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Functional diversification of sea lamprey globins in evolution and development

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ABSTRACT

Agnathans have a globin repertoire that markedly differs from that of jawed (gnathostome) vertebrates. The sea lamprey (*Petromyzon marinus*) harbors at least 18 hemoglobin, two myoglobin, two globin X, and one cytoglobin genes. However, agnathan hemoglobins and myoglobins are not orthologous to their cognates in jawed vertebrates. Thus, blood-based O_2 transport and muscle-based O_2 storage proteins emerged twice in vertebrates from a tissue-globin ancestor. Notably, the sea lamprey displays three switches in hemoglobin expression in its life cycle, analogous to hemoglobin switching in vertebrates. To study the functional changes associated with the evolution and ontogenesis of distinct globin types, we determined O_2 binding equilibria, type of quaternary assembly, and nitrite reductase enzymatic activities of one adult (aHb5a) and one embryonic/larval hemoglobin (aHb6), myoglobin (aMb1) and cytoglobin (Cygb) of the sea lamprey. We found clear functional differentiation among globin types expressed at different developmental stages and in different tissues. Cygb and aMb1 have high O_2 affinity and nitrite reductase activity, while the two hemoglobins display low O_2 affinity and nitrite reductase activity. Cygb and aHb6 but not aHb5a show cooperative O_2 binding, correlating with increased stability of dimers, as shown by gel filtration and molecular modeling. The high O_2-affinity and the lack of cooperativity confirm the identity of the sea lamprey aMb1 as O_2 storage protein of the muscle. The dimeric structure and O_2-binding properties of sea lamprey and mammalian Cygb were very similar, suggesting a conservation of function since their divergence around 500 million years ago.

1. Introduction

Hemoglobin (Hb)[2] is the circulating O_2-carrier protein of most vertebrates and many invertebrates, and has been instrumental for the investigation of protein and gene evolution and function [1,2]. Because it is so different from that of jawed (gnathostome) vertebrates, the hemoglobin of jawless (agnathan) vertebrates (lampreys and hagfish) (referred to as aHb in the following) has been a source of interest for decades. Early studies discovered that aHbs of the sea lamprey *Petromyzon marinus* are monomeric when oxygenated and dimeric when deoxygenated [3]. This reversible, oxygen-linked oligomeric association gives rise to cooperativity and Bohr effect [4–6] and replaces the well-known allosteric T-R equilibrium typical of tetrameric (jawed) vertebrate Hbs [7–9]. Later crystallographic studies discovered an unprecedented intra-dimer interface of a major lamprey aHb isoform (aHb5a) in the deoxy state, formed by each monomer's AB corner and E helix, which carries amino acid residues distal to the heme binding site and is exposed to the solvent in tetrameric vertebrate Hbs [10,11]. The same interface may also form in the heme-ligated lamprey protein at high protein concentrations [12]. These studies revealed unique stereochemical mechanisms explaining how interactions at the dimer interface regulate lamprey Hb oxygenation.

An extensive survey of the sea lamprey genome found as many as 23 intact globin genes, including at least 18 Hb genes [13] (Fig. 1). The lamprey also harbors two globin X (*GbX*)[3] genes, which are members of an ancient, membrane-bound lineage of globins [14] with putatively antioxidant function [15], and a single cytoglobin (*Cygb*) gene [16]. The biological function of Cygb is still largely unknown [13]. Surprisingly, no neuroglobin (*Ngb*) gene was found in the sequence data of the sea lamprey or any other agnathan [13]. In addition, two putative myoglobin (*Mb*) genes were identified in the sea lamprey genome and found expressed in the heart [13]. However, the lamprey Mbs are not

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[1] These authors contributed equally to this work
[2] Abbreviations: aHb, agnathan hemoglobin; Cygb, cytoglobin; GbX, globin X; Hb, hemoglobin; Mb, myoglobin; Ngb, neuroglobin; NO, nitric oxide.
[3] The italic and regular font style refer to gene and protein, respectively.

