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Crystallization, preliminary X-ray analysis and amino acid sequence studies of an "external" functional unit from the *Rapana thomasiana* grosse (mollusc, gastropod) hemocyanin

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Abstract

Rapana thomasiana hemocyanin is a representative of molluscan (gastropodan) dioxygen-transporting proteins. The cylindrical hemocyanin aggregates are composed of two structural subunits, RHSS1 and RHSS2. The 420 kDa subunit RHSS2 contains eight 50–55 kDa functional units. Each unit has a single dioxygen-binding dinuclear copper-containing active site. Molluscan hemocyanin functional units can be subdivided into "internal units", forming the so-called "arch" inside the hemocyanin cylinders, and "external" units, building the cylinder wall of the aggregates. The "external" oxygenated functional unit RtH2-e of the *Rapana* hemocyanin subunit RHSS2 was isolated and crystallized in two crystal forms. Type I crystals are small but X-ray suitable and show bipyramidal morphology. Preliminary data were collected to 3.3 Å at 120 K using synchrotron radiation. The space group is assigned to be the tetragonal $P4_12_12$ or its enantiomer with unit cell dimensions $a = b = 105.5$ Å and $c = 375.0$ Å. Type II crystals grow in thin plates and diffract to about 3.0 Å. However, they are always twinned and cannot be utilized for data collection and structure analysis.

Keywords: A1. Biocrystallization; A1. Crystal structure; A1. X-ray diffraction; A2. Growth from vapor; A2. Single crystal growth

1. Introduction

Hemocyanins (Hcs) are respiratory proteins freely dissolved in the hemolymph of invertebrates of two *phyla*: arthropoda and mollusca. They bind dioxygen reversibly and transport it from the environment to the tissues. Hcs perform the same biological function as hemoglobins but differ in the active site, the length of the polypeptide chains and structural organization. Although similar in function, arthropodan and molluscan Hcs are completely different in molecular architecture. The proteins of the first group are hexamers (1×6) or multiples of hexamers (2×6, 4×6, 6×6 or 8×6) of approximately 75 kDa structural subunits. Each subunit represents a single polypeptide chain with one dioxygen-binding dinuclear

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copper-containing active site [1]. It has been shown that the tertiary fold of the *Panulirus interruptus* and *Limulus polyphemus* Hc subunits is conserved and probably this is valid for all arthropodan Hcs [2,3]. Molluscan Hcs are more complex proteins with molecular masses ranging from 3.6×10^6 Da (cephalopodan Hcs) to 43.4×10^6 Da (gastropodan aggregates). They form decameric or multidecameric cylindrical aggregates with a diameter of $\sim$300–350 Å and different lengths. The basic building block is a cylinder made up of ten 350–450 kDa structural subunits. Each subunit has eight (gastropods and some cephalopods) or seven (cephalopods) 50–55 kDa functional units (FUs) containing a single dioxygen-binding site per unit [4]. Recently cryoelectron microscopy studies of *Vampyroteuthis infernalis* and *Benthoctopus* cephalopod hemocyanins have been reported describing the subunit arrangement at 21 Å resolution [5]. In principle most of the FUs form the wall of the cylindrical aggregates and the rest, which can be classified as "internal" units (e.g. the C-terminal FU of the *Octopus* Hc), are folded inside the cylinders forming an "arch" [4,6–8]. The first complete amino acid sequence of a molluscan Hc subunit, that from *Octopus dofleini* (cephalopod), has been reported by Miller et al. [9]. Also, the crystal structure of the C-terminal FU of the same Hc, Odg, which is an "internal" unit from the "arch", has been analyzed [10]. This is the first X-ray structure of a molluscan Hc FU, however the coordinates are not yet available from the protein data bank.

