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# Emergence of the acute-phase protein hemopexin in jawed vertebrates

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ABSTRACT

When released from damaged erythrocytes free heme not only provides a source of iron for invading bacteria but also highly toxic due to its ability to catalyze free radical formation. Hemopexin (Hx) binds free heme with very high-affinity and thus protects against heme toxicity, sequesters heme from pathogens, and helps conserve valuable iron. Hx is also an acute-phase serum protein (APP), whose expression is induced by inflammation. To date Hx has been identified as far back in phylogeny as bony fish where it is called warm-temperature acclimation-related 65 kDa protein (WAP65), as serum protein levels are increased at elevated environmental temperatures as well as by infection. During analysis of nurse shark (*Ginglymostoma cirratum*) plasma we isolated a $Ni^{2+}$-binding serum glycoprotein and characterized it as the APP Hx. We subsequently cloned Hx from nurse shark and another cartilaginous fish species, the little skate *Leucoraja erinacea*. Functional analysis showed shark Hx, like that of mammals, binds heme but is found at unusually high levels in normal shark serum. As an Hx orthologue could not be found in the genomes of jawless vertebrates or lower deuterostomes it appears to have arisen just prior to the emergence of jawed vertebrates, coincident with the second round of genome-wide duplication and the appearance of tetrameric hemoglobin (Hb).

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## 1. Introduction

Although hemoglobin (Hb) emerged over 1500 million years ago (MYA) (Hardison, 1996), the tetrameric ($\alpha_2$–$\beta_2$) form of Hb found in jawed vertebrates allows for a sophisticated regulation of oxygen binding. However this Hb form is much more harmful when released from damaged erythrocytes, having highly toxic oxidative and proinflammatory effects and providing a source of iron for invading bacteria (Lee, 1995). Therefore, several proteins have emerged in gnathostomes that bind and sequester free heme, thus neutralizing its detrimental features. Of these hemopexin (Hx), a ~60 kDa serum glycoprotein, binds heme with the highest affinity ($K_d < 10^{-12}$ M) (Hrkal et al., 1974). Hx is folded into 2 homologous domains of ~200 aa, joined by a highly flexible, ~20 aa linker. Each hx domain (N.B. herein hemopexin domains are denoted hx and the hemopexin protein Hx) folds into four β-sheets, which are arranged as the blades of a flat, four-bladed β-propeller, with the edge of the C-terminal domain locked at a 90° angle against the face of the N-terminal domain (Paoli et al., 1999). Hx binds a single heme molecule in a pocket formed between the two hx domains and bounded by the interdomain linker. The Fe(III) of the heme is coordinated by two histidine residues, $His^{213}$ from the linker and $His^{266}$ of the C-terminal hx domain and further stabilized by a host of non-covalent interactions provided by a large number of invariant aromatic and basic residues (Paoli et al., 1999). The Hx–heme complex in mammals is taken up by liver parenchymal cells through receptor-mediated endocytosis mediated by the scavenger receptor LRP1 (also known as CD91) (Hvidberg et al., 2005). The bound heme is released from Hx in the low pH environment of the endosome, likely by protonation of one or both of the coordinating histidine residues and movement of the hx domains and/or linker (Paoli et al., 1999). The heme is moved into the cell cytoplasm where it is used to build new hemoproteins or is safely catabolised (reviewed in Schaer and Alayash, 2010). Thus heme-binding and transport by Hx protects against oxidative damage, limits access by pathogens to heme, and recycles valuable iron.

In mice and humans Hx are produced by the liver and found in serum at ~0.5–1.25 mg/ml (Tolosano et al., 2010). However as Hx is an acute-phase protein the concentration of Hx greatly increases during inflammation (Wagener et al., 2001; Graca-Souza et al., 2002), its production being upregulated by proinflammatory cytokines including IL-6, IL-11, IL-1β and TNF-α (Immenschuh et al., 1995). Phylogentically, hemopexin has been identified in the mammals (placental, marsupial and monotreme), amphibians, and birds. Additionally an Hx homologue, the 65 kDa protein WAP65, has been found in teleost fish, although orthology has yet to be

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confirmed. Most teleost species possess two WAP65 genes which are differentially expressed; whilst WAP65-1 is widely and constitutively expressed, WAP65-2 expression is limited to the liver and is upregulated upon warm acclimation (e.g. an environmental temperature shift from 10 to 30 °C), following bacterial infection, or administration of LPS (Kikuchi et al., 1995; Peatman et al., 2007); however, it is not yet known whether the two forms have different roles in Hb physiology. Herein we describe the cloning and functional characterization of Hx from the nurse shark (*Ginglymostoma cirratum*) and little skate (*Leucoraja erinacea*), members of the most ancient gnathostome lineage. We will discuss the emergence of this APP and potential modifications of its function in different vertebrate taxa.

## 2. Materials and methods

### 2.1.1. Preparation and SDS-PAGE of shark serum proteins

Animals were maintained in artificial seawater at approximately 28 °C in large indoor tanks at the University of Maryland Biotechnology Institute, Center of Marine Biotechnology (COMB), Baltimore. Sharks were anaesthetized with MS222 prior to bleeding from the caudal sinus and the blood spun at 300 g for 10 min to isolate serum. From this 1 μl of serum was mixed with non-reducing Laemmli sample buffer containing 40 mg/ml SDS, loaded onto a 5% SDS-PAGE gel and electrophoresed in SGT buffer (1.5 g Tris, 7.2 g glycine, 0.5 g SDS per litre $dH_2O$). All gels were stained with Gelcode blue (Pierce) according to manufacturer's instructions.

