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The Cupredoxin-like Domains in Hemocyanins

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Abbreviations(in alphabetical order):

AcH	– <i>Aplysia californica</i> hemocyanin
CBP	– cucumber basic protein
CopC	– copper carrier protein
FU	– functional unit
HtH1, HtH2	– isoform 1 and 2 of <i>Haliotis tuberculata</i> hemocyanin
KLH	– keyhole limpet (<i>Megathura crenulata</i>) hemocyanin
KLH1, KLH2	– isoform 1 and 2 of KLH
KLH1-h	– functional unit h of KLH1
KLH1-h-D1 (-D2, -D3)	– domains 1, 2 and 3 of KLH1-h
NpH1, NpH2	– isoform 1 and 2 of <i>Nucula nucleus</i> hemocyanin
rms	– root mean square

Synopsis

Hemocyanins are multimeric oxygen transport proteins which bind oxygen to type 3 copper sites. Arthropod hemocyanins are composed of 75 kDa subunits, while molluscan hemocyanins contain 350-400 kDa subunits with 7-8 different 50 kDa “functional units” (FU-a to FU-h), each with an active site. FU-h possesses a tail of 100 amino acids not present in the other FUs. Here we show by X-ray crystallography of FU-h of keyhole limpet hemocyanin isoform 1 (KLH1) that the structure of the tail domain is cupredoxin-like but contains no copper. The copper-free domain #3 in arthropod hemocyanin subunits has recently also been reinterpreted as being cupredoxin-like. We propose that the cupredoxin-like domain in both hemocyanin types once served to upload copper to the active site of the oxygen-binding domain.

Introduction

Hemocyanins are giant oxygen transport proteins found in the hemolymph of many arthropods and molluscs. They reversibly bind oxygen, often with very high cooperativity, at binuclear type 3 copper centers (types 1 and 2 are mononuclear, respectively) [1, 2]. The copper centers of all hemocyanins are very similar, with the two copper atoms being bound to a four α -helix bundle and ligated by three histidines each [3-6]. As type 3 copper proteins, hemocyanins are structurally related to the phenoloxidases, tyrosinases and catecholoxidases; all these proteins bind oxygen in a side-on (μ - η^2 : η^2) coordination between the two copper atoms of the active site [7].

In spite of their common identity as type 3 copper proteins, the hemocyanins of arthropods and molluscs have been considered as two distinct protein families, based on differences in their amino acid sequences and quaternary structures. [8-10]. Molluscan hemocyanins are semi-hollow cylinders 35 nm in diameter representing decamers, didecamers or multidecamers of a 350-400 kDa subunit. The latter is a concatenation of 7 or 8 paralogous functional units (FU) of ~50 kDa each, termed FU-a to FU-h. Thus, 160 oxygen binding sites exist in a molluscan hemocyanin didecamer such as isoform 1 of keyhole limpet hemocyanin (KLH1) [11]. KLH is a mixture of two isoforms, KLH1 and KLH2, isolated from the hemolymph of the giant keyhole limpet *Megathura crenulata*, and widely used as an immunological tool [12]. In contrast, arthropod hemocyanins occur as hexamer (1x6mer) or oligo-hexamer (2x6mer, 4x6mer, 6x6mer, 8x6mer) assembled from several paralogous subunit types, each of molecular mass ~75 kDa and with a single active site [13]. The largest arthropod hemocyanin type occurs in horseshoe crabs such as *Limulus polyphemus*; it is a cubic 8x6mer of 25 nm length, with 48 oxygen binding sites; it has recently been analysed structurally in detail [14]. From a functional viewpoint, the arthropod hemocyanin subunit corresponds to the molluscan hemocyanin FU, each showing a single active site.

