BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 403–410 (1997)
ARTICLE NO. RC977314

Complete Amino Acid Sequence of Dioxygen-Binding Functional Unit of the *Rapana thomasiana* Hemocyanin

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Received July 25, 1997

The complete amino acid sequence of the *Rapana thomasiana* hemocyanin N-terminal functional unit Rta was determined by direct sequencing and matrix-assisted laser desorption ionization mass spectrometry of the protein and peptides obtained by cleavage with EndoLysC proteinase, TPCK-trypsin and cyanogen bromide. The single polypeptide chain consists of 407 residues. This is the first report on the primary structure of a dioxygen-binding unit from a marine gastropod hemocyanin and of an N-terminal domain from a molluscan dioxygen carrier. Comparison with the sequences of other molluscan hemocyanin functional units shows an average identity of 48 ± 5 %. Inspection of the Rta sequence revealed residues 27 and 250 as carbohydrate attachment sites. Conclusions about the molecular evolution of the molluscan hemocyanin dioxygen-binding functional units are made.

Key Words: **amino acid sequence; functional unit; gastropod; hemocyanin; *Rapana*.**

Dioxygen transport is of vital importance for the living organisms. The nature has chosen three classes of proteins which act as dioxygen carriers in animals: hemoglobins, containing Fe (II) in a heme group, hemerytrins, with two Fe(II) atoms at the active site and hemocyanins binding reversibly a dioxygen molecule at a binuclear copper-containing active site. Each of the two Cu(I) atoms is ligated to three histidyl residues from the polypeptide chains of the last group of proteins (1).

Hemocyanins (Hcs) are freely dissolved in the hemolymph of molluscs and arthropods and perform one and the same physiological function in the organisms of both *phyla*. Nevertheless, the molecular architecture and the arrangement of the subunits are quite different for the two types of respiratory proteins. Thus, the native aggregates of arthropodan Hcs are built of 1, 2, 4, 6 or 8 hexameric assemblies of 67 - 90 kDa subunits. Each subunit contains one dioxygen-binding site (2). The basic 330 - 460 kDa structural subunits of molluscan Hcs form cylinders with external diameters of 300 - 350 Å and 140 - 190 Å in height. The cylinders are composed of 10, 20 or more subunits and their moleculr masses are in the range 8.7 - 43.4 $\times$ 10^6 Da. The subunit polypeptide chain contains 7 - 8 functional units (domains) of 50 - 55 kDa. Each domain contains one binuclear active site binding reversibly a dioxygen molecule (2).

Complete or partial amino acid sequences of functional units from arthropodan (3-7) and molluscan (8-12) Hcs have been reported. X-ray crystal structures of crustacean (*Panulirus interruptus*) and cheliceratan (*Limulus polyphemus*) Hcs have been determined at 3.2 and 2.18 Å resolution, respectively (1, 13). Crystallographic analysis showed that the copper-copper distance in oxygenated *Limulus* Hc is 1Å less than that in the deoxygenated form of the same protein (14). Away from this difference, the tertiary and quaternary structures of the two forms are quite similar. Three-dimensional reconstruction of molluscan (15-19) and arthropodan (20) Hcs has been performed by the group of J. Lamy.

Hcs are glycoproteins medically applied as immunomodulators for clinical trials. The keyhole limpet hemocyanin (KLH) is an effective tool for immunotherapy of murine bladder cancer (21). Prophylactic KLH treatment reduces superficial bladder cancer relapse rate after surgical intervention (22). This respiratory protein is used also for diagnosis of *Schistosoma haematobium*, *S. mansoni* and *S. japonicum* infections (23-25).

Homology and evolutionary relationships in proteins are usually assessed by comparison and analysis of their amino acid sequences. At present, little

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Abbreviations: Hc, hemocyanin; KLH, keyhole limpet hemocyanin; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; RHSS1 and RHSS2, subunits of the *Rapana* hemocyanin; Rta, N-terminal functional unit of the *Rapana thomasiana* hemocyanin.