Molluscan Hcs are powerful antigens probably due to the high carbohydrate content and specific monosaccharide composition. Keyhole-limpet (gastropod) Hc is used in laboratories and clinics as an immune stimulant [11] and in the immunotherapy of bladder cancer [12] and renal cell carcinoma [13]. *Rapana thomasiana* grosse (gastropod) is a marine snail originally living in the Yellow Sea and in the East China Sea. In the middle of this century it was discovered on the west coast of the Black Sea where it adapted and is now widespread [14] (Fig. 1). The Black Sea is effectively a closed sea and its salinity is less than half of that of Pacific Ocean. Probably, the specific ecosystem affected some physiological properties

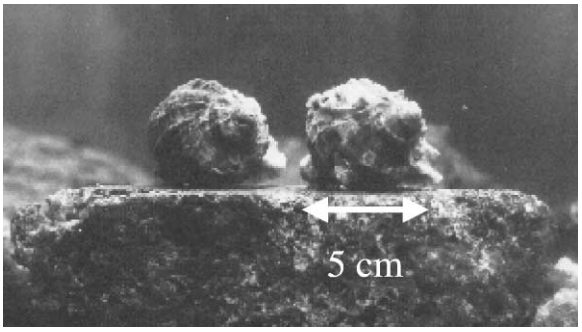

Fig. 1. Black Sea *Rapana thomasiana* grosse. Hemolymph was collected from 25–30 g species.

of this gastropod. The Hc of *Rapana thomasiana* is a glycoprotein with a carbohydrate content of 8.9% [15]. It is one of the rare examples of glycoproteins with O-methylated carbohydrate chains. The Hc aggregates are composed of two structural subunits, RHSS1 and RHSS2 [14]. Here we report the conditions for the crystallization of the "external" (from the wall of the Hc cylinders) FU RtH2-e of the *Rapana* Hc subunit RHSS2 as well as preliminary crystal parameters. Also, amino acid sequence studies have been performed and the data compared with those of other molluscan hemocyanin FUs of type e. In contrast to the "internal" molluscan (cephalopod) hemocyanin functional units, the crystallographic structure of an "external" unit or that of gastropod hemocyanin functional unit has not been analyzed so far. The cylinder walls may have a common architecture in all molluscan hemocyanins. The X-ray structure of RtH2-e will allow comparison of the tertiary structures of "internal" and "external" functional units as well as those of cephalopodan and gastropodan hemocyanins.

2. Methods and results

2.1. Crystallization and X-ray investigation of the functional unit RtH2-e

The type I and type II crystals of the Rapana Hc "external" FU RtH2-e were obtained within six and four days at room temperature by the hanging

drop vapor diffusion method, respectively. The purified sample of the protein was dissolved in water to an initial protein concentration of 20 mg/ml. For type 1 the protein solution was mixed in a ratio of 1 : 1 with the reservoir solution containing 0.2 M Mg-formate and 20 mM Tris/HCl buffer, pH 7.8. The final drop volume was 10 μl. For type II crystals the initial protein solution was mixed in a ratio of 1 : 1 with the reservoir solution containing 3% PEG 3350 and 0.1 M sodium acetate, pH 4.6. Preliminary diffraction data were collected at room temperature and at 120 K at DESY-Hamburg, beam line X11, and at the Brookhaven National Synchrotron Light Source (BNL) beam line X9A. Type I crystals have a bipyramidal form with dimensions of about $0.1 \times 0.15 \times 0.1\,mm^3$ (Fig. 2A). Diffraction data up to 3.3 Å were collected at 120 K. The diffraction data were processed with DENZO and SCALEPACK [16]. Data collection statistics are summarized in Table 1. The space group is assigned to be $P4_12_12$ or the enantiomorphic space group $P4_32_12$ and the packing parameter V_M was calculated to be 2.7 $Å^3$/Da assuming 4 molecules in the asymmetric unit [17]. Type II crystals form thin plates with dimensions $0.05 \times 0.1 \times 0.4\,mm^3$ (Fig. 2B). The crystals diffract to a resolution of about 3.0 Å. However, all crystals show a twinned diffraction pattern which could not be processed and applied for structure analysis. Diffraction patterns of type I and type II crystals are shown in Fig 3. Further experiments to analyze the twinning effect and to improve this crystal form are in progress.

