The Sequence of a Gastropod Hemocyanin (HtH1 from *Haliotis tuberculata*)

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The eight functional units (FUs), a–h, of the hemocyanin isoform HtH1 from *Haliotis tuberculata* (Prosobranchia, Archaeogastropoda) have been sequenced via cDNA, which provides the first complete primary structure of a gastropod hemocyanin subunit. With 3404 amino acids (392 kDa) it is the largest polypeptide sequence ever obtained for a respiratory protein. The cDNA comprises 10,758 base pairs and includes the coding regions for a short signal peptide, the eight different functional units, a 3′-untranslated region of 478 base pairs, and a poly(A) tail. The predicted protein contains 13 potential sites for N-linked carbohydrates (one for HtH1-a, none for HtH1-c, and two each for the other six functional units). Multiple sequence alignments show that the fragment HtH1-abcd efgh is structurally equivalent to the seven-FU subunit from *Octopus* hemocyanin, which is fundamental to our understanding of the quaternary structures of both hemocyanins. Using the fossil record of the gastropod-cephalopod split to calibrate a molecular clock, the origin of the molluscan hemocyanin from a single-FU protein was calculated as 755 ± 68 million years ago. This fits recent paleontological evidence for the existence of rather large mollusc-like species in the late Precambrian.

The blue copper-containing respiratory protein hemocyanin occurs in molluscs as a ring-like decamer with a molecular mass of 4 MDa, consisting in a wall made up of 60 globular functional units (FUs) and an internal collar complex of either 10 or 20 functional units, depending on the species. In cephalopods, such decamers are the only hemocyanin quaternary structure observed, but in gastropods two decamers are assembled face to face to form the so-called didecamer. It is well established that the cephalopod and gastropod hemocyanin decamers are the only hemocyanin quaternary structures of both hemocyanins. Using the fossil record of the gastropod-cephalopod split to calibrate a molecular clock, the origin of the molluscan hemocyanin from a single-FU protein was calculated as 755 ± 68 million years ago. This fits recent paleontological evidence for the existence of rather large mollusc-like species in the late Precambrian.

**EXPERIMENTAL PROCEDURES**

**Animals**—The European abalone *Haliotis tuberculata* is a member of the phylogenetically rather ancient Archaeogastropoda. Animals were gifts from Syndicat Mixte d’Équipement du Littoral Blainville sur Mer, France and Biosyn, Fellbach, Germany. The abalone were kept in a sea water aquarium at 17 °C and fed on brown algae.

**Construction and Screening of cDNA Libraries**—RNA was isolated from *Haliotis tuberculata* mantle tissue (1 g) using an RNeasy Maxi kit (Qiagen, Hilden, Germany) according to the instruction manual followed by mRNA isolation using paramagnetic beads from Promega (Mannheim, Germany). Two cDNA libraries were constructed using the Lambda ZAP®-CMV cDNA synthesis kit from Stratagene (Heidelberg, Germany). Pooled RNA from 19 and 2 individuals, respectively, was applied. The first library stems from Keller et al. (12) and was oligo(dT)-primed; the second library was constructed in the present study, using random primers (Life Technologies, Inc.) in addition to specific HtH1 oligonucleotides, derived from the cDNA encoding HtH1-e and HtH1-f. Screening was performed with digoxigenin-labeled DNA probes.

**Isolation and Analysis of cDNA Clones**—cDNA clones obtained from the cDNA libraries were isolated using the *in vivo* excision protocol from Stratagene. cDNA-containing plasmids were isolated using the QIAprep Spin Miniprep kit (Qiagen) and restricted with EcoRI and *XhoI* (Stratagene) for clones that were derived from the first cDNA library where the cDNA fragments were cloned in a directional manner. In the case of the random-primed cDNA library, cDNA clones were analyzed only by EcoRI restriction. cDNA clones with different restriction patterns were sequenced by Seqlab (Göttingen, Germany) using standard primers. Subsequent sequencing reactions on both strands were performed with specific oligonucleotides.

