Biochimica et Biophysica Acta 1834 (2013) 1840–1852

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Evolution of molluscan hemocyanin structures ☆

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ARTICLE INFO

Article history:
Received 24 December 2012
Received in revised form 12 February 2013
Accepted 13 February 2013
Available online 21 February 2013

Keywords:
Electron microscopy
Respiratory proteins
Hemocyanin
Keyhole limpet hemocyanin
KLH
Quaternary structure
Mollusca
Evolution

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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1570-9639
http://dx.doi.org/10.1016/j.bbapap.2013.02.020

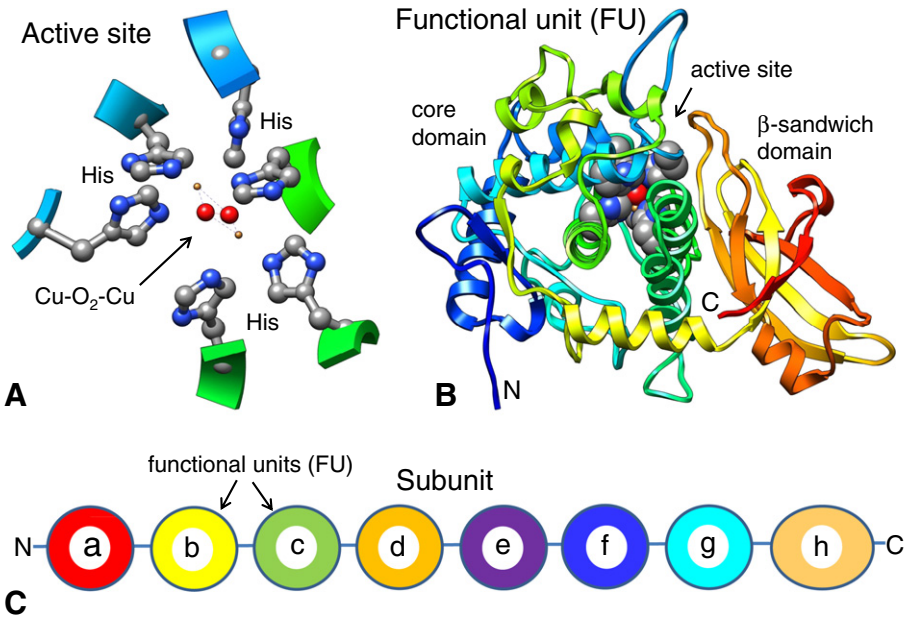

Fig. 1. Structure of the molluscan hemocyanin subunit. (A) Active site with two copper ions (orange), six histidine residues and a bound dioxygen molecule (red). (B) A functional unit, with the atoms of the single active site highlighted as spheres (PDB-ID 1JS8 [22]). The hinge connecting the α-helical 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

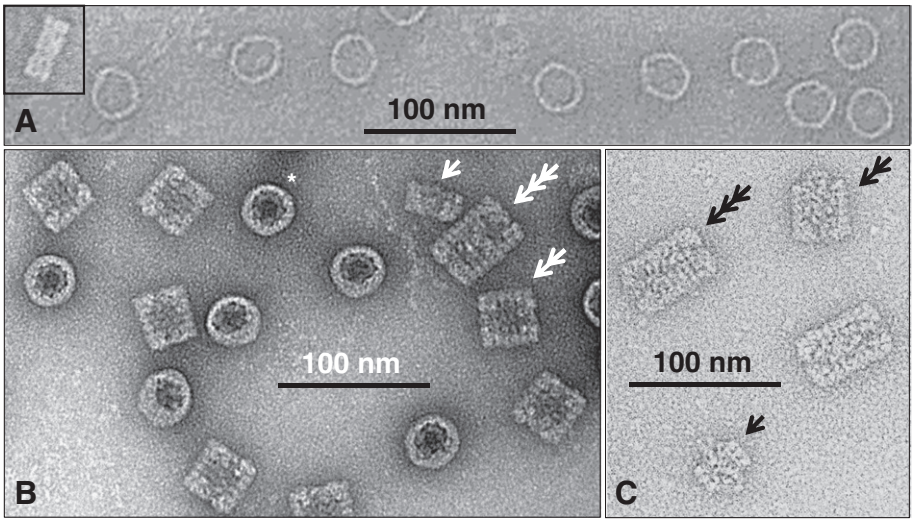

Fig. 2. Electron microscopy of three gastropod hemocyanins. (A) *Biomphalaria glabrata* hemocyanin. This hemocyanin is exclusively present as solitary decamers [32] and visible here as top views; note lack of a collar. Insert: A side view from another micrograph, exhibiting the three-tiered wall (larger 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.).

Lamy group in France (*e.g.* [57–63]), the van Holde group in the USA (*e.g.* [22,26,64–68]) and our group in Mainz, Germany (*e.g.* [7,8,27,28,30,68–86]), but also by many others (*e.g.* [31,87–99]). Over time models of the quaternary structure gradually improved with the raise of 3D electron microscopy (3D-EM), a method that was first applied to molluscan hemocyanins by the Lamy group [58–63]. Ultimately, by using shock-frozen specimens, advanced 3D-EM methods and the currently available digital imaging computer power, our group published 3D reconstructions of two molluscan hemocyanins at sub-nanometer resolution, and their atomistic models [100,101].

The different molluscan hemocyanin structures will be explained here, and the combined structural knowledge will be used to trace their evolution. Instead of original 3D reconstructions of the quaternary structure, in most cases simulated hybrid models are shown here for better clarity. They were simulated at 7 Å resolution by the software UCSF Chimera [102], using the PDB model of the respective whole molecule as template. These holistic PDB models have been obtained as published [100,101]: Homology models of the different functional units were calculated by using their amino acid sequence as target and published crystal structures as template. Then, the individual FU models were docked to the 3D density map of the quaternary structure that was obtained by 3D electron microscopy.

2. Hemocyanin sequences and gene structures are available from different molluscan taxa

The usual and most probably the ancestral number of different functional units in molluscan hemocyanins is eight, resulting in a 400 kDa polypeptide subunit (*ca.* 3400 amino acids) with the structure a–b–c–d–e–f–g–h (see Fig. 1C). The first complete sequence of a molluscan hemocyanin FU was unraveled in 1987 [103]. In 1998, the full-length sequence of a cephalopod hemocyanin subunit (from *Octopus* = *Enteroctopus dofleini*) was published [26]; this subunit has the seven-FU structure a–b–c–d–e–f–g (Table 1). The different FU types share sequence identities of ~45% and are similar in size (45–50 kDa; ~420 amino acids). The first complete sequence of a gastropod hemocyanin (from *Haliotis tuberculata*), published in 2000, allowed construction of a phylogenetic tree and calculation of a molecular clock [7]. The *Haliotis* hemocyanin sequence also included FU-h (see Table 1). This C-terminal FU type has a molecular mass of 60 kDa, due to an additional tail of *ca.* 100 amino acids. The sequence of this tail was found to be unrelated to hemocyanin and also to all other proteins in the databases. However, X-ray crystallography recently revealed a relationship to the cupredoxins [24].

Comparison of the gene structure of *Octopus* and *Haliotis* hemocyanin unraveled a concatenation of exons encoding the different FUs [68]. These exons are connected by strictly conserved phase 1 "linker introns" and may be interrupted by varying "internal introns" (Fig. 3). This suggests that the linker introns are very ancient; presumably, they are required for correct transcription of the hemocyanin gene. In contrast, the internal introns have been inserted significantly later in evolution and might lack a functional meaning [68].

Hemocyanin subunit sequences are available, in full length, also from other molluscs, *e.g.* the opisthobranch gastropod *Aplysia* [30] and the bivalve *Nucula* [29]. The present review will focus on six hemocyanins that have been completely sequenced and together allow discussion of the different molluscan hemocyanin quaternary structures and their possible evolution: *Nautilus pompilius* hemocyanin (NpH), *Sepia officinalis* hemocyanin (SoH1), two isoforms of keyhole limpet hemocyanin (KLH1 and KLH2), and two isoforms of *Melanoides tuberculata* hemocyanin (MtH1 and MtH2). A phylogenetic tree encompassing the functional units of these six hemocyanin species is shown in Fig. 4. The eight FU types constituting the subunit structure a–b–c–d–e–f–g–h evolved together in the late Precambrian, most probably by subsequent exon duplication and evolution events [7,68]. Later in evolution, several additional FU types branched off from FU-d and FU-f, respectively, and also losses of certain FU types occurred in specific taxa (see Table 1).

