Domain Structure of the *Rapana Thomasiana* (Gastropod) Hemocyanin

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Summary: Sufficient and well-dosed oxygen supply is one of the prerequisites of human and animal life and nature has developed families of respiratory proteins to master this task: Hemoglobins/ myoglobins (vertebrates), hemocyanins (molluscs, arthropods) and hemerythrins (worms). Though structurally and in molecular size completely different, these proteins have all in common that under physiological conditions, oxygen is reversibly bound to a central heavy metal ion. From these three families, the hemocyanins are one of the largest and most complex biopolymers, known so far, reaching diameters of up to 25 nm, comparable to ribosomes and detailed primary, secondary and quaternary structure determination obviously belongs to one of the most tedious tasks in biochemistry.

The subunits of molluscan hemocyanins are organized into series of globular folded regions. Their structure has been clearly resolved in the electron microscope as a string of seven or eight beads (the number depending on the source of the hemocyanin), each presenting a functional oxygen-binding unit. Between each pair of functional units is a short flexible linker region consisting of 10-15 amino acid residues. *Rapana thomasiana* hemocyanin aggregates are constituted of two different types of subunits called RHSS1 and RHSS2 which were purified by ion exchange chromatography of the dissociated *Rapana* hemocyanin. Various tri-, di- and mono-domain fragments of the subunit are obtained by "autolysis" and limited proteolysis with V8 (Glu-C) proteinase, trypsin and elastase. The individual fragments are isolated by ion exchange, gel and/or reversed-phase chromatography. Their molecular weights are determined by SDS electrophoresis and the N-terminal amino acid sequences analyzed by Edman degradation, thus revealing the precise ordering of the domains in the *Rapana* subunit. The results confirm the 8-functional unit structure. Comparison of the RHSS1 domain sequences with those of other gastropod hemocyanin subunits is made.

Introduction

Hemocyanins are extracellular copper proteins that serve to transport oxygen in many species of arthropods and molluscs [1-6]. Although arthropod and molluscan hemocyanins are functionally equivalent and have long been assumed to be closely related, sequence studies indicated that these two classes are very different and have probably evolved independently from copper proteins such as tyrosinase [7-10]. Radical differences in both primary and quarternary structures of the two hemocyanin classes are found and it is suggested to consider the arthropod and molluscan hemocyanins as distinct proteins sharing common functional characteristics [6 and the references therein].

Molluscan hemocyanins exist in the hemolymph as very large molecules, assembled as 10-mers or 20-mers of the polypeptide chain. In the electron microscope decamers appear as a cylinder with a three-tiered wall and a five- or tenfold symmetry of the collar in the central cavity [11,12]. The monomer subunits with a molecular mass of 350-440 kDa are organized into a series of globular folded regions, clearly resolved in the electron microscope as a string of seven or eight beads of ca. 50 kDa, the so called "domains", "functional subunits" or "functional domains" [3,6]. Each domain is carring one binuclear copper site. Between each pair of functional units is a short flexible linker

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region consisting of 10-15 amino acid residues [9,13,14], where specific cleavage between the domains can be achieved by limited proteolysis. Through such studies the domains of *Octopus dofleini* [15], KLH1 and KLH2 [16] have been shown to be immunologically distinct. The number of functional units depends on the source of the hemocyanin: thus, the cephalopod hemocyanins from *Octopus dofleini* [9,15] and *Octopus vulgaris* [17] have only seven functional units, on the other hand *Sepia officinalis* [18] and *Loligo paelei* [19] possess eight functional units. Chitons (*Cryptochiton stelleri*, [20]), bivalves (*Yoldia limatula*, [21]) and gastropods (*Helix pomatia* [22], *Megatura crenulata* [16,23], *Busycon canaliculatum* [24]) indicated the presence of eight functional units with an average molecular weight of about 55 kDa.

To understand the structure of the functional hemocyanin molecule, the number and roles played by different domains in the assembled structure should be determined. This will provide information to answer the questions: which domains are involved in the cylinder wall, and which in the inner projections, how is the three-tiered wall constructed.

Another important aspect is that hemocyanins find practical application as immunomodulators for clinical trials, as a hapten carrier, as an adjuvant in immunocompetence tests. It was demonstated that the keyhole limpet hemocyanin (KLH) is an effective tool for immunotherapy of murine bladder cancer [25]. Prophylactic KLH treatment reduced superficial bladder cancer relapse rate after surgical intervention [26]. This respiratory protein was used also for diagnosis of *Schistosoma haematobium*, *S. mansoni* and *S. japonicum* infections [27-29].