Representative crystal structures of the molluscan hemocyanin FU and the arthropod hemocyanin subunit are available [3-6], but questions about the evolutionary connection between the two hemocyanin families remain. The arthropod hemocyanin subunit folds into three structural domains: The N-terminal domain #1 is predominantly α -helical and shields the entrance to the active site. The central domain #2 contains the active site in a 4- α -helix bundle motif. The C-terminal domain #3 folds into a 7-stranded anti-parallel β -barrel, a feature previously interpreted as being immunoglobulin-like [15]. Whether in the basic hexamer domain #3 has a role other than structural is unknown, but in oligo-hexamers it certainly contributes to allostereism [14]. Most molluscan hemocyanin FUs fold into only two structural domains: The N-terminal core domain (D1) contains the oxygen binding site in a 4- α -helix bundle and therefore corresponds to domain #2 in arthropod hemocyanin, although their remaining tertiary structure elements are very different. The C-terminal domain (D2) is dominated by a 6-stranded β -sandwich and shields the entrance to the active site. Hence, its role in molluscan hemocyanin is comparable to that of domain #1 in arthropod hemocyanin [9].

There is one exception from the standard architecture of molluscan hemocyanin FUs, namely the C-terminal FU-h. Rather than 50 kDa, its molecular mass is 60 kDa due to a C-terminal extension of ~100 amino acids which marks the very end of the 400 kDa polypeptide [16]. The sequence of this peculiar tail is unrelated to any other protein in the databases, and

therefore its fold remained unknown as yet. In this paper we report the 4 Å crystal structure of KLH1-h, and discuss the structural and evolutionary implications of its tail domain. Thereby, we will substantially reinterpret the phylogenetic origin and functional role of the structural domains found in molluscan and arthropod hemocyanins.

Experimental

Purification. KLH1 was purified from *Megathura crenulata* hemolymph serum (Biosyn, Fellbach, Germany) by selective dissociation of KLH2 as described elsewhere [17]. Briefly, overnight dialysis against 2 % ammonium molybdate (pH 5.7) at 4 °C results in selective dissociation of KLH2 into its subunits, while KLH1 remains intact as multimers. KLH isoforms were then separated by pelleting the intact KLH1 multimers at 30,000 g for 4 h at 4 °C, while leaving KLH2 subunits in the supernatant. The pellet was resolved in stabilizing buffer (150 mM NaCl, 50 mM Tris/HCl, 5 mM MgCl₂, 5 mM CaCl₂, pH 7.4) and then dialyzed overnight against dissociation buffer (130 mM glycine/NaOH, pH 9.6) to dissociate KLH1 multimers into their subunits. Purified KLH1 subunits were subjected to limited proteolysis by *Staphylococcus aureus* V8 protease type XVII (Sigma-Aldrich, Taufkirchen). KLH1 subunits (10 mg/ml final concentration) dissolved in proteolysis buffer (100 mM NH₄HCO₃, pH 8.0) were mixed with V8 protease (2 % (w/w) final concentration) and incubated at 37 °C for 5 h. Proteolytic cleavage was terminated by adding proteolysis inhibitor (Pefabloc®, Roth, Karlsruhe, Germany) to a final concentration of 10 mM. KLH1-h was purified from the resulting protein fragments by anion exchange chromatography on a Q-Sepharose column (V = 25 ml, Pharmacia, Freiburg, Germany). To this end about 50 mg of protein fragments were applied to the column equilibrated with 20 mM Tris/HCl (pH 8.0). Fragments were then eluted by a linear sodium chloride gradient from 160 mM to 400 mM NaCl in the same buffer at a flow rate of 0.5 ml/min (Fig. S1, A). The KLH1-h content and purity of the eluting fractions was assayed by SDS-PAGE on 10 % polyacrylamide gels stained with Coomassie brilliant blue [18] and by immunoelectrophoresis using rabbit antibodies against dissociated KLH1 [19, 20] (Fig. S1, BC). Absorption spectra were measured with a U-3000 spectrophotometer (Hitachi, Japan) in quartz cuvettes with 1 cm pathlength. For obtaining deoxy spectra KLH1-h was deoxygenated by addition of sodium dithionite.