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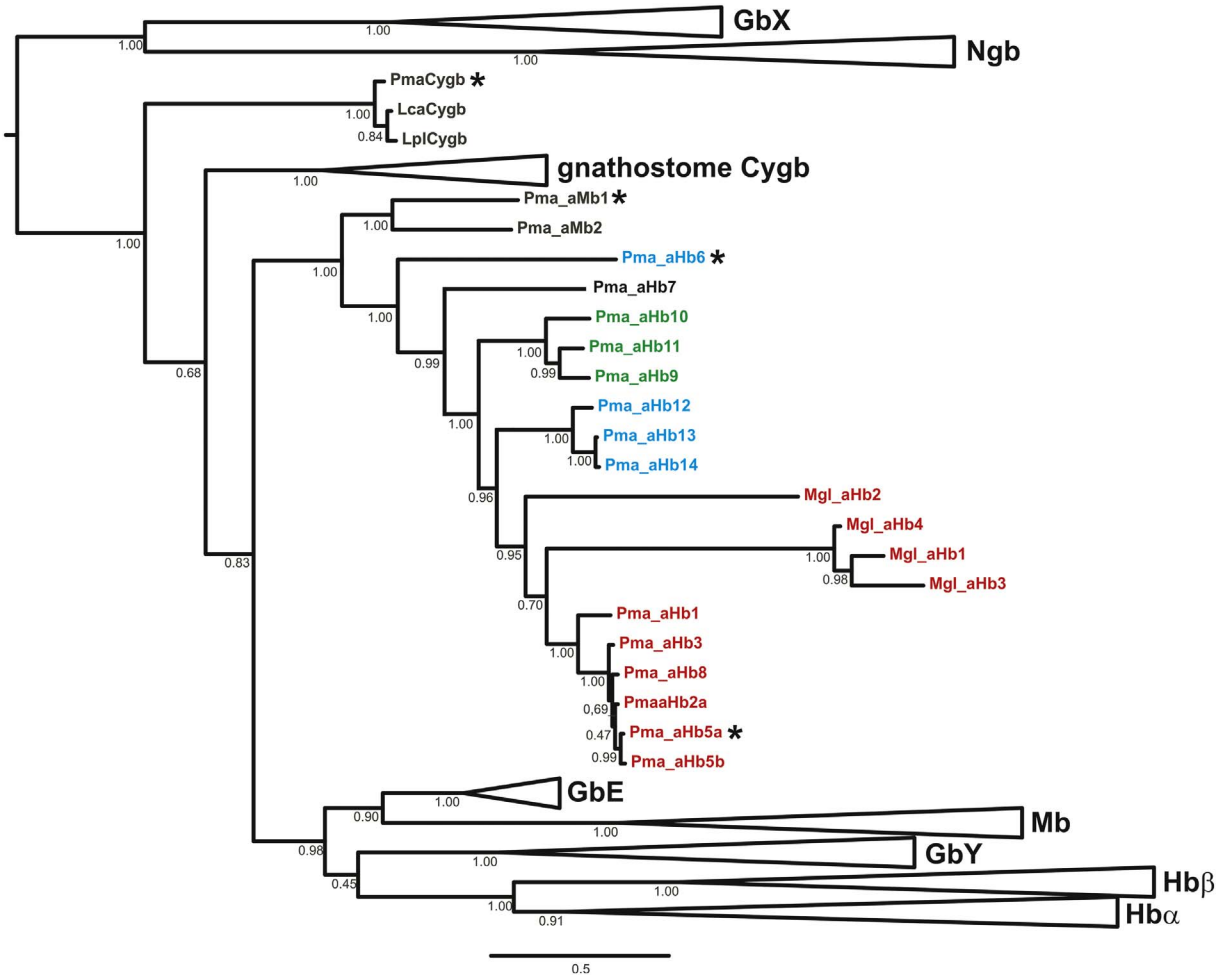

Fig. 1. Phylogenetic relationship of vertebrate globins. A Bayesian phylogenetic tree was constructed from 114 selected globins assuming the LG model of protein evolution [13]. The clades including gnathostome globins were collapsed and labeled. Sea lamprey globins with partial sequences were also excluded. The bar represents 0.5 PAM distance. The coloring of the *P. marinus* aHbs marks the developmental stages of main expression: green, embryo; blue larva; red, adult. The asterisks indicate the globins used in this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

closely related to the gnathostome Mbs. Rather, phylogenetic analysis showed that the Hbs and Mbs of agnathans form a monophyletic group that excludes the functionally analogous Hbs and Mbs of gnathostomes. This finding indicates that globin-based O_2 transport Hbs in the red blood cells, as well as O_2 storage Mbs in the heart and skeletal muscles, evolved convergently in these taxa. Thus, the paralogous globins of the agnathans were designated as aHb and aMb, respectively [13].

Initial biochemical studies identified six distinct aHb components in the adult sea lamprey [17] and the amino acid sequences of four aHb chains have been determined [5,10,18,19]. These four aHb protein chains could be matched to nine (Fig. 1) of the 18 *aHb* genes identified in the genome [13]. The other nine *aHb* genes code for aHb chains that are differentially expressed throughout development [20]. Investigations on tissue localization of these globins and gene switching during lamprey development from egg to embryo, larvae (ammocoetes) and adults (parasitic and reproductive states) have revealed a novel cluster arrangement of most globin genes that are switched on and off in consecutive blocks during development [13,20] (Fig. 1). Thus, in the sea lamprey, ontogeny of aHbs essentially recapitulates their phylogeny, as earlier evolved aHbs are also expressed first during development.

The unprecedented diversity and complexity of the sea lamprey globin system make this animal a unique model to trace the evolution of globins along with functional differentiation during development. Besides the well-known functions of Hb and Mb as reversible O_2 carriers in the blood and as O_2 storage protein in the heart and muscle cells, respectively, other functions of globins are known [8,13]. These include catalysis of redox reactions for detoxification against reactive oxygen and nitrogen species and signaling, including nitrite reduction to signaling molecule nitric oxide (NO). In this study, we have undertaken a comparative analysis of two key functional properties (O_2 binding and nitrite reduction) of selected lamprey globins, expressed as recombinant proteins, including aHbs from early developmental and adult stages, aMb, and Cygb. We find distinctive functional characteristics of tissue-specific globins expressed at different stages of lamprey development that are consistent with their putative *in vivo* biological roles and with variations in quaternary structural assembly.

2. Materials and methods

2.1. Recombinant expression and purification of sea lamprey globins

cDNA of sea lamprey larvae and muscle tissue were used from an earlier study [20]. The full-length coding sequences of the sea lamprey *aHb5a*, *aHb6*, *aMb1*, and *Cygb* were obtained by RT-PCR with the Pfu polymerase using the following gene-specific primers: aHb5a: 5′-GCG TACATATGCCTATCGTTGACACTGGAAGC-3′, 5′-GCGTAGGATCCTTAG TAGGCGGACCTGAGCAGG-3′; aHb6: 5′-GCGTACATATGGGTGCCCTGC AGGACTCGGGA-3′, 5′-GCGTAGGATCCCTAGTAGGCCGACTGCAGCTC GATG-3′; aMb1: 5′-GCGTACATATGAGCATTGCAGACAGC-3′, 5′-GCGT