For a number of years, our laboratory has been investigating the structure of the hemocyanin of the marine gastropod (snail) *Rapana thomasiana* originally living along the coast of Japan. In 1947 it was discovered in the Black Sea where it adapted. We have purified the two structural subunits building the native hemocyanin aggregates [30,31], isolated the N-terminal functional unit of one of them [32] and determined its complete amino acid sequence [33]. In order to continue the studies on the evolutionary relationships within molluscan hemocyanins and to obtain more information on their subunit organization, we have determined the number and the sequencial arrangement of the domains of the *Rapana* hemocyanin subunit RHSS1.

Results and Discussions

The strategy for the determination of the number of functional domains in the RHSS1 subunit and elucidation of their sequential arrangement includes limited proteolytic cleavage of RHSS1 into a panel of fragments containing single or multiple domains, isolation by gel-, ion exchange- and RP-chromatography of the obtained fragments, their characterization by SDS-PAGE and N-terminal sequencing and their precise ordering within the *Rapana thomasiana* subunit (Fig. 1). The domains (functional units) of subunit 1 were termed **a-b-c-d-e-f-g-h**, where **a** was defined as the N-terminal and **h** as the C-terminal domain.

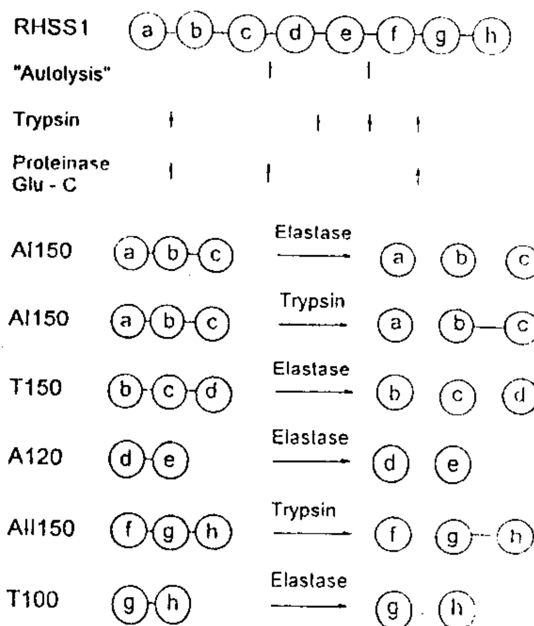

Fig. 1 Location of the proteolytic domain fragments in the polypeptide chain of the RHSS1 subunit of *Rapana thomasiana* hemocyanin. The domains **a-h** are represented by circles; A=autolysis; T=Trypsin.

RHSS1 was first isolated and characterized by Idakieva *et al* [31] to have a molecular weight of 250 kDa. In our experiments A420 (Fig. 2) shows a

molecular mass of 420 kDa and N-terminal sequence SLLRKNVDTLTEQEILRLQNTLL... corresponding to the sequence reported for the 250 kDa RHSS2 subunit. Although care was taken in order to prevent hydrolysis ("autolysis") of this large polypeptide chain during dialysis, along with the homogeneous RHSS1 (A420) four RHSS1 degradation fragments **(A250, AI150, AII150 and A120)** were isolated by ion exchange chromatography of the overnight dialyzed product (Fig. 2). The size of these fragments subsequently purified by RP-HPLC **(Fig. 2A)** was determined by SDS-PAGE to be 250, 160, 150 and 120 kDa, which represent one 5-domain fragment, two 3-domain fragments and a 2-domain fragment (Tab. I). All fragments were N-terminally sequenced, which characterized the 5- domain fragment **A250** and the 3-domain fragment **AI150** as the N-terminal part of RHSS1, consequently they were designated as **a-b-c-d-e** and **a-b-c,** respectively.

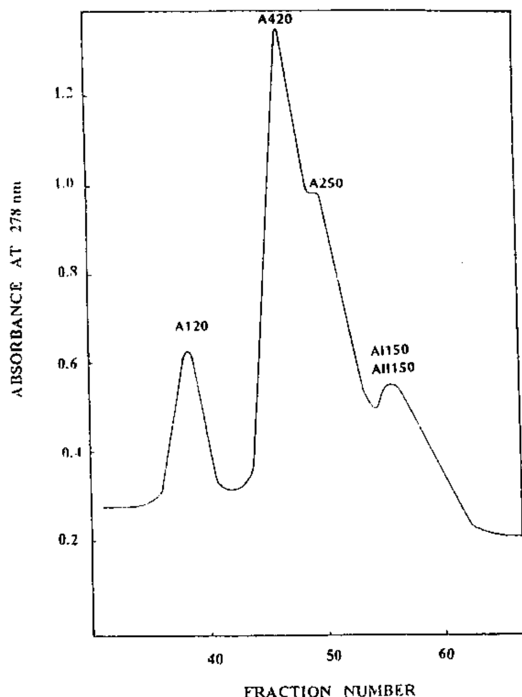

Fig. 2 Anion exchange chromatography of the RHSS1 autolytic products on DEAE-Sepharose 6B-CL. Elution buffer 50mM Tris/HCl, 10mM EDTA, pH 8.2. Gradient : 0.15 to 0.4 M NaCl in 80 min at a flow rate of 1.0ml/min. Isolation of A420 (RHSS1), A250, AI150, AII150, A120.