Table 1
Subunit structures of molluscan hemocyanins discovered up to now.

Major groups	Subtaxa	Subunit structure[1]	Ref.
Gastropoda	Patellogastropoda *Patella, Lottia*	Lack hemocyanin	DNA [2]
	Vetigastropoda *Megathura, Haliotis*	a–b–c–d–e–f–g–h	[7, 8, 27, 101]
	Caenogastropoda	a–b–c–d–e–f–g–h	[75, 99]
	Neogastropoda *Rapana*	a–b–c–d–e–f + g–h	
	Caenogastropoda	a–b–c–d–e–f–g–h	[33]; DNA [2]
	Cerithioidea *Melanoides*	a–b–c–d–e–f –f1–f2–f3–f4–f5–f6	
	Heterobranchia Opisthobranchia *Aplysia*	a–b–c–d–e–f–g–h	[30]
	Heterobranchia Pulmonata *Helix*	a–b–c–d–e–f–g–h	[31]
	Biomphalaria	a–b–c–d–e–f [3]	[32]
Bivalvia	Protobranchia	a–b–c–d–e–f–g–h	[29]
	All others	Lack hemocyanin	[1]
Cephalopoda	Nautiloidea *Nautilus*	a–b–c–d–e–f–g	[28,100]
	Octopodiformes *Octopus*	a–b–c–d–e–f–g	[26]
	Decapodiformes *Sepia*	a–b–c–d–d *–e–f–g	[59, 98]
Polyplacophora		a–b–c–d–e–f–g–h	[70,108]; DNA [2]
Monoplacophora		a–b–c–d–e–f–g–h	DNA [2]
Scaphopoda		Lack hemocyanin	DNA [2]
Caudofoveata		a–b–c–d–e–f–g–h	[85]
Solenogastres		Lack hemocyanin	[85]

[1] Note that in the different taxa only a small percentage of the species has been studied.
[2] Unpublished DNA analysis (Bernhard Lieb, Jürgen Markl and colleagues).
[3] Subunit structure as predicted from EM images of the decamer. In all other cases, the subunit structure was deduced from direct biochemical data and/or the amino acid sequence.

3. Most FU types have two domains, but FU-h has a third domain that is cupredoxin-like

For FU-g, FU-e and FU-h crystal structures are available [22–25]. They each show a single active site (see Fig. 1B) and the distribution of secondary structure elements within a core domain and a β-sandwich domain (Fig. 5A, B). This distribution is very similar in the three crystallized FU types. The other FU types share these structural features, as deduced from their sequences. This then allowed reliable homology modeling of those FU types for which no crystal structure is yet available [100,101].

FU-h is different in that its additional 100 amino acids constitute a third domain (Fig. 5C, D). Although the sequence identity is too low to detect a phylogenetic relationship by BLAST searches, comparative crystal structure analyses unraveled a cupredoxin-like fold of this tail [24,25]. Cupredoxins are usually mono-copper proteins but sometimes have lost their copper active site, which is also the case with the cupredoxin-like domain in FU-h. Originally, this domain in hemocyanin might have served to replenish lost copper to the active site as is the case with the "caddy protein" of some tyrosinases [6]. Today this domain is a structural element of the collar: It serves as a bridge within the antiparallel FU-h dimer that occurs not only in the protein crystal (see Fig. 5D), but also in the quaternary structure ([101]; see Fig. 8D). Correspondingly, the association mode of the antiparallel FU-g dimer found in the protein crystal (see Fig. 5B) exists also in the native oligomer ([100,101]; see Fig. 8B, C). In contrast, the trimeric repeating unit in the FU-e crystal [23] does not correspond to any FU arrangement within the quaternary structure [100,101].

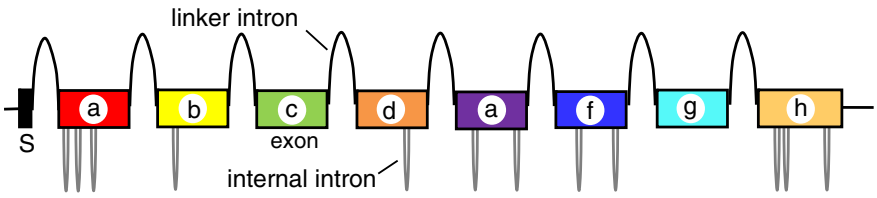

Fig. 3. Gene structure of the hemocyanin subunit from a mollusc (*Haliotis tuberculata*). The exons encoding the eight functional units are separated by phase 1 "linker" introns and interrupted by "internal" introns. The linker introns are strictly conserved in position and phase, the internal introns can vary from species to species in number, position and phase [68]. S, region encoding the signal peptide.

4. Ten copies of a–b–c–d–e–f constitute the conserved wall of the hemocyanin decamer

Biomphalaria glabrata is a planorbid snail using, as oxygen carrier, multimeric hemoglobin instead of hemocyanin [32]. In addition, traces of a "rudimentary" hemocyanin are expressed. It is a decamer exclusively consisting of only a wall (see Fig. 2A). This molecule demonstrates that the quaternary structure of molluscan hemocyanin remains stable even if the collar is lacking. *Biomphalaria* hemocyanin is based on a 300 kDa subunit with six FUs and the tentative FU structure a–b–c–d–e–f ([32]; see Table 1). As deduced from the combined biochemical and electron microscopical data collected until now, the cylinder wall of molluscan hemocyanin is generally formed by ten copies of the subunit segment a–b–c–d–e–f, whereas the remaining FU types constitute the collar [1,100,101].

Moreover, the structural repeating unit of the 35 nm molecule is not the subunit, but the subunit dimer (Fig. 6A); the decamer is constituted by five obliquely oriented subunit dimers [35,72]. The architecture of the cylinder wall was solved by 3D electron microscopy at subnanometer resolution of *N. pompilius* hemocyanin (NpH) and keyhole limpet hemocyanin (KLH1), yielding overall atomistic models. They were constructed by docking homology-modeled FUs to the respective cryo-EM structure, and revealed the following features [100,101]: (i) Within the subunit dimer of both hemocyanins, the two a–b–c–d–e–f segments are arranged in an antiparallel manner, exhibiting perfect two-fold (dyad) rotational symmetry (see Fig. 6A). (ii) The subunit pathway within the wall could be reliably traced (Fig. 6B) and was identical in the cephalopod and the gastropod hemocyanin. (iii) In the atomistic model of the wall (Fig. 6C, D), the molecular interfaces between FU types could be defined at the level of individual amino acid residues [100,101].

Docking of FUs to the cryo-EM structures was greatly facilitated by our discovery that the ovoid crystallographic dimer of OdH-g (see Fig. 5B) fitted exactly the "morphological units" that constitute the wall [100,101]. Morphological units are visible, in low resolution structures of molluscan hemocyanins, as single masses and represent FU dimers [58,80]. In other words, the specific dimeric association