0006-291X/97 $25.00

TABLE 1

Amino Acid Sequence and Molecular Mass of Peptides Obtained after Cleavage of Reduced-Alkylated N-Terminal Oxygen Binding Functional Unit Rta of the *Rapana* Hemocyanin Subunit RHSS2 with EndoLysC Proteinase (LC), Cyanogen Bromide (CN) and Digestion of Maleylated Rta with TPCK-trypsin (R)

Peptide	RT	Pos.Nr	Sequence	Expected mass [Da]	Measured mass [MH^+,Da]
LC 1	25,36	1-5	SLLRK	615.78	617.1
LC 2	58,92	6-54	NVDSLTEEEILTLQSVMRELQXDSSEHGFQSIASFHGSPPL[46]. . .	5428.89	6835.6
CN III	29,65	23-63	RELQNDSSEHGFQSIASFHGSPPLCPSPEANKKVACCVHGM	4540.86	4542.0
LC3	41,99	56-75	VACCVHGMASFPQWHRIFTK	2434.78	2435.0
CN IV 1	29,99	64-77	ASFPQWHRIFTKQM	1747.01	1748.6
LC 4	55,65	76-101	QMEAALMGHGAKVGMPYWDWTTSFTK	2944.41	2946.1
LC 5	57,78	88-137	VGMPYWDWTTSFTKLPRFIPYDDEQLNPFVRI[119]. . .	6104.87	6106.1
R IV 2	29,44	105-118	FIPYDDEQLNPFVR	1752.95	1754.0
R II 11	18,67	119-129	ITDLEDHFTTR	1347.45	1348.3
R II 9	25,43	130-137	DPQPELFK	937.10	938.1
LC 6	60,64	138-183	DPEGGDESFFFRQVLIALEQRDYCDFEVQFEVIHNSIHYWIGGHQK	5559.07	5560.9
R II 5	18,76	159-207	DYCDFEVQFEVIHNSIHYWIGGHQKYGMSTL[189]. . .	5982.56	5983.9
CN V 1	52,83	187-233	STLEYTAYDPLFFIHHSNVDRLWAIWQELQKYRGL[221]. . .	5679.26	5681.4
R IV 7	37,12	208-219	LWAIWQELQKYR	1633.92	1635.0
R II 10	32,62	220-234	GLPYDESDCGVELMR	1741.88	1742.7
CN V 2	24,94	234-280	REPLQPFAQTSATNPNXVTRAHSTPKSLFN[263]. . .	5225.91	6264.8
LC 7	51,24	260-294	SLFNYRQLAGYTYDTLTLNGMTISQL[285]. . .	4039.64	4039.9
CN IV 2	30,82	281-340	TISQLESSLLRLQKEED[297]. . .	6636.34	6635.9
LC 8	35,93	295-321	EEDRVFAGFLLRGIGSSADVTFDLCDK	3019.31	3021.4
LC 9	55,61	322-349	DEHCDFAGTFAVLGGPLEMPWSFDRLFK	3244.63	3246.2
CN IV 4	38,10	341-359	PWSFDRLFKMDVTKVFKQM	2373.81	2376.1
LC 10	44,03	358-399	QMRLRPDDSEYHFELEVTARAGTDLSPELLKPGSVSFLPGRK	4744.38	4744.7
R III 5	33,82	400-407	IQNTPDVR	942.04	943.2

Peptides not sequenced to the end are marked by (. . .).

[a] The molecular weights of the CNBr peptides were calculated for a homoserine at the C-terminus and for the Cys-containing fragments the carboxymethylated form was used.

is known about the primary and higher sructures of Hcs from marine gastropods. No amino acid sequence of functional unit from this group of respiratory proteins has been published so far. In order to continue the studies on the evolutionary relationships within molluscan Hcs, we have determinated the complete amino acid sequence of the *Rapana* Hc N-terminal functional unit which is described in the present paper. *Rapana thomasiana* (grosse) is a marine gastropod (snail), originally living along the coast of Japan. In 1947 it was discovered in the Black Sea where it adapted. The salinity of the Black Sea is less than half that of the Mediterranean Sea and Pacific Ocean and changes in the physiological functions of the organism might be expected. The hemolymph of *Rapana* contains one hemocyanin (26). We have purified the two structural subunits building the native hemocyanin aggregates (27) and isolated the N-terminal functional unit of one of them (28).

