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Rapana thomasiana hemocyanin (RtH): Comparison of the two isoforms, RtH1 and RtH2, at 19 Å and 16 Å resolution

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Abstract

Three-dimensional (3D) reconstructions of the two 8.4 MDa *Rapana thomasiana* hemocyanin isoforms, RtH1 and RtH2, have been obtained by cryoelectron microscopy of molecules embedded in vitreous ice and single particle image processing. The final 3D structures of the RtH1 and RtH2 didecamers at 19 Å and 16 Å resolution, respectively, are very similar to earlier reconstructions of gastropodan hemocyanins, revealing structural features such as the obliquely oriented subunits, the five- and two-fold symmetrical axes. Three new interactions are defined; two of them connecting the arch and the wall while the third is formed between the collar and the wall. The collar–wall connection and one of the arch–wall connections are positioned between two individual subunit dimers, while the second arch–wall connection is located between two subunits within the subunit dimer. All three interactions establish connections to the first tier of the wall. Furthermore, for each interaction we have allocated two first tier functional units most likely involved in forming the connections.

Keywords: Cryoelectron microscopy; *Rapana thomasiana*; Hemocyanin; Mollusc

1. Introduction

Hemocyanins (Hcs) are giant copper-containing multi-subunit respiratory proteins, freely dissolved in the hemolymph of many arthropod and mollusc species (for review, see van Holde and Miller, 1995). The basic quaternary structure of the molluscan hemocyanin is a hollow cylindrical molecule, about 350 Å in diameter, with an internal collar complex (composed of arch and collar elements). The decamer (ca. 4 MDa) is the smallest cooperative unit, and consists of identical structural subunits organized in an anti-parallel manner as stable homodimers (the repeating unit) (Orlova et al., 1997). Five such dimers form the decamer and thus give rise to the general C5 pointgroup symmetry of the molecule. In cephalopods, the only hemocyanin quaternary structures found are symmetrical decamers (D5 pointgroup symmetry) with a central collar complex, whereas chiton hemocyanins are asymmetrical decamers (C5 pointgroup symmetry) with the collar complex located within the first and second tier of the decameric wall. In most gastropods, two asymmetrical decamers assemble face-to-face forming didecamers (ca. 8 MDa; D5 pointgroup symmetry) with the collar complex located at each end (van Holde and Miller, 1995).

The subunit of molluscan hemocyanins is a 350–420 kDa polypeptide chain folded into seven (cephalopod) or eight (gastropods and some cephalopods) globular functional units (FUs), termed *abcdefgh* with FU-*a* at the N-terminus, connected covalently by linker regions of 10–15 amino acid residues. The ''wall'' of the cylindrical molecule is built up by the FUs *abcdef*, whereas the FUs *g* and *h* form the collar complex. Each FU carries one active site capable of reversibly

Abbreviations: Hc, hemocyanin; FU, functional unit; KLH, keyhole limpet hemocyanin; HtH, *Haliotis tuberculata* hemocyanin; RtH, *Rapana thomasiana* hemocyanin; 3D, three-dimensional; CTF, contrast transfer function; FSC, Fourier shell correlation

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binding one dioxygen molecule. The oxygen-binding unit is a pair of Cu atoms, each coordinated to the protein by three histidine side chains (Ling et al., 1994).

More than 30 years ago, the keyhole limpet hemocyanin (KLH) from the marine gastropod *Megathura crenulata* was first found to possess immunostimulatory properties. Since then, molluscan hemocyanins have been of interest in both research and clinical therapy. Moreover, KLH is widely used as a hapten carrier for tumour cell peptides and has in clinical studies proven to be a powerful tool for the treatment of bladder carcinoma, indicating the broad clinical value of this protein (for review, see Harris and Markl, 1999). Most hemocyanins are glycoproteins, and the immunological response of KLH has often been related to the specific carbohydrate content and composition rather than the protein alone. More recently, the *Haliotis tuberculata* hemocyanin (HtH) has been considered as a potential substitute of KLH. *H. tuberculata* is the European abalone and, like *M. crenulata*, belongs to the gastropod subclass of the Prosobranchia. Glycosylation studies of KLH and HtH showed that these two macromolecular assemblies differ in their carbohydrate content and monosaccharide composition (Idakieva et al., 2004). Whether or not hemocyanins from other molluscs might function equally well as immunological agents as KLH remains to be established, but it is likely that this is the case.