**Computer Software**—The obtained sequences were analyzed by the latest versions of CHROMAS, Translate Tool, ALIGN, and Signal P

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The abbreviations used are: FU, functional unit; HtH, *Haliotis tuberculata* hemocyanin; HtH1, *Haliotis tuberculata* hemocyanin; KLH, keyhole limpet (*Megathura crenulata*) hemocyanin; OdH, *Octopus dofleini* hemocyanin; RkH, *Rapana thomassiana* hemocyanin; bp, base pair.
from Ex PASY Proteomics Tools; Clustal (14); and TreeView® (15); all of these programs are freely available in the Internet.

Linearization of the Trees and Time Estimations—Corrected pairwise distances were calculated with the PROTDIST program of the PHYLIP software package (16) and scaled in expected historical events per site (17). Distance matrices were imported into the Microsoft Excel PHYLIP software package (16) and scaled in expected historical events as calculated from a percent accepted mutation matrix (18). For each functional unit (indicated on the right), the copper A site is underlined, the copper B site is double underlined, and the copper binding histidines are white letters in black boxes. The peptide bridges linking the functional units are shaded as are the 13 potential N-linked carbohydrate attachment sites. Note that functional unit HtH1-c is devoid of potential sites for N-linked sugar chains. Shaded arrow, start of the tail extension of functional unit HtH1-b.

RESULTS

From the two cDNA libraries we isolated five different cDNA clones, together encoding the previously unknown N-terminal fragment HtH1-abcd and the recently published C-terminal fragment HtH1-fgh (see Ref. 12) of the subunit of Haliotis hemocyanin isoform 1 (HtH1). Overlapping regions of about 300 bp of the different cDNA clones were analyzed to ensure that they represented a single mRNA. The complete cDNA encoding HtH1 comprises 10,758 bp (including a 3'-untranslated region of 478 bp plus a poly(A) tail of 18 bp); it is available from the EBI Data Bank (accession number Y13219). The 5'-untranslated region and the triplet ATG coding for the very first methionine of the subunit have been unsuccessful to date. We assume that there are still a few amino acids of the signal peptide missing, but from the N-terminal motif the biochemically isolated HtH1 subunit obtained by direct protein sequencing (DNVRKDVSHLTD; DEVQ; see Ref. 12), it is clear that we have now obtained the complete sequence of the hemocyanin secreted into the hemolymph.

The amino acid sequence of the Haliotis hemocyanin subunit predicted from the cDNA sequence contains 3404 amino acids plus the signal peptide of 15 amino acids (Fig. 1). Identification of the latter was confirmed using the computer program Signal P (not shown). Because the only molluscan hemocyanin subunit completely sequenced previously, from Octopus, has a length of 2896 amino acids due to its lack of FU-h (6), the present Haliotis hemocyanin primary structure is the largest ever obtained for a respiratory protein, and it is certainly among the largest polypeptides in nature. The sequence is clearly substructured into eight homologous regions of 405–420 amino acids (Fig. 1), corresponding to the eight different functional units HtH1-a–HtH1-h identified at the protein level; HtH1-h carries a unique tail extension of ~95 additional amino acids (see also Ref. 12). The N-terminal partial sequences of the biochemically isolated HtH1 functional units obtained from direct Edman degradation (12) fit the present sequence 100%, which was therefore conclusively identified as HtH1. Moreover, the ~300-bp sequence overlaps of the cDNA clones ensured that we did indeed analyze cDNAs encoding a continuous polypeptide chain. For each functional unit, some characteristics calculated from the sequence are shown in Table I. A multiple sequence alignment of the different functional units from HtH1 and OdH is shown in Fig. 2. For illustration of the structural aspects, a schematic representation of the x-ray structure of OdH-g is given in Fig. 3 (re-drawn from Refs. 6 and 7). The sequence identities and similarities, as calculated from a broader sequence alignment of molluscan hemocyanin functional units, are shown in Fig. 4, and a phylogenetic tree is presented in Fig. 5. Finally, Fig. 6 shows a time scale of the phylogenetic diversification of the sequenced molluscan hemocyanins as calculated from a percent accepted mutation matrix (17), assuming 520 million years ago for the gastropod-cephalopod split.