MATERIALS AND METHODS

Chemicals and enzymes. Tris(hydroxymethyl)-aminomethane hydrochloride, guanidine hydrochloride and urea were purchased from Merck (Darmstadt, Germany). Sephadex G-50, G-75 and G-100 were products of Pharmacia (Uppsala, Sweden). Chemicals and reagents were of analytical grade. EndoLysC proteinase and TPCK-trypsin, sequencing grade, were obtained from Boehringer (Mannheim, Germany).

Preparation of the Rapana thomasiana hemocyanin and isolation of the amino terminal oxygen-binding unit Rta. 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 (26, 27). The amino terminal oxygen-binding functional unit Rta was obtained after limited trypsinolysis of the 450 kDa subunit RHSS2 and separation of the mixture by gel chromatography on Sephadex G-75 and HPL chromatography (28). The unit was identified as N-terminal by sequence analysis.

Reduction and carboxymethylation of the oxygen-binding unit Rta. 70 mg of the N-terminal functional unit Rta of the *Rapana* hemocyanin structural subunit RHSS2 were dissolved in 4 ml 1 M Tris/HCl buffer, pH 8.2, containing 6 M guanidine/HCl and 1 mM EDTA. An ethanolic solution of 2-mercaptoethanol (100-fold molar excess regarding to the cysteinyl residues) was added. The mixture was incubated under nitrogen for 30 min at 56 °C and after that for 2 h at room temperature, in the dark. Iodoacetic acid in 0.2 ml of 1 M NaOH (100-fold molar excess regarding to the cysteinyl residues) was added and the mixture incubated under nitrogen for 30 min at room temperature, in the dark. The reaction was stopped by adding 0.1 ml of 2-mercaptoethanol. The carboxymethylated protein was chromatographed through a column of Sephadex G-100 (100 × 2.8 cm) and eluted with 8 M urea, pH 3.5. Fractions containing the

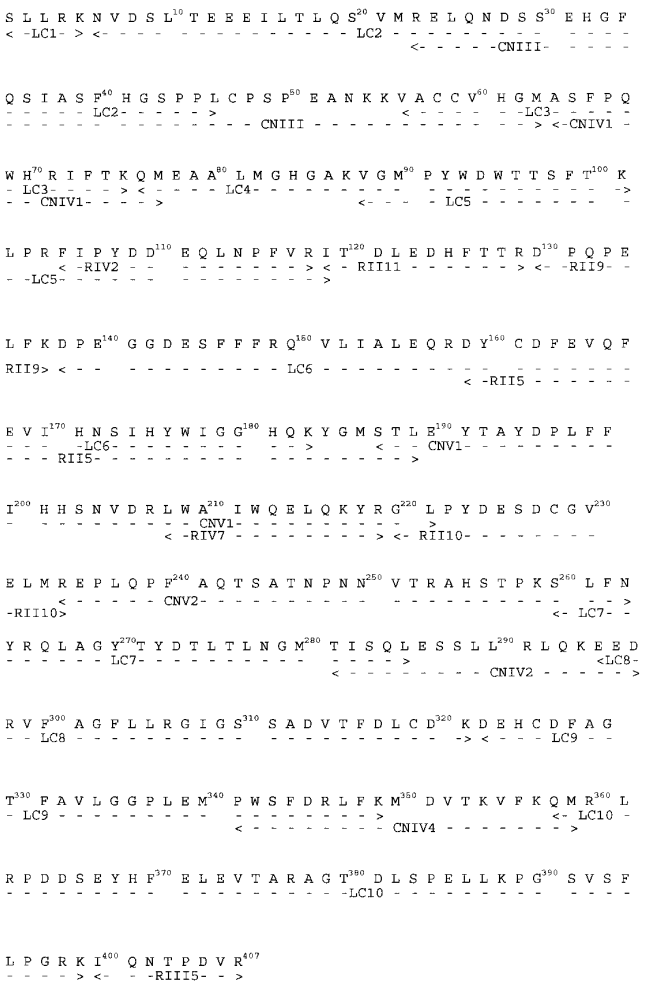

FIG. 1. Amino acid sequence of the N-terminal oxygen binding functional unit Rta of the *Rapana* hemocyanin subunit RHSS2 determined using EndoLysC (LC), cyanogen bromide (CN) and tryptic fragments (R) of Rta. Only peptides useful for the sequence and overlap determination are shown.

modified domain were collected and dialysed against 5 % acetic acid. The product was lyophylized.

