Cupredoxin-like domains in haemocyanins

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INTRODUCTION

Haemocyanins are giant oxygen transport proteins found in the haemolymph of many arthropods and molluscs. They reversibly bind oxygen, often with very high co-operativity, at bi-nuclear type 3 copper centres (types 1 and 2 are mononuclear) [1,2]. The copper centres of all haemocyanins are very similar, with the two copper atoms being bound to a four α-helix bundle ligated by three histidine residues each [3–6]. As type 3 copper proteins, haemocyanins are structurally related to the phenol oxidases, tyrosinases and catecholoxidases; all these proteins bind oxygen, often with very high co-operativity, at bi-nuclear copper centres of all haemocyanins are very similar, with the two type 3 copper centres (types 1 and 2 are mononuclear) [1,2]. The copper-free domain (types, each of an approximate molecular mass of 75 kDa and eight-hexamers) assembled from several paralogous subunit units) of approx. 50 kDa each, termed FU-a to FU-h. Thus 160 oxygen-binding sites exist in a molluscan haemocyanin didecamer such as KLH1 [KLH (keyhole-limpet-haemocyanin) isoform 1] [11]. KLH is a mixture of two isoforms, KLH1 and KLH2 (KLH isoform 2), isolated from the haemolymph of the giant keyhole limpet Megathura crenulata, and widely used as an immunological tool [12]. In contrast, arthropod haemocyanins occur as single hexamers or oligo-hexamers (two-, four-, six- and eight-hexamers) assembled from several paralogous subunit types, each of an approximate molecular mass of 75 kDa and with a single active site [13]. The largest arthropod haemocyanin type occurs in horseshoe crabs such as Limulus polyphemus; it is a cubic eight-hexamer of 25 nm in length, with 48 oxygen-binding sites, which has been structurally analysed in detail previously [14]. From a functional viewpoint, the arthropod haemocyanin subunit corresponds to the molluscan haemocyanin FU (functional unit), each showing a single active site. Representative crystal structures of the molluscan haemocyanin FU and the arthropod haemocyanin subunit are available [3–6], but questions about the evolutionary connection between the two haemocyanin families remain. The arthropod haemocyanin 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 four-α-helix bundle motif; and the C-terminal domain 3 folds into a seven-stranded anti-parallel β-sheet, a feature interpreted previously as being immunoglobulin-like [15]. For the basic hexamer it is unknown whether domain 3 has a role other than structural, but in oligo-hexamers it certainly contributes to allosterism [14]. Most molluscan haemocyanin FUs fold into only two structural domains: the N-terminal core domain [KLH1-h (KLH1 FU-h)-D1], which contains the oxygen-binding site in a four-α-helix bundle and therefore corresponds to domain 2 in arthropod haemocyanin (although their remaining tertiary structure elements are very different); and the C-terminal domain (KLH1-h-D2), which is dominated by a six-stranded β-sandwich and shields the entrance to the active site. Hence its role in molluscan haemocyanin is comparable with that of domain 1 in arthropod haemocyanin [9].

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

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Abbreviations used: CBP, cucumber basic protein; CopC, Pseudomonas syringae pv. tomato copper carrier CopC; D1/D2/D3, functional unit domains 1/2/3 of molluscan haemocyanin; FU, functional unit; KLH, keyhole-limpet (Megathura crenulata) haemocyanin; KLH1/2, KLH isoform 1/2; KLH1-h, KLH1 FU-h; OdF-h, Octopus dofleini haemocyanin FU-g.

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The structural co-ordinates reported for KLH1-h will appear in the Protein Data Bank under accession code 3L6W.
EXPERIMENTAL