Crystallization. Preliminary crystallization conditions were determined for batch-under-oil by high-throughput screening at the Hauptman-Woodward Institut (Buffalo, NY, USA) and then refined for the sitting-drop setup [21]. Crystals were grown on microbridges in Linbro plates covered with cover slides (Hampton Research, Aliso Viejo, CA, USA). Briefly, 5 µl of a 15 mg/ml KLH-h solution were mixed with 5 µl reservoir solution (9.0 % (w/v) PEG 1000, 50 mM K₂HPO₄, 50 mM sodium citratebuffer, pH 4.0) and left to equilibrate in the sealed well at 20 °C. Single, non-birefringent crystals were obtained after 10 days which were shaped like rhombic dodecahedra and had a maximum dimension of 0.1 mm (Fig. S1, D).

Structure determination. The crystals were cryocooled in liquid nitrogen after being cryoprotected by soaking them in reservoir solution containing 30% (v/v) glycerol. The crystals diffracted to 4.0 Å resolution at the PX-II beamline of the Swiss Light Source, Villigen, Switzerland. Data processing indicated I-centered cubic symmetry, and data

statistics are shown in Table 1. Molecular replacement with PHASER [22] using the structure of the homologous FU-g from *Octopus dofleini* hemocyanin (OdH-g) (pdb code: 1JS8 [3]) as the search model identified the space group as I213 and showed the asymmetric unit to contain a dimer, indicating a solvent content of 83 %. Between the two monomers of the search model, clear additional electron density indicating β -sheet topology could be seen for the C-terminal domain that is not present in the search model. Prime-and-switch density modification using RESOLVE [23, 24] starting from the phases calculated by PHASER resulted in an electron density map in which the fold of the C-terminal domain could be traced with high confidence. C_α atoms for the C-terminal domain were placed using COOT [25] and XFIT [26] and were submitted to the DALI server [27, 28] to find similar folds. Strikingly, the closest match (Z-score 7.4) was the structure of cucumber basic protein (pdb code: 2cbp [29]) which is a type I copper protein. Based on the similarity of KLH1-h with the folds of FU-g of *Octopus* hemocyanin (OdH-g) and cucumber basic protein, a homology model of the KLH1-h was built using the SWISS-MODEL server [30, 31] to aid in obtaining a tentative assignment of the sequence to the C_α trace (Fig. S2).

Results and Discussion

C_α -trace of KLH1-h. Functional unit KLH1-h was released from the KLH1 subunit by limited proteolysis, preparatively purified, crystallized and its three-dimensional structure solved to a resolution of 4 Å. This allowed tracing its C_α -backbone, encompassing 490 amino acids (Fig. 1, Fig 2A.1). It folds into three well defined structural domains: The N-terminal core domain (KLH1-h-D1) and the central β -sandwich domain (KLH1-h-D2) exhibit the canonical molluscan hemocyanin fold already known from the crystal structures of two other FU types, FU-e and FU-g [3, 6]. The additional C-terminal domain (KLH1-h-D3) contains mainly β -strands (Fig. 1, Fig. 2A.1). It shares a striking and completely unexpected structural similarity with cucumber basic protein (CBP) which has a type 1 copper site (Fig. 1C, Fig. 2A). CBP is a member of the cupredoxin protein family and belongs to the sub-group plantacyanins [29]. The cupredoxin-like fold of KLH1-h-D3 resembles a Greek key β -barrel, except that, as in CBP, the barrel is open at one side and thus might better be described as β -sandwich or " β -taco" [29]. The rms deviation between KLH1-h-D3 and CBP is 2.5 Å. Moreover, KLH1-h-D3 and CBP show a disulphide bridge at identical positions, with the two cysteines separated by 20 amino acids, accurately. On the other hand, their sequence identity is only ~10%, and consequently, they show specific physico-chemical differences (Fig. 2, A.2). In particular, the isoelectric point (pI) of KLH1-h-D3 is 6.3 (predicted from the sequence), while the pI of CBP is 11 [29]. Moreover, this low degree of sequence identity prohibits using CBP as template for molecular modeling of the KLH1-h-D3 sequence. Therefore, the present considerations are restricted to the C_α backbone.