Enzyme hydrolysis with EndoLysC proteinase. Cleavage of the reduced and carboxymethylated oxygen-binding unit Rta with EndoLysC proteinase, specifically hydrolyzing bonds C-terminally at lysine, was performed as follows: 1.5 mg of the modified Rta were dissolved in 0.3 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 LysC in water, at an enzyme/substrate ratio of 1/100 (w/w) for 22 h at 37 °C. HPLC separation of peptides was performed on a Nucleosil 7C_{18} column (250 × 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: 0 % B for 2 min than 0 % to 100 % B in 97 min at a flow rate of 2.5 ml/min.

Hydrolysis of maleylated Rta with trypsin. Maleic anhydride was used for the reversible blocking of the amino groups in the polypeptide chain of Rta. Trypsin splits the blocked protein only at arginine residues and a decreased complexity of the tryptic digest was expected. 60 mg of the reduced and carboxymethylated Rta were dissolved in 10 ml 50 mM borate buffer, pH 9.0, containing 2 M urea and treated at 2 °C with a 20-fold excess of maleic anhydride over the total amino groups. Dry maleic anhydride was added in six portions and the pH of the mixture was maintained at 9.0 by sodium carbonate. When the reaction was completed, the maleyl-Rta was desalted by dialysis against 0.5 % ammonium hydrogen carbonate. Tryptic cleavage was performed at 37 °C for 18 h by addition of TPCK-trypsin to a final concentration of 2 % (w/w). The tryptic digest was separated by gel filtration on a column of Sephadex G-50 (150 × 1.8 cm) in a 0.1 M ammonium hydrogen carbonate, pH 8.5. The fractions were further separated by HPLC on a Macherey-Nagel Nucleosil 300-7 C_4 (250 × 10 mm) reverse-phase column as described in the previous paragraph.

Cleavage with cyanogen bromide. Cleavage of the reduced and carboxymethylated functional unit Rta was made by the method described in (29). 16 mg of Rta in 3 ml 70 % formic acid were treated with CNBr (200-fold molar excess regarding to the methionyl residues). The reaction mixture was left overnight under nitrogen at room temperature, then flushed with nitrogen to remove most of the CNBr. The peptides were separated on a Sephadex G-100 column (180 × 2.6 cm), eluted with 30 % acetic acid and purified by HPLC as described in a preceeding paragraph.

Amino acid analysis. Samples were hydrolyzed in 5.7 M HCl at 110°C in vacuo for 24 h and analyzed in an amino acid analyzer LC-3000 (Biotronik/Eppendorf, Maintal, Germany). Tryptophan was determined in the presence of 6 % thioglycolic acid. Oxidation of the protein with performic acid (1vol. of 30 % (w/w) H_2O_2 to 9 vol. of 88 % (w/w) formic acid), converting cysteine and cystine into cysteic acid, was used for the quantitative determination of these residues.

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 phenylthiohydantoin derivatives. Approximately 50-500 pmol of peptides were applied on the cartridge filter previously treated with polybrene.

Mass spectrometric analysis. Mass spectra were obtained by matrix-assisted laser desorption ionization mass spectrometry (Kratos, MALDI I equipment, Shimadzu, Europe). Peptides (10-50 pmol) were dissolved in 0,1 % (v/v) TFA and applied onto a target. Analysis was carried out in α-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid. Solutions of human substance P (M=1347.7 Da) and bovine insulin (M=5733.6 Da) were used to calibrate mass scale. The mass values assigned to the amino acid residues are the average masses.