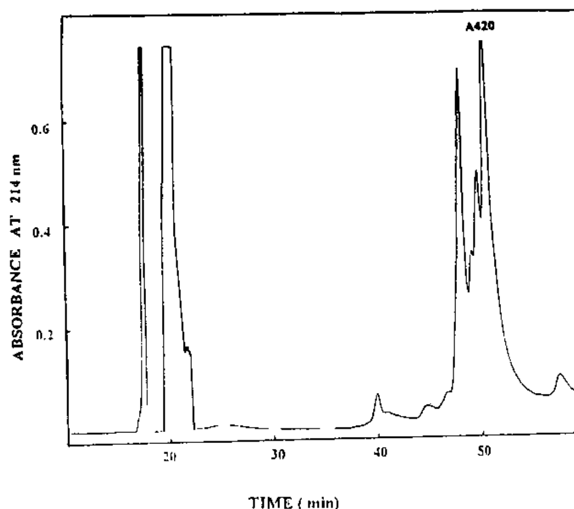

Fig. 2A HPLC purification of fraction A420 on a Nucleosil $7C_{18}$ column (250 x 10 mm; Macherey-Nagel). Eluent A, 0.1 % trifluoroacetic acid; eluent B, 80 % acetonitrile in A. Gradient program: 25% B for 5 min, then 25 % to 75 % B in 60 min at a flow rate of 2.0 ml/min. Isolation of RHSS1 (A420).

After cleavage of the RHSS1(**A420**) with trypsin three distinct fractions $\mathbf{T_1}$, $\mathbf{T_2}$ and $\mathbf{T_3}$ were isolated by gel-chromatography on Sephadex G-150 (Fig. 3). The first one $\mathbf{T_1}$ was submitted to rechromatography on DEAE-Sepharose CL-6B and further separated in two components, **T150** and **T100** (pattern not shown). Each of the fractions, thus isolated was purified by HPLC, yielding homogeneous individual compounds. $\mathbf{T_3}$ was applied to a Mono-Q 10/10 anion exchange column and eluted by a linear sodium chloride gradient (Fig. 4). The isolated functional units **R1a** and **R1f** (pattern not shown) and $\mathbf{T_2}$ (**R1e**; Fig. 5) were rechromatographed and desalted on a Nucleosil $7C_{18}$ column.

Analysis of the isolated individual fractions by SDS-PAGE revealed five different polypeptides of 150, 100, 60 and 50 kDa (Tab. I). This indicated a 3-domain, a 2-domain and three 1-domain fragments. The N-terminal amino acid sequence of the isolated fragments was determined by automatic Edman degradation.

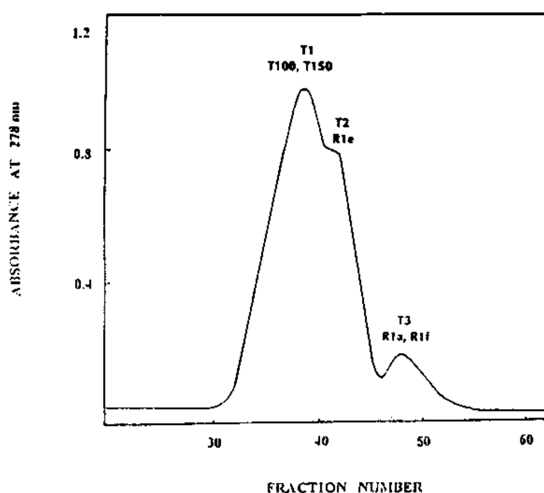

Fig. 3 Gel-chromatography on Sephadex G-150 (column 3.0x100.0 cm) of the products of limited proteolytic cleavage of RHSS1 (A420) with trypsin. Elution buffer 100mM NH_4HCO_3, pH 8.2 at a flow rate of 0.15 ml/min. Isolation of T150, T100 and three single domains R1a, R1e and R1f.

