcriptase (Invitrogen), as previously described [24].

### 2.4. PCR and 5′-RACE amplification of cDNA

All the PCR and 5′-RACE (Rapid amplification of 5′ cDNA ends) experiments were performed using the Fast Start Taq DNA polymerase (Roche Diagnostics) and a peqSTAR thermal cycler (PEQLAB Biotechnologie). PCR reactions were carried out on 2 μl of first-strand cDNA, with an initial denaturation step at 94 °C for 4 min, 30 cycles made of a step at 94 °C for 30 s, a step at 50 °C for 30 s, and a step at 72 °C for 1 min, followed by a final elongation step at 72 °C for 10 min. The 5′-ends of the cDNAs were obtained by the RACE protocol of the Marathon cDNA Amplification kit (BD Biosciences) as previously described [25]. PCR conditions were set according to the Marathon kit protocol.

Specific primers used for the PCR and 5′-RACE are listed in Table 1. The primers were designed on the nucleotide sequences of piscine ferritin M subunit available at the