RESULTS AND DISCUSSION

The complete amino acid sequence of the *Rapana* Hc functional unit Rta was determined by N-terminal sequencing of the whole protein and peptides obtained by cleavage with EndoLysC proteinase, TPCK-trypsin and CNBr. In order to generate a limited number of peptides, a CNBr cleavage was carried out on the reduced-alkylated protein. Fractionation of the obtained fragment mixture by gel filtration on Sephadex G-100 in 30 % acetic acid, followed by reversed-phase HPLC on a C_{18} column (data not shown), resulted in the isolation of about 20 fractions. The trypsin digest fragment mixture was first fractionated by gel filtration on Sephadex G-50 in 0.1 M ammonium hydrogen carbonate and then the peptides were isolated by reversed-phase HPLC on a C_4 column. The LysC digest was directly fractionated by reversed-phase HPLC on a C_{18} column yielding 10 individual peptide fragments. An aliquot

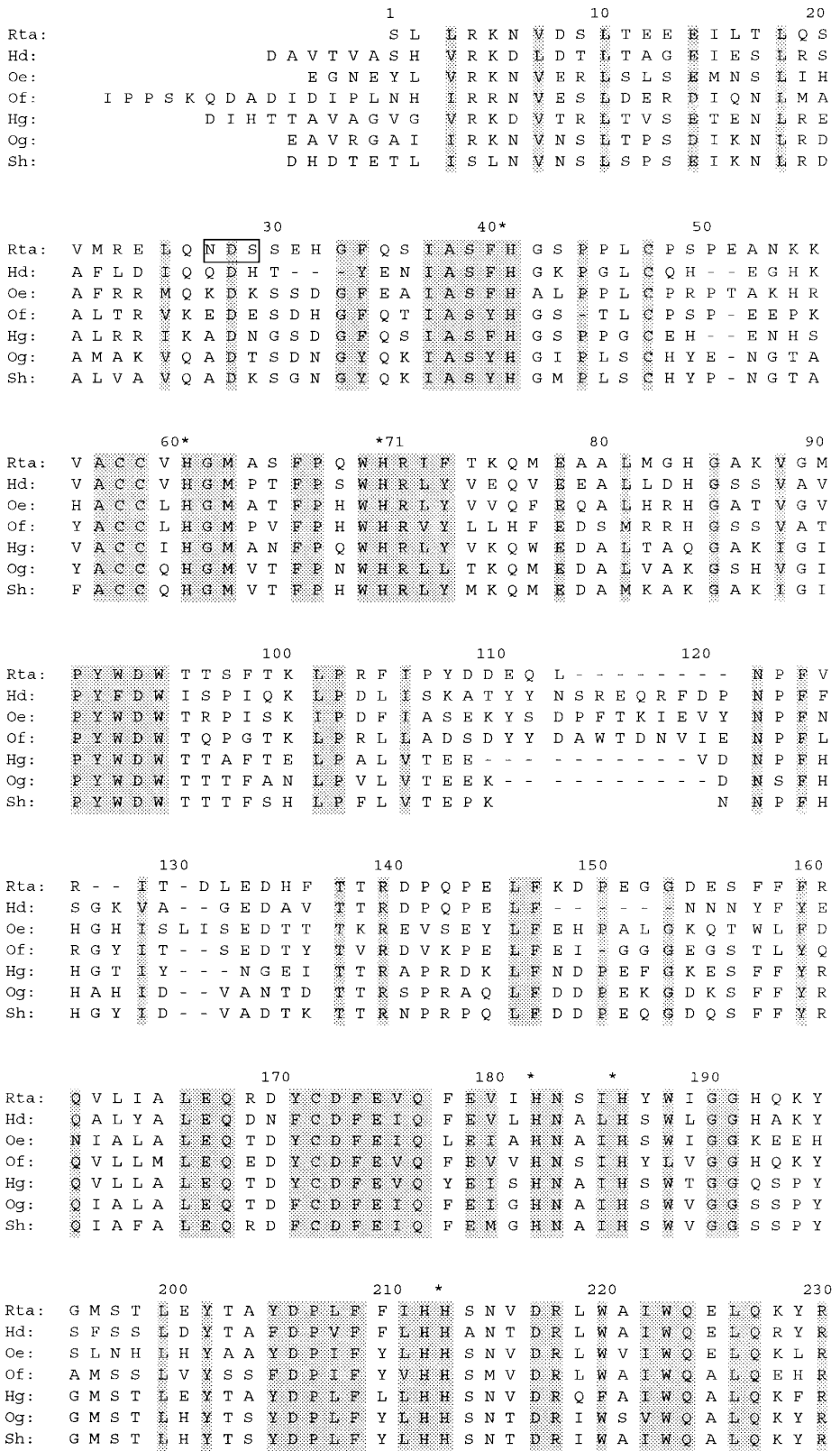

FIG. 2. Alignment of amino acid sequences of molluscan hemocyanin oxygen-binding functional units. The indications for the units are: Rta, N-terminal unit of the *Rapana thomasiana* (marine gastropod) hemocyanin; Hd and Hg (8, 10) units from the *Helix pomatia* (terrestrial gastropod) hemocyanin; Oe, Of and Og (11), units from the *Octopus dofleini* (cephalopod) hemocyanin (Og is a C-terminal unit) and Sh (12) is the C-terminal unit from the *Sepia officinalis* (cephalopod) hemocyanin. Conserved residues among these proteins are highlighted. Histidyl residues, supposed to be copper ligands, are marked with an asterisk (*) and deletions by (−). The following groups of amino acid residues are considered to be isofunctional: E and D; N and Q; S and T; S and C; M, L, I and V; F, Y and W; H, K and R. Numbering refers to the sequence of Rta.