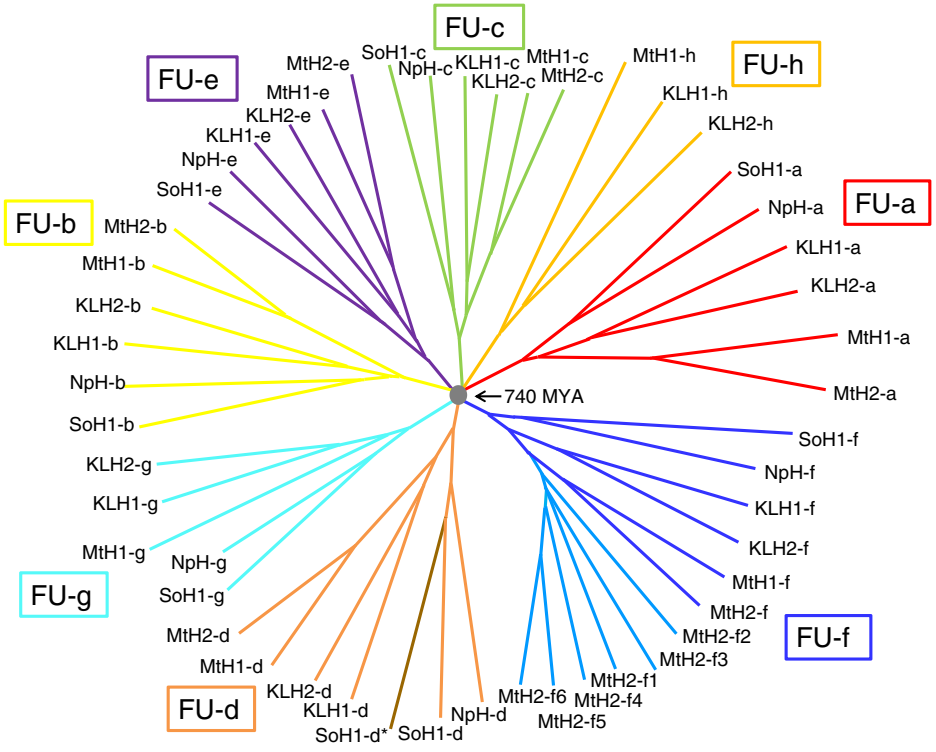

Fig. 4. Phylogeny of the functional units from different molluscan hemocyanins. Note that each FU type forms an individual branch in this tree. (The eight branches are highly bootstrap-supported; not shown.) According to molecular clock calculations, this tree originated *ca.* 740 million years ago (MYA) [7,8]. Neighbor joining tree calculated by Clustal X. KLH1/KLH2, keyhole limpet (*Megathura crenulata*) hemocyanin type 1 and type 2 (GenBank entries CAG28309 and CAG28310); MtH1/MtH2, cerithioid snail (*Melanoides tuberculata*) hemocyanin type 1 (also termed $MtH1_{400}$; entry KC405575) and type 2 (also termed $MtH2_{550}$; entry KC405576); NpH, chambered nautilus (*Nautilus pompilius*) hemocyanin (entry CAF03590); SoH1, cuttlefish (*Sepia officinalis*) hemocyanin (entry ABD47515; the second isoform SoH2 is omitted here). Note that FU-h is missing in the two cephalopod hemocyanins (NpH and SoH1). Also note that from SoH1-d and the MtH-f lineage, novel functional units evolved.

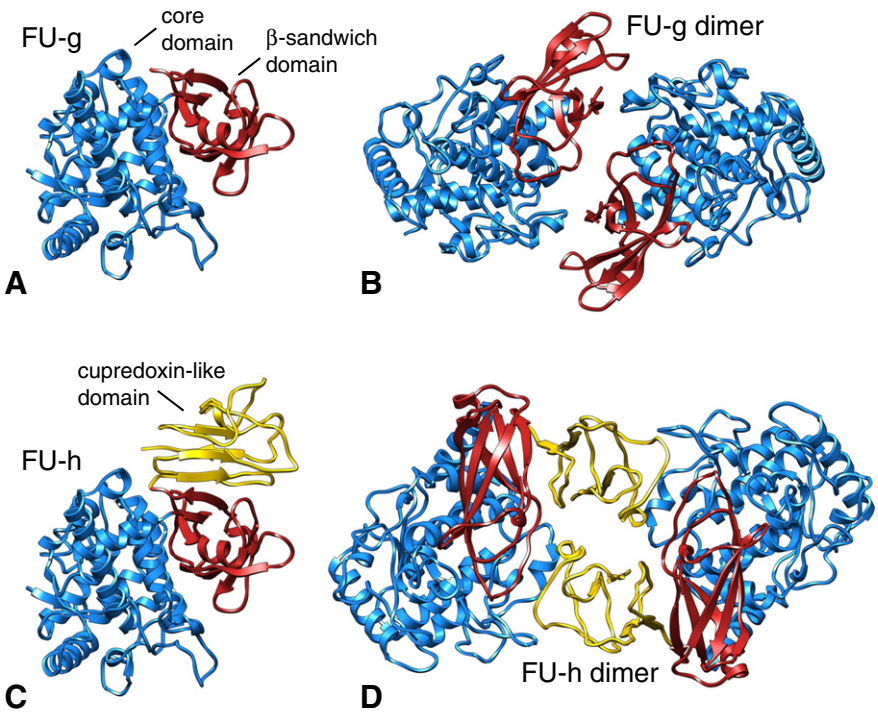

Fig. 5. Crystal structures of the collar functional units FU-g and FU-h. (A) FU-g (PDB-ID 1JS8 [22]); note the core domain (blue) and the β-sandwich domain (red). (B) Crystallographic dimer of FU-g (1JS8); within the quaternary structure, FU-g forms similar g + g dimers. Moreover, this is the association mode of the wall functional units FU-a to FU-f. (Specifically, in the wall the dimers a + b, d + e and c + f occur [100].) (C) FU-h (PDB-ID 3QJO [25]); note the third structural domain which is cupredoxin-like. (D) Crystallographic dimer of FU-h (3QJO); within the quaternary structure, FU-h dimerizes in the same way [101].

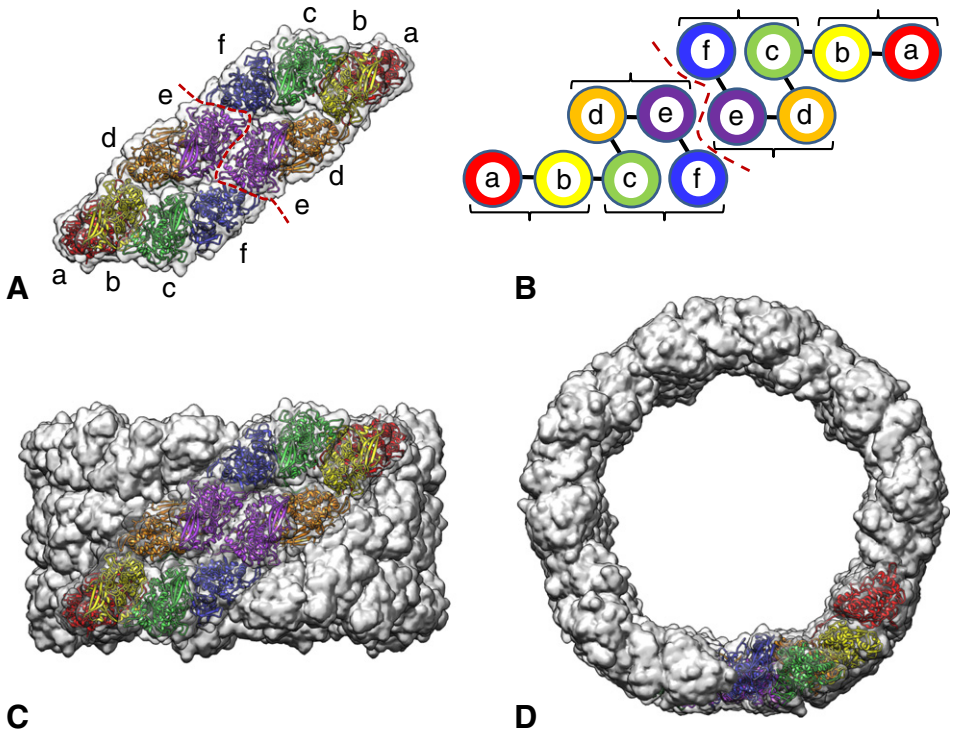

Fig. 6. Subunit dimerization and pathway within the wall of the decamer. (A) Subunit type a–b–c–d–e–f (*i.e.* lacking the collar functional units FU-g and FU-h) arranged as antiparallel dimer. The red broken line marks the borderline between the subunits. (B) Scheme of the model shown in (A). The brackets indicate FU dimers arranged according to the crystallographic dimers of OdH-g (see Fig. 5B). (C) Side view and (D) top view of a simulated 7 Å map of a decamer lacking the collar. It is composed of five subunit dimers, one of which is highlighted here as ribbon model. FU-a, FU-b, FU-c and FU-f occupy both peripheral tiers of the decamer, whereas FU-d and FU-e form the central tier. A native molluscan hemocyanin showing this quaternary structure is expressed in the snail *Biomphalaria glabrata* (see Fig. 2A). The atomistic model of KLH1 [101], and structures simulated from this model at 7 Å resolution are shown. (The atomistic model of KLH1 is available under PDB-ID 4BED).

mode of OdH-g in the crystal is also characteristic for the six wall FU types. This pairing occurs within each subunit, in the combinations a + b, c + f and d + e (see the brackets in Fig. 6B; see also Fig. 8B).