Protein purification

KLH1 was purified from *M. crenulata* haemolymph serum (Biosyn) by selective dissociation of KLH2 as described previously [17]. Briefly, overnight dialysis against 2% (w/v) ammonium molybdate (pH 5.7) at 4 °C results in selective dissociation of KLH2 into its subunits, whereas KLH1 remains intact as multimers. KLH isoforms were then separated by centrifugation of KLH2 into its subunits, whereas KLH1 remains intact as multimers at 30000 g for 4 h at 4 °C, leaving KLH2 subunits in the supernatant. The pellet was dissolved in stabilizing buffer (50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 5 mM MgCl2 and 5 mM CaCl2) and then dialysed 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). 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®; Carl Roth) to a final concentration of 10 mM. KLH1-h was purified from the resulting protein fragments by anion-exchange chromatography on a 25 ml Q-Sepharose column (Pharmacia). To this end approx. 50 mg of protein fragments were applied to the column equilibrated with 20 mM Tris/HCl (pH 8.0). Fragments were then eluted using a linear NaCl gradient, from 160 mM to 400 mM NaCl, in the same buffer at a flow rate of 0.5 ml/min (see Supplementary Figure S1A available at http://www.BiochemJ.org/bj/426/bj4260373add.htm). The KLH1-h content and purity of the eluting fractions was assayed by SDS/PAGE (10% gels) stained with Coomassie Brilliant Blue [18] and by immunoelectrophoresis using rabbit antibodies against dissociated KLH1 [19,20] (see Supplementary Figures S1A and S1B). Absorption spectra were measured with a U-3000 spectrophotometer (Hitachi) in quartz cuvettes with a 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 Institute (Buffalo, NY, U.S.A.) and then refined for the sitting-drop setup [21]. Crystals were grown on microbridges in Linbro plates covered with cover slides (Hampton Research). Briefly, 5 μl of a 15 mg/ml KLH-h solution was mixed with 5 μl of reservoir solution (50 mM sodium citrate buffer, pH 4.0, containing 9.0% (v/v) PEG [poly(ethylene glycol)] 1000 and 50 mM K3HPO4) 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 (Supplementary Figure S1D).

Structure determination

The crystals were cryo-cooled in liquid nitrogen after being cryo-protected by soaking them in reservoir solution containing 30% (v/v) glycerol. The crystals were diffraction to 4.0 Å resolution at the PX-II beamline of the Swiss Light Source, Villigen, Switzerland. Data processing indicated I-centred cubic symmetry and data statistics are shown in Table 1. Molecular replacement with PHASER [22] using the structure of the homologous OdH-g (FU-g from *Octopus dofleini* haemocyanin; PDB code 1JS8 [3]) as the search model identified the space group as I 21 3 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 the RESOLVE software [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. Cα atoms for the C-terminal domain were placed using the program COOT [25] and XFIT [26] and were submitted to the Dali server [27,28] to find similar structures. Strikingly, the closest match (Z-score of 7.4) was the structure of CBP (cucumber basic protein; PDB code 2CBP [29]) which is a type 1 copper protein. On the basis of the similarity of KLH1-h with the folds of OdH-g and CBP, 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 Cα trace (see Supplementary Figure S2 available at http://www.BiochemJ.org/bj/426/bj4260373add.htm).

RESULTS AND DISCUSSION

Cα trace of KLH1-h

KLH1-h was released from the KLH1 subunit by limited proteolysis, purified preparatively, crystallized and its three-dimensional structure solved to a resolution of 4 Å. This allowed tracing of its Cα backbone, which encompassed 490 amino acids (Figures 1 and 2A.1). It folded into three well-defined structural domains. The N-terminal core domain (KLH1-h-D1) and the central β-sandwich domain (KLH1-h-D2) exhibited the canonical molluscan haemocyanin 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) mainly contained β-strands (Figures 1 and 2A.1). It shares a striking and completely unexpected structural similarity with CBP, which has a type 1 copper site (Figures 1C and 2A). CBP is a member of the cupredoxin protein family and belongs to the subgroup plantacyanins [29]. The cupredoxin-like fold of KLH1-h-D3 resembled 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 RMSD (root mean square deviation) between KLH1-h-D3 and CBP was 2.5 Å. Moreover, KLH1-h-D3 and CBP showed a disulfide bridge at identical positions, with the two cysteine residues separated by 20 amino acids. On the other hand, the identity between the sequences is

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<td>Figure of merit after PHASER</td>
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Table 1 Data collection statistics

Values in parentheses are for highest-resolution shell.
Cupredoxin-like domains in haemocyanins

Figure 1 Structure of KLH1-h-D3, related molluscan haemocyanin domains and CBP

(A) Cartoon representation of KLH1-h-D3 rainbow-coloured from blue (N-terminus) to red (C-terminus) with the conserved disulfide 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 disulfide bridge is shown as a grey arrow. (C) Tertiary structure-based alignment of CBP and KLH1-h-D3, combined with a multiple sequence alignment of KLH2-h-D3 and several other molluscan haemocyanin FU-h-D3 domains. Secondary structure elements are defined and coloured as in (A) and (B). The conserved disulfide bridge is drawn as a grey line. Green asterisks mark amino acids co-ordinating copper at the type 1 centre in CBP. Conserved amino acids and amino acids pertaining to the same substitution group in CBP and molluscan haemocyanin are coloured red and blue respectively. Conserved amino acids and amino acids pertaining to the same substitution group within the molluscan haemocyanin sequences are coloured black and grey respectively. The secondary structure (wave, α-helix; arrow, β-strand) was assigned from the crystal structure of KLH1-h-D3 and manually by comparison with OdH-g and CBP. AcH, Aplysia californica haemocyanin; HtH, Haliotis tuberculata haemocyanin; NnH, Nucula nucleus haemocyanin.