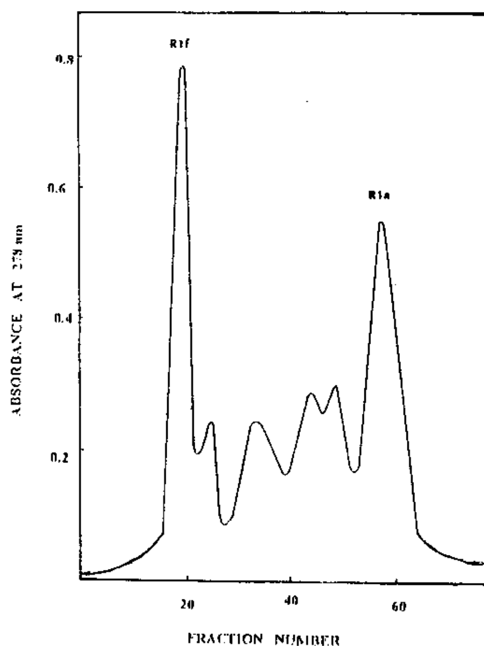

Fig. 4 Anion exchange chromatography on Mono-Q column 10/10 of gel chromatographic fraction T3. Elution buffer 50mM Tris.HCl, 10mM EDTA, pH 8.2. Gradient : 0.0 to 0.5 M NaCl in 80 min at a flow rate of 1.0 ml/min. Isolation of domains R1a and R1f.

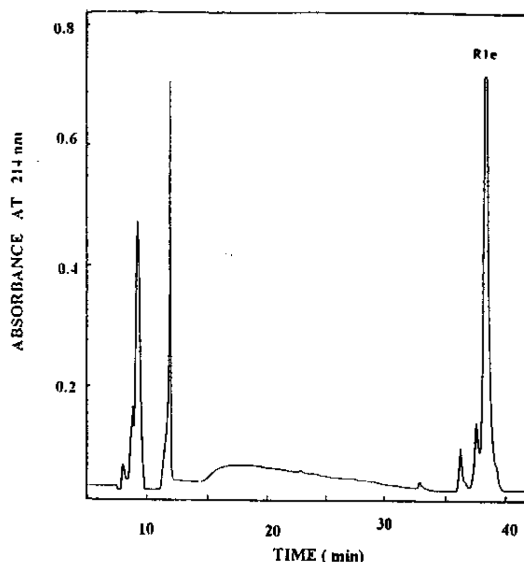

Fig. 5 HPLC purification of fraction T2 (R1e, isolated by gel-chromatography) on a Nucleosil $7C_{18}$ column (250x10 mm; Macherey-Nagel). Eluent A, 0.1 % trifluoroacetic acid; eluent B, 80 % acetonitrile in A. Gradient program: 15% B for 5 min, then 15 % to 75 % B in 60 min at a flow rate of 2.0 ml/min. Isolation of domain R1e.

Table-I: Molecular masses of RHSS1 substructures as determined by SDS-PAGE.

RHSS1	MASS (kDa)
Subunit	420 ± 40
1abc	150 ± 15
1bcd	150 ± 15
1cd	100 ± 10
1de	120 ± 10
1fgh	160 ± 15
1gh	120 ± 10
1a	50 ± 5
1b	50± 5
1c	50 ± 5
1d	50 ± 5
1e	60 ± 5
1f	50 ± 5
1g	50 ± 5
1h	60 ± 5

At the second level of the analysis in order to locate fragments T150 and T100 the isolated 3-domain fragments AI150 (a-b-c) and AII150 (f-g-h) were cleaved with trypsin into two single domains (ca. 50 kDa) and two 2-domain fragments (100 and 110 kDa; Tab. I). All components were isolated by

HPLC and N-terminally sequenced, revealing the single domains as the N-termini of the corresponding 3-domains and were termed as **a** (Fig. 6) and **f**, while the 2-domains corresponded to functional units b-c and g-h. By comparison of the N-terminal sequences of the individual domains it turned out that the 3-domain fragment **T150** corresponds to the N-terminus of the 2-domain fragment **b-c (AI150/T)** and consequently T150 was termed **b-c-d**. Likewise, the N-terminal sequence of the 2-domain fragment **T100** is identical to the sequence of the 2-domain fragment **g-h** resulting from the subsequent trypsin cleavage of AII150, therefore **AII150** is identified as the C-terminal 3-domain fragment **(f-g-h)** of RHSS1. Moreover, the comparison of the N-terminal sequences of fragment **A120** and the functional domain **d** from the the further proteolytic degradation of **T150** by elastase enabled the identification of **A120** as **d-e**.