5. A collar consisting of ten g–h segments might be archetypical

Most gastropod hemocyanins are based on a 400 kDa subunit, encompassing the canonical wall segment a–b–c–d–e–f plus the collar segment g–h (see Fig. 1C). This eight-FU hemocyanin subunit also occurs in Bivalvia [29] and has been found in members of the Polyplacophora, Monoplacophora and Caudofoveata (see Table 1). As deduced from the FU phylogeny (see Fig. 4) and molecular clock, the subunit structure a–b–c–d–e–f–g–h evolved *ca.* 740 million years ago [7,8,85]. This is significantly older than the Cambrian "explosion" of the animal kingdom that dates back *ca.* 540 million years. Therefore, decamers constituted by this 400 kDa subunit should represent the molluscan hemocyanin archetype. Within the collar of such decamers, the five FU-g pairs form five "arcs" connecting adjacent subunit dimers [58,60,80], whereas the five FU-h pairs form the single peripheral annulus [79,104,105]. This endows the semi-hollow decamer with an "open face" and a "closed face" (Fig. 7A,B).

The atomistic model of KLH1 [101] revealed the subunit pathway in the wall (Fig. 7C), and the shape and FU topology of the subunit dimer (Figs. 7D, E and 8A). It also showed the FU pairing within the three types of morphological unit constituting the wall (Fig. 8B; see also Fig. 6B). This association mode is known from the crystal structure of OdH-g (see Fig. 5B); it is also observed within the five arcs (FU-g pairs) of KLH1 (Fig. 8C). The structure of the FU-h ring is different, due to the additional third domain (Fig. 8D). The peculiar switch from an antiparallel subunit pairing in the wall to a parallel pairing in the collar could be explained on the basis of two distinct subunit conformers [100,101]. They possess the same primary structure but differ in the spatial arrangement of their collar FUs (see Fig. 8A). These FU conformers have previously been designated as g_1–h_1 and g_2–h_2 [100,101]. However, this led to their confusion with hemocyanin isoforms such as KLH1 and KLH2. (For example, in ref. [106] Fig. 11, subunit structure images that have been taken from ref. [101] Fig. 3, are misinterpreted.) Therefore, I use here g–h and g′–h′ for discriminating the conformers (see Fig. 8).

The atomistic model of KLH1 (available under PDB-ID 4BED) fits also the 7.8 Å cryo-EM structure of *Haliotis diversicolor* hemocyanin (HdH1) recently deposited by a group in China (EMDB-ID 1648). The cryo-EM structures of KLH1 and HdH1 differ slightly in size, but reducing the voxel size of the HdH1 structure from 1.05 to 1.03 yields a perfect fit. (This size difference might result from mutual tiny calibration deviances during the independent reconstruction processes.) The 7.8 Å cryo-EM structure of HdH1 shows many α-helices and other striking details. They are perfectly correlated with corresponding structures in our atomistic model of KLH1, thereby fully confirming the latter.

6. The subunit type a–b–c–d–e–f–g–h allows formation of di- and multidecamers

In samples of gastropod hemocyanin, single decamers are occasionally observed, but the majority of molecules are didecamers in

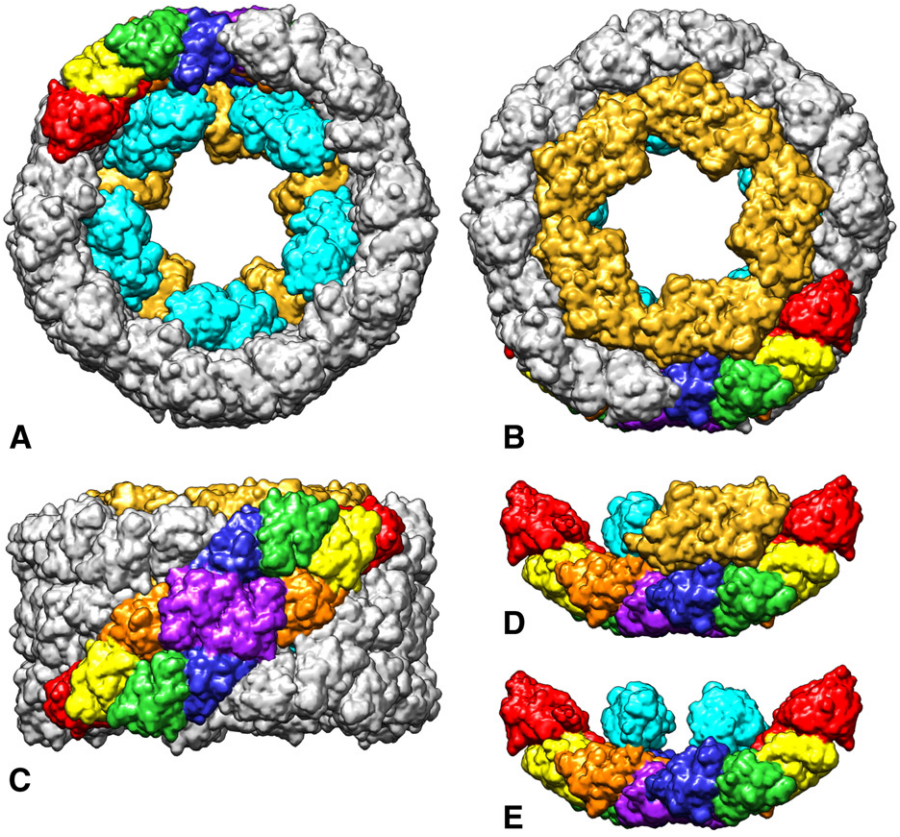

Fig. 7. The typical gastropod hemocyanin decamer. Structure simulated at 7 Å resolution from the atomistic model of KLH1 [101]. (A) Top view, exposing the open face; note the ten FU-g copies (cyan) assembled in pairs (as in Fig. 5B). (B) Top view, exposing the closed face; note the annulus consisting of ten FU-h copies (golden) assembled in pairs (as in Fig. 5D). (C) Side view, with a subunit dimer and the FU-h ring highlighted in colors. Note that FU-a (red), FU-b (yellow), FU-c (green) and FU-f (blue) occupy both peripheral tiers, whereas FU-d (orange) and FU-e (purple) occupy the central tier of the wall. Also note the FU-h ring protruding from the closed face of the decamer. (D) Extracted subunit dimer. Note that the FU-h pair (for the latter, see Fig. 5D) connects the two constituent subunits. (E) Extracted subunit dimer with FU-h removed (thereby corresponding to the subunit dimer of *Nautilus* hemocyanin; see Fig. 10). Note the gap between both FU-g copies (cyan), illustrating that they have no contact within the subunit dimer. Instead, the FU-g pairs visible in (A) connect adjacent subunit dimers. The original 9 Å cryo-EM structure is available under EMDB-ID 1569.

