

Research Communication

The Refined Structure of Functional Unit h of Keyhole Limpet Hemocyanin (KLH1-h) Reveals Disulfide Bridges

Elmar Jaenicke¹, Kay Büchler², Heinz Decker¹, Jürgen Markl² and Gunnar F Schröder³

¹Institut für Molekulare Biophysik, Johannes Gutenberg-Universität, Jakob Welder Weg 26, Mainz, Germany

²Institut für Zoologie, Johannes Gutenberg-Universität, Müllerweg 6, Mainz, Germany

³Institut für Strukturbiologie und Biophysik (ISB-3), Forschungszentrum Jülich, Jülich, Germany

Summary

Hemocyanins are multimeric oxygen-transport proteins in the hemolymph of many arthropods and mollusks. The overall molecular architecture of arthropod and molluscan hemocyanin is very different, although they possess a similar binuclear type 3 copper center to bind oxygen in a side-on conformation. Gastropod hemocyanin is a 35 nm cylindrical didecamer (2×10 -mer) based on a 400 kDa subunit. The latter is subdivided into eight paralogous “functional units” (FU-a to FU-h), each with an active site. FU-a to FU-f contribute to the cylinder wall, whereas FU-g and FU-h form the internal collar complex. Atomic structures of FU-e and FU-g, and a 9 Å cryoEM structure of the 8 MDa didecamer are available. Recently, the structure of keyhole limpet hemocyanin FU-h (KLH1-h) was presented as a C_{α} -trace at 4 Å resolution. Unlike the other seven FU types, FU-h contains an additional C-terminal domain with a cupredoxin-like fold. Because of the resolution limit of 4 Å, in some loops, the course of the protein backbone could not be established with high certainty yet. Here, we present a refined atomic structure of FU-h (KLH1-h) obtained from low-resolution refinement, which unambiguously establishes the course of the polypeptide backbone and reveals the disulfide bridges as well as the orientation of bulky amino acids. © 2011 IUBMB

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Abbreviations CopC, *Pseudomonas syringae* (pv. tomato) copper carrier CopC; D1/D2/D3, functional unit domains 1/2/3 of molluscan hemocyanin; FU, functional unit; KLH, keyhole-limpet (*Megathura crenulata*) hemocyanin; KLH1, KLH isoform 1; KLH1-h, KLH1 FU-h

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Address correspondence to: Prof. Elmar Jaenicke, Institut für Molekulare Biophysik, Johannes Gutenberg-Universität, Jakob Welder Weg 26, 55128 Mainz, Germany. Tel: +49/6131/3923567, Fax: +49-6131-3923557. E-mail: elmar.jaenicke@uni-mainz.de

INTRODUCTION

Hemocyanins are extracellular oxygen-transport proteins frequently found in the hemolymph of arthropod and mollusc species (1–3). As type 3 copper proteins and their active sites are embedded in a four α -helix bundle with six histidine residues, which coordinate two copper atoms (4–8). Between them one molecule oxygen is reversibly bound in side-on (μ - η^2 : η^2) coordination (4, 7, 9, 10). In most hemocyanins, oxygen binding is highly cooperative and tightly controlled by allosteric effectors (1, 2, 11, 12).

Unlike their active site, the general structures of hemocyanins from arthropods and molluscs are very different, which gave rise to the assumption that hemocyanins of both phyla form two distinct protein families (13, 14). Arthropod hemocyanins form hexamers or oligo-hexamers from several paralogous subunit types, which possess three distinct domains. Each subunit bears a single active site and has a molecular mass of 75 kDa (5, 15). In contrast, mollusc hemocyanins form hollow cylinders made up from 10 copies of the same subunit type. These cylinders either exist as decamers or aggregate further to form didecamers or multidecamers. The mollusc hemocyanin subunit has a molecular mass of 350–400 kDa and is a concatenation of seven or eight paralogous FUs of approximately 50 kDa each (FU-a to FU-h) on a single polypeptide chain (2, 16, 17). Each FU is typically made-up from two domains and bears one active site. Thus, from a functional point of view, the arthropod subunit and the mollusc FU correspond to each other.