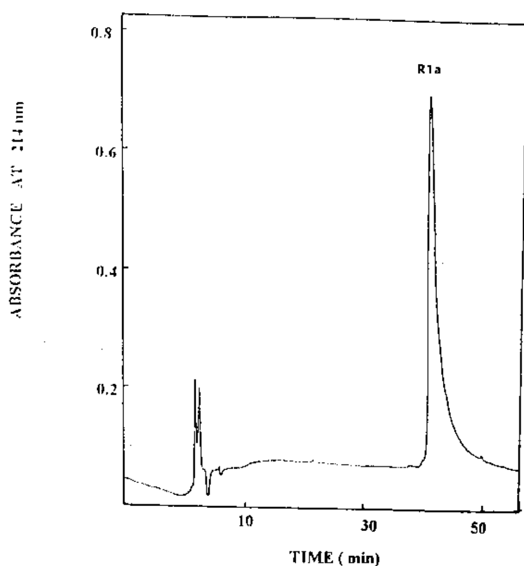

Fig. 6 HPLC purification of fraction R1a on a Nucleosil $7C_{18}$ column (250x10 mm; Macherey-Nagel). Eluent A, 0.1 % trifluoroacetic acid; eluent B, 80 % acetonitrile in A. Gradient program: 15% B for 5 min, then 15 % to 75 % B in 60 min at a flow rate of 2.0 ml/min. Isolation of domain R1a.

At the third level of the experiments the isolated fragments **AI150, A120, T150** (Fig. 7) and **T100** were cleaved by elastase into single domains, which were fractionated by HPLC. The N-terminal primary structures of domains **c**, **e** and **h** were thus determined and positioned by overlapping in the RHSS1 structure (Fig. 8).

Cleavage of RHSS1 (A420) with the endoproteinase Glu-C generated four distinct polypeptides, which after separation by ion exchange chromatography (pattern not shown) were found by SDS-PAGE to represent one 3-domain fragment of 160 kDa, two 2-domain fragments of 110 and 100 kDa and one 1-domain fragment of 50 kDa (Fig. 1; Tab. I). The N-terminal sequence of the individual fragments was analyzed and served as confirmation of the overlapping of the domains. Combined interpretation of the results completed the domain arrangement of RHSS1 as shown in Fig. **8**. The sequencing results of the limited proteolytic RHSS1 substructures and functional units indicate that is constituted of eight individual domains. Comparison of the RHSS1domain sequences with those of other gastropod functional units (KLH1 and KLH2) [16] revealed a definite homology, especially the motif -V(I,L)-R-x-x-V(I,L)-x-x-L-, which represents the conserved sequence of the N-terminal region of molluscan hemocyanins (Fig. 9).

To characterize the obtained RHSS1 substructures spectroscopic analysis (UV and CD) was performed. Table II presents a summary of spectroscopic parameters of R1a (as a representative domain) and the native *Rapana* hemocyanin. Besides the absorption band at 280 nm, due to the aromatic chromophores, a band with a maximum at 345 nm, resulting from transitions of the oxygen-copper-protein active site and characteristic for oxyhemocyanins, was observed. The presence of the band at 345 nm proves that on proteolysis the functional groups are preserved and the binuclear active site is conserved in R1a. The spectroscopic ratio 345nm/280nm is informative for the oxygen saturation of the hemocyanin molecule and can serve as a measure for the oxygen-binding at the copper site. The value calculated for the functional unit R1a, 0.30, is practically the same as that determined for the whole *Rapana* hemocyanin (Table II). This observation characterizes the isolated domain R1a as a fuctional unit with preserved capability of binding dioxygen with the same affinitv as the native