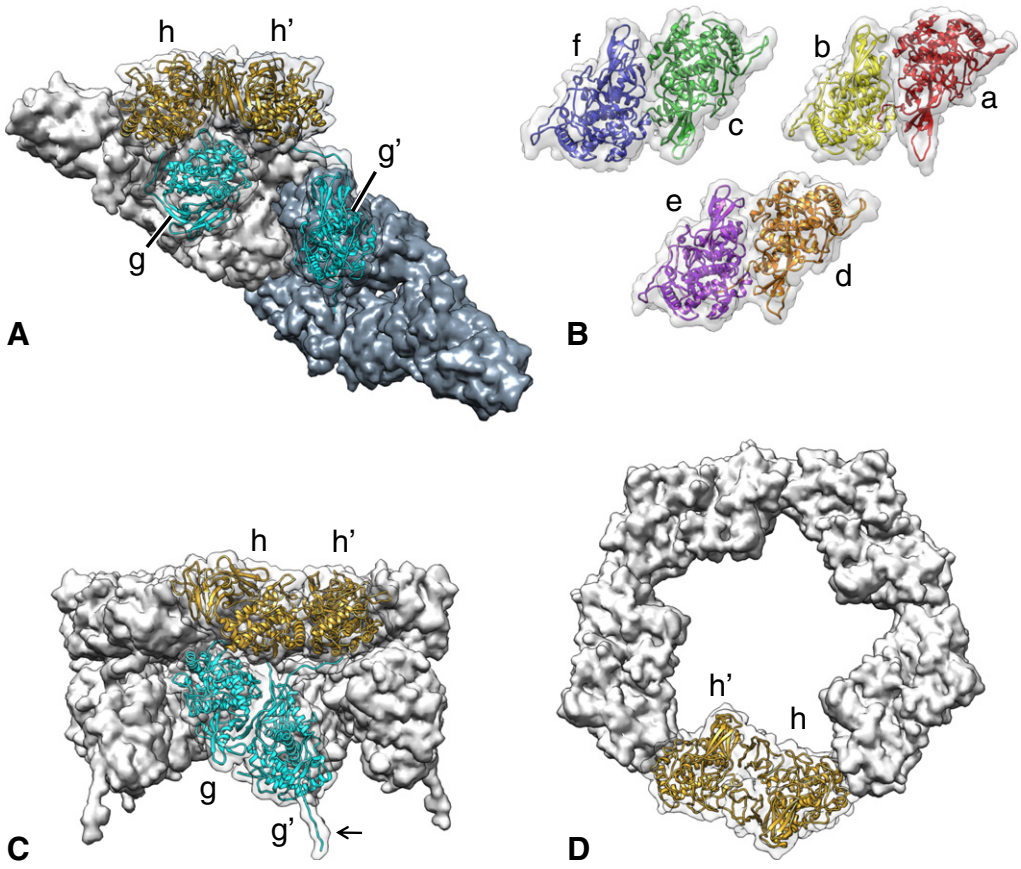

Fig. 8. Wall and collar elements of the typical gastropod hemocyanin. Structures simulated at 7 Å resolution from the atomistic model of KLH1, and ribbon representations of the latter. (A) Subunit dimer viewed from inside the cylinder cavity. The two symmetrically assembled wall segments a–b–c–d–e–f are depicted in light and dark gray, respectively. The asymmetrically arranged collar segments g–h (light subunit) and g′–h′ (dark subunit) are different conformers of the same type of polypeptide. Note that FU-g and FU-g′ are well separated, in contrast to the situation in (C). (B) The six wall FUs in their typical pairing that occurs within each subunit (see also Fig. 6B). Each pair represents a morphological unit and is assembled like the OdH-g pair in the crystal structure (see Fig. 5B). (C) The complete collar, with the FU-h ring viewed from the side and the five FU-g pairs underneath. Note that g–h and g′–h′ stem from adjacent subunit dimers, in contrast to the situation in (A). Also note that dimerization of FU-g occurs as in the crystal structure of OdH-g (see Fig. 5B). The protrusion (arrow) of FU-g′ is the stretched linker to FU-f which in the adjacent conformer FU-g is folded in a different way. (D) Terminal annulus of the collar composed of five FU-h dimers; note that the arrangement of the constituent FUs is exactly as in the crystal structure of KLH1-h (see Fig. 5D).

which the two decamers dimerize at their open faces (see Fig. 2B). To a "nucleating" didecamer, an additional decamer is often attached by its open face, resulting in a tridecamer (see Fig. 2B); attachment of further decamers yields multidecamers that can be remarkably long (for images, see [73]). Also, some bivalve hemocyanins are capable of forming such multidecamers [29,96]. The biological role of these tubular multimers is unclear, but their structure has been elucidated by 3D electron microscopy [33,101]: Within the didecamer, the two decamers are rotated, with respect to each other, by 36° around the fivefold axis of symmetry (Fig. 9A, B). In tri- and multidecamers (Fig. 9C), the additional decamers are attached with the same rotation angle of 36° [33].

Within the Gastropoda, there are species with excessive multidecamer formation, and other species with complete restriction to the didecamer level. Even within the same animal, different hemocyanin isoforms can behave differently in this respect: In case of keyhole limpet (*Megathura crenulata*) hemocyanin, isoform KLH2 forms a mixture of di- and multidecamers, whereas isoform KLH1 is limited to the didecamer level, at least *in vivo*. Only in some experimental *in vitro* buffer systems, KLH1 produces multidecamers as well [76]. Structural comparison of KLH1 and KLH2 demonstrated that their FU complement coincides [78,83]. Moreover, due to the D5 pointgroup symmetry of the wall, the amino acid residues at the potential inter-decamer interfaces are identical at both cylinder openings of a given hemocyanin; from this it would not matter at which face the two decamers assemble. The only difference between the two faces is the FU-h ring. Indeed in KLH1, a sugar side chain protruding from FU-h seems to impede docking of a decamer at the closed face of the cylinder [101].

The opposite phenomenon occurs in some polyplacophoran (chiton) hemocyanins in that they are restricted to the decamer level, whereas the hemocyanin of other chiton species is didecameric. Despite this difference, they all possess the classical eight-FU subunit. Sequence analyses revealed that the chiton taxon with decameric hemocyanin shows a specific deletion at the inter-decamer interface (Bernhard Lieb, Jürgen Markl and coworkers, unpublished data).

7. The cephalopod hemocyanins lost FU-h, and the ten-armed cephalopods doubled FU-d

Cephalopod hemocyanin does not assemble beyond single decamers and lacks FU-h [1]. *Octopus* and *Nautilus* hemocyanin are based on a seven-FU subunit a–b–c–d–e–f–g of 350 kDa ([26,28]; see Table 1). The exact pathway of this subunit within the subunit dimer, with the segment a–b–c–d–e–f in the wall and FU-g in the collar (Fig. 10A-C), has been traced by docking homology-modeled FUs to a sub-nanometer cryo-EM structure of the *Nautilus* hemocyanin decamer [72,100]. This subunit pathway has been confirmed by our study on KLH1 [101] and differed in some aspects from all the models published previously (see discussion in [100]).

Moreover, we discovered that like in KLH1, the five FU-g pairs (arcs) break the perfect dyad symmetry of the decamer by being

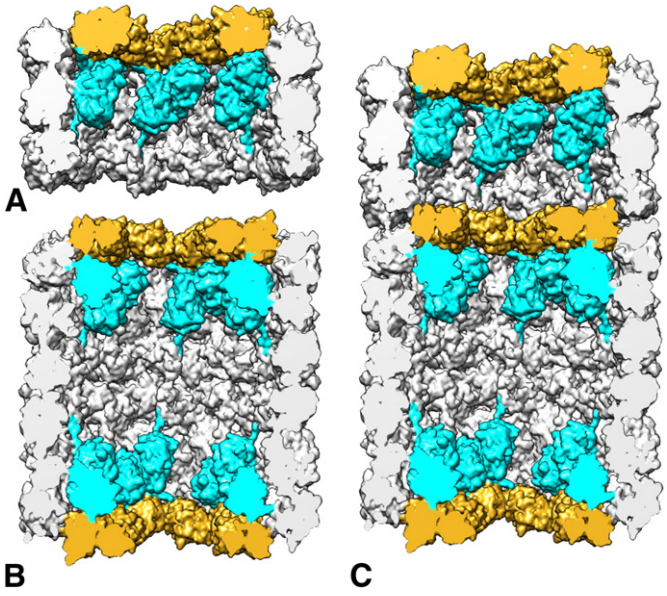

Fig. 9. Assemblies of the typical gastropod hemocyanin decamer. Structures simulated at 7 Å resolution from the atomistic model of KLH1 [101]. (A) Cut-open view of a decamer, with the open face pointing downwards. (B) Cut-open view of a didecamer. Note association at the open faces, and the shift of both collars towards the cylinder openings. The rotation angle between the decamers around their major fivefold axis of symmetry is 36°. (C) Cut-open view of a tridecamer. Note that the additional decamer is attached to the didecamer with its open face; the rotation angle is again 36° [33].

shifted towards one of the two cylinder openings (see Fig. 10B). None of the previous models predicted this asymmetry in cephalopod hemocyanin [58,60,72], which is established by the capability of the subunit to fold into two different conformers [100]. This is apparently a relic of the primordial subunit containing FU-h, because in *Nautilus* hemocyanin the ten copies of FU-g occupy exactly the same topologic positions as in KLH1 (see Fig. 8A). Thus, apart from the lack of the peripheral FU-h ring, the cephalopod and the gastropod hemocyanin decamer are completely equivalent. The absence of FU-h in cephalopods might represent a secondary loss as proposed [26,28], because the phylogenetic tree and the molecular clock suggest that FU-h evolved together with FU-a to FU-g in the late Precambrian, whereas the cephalopods occurred much later, *ca.* 520 million years ago in the Cambrian (see Fig. 4 and [7,8]). Revealing this local asymmetry within the otherwise D5 symmetrical decamer by 3D-EM was technically difficult [100]. However, this experience helped us when we subsequently reconstructed KLH1 (see Figs. 7 and 8), and *Sepia* hemocyanin (Fig. 10D, E).