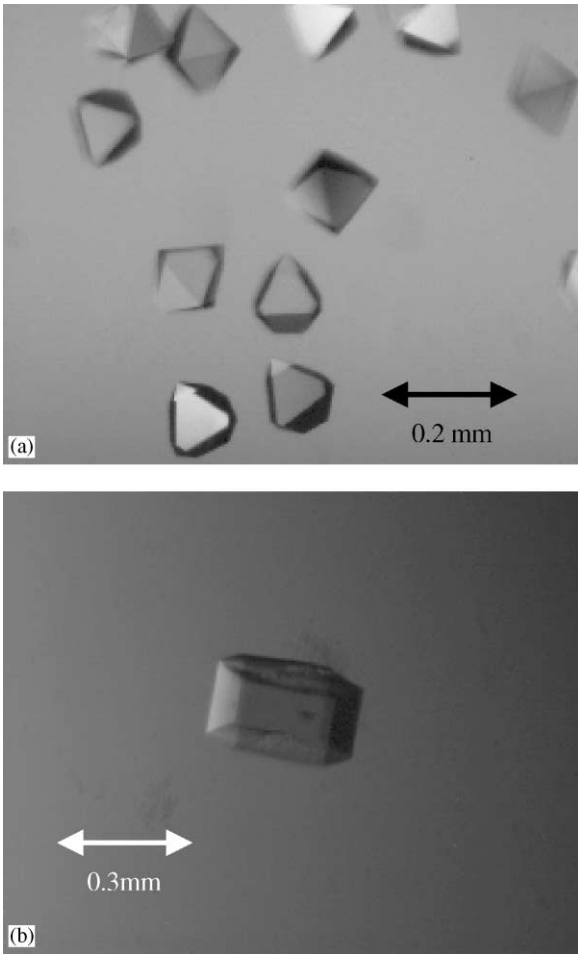

Fig. 2. (A) Type I (bipyramidal) crystals of the *Rapana thomasiana* hemocyanin functional unit R2e. The size of the crystals is about $0.1 \times 0.15 \times 0.1\,mm^3$; (B) a representative type II crystal (thin plate) of the same functional unit. The size of this crystals is about $0.05 \times 0.4 \times 0.2\,mm^3$.

Amino acid sequence data (Fig. 4) about the *Rapana* Hc FU RtH2-e were obtained by N-terminal sequencing of the whole protein and peptides obtained by cleavage with chymotrypsin. The peptides were localized in the sequence by comparing with the sequences of other molluscan hemocyanin functional units, those of the *Haliotis tuberculata* Hc type 2 unit HtH2-e and the *Octopus*

Table 1
Data collection statistics

Crystal data	
Space group	$P4_{(1)3}2_12$
Cell parameters	$a = b = 105.5$ Å, $c = 375.0$ Å
V_M	2.7 $Å^3$/Da
	4 molecules/asymmetric unit
Data collection	
Resolution (Å)	30–3.3 Å
Wavelength	0.98 Å
X-ray source	BNLS/X9A
Number of observations	792618
Number of unique reflections	33066
R_{merge} (%)	10.8
Completeness (%)	99.8