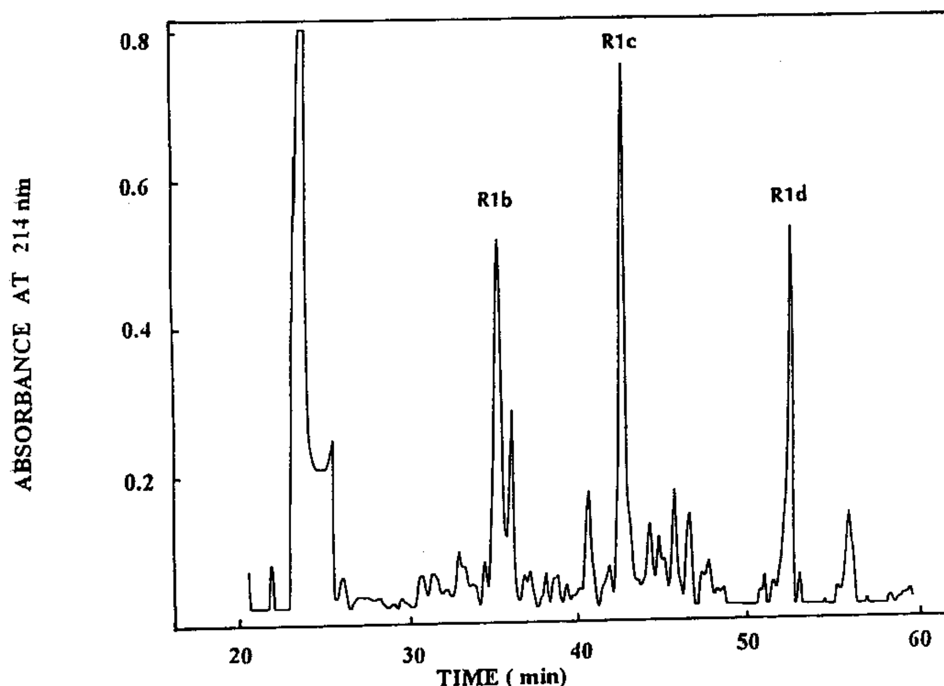

Fig. 7 HPLC of the products of limited proteolytic cleavage of T150 with elastase on a Nucleosil $7C_{18}$ column (250x10 mm; Macherey-Nagel). Eluent A, 0.1 % trifluoroacetic acid; eluent B, 80 % acetonitrile in A. Gradient program: 5% B for 5 min, then 55 % to 75 % B in 60 min at a flow rate of 2.0 ml/min. Isolation of three single domains R1b, R1c and R1d.

RHSS1	S L L R K N V D T L T E Q E I L R L Q N T L L ...
AI150 (abc)	S L L R K N V D T L T E Q E I L R L Q N T L L ...
R1a	S L L R K N V D T L T E Q E I L R L Q N T L L ...
T150 (bcd)	A L T R K N V D T L N E ...
R1b	A L T R K N V D T L N E ...
R1c	S L T R K N V E F L ...
A120 (de)	A P V R V R K N L N V L T D ...
R1d	A P V R V R K N L N V L T D ...
R1e	A E V R H N V D R L T D Q E I L D ...
AII150 (fgh)	G V G V R K D L N T L T A A E M K N L R D A L D ...
R1f	G V G V R K D L N T L T A A E M K N L R D A L D ...
R1g	V T A L E E E H R K E V D D L N D R D I ...
R1h	V T H L L E E V R K E V D D L ...

All RHSS1 functional units share the motif -V(I,L)-R-x-x-V(I,L)-x-x-L-, which represents the conserved sequence of the N-terminal region of molluscan hemocyanins.

Fig. 8 N-Terminal amino acid sequences of RHSS1 substructures and functional domains. The isolation of the RHSS1 substructures and individual functional units is illustrated in Figs 2-7; sequence analysis is described in Methods.

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R1a      SLLRKNVDTLTEQEILRLQNTLL ...
KLH1a    ENLVRKDVERL ...
KLH2a    VDTVVRKNVDSLSXD ...

R1b      ALTRKNVDTLNE ...
KLH2b    NLAVRKNINDLTAN ...

R1c      SLTRKNVEFL ...
KLH1c    KVPRSRLIRKNVDRLTPSE ...
KLH2c    DFGHSKKIRKNVHSLTAADDQ ...

R1d      APVRVRKNLNVLTD ...
KLH1d    EVTSANRIRKNIENLS ...
KLH2d    AVTSASHIRHNIRDLGEG ...

R1e      AEVRHNVDRLTDQEILD ...
KLH2e    VPXIRKNIK ...

R1f      GVGVRKDLNTLTAAEMKNLRDALDAVQ ...
KLH1f    HHLSXNKVRHDLSTL ...
KLH2f    HVGRNRIRMDLSDLTXXDLA ...

R1g      VTALEEEHRKEVDDLNDRDI ...
KLH1g    SSMAGHFVRKDINTLTP ...
KLH2g    IAGSGVRKDV ...

R1h      VTHLLEEVRKEVDDL ...
KLH1h    HEDHHEDILVRKNIHSL ...
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Fig. 9 Comparison of N-terminal amino acid sequences of individual functional units of RHSS1 and KLH1 and KLH2 as reported by Gebauer et al [16]. The motif **-V(I,L)-R-x-x-V(I,L)-x-x-L**, which represents the conserved sequence of the N-terminal region of molluscan hemocyanins. 5-25% polyacrylamide gradient gels according to Laemmli [34] and various marker proteins were applied. Each value is calculated from several independent experiments.

hemocyanin. Evidently, the experimental conditions used lead to limited proteolysis of the RHSS1 subunit without disturbing the oxygen binding properties of the R1a active site.