Hemocyanin of the cuttlefish *S. officinalis*, and also of other ten-armed cephalopods such as *Loligo*, contains an additional functional unit (see Fig. 10D) that is inserted between FU-d and FU-e [1,59]. Two similar isoforms of *Sepia* hemocyanin, termed SoH1 and SoH2, have been sequenced by the Preaux/Gielens group in Belgium (GenBank entries ABD47515 and ABD47516). These primary structures suggest that the additional FU type evolved by gene duplication of FU-d, and that the remaining seven FU types correspond to those in *Nautilus*/*Octopus* hemocyanin (see Fig. 4). Since the original FU nomenclature of *Sepia* hemocyanin is confusing, I designate here the

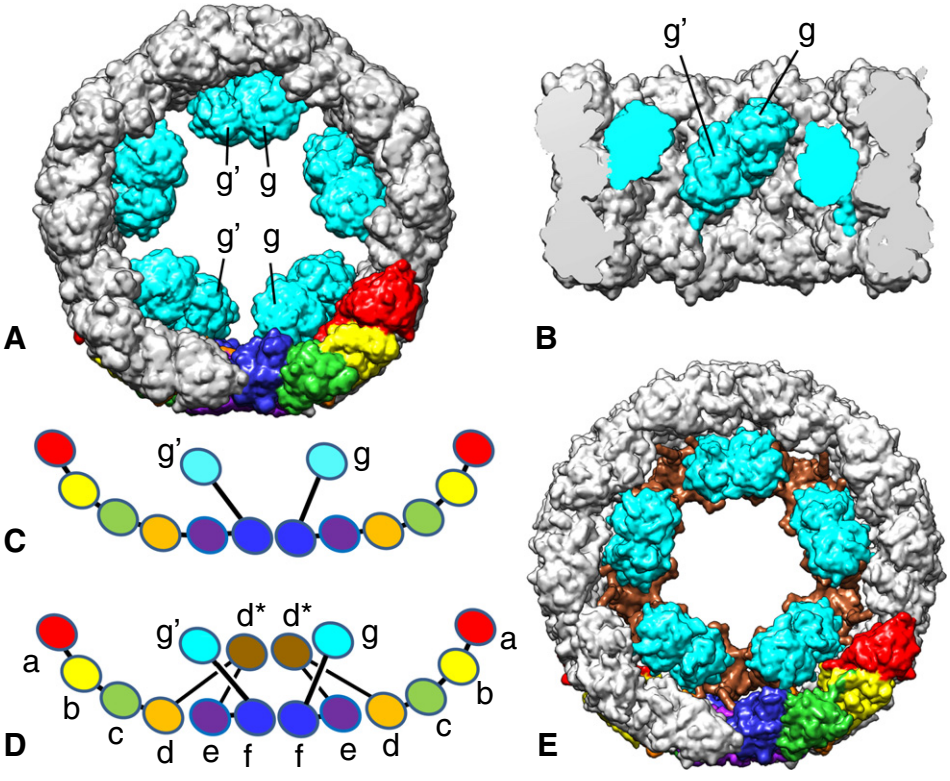

Fig. 10. Cephalopod hemocyanin decamers. (A) Top view of a decamer simulated at 7 Å resolution from the atomistic model of NpH [100]; a subunit dimer and the additional collar FUs are highlighted in colors. (The original 9 Å cryo-EM structure of *Nautilus* hemocyanin is available under EMDB-ID 1434). (B) Cut-open side view of the decamer in (A). Note shifting of the collar towards one of the cylinder openings. This is based on two FU-g conformers [100], termed here g and g′. They differ mostly in the fold of the FU-f→FU-g linker. This asymmetry breaks the perfect dyad symmetry of the wall. (C) Scheme of the subunit dimer of NpH (for a more realistic model, see Fig. 7E). (D) Scheme of the subunit dimer of *Sepia officinalis* hemocyanin (SoH); note FU-d* as an additional collar component. (E) Top view of a decamer simulated at 7 Å resolution from the atomistic model of SoH (Arne Moeller, Christos Gatsogiannis and Jürgen Markl, unpublished); a subunit dimer and the additional collar FUs are highlighted in colors.

additional functional unit as FU-d*. This yields the subunit structure a–b–c–d–d*–e–f–g (see Table 1 and Fig. 10D).

Sepia hemocyanin is a decamer, but its collar has a more compact appearance than in *Octopus* or *Nautilus* hemocyanin; it has therefore been assumed that the ten copies of FU-d* are incorporated in the collar [1]. This has been confirmed by the Lamy group through 3D-EM of the *Sepia* hemocyanin decamer [57,59,62,98]; however, at the resolution reached at that time, the subunit pathway remained obscure. We recently obtained a better resolved cryo-EM structure, and an atomistic model of *Sepia* hemocyanin, revealing the architecture of the collar (see Fig. 10E) and the exact subunit pathway (Arne Moeller, Christos Gatsogiannis and Jürgen Markl, unpublished). The additional FU type makes the decamer more rigid against deformation, as deduced from its significantly reduced flattening on EM grids, and probably relates to the exceptionally high cooperativity of *Sepia* hemocyanin (n_{50} up to 5.9; see [107]).

8. Expression of two distinct hemocyanin isoforms is an evolutionary trend in molluscs

Expression of different hemocyanin isoforms, in most cases two like KLH1 and KLH2, occurs in several molluscan species (see Fig. 2 in [8]). On the other hand, some well-studied examples like *Nautilus* and *Aplysia* possess only a single hemocyanin [28,30]. *Octopus* expresses two hemocyanin sequences with 97% identity (GenBank entries P12659 and O61363) that might represent alleles rather than isoforms [26]. In *Helix lucorum*, three different isoforms have been sequenced [31]. In the abalone *Haliotis tuberculata*, two distinct hemocyanin isoforms (termed HtH1 and HtH2) have been detected and sequenced [7,27]. Their sequence identity is *ca.* 70%, and molecular clock estimations revealed that they diverged *ca.* 360 million years ago (see Fig. 2 in [8]). KLH1 and KLH2 are their respective correlates in *Megathura*, with the *Megathura*/*Haliotis* split dating back *ca.* 260 million years (see Fig. 2 in [8]). The significant difference between HtH1/KLH1 and HtH2/KLH2 strongly suggests that they represent distinct isoforms and not different alleles. Apparently, they evolved by duplication of the entire hemocyanin gene and subsequent independent evolution of the daughter genes. KLH1 and KLH2 are differentially expressed; under certain physiological conditions, KLH1 completely disappears from the hemolymph [83].

In the present phylogenetic context, three observations are interesting to note with respect to molluscan hemocyanin isoforms: (i) The pattern of internal introns in hemocyanin genes varies between the major molluscan taxa [68], but this pattern remained virtually unchanged in the cluster HtH1/KLH1 + HtH2/KLH2 [8]. This means that in the genes of these vetigastropod hemocyanins, all "intron late" events were terminated 360 million years ago. (ii) Several molluscan taxa evolved their hemocyanin isoforms independently. For example, HtH1/KLH1 and HtH2/KLH2 are not equivalent to MtH1 and MtH2 of *Melanoides* hemocyanin (see Fig. 4). Likewise, the two hemocyanin isoforms in the bivalve *Nucula nucleus* bifurcated independently ([29]; see also Fig. 2 in [8]). (iii) As deduced from the degree of sequence identity, the hemocyanin isoform pairs in gastropods and bivalves evolved much earlier than the hemocyanin duplets in cephalopods.

After our publication of the KLH1 didecamer structure [101] we have also reconstructed the KLH2 didecamer (Christos Gatsogiannis, Erik Schnittger, Julia S. Markl, Frank Depoix, Wolfgang Gebauer and Jürgen Markl; cryo-EM structure deposited under EMDB-ID 2320). Although both isoforms differ in many amino acid residues, their 3D structures are very similar. The atomistic model of KLH1 could be convincingly docked to the cryo-EM structure of KLH2. However, the number and spatial distribution of potential attachment sites for N-glycans is significantly different in KLH1 and KLH2, and this might be the basis of their differential katabolic regulation [83].

9. Did mega-hemocyanin boost the adaptive radiation of cerithioid snails?

A large variety of molluscan hemocyanins has been studied [1], and several species of the gastropod superfamily Cerithioidea (supertaxon Heterobranchia) are very abundant, even populating many fish tanks. Nevertheless, the existence of an atypical hemocyanin that we now call mega-hemocyanin remained hidden. In 2008 we studied, by chance, a sample of *Leptoxis carinata* hemocyanin in the electron microscope, and found that it differs significantly from a typical gastropod hemocyanin such as KLH1 [33]. It occurs as a tridecamer (see Fig. 2C), with two "normal" decamers at either side of a central deviant mega-decamer (Fig. 11A-C). The subunit constituting the flanking decamers is a 400 kDa polypeptide (termed $MtH1_{400}$) and has a structure like KLH1 (Fig. 11D, D′). Isolated $MtH1_{400}$ readily reassembled into typical decamers, didecamers and even multidecamers [33].