DISCUSSION

Significance of the Haliotis Hemocyanin Sequence—Both Octopus and Sepia hemocyanin have been studied to much detail (see Refs. 1, 20–22), but the established cephalopod reference hemocyanin is clearly that from Octopus, because it has been completely sequenced (6). However, cephalopod hemocyanins...
are restricted to decamers, and therefore, their analysis is inefficient to explain the didecamers and multidecamers observed in other molluscan classes, notably the Gastropoda. In gastropods, the “classical” hemocyanin studied is that from *Helix pomatia* (see Ref. 1), but its sequence is only partially known (see Fig. 5). The recent biochemical characterization of *Halioitis* hemocyanin (12, 13), in combination with the complete amino acid sequence presented here, now establishes HtH1 as the gastropod reference hemocyanin. Together with the partial sequence of HtH2 (13), which will soon be completed, questions of the biological significance and regulation of the two physiological isoforms found in prosobranch species (see Refs. 4, 5, 23, and 24) can now be approached at the single amino acid level.

More importantly, the combined present and future data from *Octopus* and *Halioitis* hemocyanin will enable fundamental questions on the structure-function relationships of molluscan hemocyanin to be solved, which could not be efficiently addressed if only one of the two reference sequences was available. For example, the sequence of *Octopus* hemocyanin alone allowed the identification of amino acid residues that are conserved in all seven functional units (6), whereas together with the *Halioitis* hemocyanin sequence, residues can now be identified that have been specifically conserved in corresponding functional units over more than 500 million years but are different in the other functional unit types (Fig. 2). In addition, gastropod- and cephalopod-specific sequence motifs are now discernible (Fig. 2). Thus, efficient strategies for the analysis of specific functions of the different functional units (for example, with recombinant hemocyanin) can now be designed, which was impossible on the basis of the *Octopus* sequence alone. Moreover, tracing the as yet unclear path of the elongated subunit within the native quaternary structures is now greatly facilitated, because electron microscopical and biochemical data from the decameric cephalopod and the didecameric gastropod hemocyanin can now be combined on the basis of sequence alignments. Ultimately, the date of the evolutionary origin of molluscan hemocyanin, which was previously only roughly estimated (1, 13), can now be traced with greatly improved accuracy by using the complete cephalopod and the complete gastropod hemocyanin primary structure in combination.

**Localizing Potential Sugar Sites**—The total molecular mass of the secreted polypeptide calculated from the sequence (therefore neglecting possible carbohydrate side chains) is 392 kDa. This is very close to the value of ~400 kDa measured in SDS-polyacrylamide gel electrophoresis for both HtH1 and KLH1 (12, 24). From the multitude of conserved and variable structural features visible in Fig. 2, the potential N-linked carbohydrate sites (NXT/S) will now be discussed in more detail. 13 such sites exist in the sequence of *Halioitis* hemocyanin isoform HtH1 (Fig. 1), which, according to the X-ray structure of functional unit OdH-g from *Octopus* hemocyanin (Fig. 3), would all be accessible on the protein’s surface. If they all carry an oligosaccharide side chain similar to that confirmed for OdH-g (−1 kDa; see Ref. 6), they would together increase the molecular mass of the *Halioitis* hemocyanin subunit to ~405 kDa.