Crystal structures of molluscan hemocyanin FUs representing different topological positions in the quaternary structure (wall, inner collar, outer collar) are available (4–8). The standard molluscan hemocyanin FU (FU-a to FU-f) folds into two domains. The N-terminal core domain is predominantly α -helical and bears the active site in a four- α -helix bundle, whereas the C-terminal domain is dominated by a six-stranded β -sandwich and shields the entrance to the active site (18). From a functional point of view, the central domain of arthropod hemocyanin and

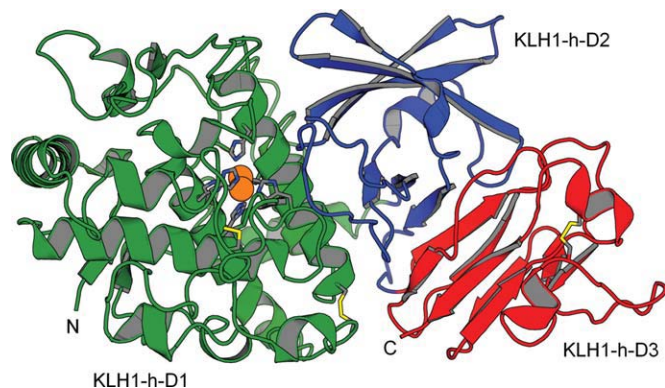


Figure 1. Structure of KLH1-h. The structure of KLH1-h is composed of three distinct domains. The N-terminal domain (KLH1-h-D1, green) is predominantly made up from α -helices and harbors the type 3 copper active site. The adjacent domain (KLH1-h-D2, blue) is composed of a β -sandwich and covers the entrance to the active site. Both, KLH1-h-D1 and KLH1-h-D2, are present in all molluscan hemocyanin FUs. Unlike other FUs, KLH1-h contains a 100 amino acid C-terminal domain (KLH1-h-D3, red), which has a cupredoxin-like fold, but seems to have lost its active site in the course of evolution. Color-coding: carbon atoms (grey), nitrogen atoms (blue), copper atoms (orange), sulfur atoms (yellow). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the N-terminal domain of mollusc hemocyanin perform the same function because both bear the active site (19).

The only exception to the standard architecture of mollusc FUs is FU-h, which forms the outer collar of the decamer (20). Unlike all other FUs, FU-h has an additional C-terminal extension of approximately 100 amino acids, which marks the very end of the molluscan hemocyanin subunit polypeptide (21). The sequence of this extension could not be related to any other sequence or fold in the databases for a long time. Recently, we reported the structure of keyhole limpet hemocyanin (KLH1-h), which is FU-h of isoform 1 of keyhole limpet (*Megathura crenulata*) hemocyanin, at 4.0 Å resolution as a C_α -trace (6). KLH1-h is a widely used immunological tool (22).

The structure of KLH1-h shows three well-defined domains (Fig. 1). The N-terminal core domain (KLH1-h-D1) contains the type 3 copper active site as in other known FU structures (4, 8). The central domain (KLH1-h-D2) is equivalent to the C-terminal domain of other FUs (FU-a to FU-g) and contains a β -sandwich as its main structural element. The additional C-terminal extension of KLH1-h-D3 folds into one distinct domain with a very high structural similarity to cupredoxins (6). The closest structural neighbor for KLH1-h-D3 identified by submitting the C_α -trace to the DALI server is cucumber basic protein. It shares a sequence identity of only 10% with KLH1-h-D3 and belongs to the cupredoxin subfamilies of phycocyanins and plantacyanins (23–25). However, KLH1-h-D3 lacks all residues, which form the type-1 active site in cupredoxins. Thus, owing

to its similar fold but lack of the active site, the C-terminal domain of KLH1-h was named “cupredoxin-like domain” (6).