In contrast, the subunit constituting the central mega-decamer has a mass of 550 kDa and contains 12 functional units. In the 550 kDa subunit (termed $MtH2_{550}$), the segment a–b–c–d–e–f is present and constitutes the canonical molluscan hemocyanin cylinder wall. FU-g and FU-h are missing; instead, six consecutive variations of FU-f (termed FU-f1 to FU-f6) form an atypical collar (Fig. 11C, E; see also Table 1). Consequently, instead of only 20 FUs as in the collar of the typical decamer, the collar of the mega-decamer contains 60 FUs. The published biochemical and electron microscopical data on this novel hemocyanin [33] have more recently been confirmed by the complete amino acid sequence of $MtH1_{400}$ and $MtH2_{550}$ (see Fig. 4; GeneBank entries KC405575 and KC405576 by Meik Neufurth, Bernhard Lieb and Jürgen Markl). Moreover, an improved 3D reconstruction showing the FU structure of the atypical collar much better than in Fig. 11C has been elaborated by Christos Gatsogiannis. Manuscripts on these data are in preparation.

With respect to the oxygen binding properties, typical molluscan hemocyanins seem to be unable to evolve into high affinity forms that are required in hypoxic environments. This is the putative reason why planorbid snails possess hemoglobin instead of hemocyanin [32]. However, mega-hemocyanin has the capacity to evolve into high affinity, medium affinity and low affinity forms, depending on the animal species [33]. Moreover, differential expression of the 400 kDa *versus* the 550 kDa polypeptide might allow acclimatization of individual snail populations to changing environmental conditions [33]. Phylogenetically, we assume that the twelve-FU subunit $MtH2_{550}$ evolved from an ancestral eight-FU precursor (400 kDa) in two steps: (i) loss of FU-h and FU-g, respectively of the collar, resulting in a molecule as shown in Figs. 2A and 6; (ii) gain of FU-f1 to FU-f6, creating the mega-decamer collar, by subsequent duplication and fusion events of the exons encoding FU-f. Preliminary molecular clock calculations suggest that this was correlated with the radiation of the Cerithioidea, and therefore might be adaptive.

10. Evolved molluscan hemocyanin from an FU dimer preceding the central wall tier?

In 2001, we proposed a model for the evolution of the 35 nm hemocyanin cylinder that started with an FU-h dimer forming, as the crucial step, a decameric ring [68]. With the recent more detailed insight in the structure of these molecules, notably the common modes of FU dimerization and subunit dimerization in the wall, I propose here a different scenario (Fig. 12) with the following steps: (i) An ancestral one-FU protein, termed here y, formed an antiparallel homodimer y ↔ y, with the same assembly mode as in the crystallographic FU-g dimer (Fig. 12A, B). (ii) A gene duplication yielded heterodimer x ↔ z that was then transformed, by gene fusion, into the two-FU protein x–z (Fig. 12C, D). (iii) Subunit x–z acquired the ability to dimerize and to form a 35 nm ring from five such dimers (Fig. 12E). For symmetry reasons, this primordial hemocyanin might

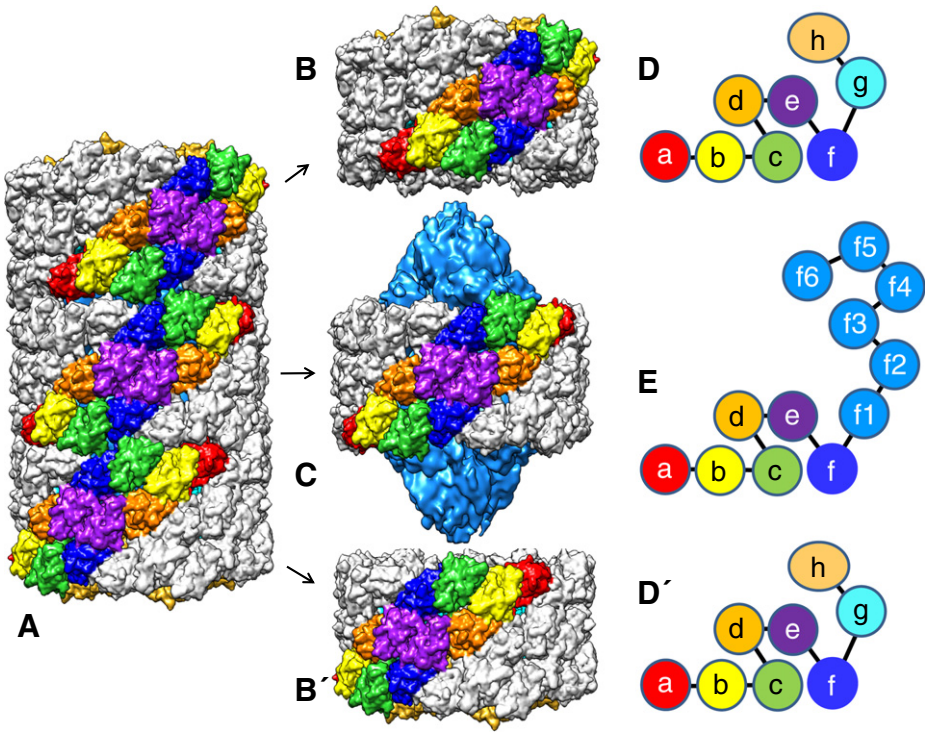

Fig. 11. Mega-hemocyanin from cerithioid snails. Structures simulated at 7 Å resolution from the atomistic model of KLH1 [101], with exception of the collar of the mega-decamer (dodger blue) that was extracted from a 13 Å cryo-EM structure [33]. (A) Mega-tridecamer, exhibiting the same wall structure as in typical hemocyanins. (B), (B′) Peripheral decamers, with the wall and collar structure of a typical gastropod hemocyanin. (C) The central mega-decamer, with the wall structure of a typical hemocyanin, but an atypical collar (see also Fig. 2C). (D), (D′) Scheme of the 400 kDa subunit constituting the peripheral decamers. (E) Scheme of the 550 kDa subunit forming the central mega-decamer. It encompasses the typical wall segment a–b–c–d–e–f, plus six atypical collar functional units that evolved from FU-f (see Fig. 4).