only approx. 10% and, consequently, they show specific physicochemical differences (Figure 2A.2). In particular, the pl of KLH1-h-D3 is 6.3 (predicted from the sequence), whereas the pl of CMP is 11 [29]. Moreover, this low-degree of sequence identity prohibited the use of CBP as a template for molecular modelling of the KLH1-h-D3 sequence. Therefore the present considerations are restricted to the Cα 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 there was a second copper centre 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 stabilized by a constrained His–His–Cys co-ordination environment. Unfortunately the 4 Å resolution, as presently achieved, did not resolve the binuclear copper centre 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 an appropriate position to allow copper to bind to the protein in this type of co-ordination environment. Consequently, we assume that KLH1-h-D3 is devoid of a type 1 copper centre and propose this might have been lost at an early stage of molluscan haemocyanin evolution (Figure 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 disulfide bridge in KLH1-h-D3 and the adrenaline ectodomain at a conserved position is remarkable, because in CBP it is thought to stabilize the type 1 copper centre and is a typical feature of phytocyanins [29]. Therefore, as no type 1 copper centre is present, it might be required for the structural integrity of the entire cupredoxin-like fold. In the present context, this disulfide 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 colour due to a broad absorption peak in the visible range with a maximum at 554 nm (see Supplemental Figure S3 available at http://www.BiochemJ.org/bj/426/bj4260373add.htm). This peak disappears completely upon deoxygenation of KLH1-h, which is typical for all haemocyanins and has been thoroughly characterized as resulting from the π* → Cu(II) charge transfer transition, where π* is the peroxide orbital orthogonal to the Cu2O2 plane of the type 3 copper centre [33]. Consequently we can rule out the possibility that the cupredoxin-like domain of KLH1-h contains any additional ligands or active sites that absorb light in the visible range.

Cupredoxin-like domains in haemocyanin

The unexpected presence of two copper protein folds within a molluscan haemocyanin functional unit raises the question as to whether the cupredoxin-like domain is a structural feature only encountered in FU-h of molluscan haemocyanins. Moreover, the structural and possibly functional role of this peculiar tail domain of the molluscan haemocyanin subunit should be addressed. A database search for structural correlates of the...
C-terminal domain 3 of arthropod haemocyanin unearthed another member of the cupredoxin family, the bacterial CopC protein [34] (Figure 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 monocopper-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 haemocyanins (Figures 2 and 3), because, as already mentioned, there are convincing arguments that both haemocyanin families evolved convergently from tyrosinase and phenol oxidase-like ancestors [8]. Indeed, haemocyanins can be considered as permanently inactivated phenol oxidases [1,36]. From the present results it now appears that both haemocyanin families evolved from proteins containing a type 3 and a type 1 copper site. In principle, the combination of several copper centres is not unusual. Blue oxidases, such as laccase, ascorbate oxidase and ceruloplasmin, combine a mixed type 2 and type 3 copper centre along with a type 1 copper centre [37–39]. However, the combination of a pure type 3 copper centre and a type 1 copper centre in a single protein, as we suggest in the present paper for ancestral haemocyanins, is unprecedented. Moreover, it is remarkable that the copper 1 site seems to have subsequently been lost in both haemocyanin families.

Possible role(s) of the cupredoxin-like haemocyanin domains

If a functional type 1 copper site existed in ancestral haemocyanins, one might suppose that its co-operation with the type 3 copper centre could significantly improve oxidative processes. However, the large distance between the two copper centres (more than 30 Å in both arthropod haemocyanin and FU-h) does not support this scenario. In blue oxidases in which an electron transfer between copper centres is documented, they are at maximum distance of approx. 13 Å apart [39]. Thus a similar coupling of the two sites in the framework of an ancient enzymatic function seems unlikely in the case of both haemocyanins.