The X-ray structure of functional unit OdH-g (7) consists in an α-helix-rich “core domain” and a “β-sandwich domain” rich in β-strands (Fig. 3). In OdH-g between strands β2 and β3 of the core domain, a carbohydrate side chain is anchored that protrudes toward strand β12 in the β-sandwich domain. This view of the 3-dimensional structure has therefore been called the “carbohydrate face” (7). In functional unit HtH1-g a potential N-linked sugar site is present in a similar position (Figs. 2 and 3). Interestingly, in FU-a, FU-d, FU-e, and FU-f of both *Halioitis* hemocyanin isoform HtH1 and *Octopus* hemocyanin, a potential N-linked sugar site is present in the region of strand β12, suggesting that a carbohydrate chain anchored there is likely to decorate the carbohydrate face from the opposite direction (Figs. 2 and 3).

The second region in which some functional units contain potential N-linked carbohydrate sites lies in the core domain as a group of exposed loops (the connections between α2/α3, α5/β4, β4/β5, and α11/α12; see Fig. 3). There, FU-d and FU-f of both species as well as HtH1-e and HtH1-g possess a second potential N-glycosylation site (Figs. 2 and 3). This suppression is interesting, because it avoids a second sugar chain in the carbohydrate face that would sterically interfere with the sugar chain potentially anchored to the accessible site in strand β12 (see Fig. 3). Another specific difference between *Halioitis* hemocyanin isoform HtH1 and *Octopus* hemocyanin concerns FU-b. Whereas functional unit OdH-b shows a single potential sugar site in the standard β12 position, in HtH1-b two such sites occur in the core domain, but none occur in the carbohydrate face (Figs. 2 and 3). Surprisingly, functional units HtH1-c and OdH-c both lack a potential N-glycosylation site (Fig. 2); in OdH-c the sequence NPT exists (in the position of helix α6), but it should be masked by the central proline (cf. Ref. 25). Indeed, carbohydrate analysis

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**TABLE I**

**Properties of the *Halioitis* hemocyanin isoform HtH1 and its functional units as predicted from the cDNA sequence**

The cleavage points between the various functional units are somewhat arbitrary. *, values estimated from SDS-polyacrylamide gel electrophoresis of proteolytic fragments of HtH1 (12). The molecular mass discrepancies in the case of HtH1-a and HtH1-d suggest that the two proteolytic fragments did not correspond exactly to a single functional unit, respectively. Note that except for HtH1-c, 1000–2000 Da should be added per functional unit for carbohydrate side chains, as deduced from the situation in OdH-g (6,7).

| Structure          | Base pairs | Amino acids | pI  | Molecular mass | Molecular massa |
|--------------------|------------|-------------|-----|----------------|----------------|
| signal sequence    | 47         | 15          | 5.52| 1,489.8        |                |
| HtH1-abcdgfh       | 10,215     | 3,404       | 5.69| 391,632.6      | 400            |
| HtH1-a             | 1,221      | 407         | 5.70| 46,527.2       | 53             |
| HtH1-b             | 1,257      | 419         | 5.41| 48,078.8       | 48             |
| HtH1-c             | 1,242      | 414         | 5.64| 47,889.5       | 46             |
| HtH1-d             | 1,239      | 413         | 5.25| 47,715.2       | 40             |
| HtH1-e             | 1,260      | 420         | 5.84| 48,363.2       | 49             |
| HtH1-f             | 1,251      | 417         | 6.04| 47,624.7       | 50             |
| HtH1-g             | 1,209      | 403         | 5.52| 46,226.6       | 45             |
| HtH1-h             | 1,535      | 511         | 5.98| 59,205.9       | 60             |
| 3'-untranslated region | 478       | 9           |     | 9              |                |
| poly(A) tail       | 18         |             |     |                |                |
of keyhole limpet hemocyanin has revealed that in contrast to all other functional units, KLH1-c is devoid of any sugar moiety, whereas KLH2-c lacks N-linked carbohydrates yet contains O-linked sugars (26). Functional unit HtH1-h has two sugar sites in quite unusual positions (Fig. 2 and 3), which has already been discussed in detail elsewhere (12, 13). Significantly, Haliotis hemocyanin isoform HtH1 shows 13 potential N-glycosylation sites per subunit, and Octopus hemocyanin shows only seven (excluding the NPS/T sites). It should be noted that HtH1 but not Octopus hemocyanin shows three NXC sequences in addition (in positions 1412, 1578, and 2956; see Fig. 2), which, according to Miletich and Broze (27), also might have the potential to bind carbohydrates. However, as deduced from the x-ray structure of functional unit OdH-g, all three sulfhydryl groups form a disulfide bond (Fig. 2) and are therefore inefficient as a hydrogen bond acceptor for the glycosylation reaction.