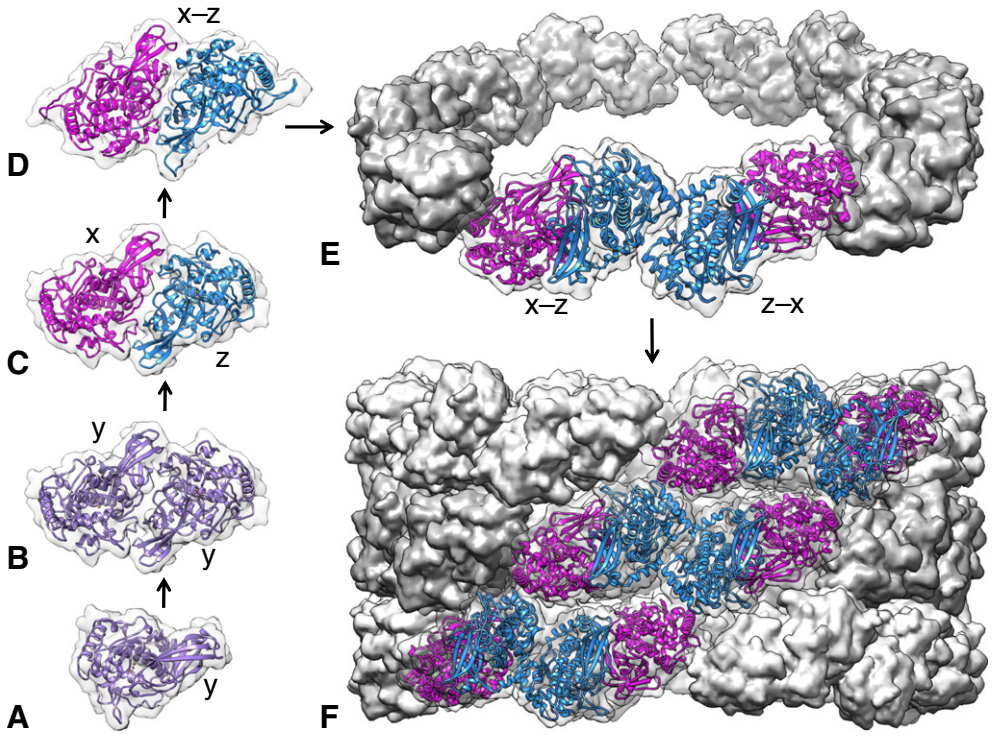

Fig. 12. Evolution of the molluscan hemocyanin decamer — a new hypothesis. (A) The putative phylogenetic origin: an ancestral FU-like tyrosinase "y" with a single copper active site. (B) Evolution of a homodimer y ↔ y, with its constituents associated as in the crystal structure of OdH-g (see Fig. 5B). This primordial dimerization mode has been strictly conserved in the later stages. (C) Gene duplication and individual evolution of the daughter genes, leading to a heterodimer x ↔ z. (D) Gene fusion resulted in a single polypeptide x–z, with the two FUs concatenated *via* a peptide linker. (E) The protein x–z gained the ability to form an antiparallel dimer x–z ↔ z–x, and five such dimers assembled to form a decameric ring, 35 nm in diameter. This hypothetical ancestral hemocyanin with 20 active sites might correspond to the central wall tier of molluscan hemocyanin. (F) Assembly of three identical rings (staggering angle 18°), yielding a primitive three-tiered hemocyanin molecule with all final symmetries already established. From here, fusing three adjacent two-FU subunits x–z into a six-FU subunit x–z–z–x–z–x, modifying the latter into a–b–c–d–e–f (see Fig. 6), and evolving a collar could happen gradually in small steps. For direct visualization, and simulation of 7 Å structures, 30 copies of the crystallographic dimer of OdH-g (PDB-ID 1JS8) were docked to the cryo-EM structure of KLH1 (EMDB-ID 1569).

be represented today by the central wall tier of the decamer, and consequently, FU-x and FU-z would be ancestral forms of FU-d and FU-e. (iv) Stacks of three rings yielded a primitive three-tiered hemocyanin molecule (Fig. 12 F). Subsequently, from the two-FU subunit the six-FU wall segment a–b–c–d–e–f (see Fig. 6) evolved by gene duplication, fusion, and diversification events. (v) Starting from FU-f, the collar evolved by gene duplication, fusion and diversification events, and ultimately, the cupredoxin-like domain of FU-h was adopted. The shift of the collar towards one cylinder opening has been explained by the need for the ten FU-h copies to form a closed ring within the collar [101]. Logically, this can only occur at one and not at both ends of the cylinder, and it also shifts the five FU-g dimers (arcs) into an asymmetric position (see Fig. 8).

Whether this specific scenario is realistic remains hidden in the Precambrian, but it is based on our current structural knowledge of this highly complex oxygen transporter. Moreover, it subdivides its evolution into steps such as duplication, fusion and diversification of genes, or antiparallel association and ring formation of polypeptide subunits that are all very common in the protein world.

11. Concluding remarks

Science is full of strange examples of coincidence, and I add here another to the list. After decades of hemocyanin research, we were able to publish the atomistic model of a gastropod hemocyanin didecamer (KLH1), thereby solving many long-standing structural questions [101]. We received the article proofs in November 2008 and were convinced that the structure of gastropod hemocyanin was now clarified. On the same day, Bernhard Lieb returned from a field trip to Potomac River, near Washington D.C., and brought some mysterious little snails into our laboratory. Another day later we discovered mega-hemocyanin. Its atypical composition was unraveled within a couple of weeks by using the structural knowledge just collected for KLH1.

The major primary, ternary and quaternary structures of molluscan hemocyanins are now solved, and atomistic models of the 35 nm molecules are available. Moreover, we now have hypotheses of the evolution of these structures that are less speculative than formerly. According to molecular clock calculations, the eight typical molluscan hemocyanin functional units (FU-a to FU-h) evolved together *ca.* 740 million years ago [7]. However, the chronological order of their appearance remains obscure: neither the exon nor the intron sequences helped us to elucidate this aspect. Nevertheless, structural considerations led to two scenarios for the stepwise evolution of the 35 nm hemocyanin, both with the formation of a ring-like decamer as the crucial step: Previously, we proposed the peripheral FU-h ring (see Fig. 8D) as the nucleating structure [68], whereas on the basis of the more recent findings I now propose that the molluscan hemocyanin cylindrical decamer started from the FUs of the central wall tier (see Fig. 12).

With respect to the phylogenetic tree of functional units (see Fig. 4), it should be mentioned that the cupredoxin-like domain of FU-h is excluded from such analyses because it is absent in the other FU types. It is therefore not certain whether it is as ancient as the other two domains of FU-h. However, its existence not only in gastropods, but also in the hemocyanins of bivalves, polyplacophorans, monoplacophorans and the Caudofoveata (see Table 1) suggests that it indeed belonged to the molluscan hemocyanin archetype.

It has emerged as a general scheme that the architecture of the molluscan hemocyanin wall is strictly conserved, whereas the collar is more variable in that collar functional units disappeared or secondarily evolved. In addition to their overall fivefold symmetry, the decamers show a perfect dyad symmetry in the wall, but possess an asymmetric collar (see Fig. 8). Also, many other structural questions heavily debated at previous oxygen carrier meetings are now solved, as for example the exact shape and pathway of the subunits within the quaternary structure [100,101]. This is useful knowledge for disassembly–reassembly studies [93], and applications of KLH as immunological tool [104,105]. In particular, the atomistic models identified the amino acid appositions at the interfaces between adjacent FUs, subunits, subunit dimers, and decamers [100,101]. This provides a firm basis for unraveling at these interfaces changes during oxygen binding, in order to elucidate the chemo-mechanical force transfer within the hemocyanin molecule during allosteric interaction.

In combination with the wealth of functional data available on molluscan hemocyanins, this structural knowledge should allow deeper insight in their biological function as oxygen carriers. On the other hand, even the small vertebrate hemoglobins still bury many surprises (see this Special Issue), although they have been studied incomparably more detailed than the hemocyanins. Therefore, it might be too ambitious at the moment to expect a deep chemo-mechanical understanding of an oxygen transporter such as KLH1 that contains *ca.*1 million atoms. In my opinion, what we can hope by applying techniques already available is a structural understanding of the fully oxygenated *versus* the fully deoxygenated hemocyanin state. In this context, we compared two sub-nanometer 3D reconstructions of *Nautilus* hemocyanin that had been obtained under oxy and deoxy conditions, respectively, and we found significant differences. What we saw when comparing the atomistic models of both conformations was a rearrangement of numerous salt bridges (Arne Moeller and Jürgen Markl, unpublished). To verify this, and to reliably pin down structural details at the various molecular interfaces, we require 3D reconstructions of the oxy and the deoxy state at significantly higher resolution, preferably close to 5 Å. The techniques to achieve this are available today.

Acknowledgements

I thank Prof. Dr. J. Robin Harris for critical reading of the manuscript and for our fruitful cooperation on molluscan hemocyanin during his time in Mainz. My local colleagues Prof. Dr. Bernhard Lieb, Prof. Dr. Elmar Jaenicke, Dr. Wolfgang Gebauer, Dr. Frank Depoix and the following former PhD students contributed with their published and unpublished work to this review: Dr. Ute Albrecht, Dr. Benjamin Altenhein, Dr. Sandra Bergmann, Dr. Valesca Boisguerin, Dr. Kai Büchler, Dr.Dr. Ralf Dürr, Dr. Christos Gatsogiannis, Dr. Henning Keller, Dr. Ulrich Meissner, Dr. Arne Moeller, Dr. Vanessa Moeller, Dr. Meik Neufurth, Dr. Sabine Söhngen, and Dr. Klaus Streit. Unpublished data on KLH2 stem from Erik Schnittger and my daughter Julia Markl. Our molluscan hemocyanin projects have been financially supported by the Deutsche Forschungsgemeinschaft (DFG grants Ma 843 and DFG graduate school GK1043), the *biosyn* company in Fellbach, Germany, and the Forschungszentrum Immunologie (FZI) of the University Medicine of the Johannes Gutenberg University.

During the final proof stage of this paper, a 3D reconstruction with a resolution of 4.5 Å of the hemocyanin didecamer of another gastropod, the abalone Haliotis diversicolor, was published [109]. This superior structure confirmed our atomistic model derived from KLH1 except of the two d→e connections within the subunit dimer. This single difference yields an alternative subunit pathway. Even so, the localization, spatial orientation and mutual contacts of the eight types of functional unit are identical in both models.

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