Copper active sites. As CBP contains a type 1 copper site [29], this was also to be expected for the cupredoxin-like fold of KLH1-h-D3 and would mean a second copper center in FU-h, in addition to the canonical type 3 copper site. In blue copper proteins such as cupredoxins, the copper (I) ion form in the type 1 copper site is stabilised by a constrained His₂Cys coordination environment. Unfortunately 4 Å resolution as presently achieved did not resolve the binuclear copper center in KLH1-h-D1, and therefore, the chance of detecting a putative single copper in KLH1-h-D3 is very low. However, the amino acid sequence of KLH1-h-D3 lacks any histidine or cysteine residues in appropriate position that could allow copper

binding to the protein (a His₂Cys coordination environment in CBP and other copper-containing cupredoxins). Consequently, we assume that KLH1-h-D3 is devoid of a type 1 copper center which might have been lost at an early stage of molluscan hemocyanin evolution (Fig. 1C). A similar situation is encountered in the murine ephrin ectodomain, which also shares the cupredoxin fold with CBP, but lacks the copper binding site [32]. In this context, the presence of a disulphide bridge in KLH1-h-D3 and the ephrin ectodomain at a conserved position is remarkable, because in CBP it is thought to stabilize the type 1 copper center and is a typical feature of phytocyanins [29]. Therefore, since no type 1 copper center is present, it might be required for the structural integrity of the entire cupredoxin-like fold. In the present context, this disulphide bridge is a strong argument in favour of our interpretation of KLH1-h-D3 as a cupredoxin-like protein.

Oxygenated KLH1-h has a blue/purple color due to a broad absorption peak in the visible range with a maximum at 554 nm (Fig. S3). This peak disappears completely upon deoxygenation of KLH1-h which is typical for all hemocyanins and has been thoroughly characterized as resulting from the $\pi_v^* \rightarrow \text{Cu(II)}$ charge transfer transition, where π_v^* is the peroxide orbital orthogonal to the Cu₂O₂ plane of the type 3 copper center [33]. Consequently it can be ruled out that the cupredoxin-like domain of KLH1-h contains any additional ligands or active sites, which absorb light in the visible range.

Cupredoxin-like domains in hemocyanin. The unexpected presence of two copper protein folds within a molluscan hemocyanin functional unit raises the question as to whether the cupredoxin-like domain is a structural feature only encountered in FU-h of molluscan hemocyanins. Moreover, the structural and possibly functional role of this peculiar tail domain of the molluscan hemocyanin subunit should be addressed. A recent database search for structural correlates of the C-terminal domain #3 of arthropod hemocyanin unearthed another member of the cupredoxin family, the bacterial CopC protein [34] (Fig. 2B). CopC is one of four proteins encoded by the copABCD operon of *Pseudomonas syringae* pv *tomato* and confers resistance to the pathogen against high copper levels [35]. The CopC protein has a β -barrel structure and features two distinct mono-copper binding sites. It has been considered to represent a link between copper-trafficking proteins and cupredoxins [34, 35].

From an evolutionary point of view, it is remarkable that the cupredoxin fold motif can be found in *both* molluscan and arthropod hemocyanins (Fig. 2, Fig. 3), because as already mentioned, there are convincing arguments that both hemocyanin families evolved convergently from tyrosinase/phenoloxidase-like ancestors [8]. Indeed, hemocyanins can be considered as permanently inactivated phenoloxidases [1, 36]. It now appears that both hemocyanin families evolved from proteins containing a type 3 and a type 1 copper site. In principal, the combination of several copper centers is not unusual. There exist blue oxidases such as laccase, ascorbate oxidase or ceruloplasmin which combine a mixed type 2 and type 3 copper center along with a type 1 copper center [37-39]. However, the combination of a pure type 3 copper center and a type 1 copper center in a single protein as it is suggested here for ancestral hemocyanins is unprecedented. Moreover, it is remarkable that the copper 1 site seems to have been lost in both recent hemocyanin families.

Possible role(s) of the cupredoxin-like hemocyanin domains. If in fact a functional type 1 copper site in ancestral hemocyanins did exist, one might suppose that its cooperation with the type 3 copper center could significantly improve oxidative processes. However, the large distance between the two copper centers (more than 30 Å in arthropod hemocyanin and FU-h,

respectively) does not support this scenario: In blue oxidases such as laccase, ascorbate oxidase or ceruloplasmin, in which an electron transfer between copper centers is documented, their maximum distance is ~ 13 Å [39]. Thus, a similar coupling of the two sites in the framework of an ancient enzymatic function seems unlikely in case of both hemocyanins.