In comparing the two hemocyanin isoforms from Haliotis, the subunit fragment HtH1-defgh contains ten potential N-linked carbohydrate sites, and HtH2-defgh contains only six (see Ref. 13 and this study). Such fundamental differences in glycosylation could well play a biological role; the counterparts of the two hemocyanin isoforms HtH1 and HtH2 in the keyhole limpet, KLH1 and KLH2, are differentially regulated physiologically. They prevail in the hemolymph for different periods, depending on the physiological condition of the animal, with KLH1 selectively disappearing from the hemolymph during starvation (see Refs. 4 and 5). In contrast Haliotis hemocyanin, the relative proportion of the two isoforms also varies considerably between individuals (12). The present sequence data suggest that the two hemocyanin isoforms found in the prosobranch gastropods might be selectively recognized and sequestered via their differential glycosylation.

Implications for the Quaternary Structure—Despite the 15-Å structure of the didecamer of keyhole limpet hemocyanin isoform KLH1 (8), the 2.3-Å structure of functional unit OdH-g
that the six wall-forming functional units from *Haliotis* hemocyanin isofrom HtH1 and *Octopus* hemocyanin form six discrete branches (Fig. 5). This means that functional units from *Haliotis* and *Octopus*, which occupy the same position in the elongated subunit and therefore carry the same designation, correspond to each other structurally. Indeed, if the hemocyanin sequences of *Haliotis* and *Octopus* are aligned and functional unit HtH1-h is chopped off at exactly the point where the *Octopus* hemocyanin ends (Fig. 2), the number of amino acids constituting the remaining fragment HtH1-abedefg is 2897, which is surprisingly close to the value of 2896 for the whole *Octopus* hemocyanin. Only 19 gaps for a single amino acid and two gaps for an amino acid pair have to be introduced for a continuous alignment of both sequences (not shown). Moreover, as judged from the x-ray structure of functional unit OdH-g, most of these small gaps are not within α-helical or β-strand regions. This is all very strong evidence that the wall architecture is similar in these two phylogenetically distant molluscan hemocyanins, and consequently, it is highly unlikely that from the proposed models of subunit arrangement the parallel version will hold true for some hemocyanins and the anti-parallel for others. Because computer-processed 3-dimensional reconstructions derived from electron micrograph images strongly support an anti-parallel arrangement in the cases of KHL1 and *Octopus vulgaris* hemocyanin (8, 21), the parallel models proposed from other evidence for *Helix pomatia* and *Sepia officinalis* hemocyanin (36) and later by our own group for KHL2 (28) are likely to be incorrect.

The newly available *Haliotis* hemocyanin sequence is especially stimulating in view of upcoming higher resolution 3-dimensional reconstructions of gastropod hemocyanin molecules, including a 12Å structure of the HtH1 didecamer, which is already available in our laboratory. Using the present sequence from the *Haliotis* hemocyanin subunit and the x-ray structure of functional unit OdH-g, molecular modeling experiments are in progress to predict the ternary structure of each functional unit, which then could be fitted into the HtH1 quaternary structure already obtained from electron microscopy. In this context, the data on possible glycosylation sites (Fig. 3) should also help, because glycosylated regions should be exposed to the free solvent and not directed toward a closely apposed functional unit. The next goal will then be to identify the locations of those amino acids that establish the intersubunit contacts within the decamer and didecamer.