By using crystal structures of FU-g and cucumber basic protein as templates in a homology modeling approach, the course of the polypeptide backbone could be established in most parts of FU-h (6). The resulting structure could be stably docked, by automated rigid-body fitting, to a 9 Å cryo-electron microscopical structure of the KLH1 decamer, which revealed the architecture of the outer collar (20). This was, especially, straightforward, because the morphological unit of FU-h in the crystal is a dimer, which, according to the docking results, exactly corresponds to the FU-h dimer naturally occurring in the KLH1 molecule (Fig. 2) (20).

However, due to the resolution limit of 4.0 Å, some loops in the domain KLH1-h-D1, KLH1-h-D2, and considerable parts of KLH1-h-D3 remained uncertain with respect to the exact course of the backbone. An offset of one or two amino acids could not be excluded, notably in the cupredoxin-like domain in which the sequence similarity with the template (cucumber basic protein) was very low (6). Consequently, in the description of the putative inter-FU contacts within the KLH1 molecule, the amino acid side chains of the cupredoxin-like domain were not considered (20).

Here, we present a refinement of 4.0 Å dataset by low-resolution refinement methods. It reveals disulfide bridges and the position of bulky amino acids. Therefore, finally the course of the polypeptide backbone in all parts of FU-h can be unambiguously established.

MATERIAL AND METHODS

Previous Structure Determination

The structure of KLH1-h was determined as described earlier (6). Briefly, KLH1-h was prepared from purified KLH1 by limited proteolysis with V8 protease and subsequently purified by ion exchange chromatography (20, 26). KLH1-h was then crystallized at pH 4.0 in sodium citrate buffer in the presence of 9.0% PEG 1000 and 50-mM K_2HPO_4 . A dataset with resolution limit of 4.0 Å was recorded from a 0.1 mm crystal under cryo-conditions at the PX-II beamline of the Swiss Light Source, Villingen, Switzerland. The structure was solved as a C_α -trace by molecular replacement from the dataset using the structure of *Octopus dofleini* hemocyanin FU-g (PDB ID: 1JS8) as search model.

The previous structure is available in the Protein Data Bank under accession code 3L6W.

Present Structural Refinement

An homology model of KLH1-h based on *Octopus dofleini* hemocyanin FU-g (PDB ID: 1JS8) and cucumber basic protein (PDB ID: 2CBP) was built using MODELLER 9v8 (27). This model was then refined with the DEN refinement method using CNS with the homology model as the DEN reference model (28, 29). The lowest R_{free} value was obtained with a γ -parame-