### 2.2. IMAC-purification and N-terminal sequencing of shark serum proteins

$Ni^{2+}$-binding serum proteins were purified by immobilized metal-affinity chromatography (IMAC). Serum (1 ml) was diluted 1/10 in PBS and passed over a $Ni^{2+}$-sepharose column. The column was subsequently washed with 5 vol of PBS, 2× 10 ml 20 mM imidazole followed by a further 5 vol of PBS. Bound proteins were eluted with 5 ml of 200 mM imidazole. Eluted proteins were mixed with reducing Laemmli sample buffer containing 40 mg/ml SDS and characterized by SDS-PAGE on 12% gels, run as above. His-purified samples were sent to Emory University, Microchemical and Proteomics core facility for N-terminal sequencing by Edman degradation.

### 2.3. Cloning of Hx from shark and skate

The SMART RACE cDNA amplification kit (Clonetech) was used as per the manufacturer's instructions to generate 5′ and 3′ RACE-primed cDNA with 2.5 μg of liver RNA as the template per reaction. The degenerate primer GcP75-f1 was designed based upon a reverse translation of the nurse shark p75 N-terminal sequence and 5′ RACE yielded a single band of ~1200 bp. The sequence was completed using the primers GcP75-f2 and GcP75-r1 for nested 3′ and 5′ RACE (respectively). The full sequence was confirmed with the primers GcP75-f3 and GcP75-r3 (Genbank accession number HM347595).

The partial Hx sequence constructed from the little skate EST database was confirmed using the primers LeHx-f1 and LeHx-r1, then nested 3′ RACE performed with the primers LeHx-f1 for the first round and LeHx-f4 for the second round (Genbank accession number HM347594).

### 2.4. Northern blots

Total RNA was prepared for Northern blotting as detailed previously (Bartl et al., 1997) and 10 μg loaded per lane. The primers GcP75-f3 and GcP75-r3 were used to amplify the full-length coding sequence of Hx. The PCR product was radiolabelled with $^{32}P$ dCTP according to usual methods (Mertz and Rashtchian, 1994), cleaned of free nucleotides and used as the probe for Hx under high (final wash: 20 min agitation in 0.2× SSC + 0.1% SDS at 65 °C) stringency conditions. Expression of the housekeeping gene NDPK (nucleoside diphosphate kinase; Genbank accession number M63964.1) was also probed on the blot as a loading control (data not shown).

### 2.5. Hemoglobin- and IMAC-precipitation of plasma proteins

Shark Hb (sHb)-sepharose was prepared by lysing shark red blood cells in 10 vol of PBS at a 1/10 dilution then the liquid spun at 13 K rpm for 10 min to remove any debris or clots. The resultant supernatant was diluted 1/10 in binding buffer and conjugated to cyanogen bromide-sepharose (Sigma–Aldrich) according to manufacturer's instructions. Prepared Hb-sepharose was washed extensively with PBS to remove any loosely bound Hb.

Plasma from shark was diluted 1/10 in PBS and 1 ml added to 100 μl $Ni^{2+}$-sepharose (Qiagen) or 200 μl species matched Hb-sepharose and incubated for 1 h with rotation. $Ni^{2+}$-sepharose was washed 4× with 1 ml PBST (PBS + 0.05% Tween 20), 2 times with 1 ml 20 mM imidazole and 4× with PBST. Hb-sepharose was washed 10 times with PBST. Following the final wash all supernatant was carefully aspirated, 200 μl of 2× reducing Laemmli buffer containing 40 mg/ml SDS added to the sepharose, and the resultant slurry boiled for 5 min to remove bound proteins. The boiled slurry was zip-spun and the supernatant loaded onto 12% SDS-PAGE gels and run as above.

### 2.6. Phylogenetic tree generation

The evolutionary histories reported in Fig. 5 were inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the sequences analyzed. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Dayhoff matrix based method (Schwarz and Dayhoff, 1979) and are in the units of the number of amino acid substitutions per site. All columns containing alignment gaps and missing data were eliminated in all comparisons (complete deletion option). There were a total of 235 positions in the final dataset. Genbank accession numbers for sequences used are as follows: *Homo sapiens* Hx (NP_000604.1), *Mus musculus* Hx (AAH19901.1), *Monodelphis domestica* Hx (predicted; XP_001380277), *Bos taurus* Hx (NP_001029784.1), *Gallus gallus* Hx (partial; AAL29887.1), *Xenopus tropicalis* Hx (putative, partial; jgi|Xentr4|435537|e_gw1.3417.2.1), *Ictalurus punctatus* WAP65-1 (ABW07853.1) and WAP65-2 (ABW07852.1), *Takifugu rubripes* WAP65 (BAD18109.1) and WAP65-2 (BAD18110.1), *Dicentrarchus labrax* WAP65-1 (DAA12503.1) and WAP65-2 (DAA12504.1), *Oryzias latipes* WAP65 (BAB97303.1) and WAP65-like (BAB97304.1). The tree was rooted with *Homo sapiens* vitronectin (VTN; Genbank accession number NP_000629.3). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Evolutionary histories were also inferred using the Maximum Parsimony method in MEGA and the trees obtained with this method did not differ significantly, and supported the conclusions drawn from the Neighbour-Joining trees.