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Evolution of molluscan hemocyanin structures ☆

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ABSTRACT

Hemocyanin transports oxygen in the hemolymph of many molluscs and arthropods and is therefore a central physiological factor in these animals. Molluscan hemocyanin molecules are oligomers composed of many protein subunits that in turn encompass subsets of distinct functional units. The structure and evolution of molluscan hemocyanin have been studied for decades, but it required the recent progress in DNA sequencing, X-ray crystallography and 3D electron microscopy to produce a detailed view of their structure and evolution. The basic quaternary structure is a cylindrical decamer 35 nm in diameter, consisting of wall and collar (typically at one end of the cylinder). Depending on the animal species, decamers, didecamers and multidecamers occur in the hemolymph. Whereas the wall architecture of the decamer seems to be invariant, four different types of collar have been identified in different molluscan taxa. Correspondingly, there exist four subunit types that differ in their collar functional units and range from 350 to 550 kDa. Thus, molluscan hemocyanin subunits are among the largest polypeptides in nature. In this report, recent 3D reconstructions are used to explain and visualize the different functional units, subunits and quaternary structures of molluscan hemocyanins. Moreover, on the basis of DNA analyses and structural considerations, their possible evolution is traced. This article is part of a Special Issue entitled: Oxygen Binding and Sensing Proteins.

1. Introduction

Hemocyanins are the blue respiratory proteins in the hemolymph of many molluscs and arthropods. They have a binuclear copper active site, with two copper ions complexed by six histidine residues. Between the two coppers, a dioxygen molecule is reversibly bound (Fig. 1A). This copper type-3 center is also present in the tyrosinases, catecholoxidases and phenoloxidases, and it is assumed that the hemocyanins evolved from tyrosinase-like ancestral oxygen-binding proteins [1–6]. According to DNA sequencing, molecular phylogeny and molecular clock calculations this occurred *ca.* 740 million years ago in case of molluscan hemocyanin [7,8] and, independently, less than 600 million years ago in case of arthropod hemocyanin [9]. Apart from their similar active site, molluscan and arthropod hemocyanins have in common that they are very large, multimeric, extracellular proteins. Moreover, they readily dissociate at alkaline pH (*e.g.* pH 9.6) into functional subunits and reassemble at near-to-neutral pH (*e.g.* pH 7.5) into their original quaternary structure; in many cases, the latter additionally requires the presence of Ca^{2+} and Mg^{2+} ions (for review, see [1]). On the other hand, the primary, ternary and quaternary structure of arthropod and molluscan hemocyanin is so different that they are considered as two distinct protein superfamilies.

In this review, arthropod hemocyanins are not further discussed. For recent data on the structure and evolution of arthropod hemocyanins, see [4,10–18]. The present report will exclusively focus on molluscan hemocyanins, combine recent results of DNA sequencing and 3D electron microscopy and trace the evolution of the different molluscan hemocyanin structures that have been discovered to date. I will not review here the wealth of functional studies on molluscan hemocyanins. They reveal the specific oxygen-binding properties of many different hemocyanins, and attempt to connect these specificities to specific physiological and/or environmental constraints of the respective animal (for a review of such data, see [1]). Molluscan hemocyanin is synthesized in special cell types [19–21] and then released into the hemolymph.

Molluscan hemocyanins are based on a subset of paralogous functional units (FUs). They usually have a molecular mass of 45–50 kDa corresponding to *ca.* 420 amino acids (Fig. 1B). Crystal structures of several FU types are in the databases [22–25]. The usual number of FU types within the subunit is eight, termed FU-a to FU-h. They are sequentially arranged like a pearl chain along the polypeptide subunit, with connecting peptide linkers 10–20 amino acids in length (Fig. 1C). From a variety of molluscan hemocyanins, the complete subunit sequence is now available (*e.g.* [7,26–31]). The subunits form very large quaternary structures that can be readily seen in the

Abbreviations: 3D-EM, three-dimensional electron microscopy; FU, functional unit; PDB, Protein Data Bank; EMDB, Electron Microscopy Data Bank; KLH, keyhole limpet hemocyanin; HdH, *Haliotis diversicolor* hemocyanin; MtH, *Melanoides tuberculata* hemocyanin; NpH, *Nautilus pompilius* hemocyanin; OdH, *Octopus* (=*Enteroctopus*) *dofleini* hemocyanin; SoH, *Sepia officinalis* hemocyanin

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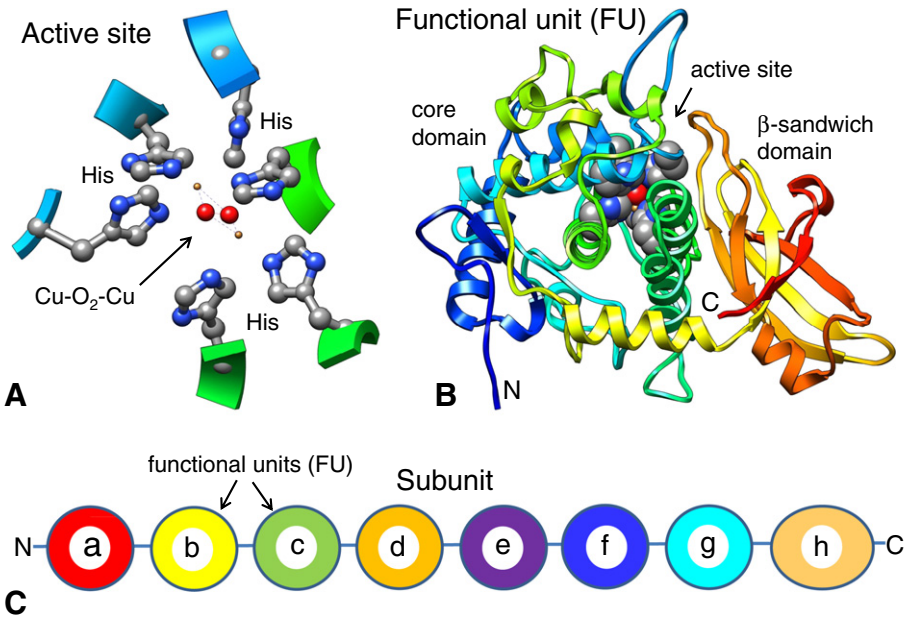