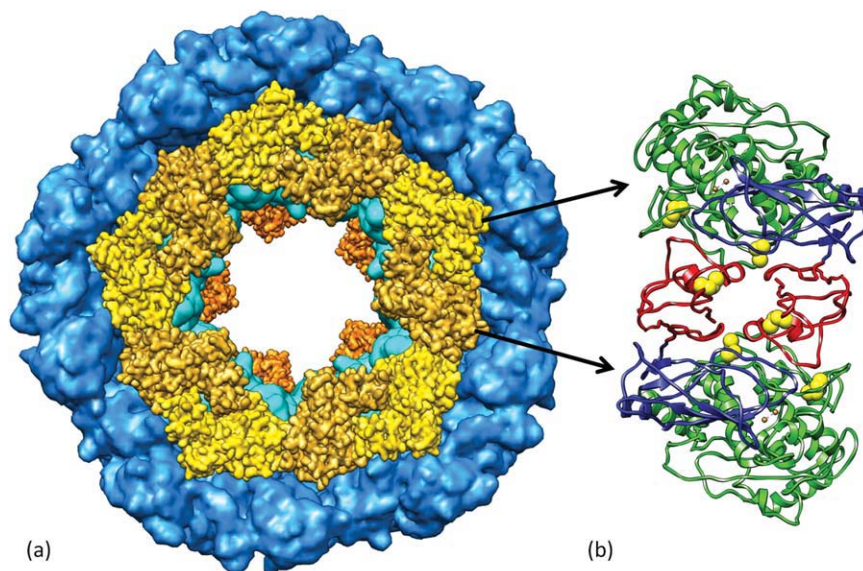


Figure 2. Structure of KLH1. KLH1 (top-view) density map simulated from the molecular model (a), and the refined FU-h crystallographic dimer (b). The wall (blue) and the arc (FU-g, cyan) have been simulated at 9 Å resolution, and the upper collar (FU-h, yellow/golden) at 4 Å resolution, respectively. Note the position of the disulfide bridges (yellow) in the FU-h dimer. Figure according to Jaenicke et al (2010)/Gatsoyiannis & Markl (2009) and this study (6, 20). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ter of 1.0 and a DEN weight (w_{DEN}) of 100. The loop region between residues 3307 and 3312 was manually modeled into the density by using COOT (30). The obtained model was energy minimized (without a crystallographic target function) for 1,000 steps with all hydrogen atoms included. The minimization included electrostatic interactions between all backbone atoms to improve the hydrogen bonding network, statistical Ramachandran restraints to improve the backbone geometry, and weak position restraints on all C_{α} -atoms to maintain overall absolute positioning. Additional restraints were applied to the histidines at the copper binding sites to improve the coordination of the copper atoms. The final structure was obtained by another standard DEN refinement round using this geometry optimized structure as a reference model; the lowest R_{free} value was obtained with $\gamma = 0.6$ and $w_{\text{DEN}} = 100$.

The refined structure has been deposited in the Protein Data Bank under accession code 3QJO.

RESULTS AND DISCUSSION

Refined Structural Model of KLH1-h

Using a homology model of KLH1-h based on the structures of FU-g (*Octopus dofleini*) and cucumber basic protein and subsequent refinement with methods developed to refine low-resolution structures, it was now possible to calculate a more detailed model of KLH1-h (Fig. 1, Table 1) (4, 23, 29).

To this end prominent “landmark” features such as disulfide bridges and bulky amino acid residues were used to avoid offsets in the amino acid backbone. Our refinement clearly shows that all three disulfide bridges present in KLH1-h were easily

identified in the electron density. Two of them are located in the N-terminal core domain and one in the cupredoxin-like domain (Fig. 3a). The disulfide bridge in the cupredoxin-like domain is also present in cucumber basic protein and is considered as a distinguishing feature of phytocyanins (31). In cucumber basic protein, the disulfide bridge is in the direct vicinity of the active site with one cysteine being the neighbor of a copper coordinating histidine of the active site (32). In the cupredoxin-like domain of hemocyanin, it seems that this disulfide bridge has been conserved to maintain the integrity of the tertiary

Table 1
Crystallographic/refinement parameters

Space groups	I2 ₁ 3
Cell dimensions	
a,b,c (Å)	251.02
α,β,γ (°)	90.0
Refinement	
Resolution (Å)	4.0
No. reflections	22294
$R_{\text{work}}/R_{\text{free}}$	27.1/29.3
No. atoms	7994
Average B-factor	142.1
R.m.s. deviations	
Bond lengths (Å)	0.0034
Bond angles (°)	0.855
Ramachandran statistics	
Favored/allowed/outlier (%)	73.0/9.4/7.6