Table II: Spectroscopic parameters of the N-terminal functional unit R1a of the *Rapana thomasiana* RHSS1 hemocyanin subunit and the whole native hemocyanin

Parameters	Functional unit R1a in oxy-form	Oxy-hemocyanin of *R. thomasiana**
Absoption	278 (70 300)	278 (68 800)
λ_{max} (ε)	345 (21 000)	345 (19 200)
A_{345}/A_{280}	0,30	0,28
Circular dichroism	208 (-6 940)	208 (-7 130)
λ_{max} (Θ)	222 (-6 370)	222 (-6 850)
	253 (126)	253 (130)
	280 (60)	280 (99)
	287 (57)	287 (76)
	345 (-163)	345 (-149)

*Data from [30,32].

The far-ultraviolet (UV) CD spectrum of the functional unit R1a which reflects the backbone conformation of the protein molecule, is characterized by negative bands at 208 and 222 nm connected mainly with the presence of α-helical structure. β-sheet also contributes to the ellipticity around 222 nm, though to a lesser extent than the α-helix. The far-UV dichroic spectra of R1a and the native *Rapana* hemocyanin are essentially identical, suggesting a closely similar folding of the polypeptide chains of the isolated functional unit R1a and the domains within the subunit RHSS1 of the native hemocyanin. The differences in the intensity of the bands at 208 and 222 nm for the unit and for the whole hemocyanin are practically in the region of the experimental errors. The CD data show that little or no conformational change occurs in the polypeptide backbone as a result of the limited proteolysis and the functional unit R1a preserve the

native backbone conformation. The near-UV CD spectrum of R1a and that of the *Rapana* hemocyanin are qualitatively identical. The spectra in the region 250 - 300 nm reflect an asymmetric environment for, both, tyrosyl and tryptophyl amino acid residues and give information on the conformation and flexibility of their side chains. However, the tryptophyl residues are mainly responsible for dichroic transitions above 280 nm. The intensities of the positive bands at 280 and 287 nm in the CD spectrum of R1a are reduced in comparison with those of the respective bands in the spectrum of the *Rapana* hemocyanin (Table II). This suggests that minor changes occur in the environment of the aromatic chromophores of the functional unit as a result of its separation from the large polypeptide chain. Probably, the whole structural subunit creates a specific environment for aromatic residues located in the N-terminal domain R1a which is somewhat different from that after the separation of R1a as an individual unit. The negative CD band at 345 nm in the spectrum of the functional unit should be attributed to the complex between the copper atoms from the binuclear active site and a dioxygen molecule. The intensity of this band is the same as that of the respective band in the spectrum of the native oxyhemocyanin (Table II). This result confirms the conclusion that the oxygen-binding capability of the N-terminal functional unit is completely preserved. The intensity of the dichroic band at 253 nm in the two spectra is also identical (Table II).

The most important feature to be noted in the comparison of the UV and CD spectra of Rta and *Rapana* hemocyanin is that the secondary structure and the native form of the oxygen-binding binuclear copper site of the N-terminal domain are conserved in the separated functional unit and only minor changes occur in the environment of the aromatic chromophores. The spectroscopic parameters obtained show that little happens to the structural integrity of the functional unit R1a upon the limited proteolysis of the whole *Rapana* RHSS1 hemocyanin structural subunit.

Further studies by immunoelectrophoresis of the accessibility of the various functional units based on the ability of whole hemocyanin to produce immunocomplexes with functional unit-specific antibodies in addition to electron microscopy will allow more precise characterization of the direction of the polypeptide chains and the location of the functional units of *Rapana thomasiana* hemocyanin within the cylinder.

Experimental

Chemicals and enzymes

Tris(hydroxymethyl)-aminomethane hydrochloride and urea were purchased from Merck (Darmstadt, Germany). Sephadex G-150 and DEAE-Sepharose CL-6B were products of Pharmacia (Uppsala, Sweden). Endoproteinase Glu-C (*Staphylococcal aureus* V8 proteinase) and TPCK-trypsin, sequencing grade, were obtained from Boehringer (Mannheim, Germany). Bovine pancreatic elastase type IV and the molecular weight markers were from Sigma (Deisenhofen, Germany). All other chemicals and reagents used were of analytical grade.