A more plausible explanation for the presence of the cupredoxin-like domain in hemocyanins may be deduced from *Streptomyces* tyrosinase that has a hemocyanin-like type 3 copper site. The structure of this tyrosinase complexed with its “caddie” protein (MelC1) was recently solved (Fig. 2C) [7]. The small copper binding caddie protein is coexpressed with tyrosinase and apparently responsible for correct copper loading to the active site of the latter [7]. In other words, the “caddie” protein appears to serve as copper chaperone of the tyrosinase. Copper insertion into proteins is an incompletely understood phenomenon and little is known about copper loading to type 3 copper proteins. Typically copper concentrations within the cell are so low that most copper proteins need copper chaperones to help load the active site [40]. We therefore assume that in the case of the hemocyanins, the ancestral tyrosinases that gave rise to arthropod and molluscan hemocyanin, respectively, combined with their copper chaperones by gene fusion resulting in a more efficient copper loading onto their active sites.

If so, then why did the copper binding site of the cupredoxin-like domain in both hemocyanin families degenerate? In tyrosinase, the active site is solvent accessible, allowing bulky substrates to approach the type 3 copper site for catalysis [41]. Thus, loss of copper atoms could easily occur during the lifetime of the tyrosinase molecule which would make it necessary to reload copper with the help of the “caddie” protein. In hemocyanins as oxygen carriers, the type 3 copper site is protected from the solvent because it is covered by additional domains (domain D2 in molluscan and domain #1 in arthropod hemocyanin), leaving only a narrow oxygen diffusion channel [9]. Thus, the probability for hemocyanins to lose copper during their lifetime is very low and the necessity to reload lost copper atoms would have faded. In such an instance, we assume that copper loading during hemocyanin biosynthesis was outsourced to dedicated copper chaperones and consequently, selective pressure to conserve the copper center decreased in the cupredoxin-like domains of hemocyanins.

The further evolution of hemocyanin as an extracellular, highly allosteric oxygen carrier was then dominated by the process of forming large macromolecular assemblies. In arthropods, the basic quaternary structure is the hexamer (1x6mer). For this, domain #3 (the cupredoxin-like domain) was probably not directly required, apart from generally stabilizing the individual subunits, because it is not involved in subunit/subunit bonding [4]. However, in 4x6mer/8x6mer chelicerate hemocyanins, domain #3 is directly involved in a variety of inter-hexamer interfaces that also might transfer allostereism [14]. Even in the “simple” case of a spider 2x6mer formed from two hexamers by a single disulfide bridge, domain #3 is directly involved [42]. Consequently, domain #3 is an indispensable structural and functional element in the architecture and allostereism of oligo-hexameric arthropod hemocyanins.

In molluscan hemocyanins, domain D3 was abandoned in seven of the eight types of functional unit, namely in FU-a to FU-g [16, 43]. Indeed, a detailed analysis of the FU topology of the cephalopod hemocyanin decamer revealed that for spatial reasons such a component would not be compatible with the architecture of the cylinder wall [44]. However, in the internal collar of the gastropod hemocyanin decamer, the characteristic dipentameric ring made from ten copies of FU-h clearly requires domain D3 as a connecting element [11].

In this context it should be noted that according to a previous hypothesis based on gene sequence data, the molluscan hemocyanin quaternary structure originated from a primordial dipentameric FU-h ring [10]. This is further support for the present idea that the cupredoxin-like domain is an ancestral feature of hemocyanins.

In summary we describe cupredoxin-like domains encountered in arthropod and molluscan hemocyanins. Today they specifically function, within the various quaternary structures, as connecting elements and notably in arthropods they apparently contribute to allosteric force transfer. With respect to evolution we suggest that their original function was associated with copper loading of the active site. Thus, the cupredoxin-like domains open new insights in the adaptive evolution and function of hemocyanins.