A more plausible explanation for the presence of the cupredoxin-like domain in haemocyanins may be deduced from *Streptomyces tyrosinase*, which has a haemocyanin-like type 3 copper site. The structure of this tyrosinase complexed with its ‘caddie’ protein (MelC1) was solved previously [7] (Figure 2C). The small copper-binding caddie protein is co-expressed 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 a copper chaperone for the tyrosinase. Copper insertion into proteins is an incompletely understood phenomenon and little is known about copper loading of type 3 copper proteins. However, 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 haemocyanins, the ancestral tyrosinases that gave rise to the caddie.
of forming large macromolecular assemblies. In arthropods, the basic quaternary structure is the hexamer. In this structure domain 3 (the cupredoxin-like domain) is probably not directly required, apart from generally stabilizing the individual subunits, because it is not involved in subunit–subunit bonding [4]. However, in four- and eight-hexamer chelicerate haemocyanins, domain 3 is directly involved in a variety of inter-hexamer interfaces that might also transfer allosterism [14]. Even in the ‘simple’ case of a spider two-hexamer, formed from two hexamers joined 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 allosterism of oligo-hexameric arthropod haemocyanins.

In molluscan haemocyanins, 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 haemocyanin 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 haemocyanin decamer, the characteristic diphenolic ring made from ten copies of FU-h clearly requires 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 haemocyanin quaternary structure originated from a primordial diphenolic diphenolic FU-h ring [10]. This is supported further by the present idea that the cupredoxin-like domain is an ancestral feature of haemocyanins.

In summary we describe cupredoxin-like domains encountered in arthropod and molluscan haemocyanins. 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 structure of the cupredoxin-like domains offer new insights into the adaptive evolution and function of haemocyanins.

AUTHOR CONTRIBUTION
Elmar Jaenicke, Jürgen Markl and Heinz Decker designed the research. Elmar Jaenicke, Kay Büchler and Thomas Barends performed the research. All authors analysed the data. Elmar Jaenicke, Jürgen Markl and Heinz Decker wrote the paper.

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REFERENCES
**SUPPLEMENTARY ONLINE DATA**

**Cupredoxin-like domains in haemocyanins**

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Figure S1  Purification and crystallization of KLH1-h

(A) After proteolytic cleavage with V8 protease, KLH1 was applied to a Q–Sepharose column and eluted with an NaCl gradient. (B) Fractions containing KLH1-h were identified by SDS/PAGE. M, molecular-mass markers; C, KLH1 sample. (C) KLH1-h-containing fractions 48–51 were concentrated and their purity again checked by immunoelectrophoresis. (D) Crystals were grown from concentrated pure fractions of KLH1-h. The scale bar denotes 200 μm.

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The structural co-ordinates reported for KLH1-h will appear in the Protein Data Bank under accession code 3L6W.
Figure S2  Sequence comparison of KLH1-h with related proteins

Structural analysis of KLH1-h revealed the structural relationship of its major part to OdH-g and its tail to CBP. Whereas the relationship with Octopus haemocyanin is easily seen by sequence alignment, the sequence identity with CBP is as low as 10%. Consequently, before the crystal structure of KLH1-h was solved, sequence database searches using the tail domain sequence (D3) of KLH1-h did not retrieve any member of the cupredoxin family. The aligned sequences of OdH-g and CBP (blue, OdH-g; green, CBP) are presented here concatenated to allow easy comparison with the KLH1-h sequence. Conserved amino acids and those conserved within the same similarity group are coloured black and light grey respectively. Cysteine residues forming disulfide bonds are coloured yellow. The leucine residue of D2, which obstructs the entrance to the active site, is coloured green. Histidine residues forming the type 3 copper centre are coloured red, and the amino acids forming the type 1 copper site of CBP are coloured light blue. Note that in the tail domain of KLH1-h, there are no amino acids in appropriate positions that could form a type 1 copper site. It is therefore concluded that D3 lacks such a site. The secondary structure (bar, α-helix; arrow, β-strand) of KLH1-h was predicted from the crystal structure and manually assigned by comparison with OdH-g and CBP respectively. The secondary structure elements are numbered according to OdH-g [1]. It should be noted that strand β1 and helix α6 are not observed in KLH1-h. The following sequences were used: KLH1-h, Genbank® AJ698341 [1]; OdH-g [2], PDB code 1JS8; and CBP, PDB code 2CBP [3].
Owing to a broad absorption band with a maximum at 554 nm oxygenated KLH1-h has a purple–blue colour. Upon deoxygenation this absorption band disappears completely proving that it is caused by the oxygen copper complex of the type 3 copper active site [4]. For the spectra a 2.5 mg/ml solution of KLH1-h was measured in 50 mM Tris/HCl (pH 8.0).

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