Fig. 1. Structure of the molluscan hemocyanin subunit. (A) Active site with two copper ions (orange), six histidine residues and a bound dioxygen molecule (red). (B) A functional unit, with the atoms of the single active site highlighted as spheres (PDB-ID 1JS8 [22]). The hinge connecting the α-helical core domain and the β-sandwich domain allows some movement which influences the oxygen binding [4]. (C) Scheme of a molluscan hemocyanin subunit with eight different functional units (as in many gastropods). N, N-terminus; C, C-terminus.

transmission electron microscope (Fig. 2). The basic molluscan hemocyanin quaternary structure is the decamer, a cylinder 35 nm in diameter and 18 nm in height, containing ten subunits with identical sequence. In its most simple but rarely seen form [32], the decamer is exclusively consisting of a wall (Fig. 2A). Usually, it is not hollow but partially filled by a structure designated as the collar. Moreover, in the majority of taxa two decamers are assembled into didecamers, and also tubular multidecamers occur (Fig. 2B). An exception from this common scheme is the recently detected mega-hemocyanin [33] that is completely filled with an internal structure (Fig. 2C).

For studying molluscan hemocyanin quaternary structures, transmission electron microscopy (EM) is the method of choice. The first molluscan hemocyanin model based on EM images was published in 1972 in the classical work of Mellema and Klug [34] and was refined two years later in a fundamental paper by Siezen and van Bruggen [35]. In the following decades the overall subunit and quaternary structure, and disassembly/reassembly properties, of many molluscan hemocyanins were studied, notably by the van Bruggen group in the Netherlands (*e.g.* [36–40]), the Lontie group in Belgium (*e.g.* [41–45]), the Herskovits group in the USA (*e.g.* [46–56]), the

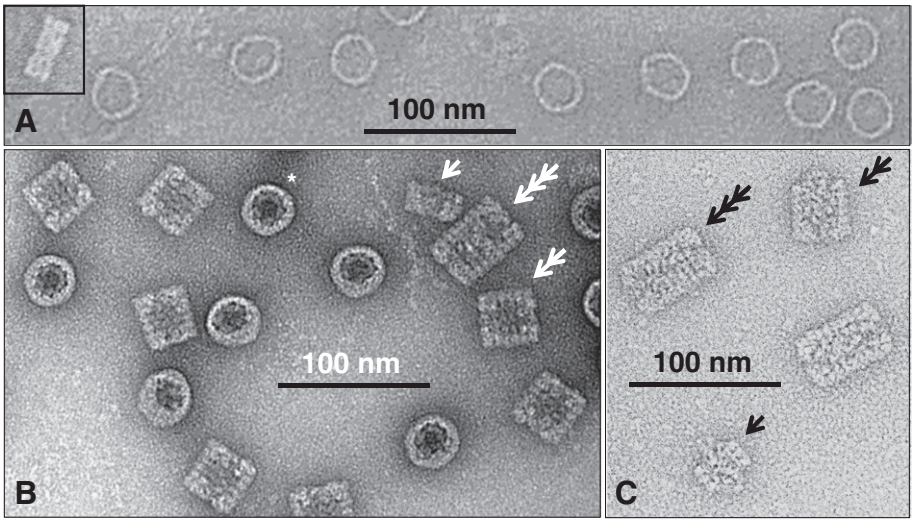

Fig. 2. Electron microscopy of three gastropod hemocyanins. (A) *Biomphalaria glabrata* hemocyanin. This hemocyanin is exclusively present as solitary decamers [32] and visible here as top views; note lack of a collar. Insert: A side view from another micrograph, exhibiting the three-tiered wall (larger diameter due to flattening on the EM grid). (B) *Lymnaea stagnalis* hemocyanin. In top view orientation (asterisk), the outer wall and the internal collar are directly visible. As in most gastropods, the major hemocyanin particle is the didecamer (double arrow), but solitary decamers (arrow) and tridecamers (triple arrow) are also present. Tridecamers consist of a didecamer (*i.e.* two decamers assembled at their open faces, with their closed faces pointing outwards) and a decamer (attached with its open face to a closed face of the didecamer). Attachment of additional decamers yields tubular multidecamers of varying length (not shown). (C) *Leptoxis carinata* mega-hemocyanin. Note the presence of tridecamers (triple arrow) that are completely filled with a mass [33]. See the semi-hollow tridecamer in (A) for comparison. Mega-didecamers (double arrow) and mega-decamers (arrow) are also present. (The samples were negatively stained with 2% uranyl acetate and images recorded in a Tecnai-12 electron microscope by Dr. Wolfgang Gebauer.).