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Tables

Table 1. Data collection statistics

Wavelength	0.97941 Å
Space group	$I2_13$
Unit cell	$a=b=c=251.0$ Å
Resolution limits	30 - 4.2 Å (4.2-4.0 Å)
No. unique reflections	21995 (2893)
R_{merge}	0.189 (0.847)
$I/\sigma I$	14.2 (3.5)
Completeness	96.4 % (96.4 %)
FOM after	
PHASER	0.51
RESOLVE	0.61

Figure legends

Fig. 1. Structure of KLH1-h-D3, related molluscan hemocyanin domains, and CBP

A, Cartoon representation of KLH1-h-D3 rainbow-coloured from blue (N-terminus) to red (C-terminus) with the conserved disulphide bridge shown as grey sticks. **B**, Diagram of KLH1-h-D3 showing the Greek key topology. Secondary structure elements are coloured as in **A**. The conserved disulphide bridge is drawn as a grey arrow. **C**, Tertiary structure-based alignment of cucumber basic protein (CBP) and KLH1-h-D3, combined with a multiple sequence alignment of KLH1-h-D3 and several other molluscan hemocyanin FU-h-D3 domains. Secondary structure elements are defined and coloured as in **A**, **B**. The conserved disulphide bridge is drawn as a grey line. Green asterisks mark amino acids coordinating copper at the type 1 center in CBP. Conserved amino acids and amino acids pertaining to the same substitution group in CBP and molluscan hemocyanin are coloured red or blue, respectively. Conserved amino acids and amino acids pertaining to the same substitution group within the molluscan hemocyanin sequences are coloured black or grey, respectively. The secondary structure (bar = α -helix, arrow = β -strand) was assigned from the crystal structure of KLH1-h-D3 and manually by comparison with *Octopus dofleini* hemocyanin FU-g and CBP.

Fig. 2. Cupredoxin-like domains in hemocyanins

A.1, Superposition of KLH1-h (red, domain D3) and CBP (pdb code: 2CBP, yellow). **A.2**, Superposition of KLH1-h-D3 and CBP. **B.1**, Superposition of *Limulus* hemocyanin subunit II (pdb code: 1LLA; red, domain #3) and CopC (pdb code: 2C9Q, yellow). **B.2**, Superposition of domain #3 of *Limulus* hemocyanin subunit II and CopC (with several loops of #3 omitted for clarity). **C.1**, *Streptomyces* tyrosinase (pdb code: 1WX4, blue) associated with the caddie protein (pdb code: 1WX4, yellow). Orange spheres represent copper atoms; CopC binds a second copper atom if present as a subunit dimer (not shown).

Fig. 3. Putative phylogenetic origin of the hemocyanin domains

The oxygen binding domain (white) of arthropod and molluscan hemocyanin presumably evolved independently from ancestral tyrosinases. To guarantee efficient copper loading to the active site in the oxygen binding domain a cupredoxin-like domain (dark grey) was added, endowing the subunit with an additional monocopper center. A high specificity for oxygen was established by an additional domain (grey), which shields the binuclear copper site from regular tyrosinase substrates, but still allows entry and exit of oxygen molecules. Moreover, this established allosteric regulation *via* slight tilting movements of the shielding domain which opens and closes the narrow oxygen diffusion channel [9]. Later in evolution of hemocyanin, the cupredoxin-like domain assumed new functions in assembly and allostereism of the hemocyanin multimers, and its monocopper center became obsolete and was lost. Today the cupredoxin-like domain is still ubiquitous in the 75 kDa arthropod hemocyanin subunits, whereas in molluscan hemocyanin it is restricted to FU-h where it marks the tail of the 400 kDa subunit. Nevertheless, this means that each molluscan hemocyanin dodecamer carries 20 copies of this peculiar domain [44].

Fig. 1: Jaenicke et al, Cupredoxin-like domains in hemocyanins

Fig. 2: Jaenicke et al, Cupredoxin-like domains in hemocyanins

Fig. 3: Jaenicke et al, Cupredoxin-like domains in hemocyanins