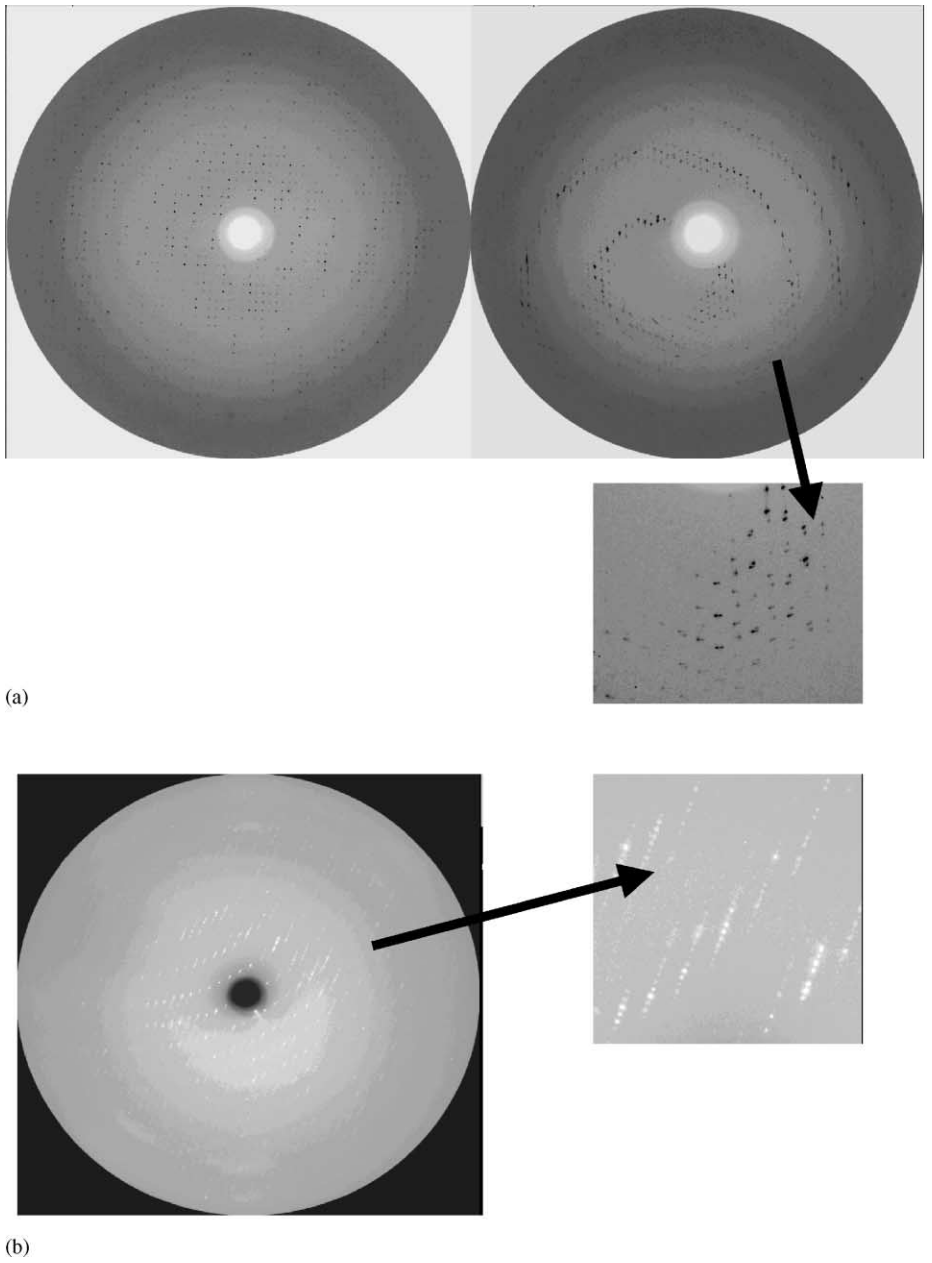

Fig. 3. (A) Diffraction pattern of type II crystals. The zoom is showing the twinning effect showing up in one direction of the reciprocal lattice; (B) diffraction pattern of type I crystals, indicating the long cell axis.

dofleini unit OdH-e. The compared units are derived from species of two classes of molluscs, gastropod (*Rapana thomasiana*, *Haliotis tuberculata*) and cephalopod (*Octopus dofleini*). These units occupy the same positions in the respective whole subunits.

In contrast to the FU Odg of the *O. dofleini* (cephalopod) Hc, which is an "internal" unit and

Fig. 4. Alignment of amino acid sequences of molluscan hemocyanin functional unit e. The indications for the units are as follows: RtH2-e, *Rapana thomasiana* hemocyanin functional unit e; HtH2-e, *Haliotis tuberculata* hemocyanin type 2 functional unit e [21] and Odh, *Octopus dofleini* hemocyanin functional unit e [9]. The following groups of amino acid residues are considered to be isofunctional: L, I, V and M; E and D; N and Q; T, S, and C; F, Y and W and K, R and H.

participates in the formation of the "arc" inside the Hc cylinder, the *Rapana* (gastropod) Hc FU RtH2-e is "external" and participates in the building of the cylinder wall. No crystallographic structure of this kind of molluscan Hc FUs exists. Also, no three-dimensional structure of gastropodan Hc FU has been published so far. The subunits of cephalopodan Hcs consist of 7 FUs and those of their gastropodan counterparts have 8 FUs. It has been shown [18] that the decameric forms (the whole molecule of cephalopodan Hc and the half-molecule of gastropodan Hc) have different symmetries. "Arches" are differently disposed in cephalopoda and gastropoda but the walls may have a common architecture in all molluscs. This adds importance to the determination of the three-dimensional structure of a unit from the Hc cylinder wall. The X-ray structure of RtH2-e will allow comparison of the tertiary structures of cephalopodan and gastropodan Hc FUs.