Rapana thomasiana (prosobranch gastropod) is a marine snail living along the west coast of the Black Sea. The *R. thomasiana* hemocyanin (RtH) exists in the hemolymph of this organism as two distinct isoforms, termed RtH1 and RtH2 (Idakieva et al., 1993; Idakieva et al., 2002), with a molecular mass of about 420 kDa for both subunits (Stoeva et al., 1997; Idakieva et al., 2000). The presence of two distinct hemocyanin isoforms within the same organism has also been shown for *M. crenulata* (termed KLH1 and KLH2) (Swerdlow et al., 1996; Markl et al., 1991; Gebauer et al., 1994) and *H. tuberculata* (termed HtH1 and HtH2) (Keller et al., 1995; Keller et al., 1999; Lieb et al., 1999). Biochemical and immunological studies performed on RtH showed that the two RtH subunits each consist of eight oxygen-binding FUs (Stoeva et al., 1997; Idakieva et al., 2000) and that both RtH1 and RtH2 are immunologically related to KLH2 (Gebauer et al., 1999). Recently, the crystal structure of FU-*e* from RtH2 in the deoxygenated form was determined at 3.38 Å resolution (Perbandt et al., 2003). We now present three-dimensional (3D) reconstructions of RtH1- and RtH2-didecamers at resolutions of about 19 Å and 16 Å, respectively, determined by cryoelectron microscopy.

2. Materials and methods

2.1. Purification and isolation of RtH1 and RtH2

Living marine snails, *R. thomasiana*, were caught at the northern Bulgarian coast of the Black Sea and stored in sea water before the collection of the hemolymph. The native RtH was purified from the freshly obtained hemolymph by ultracentrifugation at 180,000 × *g* for 2 h at 4 °C. The two isoforms, RtH1 and RtH2, were isolated by anion-exchange chromatography of RtH on DEAE–Sepharose CL-6B as described in Idakieva et al. (2002).

2.2. Preparation of unstained vitrified specimens and data collection

Specimens were prepared for cryo-EM by applying hemocyanin in solution (0.5–1.0 mg/mL, in 50 mM Tris–HCl (pH 7.2), 5 mM $CaCl_2$, 5 mM $MgCl_2$) to glow-discharged copper grids coated with a thin holey carbon film, and subsequently plunge-frozen in liquid ethane. Imaging was performed using a Philips CM 120 electron microscope at 120 kV with a condenser aperture of 70 μm, spot size of 4, and an emission of 2. Images were recorded onto Kodak SO-163 films at a magnification of 45,000× and at 0.5–2.5 μm underfocus. Films were developed in concentrated Kodak D-19 developer for 12 min. Optical diffraction was used to determine the quality of the micrographs to be used for subsequent processing.

3. Image processing

3.1. Particle selection and CTF/envelope-function parameter determination

Micrographs were digitised using a Zeiss SCAI scanner with a sampling size corresponding to 6.22 Å/pixel and 3.11 Å/pixel at the specimen level for RtH1 and RtH2, respectively. The programs used for image processing of the micrographs and surface rendering of the 3D-models were EMAN (Ludtke et al., 1999) and Khoros (Konstantinides and Rasure, 1994), respectively. Particles with different orientations were selected from the digitised micrographs to determine the symmetry of the molecule, and to generate an initial model. The top views were later removed, to improve the fitting of the contrast transfer function (CTF), leaving only side views and tilted views of the individual hemocyanin didecamers of RtH1 (5916 particles) and RtH2 (6101 particles) for subsequent image processing. The CTF and envelope-function parameters for each micrograph were determined using *ctfit*, the CTF-determination program in EMAN. A structure factor curve generated in *ctfit* was used to give a more accurate fit of the CTF/envelope-function parameters. The low-resolution part of the curve (up to ∼25 Å) was generated by performing a multiple fitting procedure on CTF curves of a few particle sets, picked from micrographs with different defocus values. This was then merged with the high-resolution part of the X-ray solution scattering structure factor of GroEL (Ludtke et al., 2001) (up to ∼10 Å) to give the structure factor curve used in the fitting procedure. Correction of the CTF-phase flips was carried out before any reconstruction was performed, whereas the CTF's amplitude was corrected in a later step.

3.2. Initial model

An initial model for subsequent refinement was generated using a technique based on the rotational symmetry (C5) of the