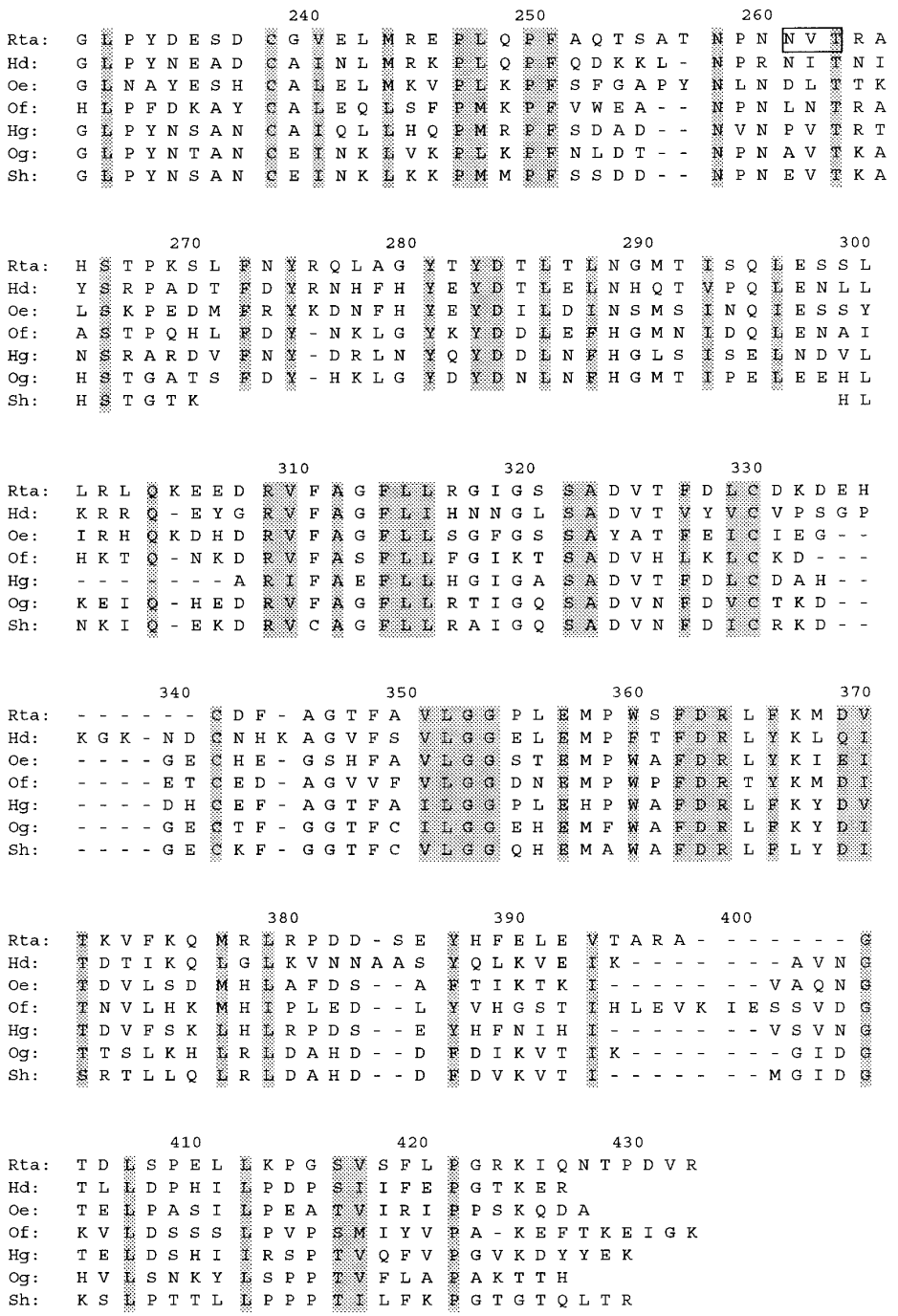

FIG. 2—*Continued*

of each fraction (5-10 pmol) was analysed by LDMS. Screening of HPLC fractions by LDMS was very useful before Edman degradation in providing information on fraction purity, peptide size and for the choice of peptides to be sequenced. Edman degradation was carried out on individual fractions. All fragments could be sequenced to the end except LC2, LC5, RII5, CNV1, CNV2, LC7 and CNIV2 (Table 1, Fig. 1).

The sequence studies showed that LysC cleavage was not complete, leading to fragments with an internal lysine (LC 4, Lys 87; LC 5, Lys 101), which were very useful for the alignment of the neighboring peptides. LC 4 (residues 76-101) allowed the overlap of CN IV 1 and LC 5. Similarly, LC 5 (residues 88-119) provided information for the positioning of R IV 2 and R II 11. The C-terminal residue of

TABLE 2

Amino Acid Compositon of the N-Terminal Oxygen Binding Functional Unit Rta of the *Rapana* Hemocyanin Subunit RHSS2

Amino acid	Rta
Asp	29.4 (29)
Asn	(12)
Thr	23.7 (25)
Ser	27.8 (29)
Glu	29.9 (29)
Gln	(20)
Pro	23.8 (25)
Gly	26.4 (24)
Ala	20.4 (20)
Cys	6.8 (7)
Val	18.8 (20)
Met	11.5 (11)
Ile	13.2 (14)
Leu	38.7 (41)
Tyr	12.6 (13)
Phe	27.6 (28)
His	15.2 (14)
Lys	17.4 (17)
Arg	21.6 (22)
Trp	6.5 (7)

Residues were calculated from 5,7 N HCl hydrolysates at 110°C for 24 h. Aspartic and glutamic acid values are the sum of their acids and amides. Tryptophan was determined in the presence of 6% thioglycolic acid. Cysteine determination was performed after oxidation with performic acid. Values in parentheses are calculated from the established sequence of Rta as shown in Fig. 1.

R II 9 is a lysine showing incomplete maleylation of Rta.

The alignment of the first two LysC peptides and CNIII was achieved on the basis of the N-terminal sequence of the intact polypeptide chain (33 steps) (Fig. 1). The other peptides were located by homology with the known sequences of the molluscan Hc functional units. For reasons of simplicity only 23 peptides from the three hydrolysates, isolated by HPLC and analyzed by MALDI-MS and automatic Edman degradation, were used for the construction of the Rta primary structure as shown in Table 1 and Fig. 1. The peptides were chosen to achieve maximal overlap of the fragments. Two fragments (RII9 and RIII5) were aligned only by homology (Fig. 1, Fig. 2). A good agreement between the measured masses of all main peptides and the masses calculated from the respective sequences was observed (Table 1). Moreover, the amino acid composition determined by acid hydrolysis of the protein, is in complete agreement with that calculated from the established amino acid sequence (Table 2).