2.2. Isolation of the Rapana thomasiana hemocyanin, the structural subunit RHSS2 and the functional unit RtH2-e

Marine snails, *Rapana thomasiana* grosse, were caught near the Bulgarian coast of the Black Sea and stored in sea water (Fig. 1). Hemolymph was collected from specimens of 20–35 g, filtered through gauze and centrifuged 20 min at 6000 rpm. 1 mM phenylmethanesulphonyl fluoride was added to the crude material to avoid possible proteolysis. Hemocyanin was isolated by preparative ultracentrifugation as described in Ref. [19] and stored in the presence of 18% sucrose at −20°C until used.

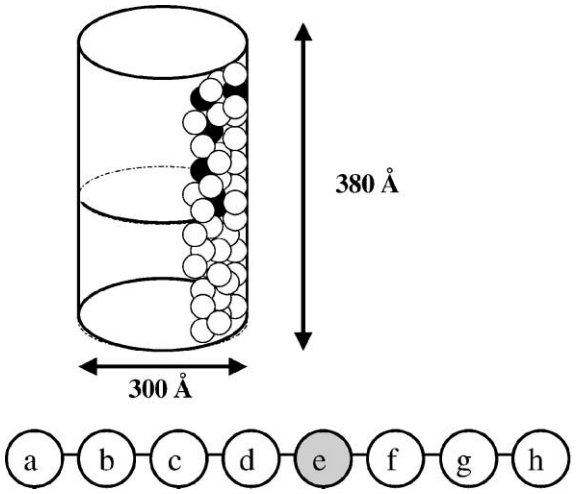

Fig. 5. Schematic representation of the *Rapana thomasiana* hemocyanin aggregates. The cylinder is a tail-to-tail dimer of decamers. Each decamer contains 10 structural subunits (80 functional units). The "external" and "internal" functional units are represented by white and black circles, respectively. The 420 kDa structural subunit RHSS2 consists of eight ~50 kDa functional units labeled "a" through "h".

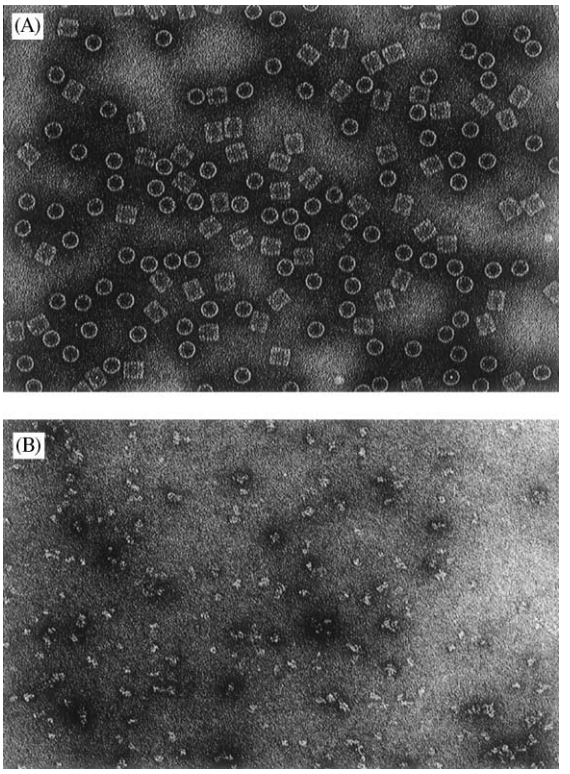

Fig. 6. (A) Electron micrograph of the native *Rapana thomasiana* hemocyanin. Rectangles and circles correspond to the lateral and axial projections of the hemocyanin cylinders, respectively; (B) electron micrograph of the dissociated *Rapana thomasiana* hemocyanin in 50 mM Tris–HCl buffer, pH 9.2, containing 10 mM EDTA.

Rapana hemocyanin structural subunits form cylindrical structures which represent tail-to-tail dimers of decamers (Fig. 5). The diameter of the cylinders is 300 Å and the height is 380 Å [19]. An electron micrograph of the native aggregates is shown in Fig. 6A demonstrating homogeneity of the starting material used for the further experimental work. Rectangles and circles correspond to the lateral and axial projections of the hemocyanin cylinders, respectively. Each di-decamer contains a total of 20 structural subunits (160 FUs). One subset of these FUs builds up the cylinder wall ("external" FUs) and the other subset forms the so-called "arch" inside the cylinder ("internal" FUs). Fig. 6B shows an electron micrograph of the dissociated *Rapana* Hc before the separation of the two structural subunits, RHSS1 and RHSS2.