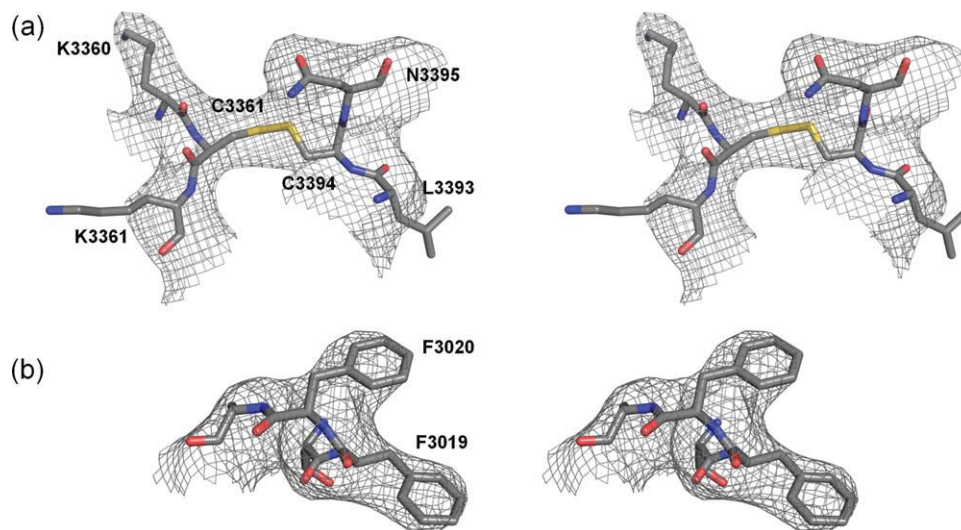


Figure 3. Typical examples of disulfide bridges and bulky amino acids in the electron density. (a) The disulfide bridge in the cupredoxin-like domain (D3) connecting C3361 and C3394 can be clearly discerned in the electron density (1σ contour level, stereo view). This disulfide bridge is also present in cucumber basic protein, which is the closest structural neighbor of D3, and is considered as a distinguishing feature of phytoeyanins (31). (b) Almost all aromatic amino acids (Trp, Tyr, Phe) either lie within consecutive regions of the electron density or can even be identified as protrusions in the electron density. Phenylalanines F3020 and F3019 are typical examples, which can be identified as protrusion in the electron density (1σ contour level, stereo view), and serve as landmarks to clearly establish the course of the polypeptide backbone. Color-coding: carbon atoms (grey), nitrogen atoms (blue), copper atoms (orange), and sulfur atoms (yellow). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

structure, whereas the active site has been lost in the course of evolution. Bulky amino acid side chains such as tryptophane, tyrosine, phenylalanine and histidine can be detected in electron densities even at 4.0-Å resolution, when they are not part of continuous regions of electron density along the polypeptide backbone but directed away from the latter. In this case, they are visible as protrusion in the electron density (Fig. 3b). Along the course of the polypeptide backbone in KLH1-h, several bulky amino acid residues can be easily identified.

For further usage of the structural model (e.g., as template for homology modeling), it has to be kept in mind that the orientation of all side chains and backbone atoms in the present model result from the optimization procedure in the refinement process, because the electron density does not provide sufficient detail at 4.0-Å resolution. The only exception is the bulky amino acid side chains, where, in some cases, a specific rotamer could be identified.

The present refinement confirms and enhances the previously published C_α -trace of KLH1-h. The course of the polypeptide backbone and features such as disulfide bridges and bulky amino acids now could be traced without a problem. This is of special relevance with respect to using the FU-h structural model as component of a molecular model of the quaternary structure (Fig. 2): With the exact position of each amino acid in the FU-h backbone now being traced, inter-FU contacts involving FU-h can be predicted more convincingly.

Cupredoxin-like Domains in Hemocyanins

Structural comparison of the C-terminal domain of arthropod hemocyanin with known structures of members of the cupredoxin family revealed an unexpected similarity with the CopC protein, which is a cupredoxin acting as copper transporter in *Pseudomonas syringae* (pv. tomato) (6, 33, 34). Although arthropod and mollusc hemocyanins represent two protein families, which evolved convergently from tyrosinase and phenoloxidase-like ancestors (35), it is remarkable that a cupredoxin-like domain can be found in both hemocyanin architectures. In both families, the cupredoxin-like domain has lost the type 1 copper active site in the course of evolution and, thus, seems to be present in hemocyanins only for structural reasons. However, it has been suggested that in the beginning of the evolution of the hemocyanin protein family, the cupredoxin-like domain may have served as a copper chaperone facilitating copper loading to the type 3 copper active site (6).

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