Rapana Hc is a glycoprotein with a carbohydrate content of 8.9 % (w/w) and monosaccharide constituents xylose, fucose, 3-O-methyl-galactose, mannose, galactose, N-acetylgalactosamine and N-acetylglycosamine residues (30). Carbohydrates have important biological functions: they stabilize membranes, participate in the biological recognition, influence the protein folding (31) and probably, the antigenic properties of Hcs are stipulated, at least in part, by their specific oligosaccharide content. In molluscan Hcs the carbohydrates are N-glycosidically linked via N-acetylglycosamine to the amide nitrogen of asparagine (32), where the asparagine is part of the triplet amino acid sequence N-X-T/S and X is any amino acid other than proline (31). Inspection of the Rta sequence (Fig. 1) revealed Asn_{27} and Asn_{250} (Asn_{261} in Fig. 2) as glycosilation sites and the respective three-residues segments as Asn^{27}-Asp^{28}-Ser^{29} and Asn^{250}-Val^{251}-Thr^{252}. The bound carbohydrate moieties showed molecular weights of 1405.7 Da and 1037.9 Da, resulting from the mass difference between the observed mass for the fractions LC 2 (MH^+=6835.6 Da) and CN V 2 (MH^+=6264.8 Da) and the calculated masses from the peptide sequence (M=5428.9 Da and M=5225.9, respectively). Although many hemocyanins (arthropodan and molluscan) are known to be glycoproteins, details on the structure and location of the carbohydrates have only been studied in a few cases (32-35). Thus, the functional units g and d of the *Helix pomatia* β_c-Hc were found to contain three and two carbohydrate attachment sites at positions 29, 53, 404 and 261 and 404, respectively (Fig. 2) (33).

The molecular mass of the polypeptide part of Rta was calculated from the amino acid sequence (Fig. 1) to be 47 250 Da. On subtracting the polypeptide contribution from the molecular mass of 49 698 Da determined by laser desorption mass spectrometry for the functional unit (28), a value of 2448 Da or 5.1 % was obtained for the carbohydrate content of Rta.

It was demonstrated that in several molluscan hemocyanin functional units the positions of the histidyl residues, serving as ligands for copper ions, are conserved (11). Comparison of the Rta primary structure with the published sequences (11) showed that seven of the fourteen histidyl residues are conserved and histidines 41, 61, 70 (A site), 182, 186 and 213 (B site) (Fig. 2) are candidates for copper ligands at the respective dioxygen-binding site. The fact that both, A and B dioxygen-binding sites, in molluscan hemocyanins involve three histidine ligands is in good agreement with studies showing similarity in the resonance Raman spectra between arthropodan and molluscan hemocyanins (36). Determination of the three-dimensional structure of a molluscan hemocyanin will provide more detailed information on its active site. In order to elucidate evolutionary relationships in molluscan Hc functional units, the amino acid sequences of six domains (8-12), four from cephalopodan (*Octopus dofleini* and *Sepia officinalis*) and two from a terrestrial gastropod Hc (*Helix pomatia*)

TABLE 3

Percent Sequence Identity Scores between the *Rapana* Hemocyanin N-Terminal Functional Unit and Other Molluscan Dioxygen-Binding Units

Functional unit	% Homology
Rta-Hd	50 (60)
Rta-Oe	47 (58)
Rta-Of	49 (60)
Rta-Hg	43 (51)
Rta-Og	50 (61)
Rta-Sh	48 (59)

Abbreviations used: Rta, *Rapana* Hc N-terminal functional unit; Hd and Hg, *Helix pomatia* Hc functional units d and g (8, 10); Oe, Of and Og, *Octopus dofleini* Hc functional units e, f and g (11) and Sh, *Sepia officinalis* Hc functional unit h (12). The values in brackets show the percent identity scores when isofunctional residues were also taken into consideration.

species were aligned (Fig. 2). The *Rapana* Hc is the only representative of marine gastropods. The data indicate that domains are well conserved between Hcs from different molluscs: an average identity of 48±5% or 58±7% including isofunctional residues was found (Table 3). The highest identity score between the *Rapana* Hc functional unit and the other units was observed for the pairs Rta - Hd and Rta - Og, where Hd and Og are the functional units d and g of the Hcs from *Helix pomatia* (8) and *Octopus dofleini* (11), respectively. The lowest identity is between Rta and Hg, where Hg is the dioxygen-binding unit g of the *H. pomatia* Hc (10). However, it should be taken into account that no other N-terminal domain sequence of molluscan Hc has been published yet and one can speculate that the highest identity score will be found with other N-terminal functional units.

In conclusion, the high degree of homology observed for functional units derived from Hcs of different species suggests that probably the dioxygen-binding units of the molluscan respiratory proteins evolved from a common ancestral gene.

ACKNOWLEDGMENT

We express our gratitute to the Volkswagen-Stiftung (Hannover, Germany) for financial support (research grant No. I/70524).

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