The two subunits were isolated by ion-exchange chromatography of the hemocyanin dissociated at pH 9.2 on DEAE-sepharose CL-6B according to the procedure described in Ref. [14]. RHSS2 is a 420 kDa polypeptide folded into eight FUs. Each unit has a single dinuclear copper-containing active site reversibly binding a dioxygen molecule. The order of FUs within the structural subunit was determined by comparison of the N-terminal sequences of RHSS2, multiunit fragments and individual FUs obtained by limited proteolysis. The *Rapana* Hc subunit RHSS2 was subjected to limited proteolysis by trypsin, endopeptidase Glu-C and plasmin. The multiunit fragments were separated and purified by FPLC and sequenced from the amino terminus. Individual FUs were obtained by mild hydrolysis of the multiunit fragments with subtilisin DY, purified and N-terminally sequenced. All fragments and FUs were characterized by SDS-polyacrylamide gel electrophoresis as described by Laemmli [20], using 7.5% gel. Comparison of the sequences of the

whole subunit, multiunit fragments and FUs allowed determination of the order of FUs within the structural subunit. The units were labeled "a" through "h" sequentially from the amino terminus. The "external" FU RtH2-e from the Hc cylinder wall is a 50 kDa polypeptide chain. The protein is homogeneous by SDS-polyacrilamide gel electrophoresis and sequence analysis.

2.3. Electron microscopy studies

Electron micrographs of the native and dissociated *Rapana thomasiana* Hc were obtained with a Philips CM 10 electron microscope using a 30 μm objective aperture. The micrographs were routinely recorded at instrument magnification of $52000\times$. The specimens were adsorbed on a support film. The protein was negatively stained with 1% uranyl acetate.

2.4. Amino acid sequence studies of the functional unit RtH2-e

The *Rapana* hemocyanin functional unit RtH2-e was reduced by an ethanolic solution of 2-mercaptoethanol (100-fold molar excess regarding the cysteinyl residues). The reduced protein was S-pyridylethylated with 4-vinylpyridine and lyophylised. Sample of the S-pyridylethylated unit was dissolved in 1 M NH_4HCO_3 buffer, pH 7.8, and hydrolysed with chymotrypsin at an enzyme : substrate ratio of 1 : 40 (w/w). The mixture was incubated for 50 min at 37°C. The reaction was stopped by lowering the pH and the digest was chromatographed through a superdex peptide HR 10/30 column eluted with 20% acetonitrile in 0.1% trifluoroacetic acid. HPLC separation of peptides was performed on a Lichrospher 60 RP (250×4 mm) column using eluent A, 0.1% trifluoroacetic acid in water and eluent B, 80% acetonitrile in A, at a flow rate of 1 ml/min.

Amino acid sequence data were obtained by N-terminal sequencing of the whole protein and peptides from the chymotryptic digest. Automatic Edman degradation was performed using an applied biosystems pulsed liquid sequencer model 473 A with on-line analysis of the phenylthiohydantoin derivatives. The peptides were localized in the sequence by comparing with the sequences of other molluscan hemocyanin functional units e, those of the *Haliotis tuberculata* Hc type 2 unit HtH2-e [21] and the *Octopus dofleini* unit OdH-e [9]. The compared units are derived from species of two classes of molluscs, gastropod (*Rapana thomasiana* and *Haliotis tuberculata*) and cephalopod (*Octopus doflini*). These units occupy the same positions in the respective whole subunits. Fig. 4 demonstrates an extension in the amino terminal region of the polypeptide chains of HtH2-e and OdH-e in comparison to RtH2-e. There are two explanations of this phenomenon: (a) the extension represents a "linker region" between FUs and (b) the limited proteolysis used for the separation of the other FUs caused hydrolysis of part of the respective unit.

The single polypeptide chain of RtH2-e consists of 415 residues. We have determined 350 amino acid positions, i.e. 85% of the primary structure of this unit. There are many regions highly homologous to other e-type sequences and these can be enlarged if isofunctional residues are also taken into consideration. Functional units RtH-e of gastropodan and cephalopodan Hcs showed a minimum of 55% identity. They are closely related proteins with similar three-dimensional structure.

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