Preparation of the Rapana thomasiana hemocyanin, purification of RHSS1 and isolation of the autolytic fragments

Living marine snails, *Rapana thomasiana* grosse, were caught near to the northern Bulgarian coast of the Black Sea and stored in sea water before the collection of the hemolymph. The isolation of the hemocyanin and its structural subunits, RHSS1 and RHSS2, was performed as described previously [30, 31]. Then RHSS1 was dialyzed overnight versus 0.13 M glycine/NaOH, pH 9.6 at room temperature and fractionated by anion exchange chromatography on DEAE-Sepharose CL-6B column (37.0 x 3.0 cm), equilibrated with 50 mM Tris/HCl + 10m M EDTA, pH 8.2, by elution with a linear sodium chloride gradient (0.15-0.40 M NaCl) in the same buffer and a flow rate of 1ml/min. The isolated fractions were submitted to rechromatography on a Nucleosil 7C_{18} column (250 x 10 mm; Macherey-Nagel, Düren, Germany). The following conditions for the HPL chromatography were used: eluent A, 0.1 % trifluoroacetic acid; eluent B, 80 % acetonitrile in A; gradient program: 25 % B for 5 min then 25 % to 75 % B in 60 min at a flow rate of 2.0 ml/min. The column eluate was monitored at 214 nm.

Limited proteolysis of RHSS1 and isolation of the fragments

A series of trial digestions was carried out for various times and with several enzyme concentra-

tions to determine the optimal conditions to obtain individual domains and multidomains with minimal further cleavage.

Cleavage with trypsin

Tryptic digestion of 5 mg RHSS1 (A420) was performed in 2 ml 100mM NH_4HCO_3 buffer (w/v) + 10 mM EDTA, pH 8.2, for 30 min at room temperature with an enzyme to substrate ratio of 1:400 (w/w). The reaction was terminated by the addition of PMSF in dioxane in a fivefold molar ratio with respect to the enzyme. The tryptic hydrolysate was centrifuged at 30000 x g for 20 min at room temperature and the supernatant was separated on a Sephadex G-150 column (100 x 3.0 cm), equilibrated with 100mM NH_4HCO_3 buffer + 10 mM EDTA, pH 8.2, at a flow rate of 0.15 ml/min, followed by rechromatography on DEAE-Sepharose CL-6B column equilibrated with 0.13 M glycine/NaOH, pH 9.6 and eluted by a linear sodium chloride gradient (0.15-0.35 M NaCl) in the same buffer and a flow rate of 1ml/min, or on Mono-Q 10/10 anion exchange column (Pharmacia, Freiburg, FRG), equlibrated with 50 mM Tris/HCl buffer, pH 8.2, containing 10 mM EDTA, and eluted by a linear sodium chloride gradient (0.00-0.50 M NaCl) in the same buffer and a flow rate of 1ml/min. The fractions thus isolated were further purified by HPLC as described in a preceding paragraph.

For production of mono- and didomain-fragments AI150 and AII150 were treated with trypsin under the conditions described above. The individual functional units and the didomains were isolated using HPLC on a Nucleosil $7C_{18}$ column.

Cleavage with endoproteinase Glu-C

Cleavage of RHSS1 with endoproteinase Glu-C, specifically hydrolyzing bonds C-terminally at glutamic acid, was performed as follows: 1.5 mg of RHSS1 were dissolved in 0.75 ml of 25 mM Tris/HCl buffer, pH 8.5, containing 1 mM EDTA and 1 M urea and treated with 15 µg of endoproteinase Glu-C in water, at an enzyme/substrate ratio of 1/100 (w/w) for 5 h at 37 °C. Separation of the resulting mono- and polydomain fragments was performed by anion exchange chromatography on DEAE-Sepharose CL-6B and on a Nucleosil $7C_{18}$ column as described above.

Cleavage with elastase

The only proteinase found to be efficient at cleaving the subunit and the multidomain structures into single domains without further damage, the elastase, was used to produce individual domains of AI150, T150, A120 and T100. Limited proteolysis with elastase was performed in 0.13 M glycine /NaOH, pH 9.6, for 5 h at 37°C with an enzyme/ substrate ratio of 1/100 (w/w). All cleavage fragments generated by the action of elastase were isolated by HPLC on a Nucleosil $7C_{18}$ column as described.

Amino acid sequence determination

Automated Edman degradation was performed using an Applied Biosystems pulsed liquid sequencer model 473 A (Weiterstadt, Germany) with on-line analysis of the phenylthio-hydantoin derivatives. Approximately 50-500 pmol of peptides were applied on the cartridge filter previously treated with polybrene.

Polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was made under reducing conditions following the method of Laemmli [34] using a 10% gel. For molecular mass determination the following protein markers were used: myosin (205 kDa), ß-galactosidase (116 kDa), phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). A plot of migration versus log molecular weight of the protein standards was linear (not shown).

Spectroscopic measurements

Absorption spectra were recorded with a Shimadzu spectrophotometer, model 3000. Circular dichroism was measured with a Roussel Jouan Dichrographe III instrument. The data were expressed in terms of mean ellipticity using a specific absorption coefficient at 280nm $\varepsilon_{280} = 6.88 \times 104\ M^{-1}\ cm^{-1}$.

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