*Tracing the Evolution of Molluscan Hemocyanins and of the Phylum Mollusca*—For the phylogenetic tree we chose an unrooted radial representation (Fig. 5), because no suitable outgroup is yet available; as shown by our previous work (13), the relationship to tyrosinase was found to be too remote for this purpose. In our recent phylogenetic analysis we found it impossible to resolve the evolutionary branching orders among the different functional units, indicating that they evolved very rapidly from their ancestral precursor (13). Indeed, even with the five additional functional unit sequences included, this aspect of the tree is not improved, with the branching pattern of the different functional unit types still being highly unstable (Fig. 5). However, the stable branches of the eight different functional units demonstrate that FU-a–FU-h existed individually long before gastropods and cephalopods separated, which according to fossil records was about 520 million years ago in the late Cambrian (18). This also means that in cephalopods, the lack of FU-h is the result of a secondary loss. The only exception is functional unit “a” from *Rapana thomasi*ana he-
mocyanin (RtH2-a), which groups together with the functional units of type "g" of the other hemocyanins and moreover, branches off from this line before the gastropod-cephalopod separation (Fig. 5), although Rapana is a prosobranch gastropod. This phenomenon has already been discussed (12, 13), but in view of the present "correct" grouping of functional units "a" from Haliotis and Octopus hemocyanin together in one branch it is even more difficult to interpret. A recent immunochemical analysis of Rapana hemocyanin also revealed some unusual features (37). Functional unit RtH2-a has been analyzed by direct Edman degradation (11), and confirmation by cDNA sequencing might be appropriate in this confusing case, because if the sequence holds true, Rapana hemocyanin could become extremely interesting in terms of evolution.

Using the gastropod-cephalopod split to calibrate a molecular clock, from the structural divergencies of the different functional units we calculated the HtH1-HtH2 split 320 ± 60 million years ago (Fig. 6), and the prosobranch-pulmonate split 359 ± 24 million years ago (Fig. 6); the latter corresponds to the occurrence of the first pulmonate fossils in the early Carboniferous (18). The common origin of the different functional units could now be dated back, with much better statistics than in the previous rough estimations (1, 13), 753 ± 68 million years ago (Fig. 6). We believe that this early event does indeed signal the birth of a protein for extracellular oxygen transport. The different functional units arranged in an elongated subunit are the prerequisite for hemocyanin oligomerization; in turn, oligomerization is required for hemocyanin to function efficiently as an extracellular blood oxygen carrier (for colloid-osmotic and rheological reasons as well as for establishing allosteric effects of the respiratory protein; see Ref. 38). On the other hand, extracellular oxygen carriers only make sense for compara-

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**Fig. 5.** Radial phylogenetic tree of molluscan hemocyanin functional units. This unrooted tree is based on a Clustal multiple alignment of the currently available, complete functional unit sequences. They stem from hemocyanins of the prosobranch gastropod Haliotis tuberculata (HtH1, HtH2), the cephalopod Octopus dofleini (OdH), the pulmonate gastropod Helix pomatia (HpHβ), the prosobranch gastropod Rapana thomassiana (RtH2), and the cephalopod Sepia officinalis (SoH). For sources, see Refs. 6, 9–13, and 42. Because functional unit "h" from Sepia corresponds to functional unit "g" from other molluscan hemocyanins, it is termed here SoH/h-g as recently proposed (see Refs. 12 and 13). Bootstrap percentages are based on 1000 replicates. Bootstrap values (33) are shown only if >50. Note that with the exception of RtH2-a (see “Discussion”), topologically corresponding functional unit types group together to form eight distinct branches.

**Fig. 4.** Matrix of percent identity (top) and percent similarity (bottom) in compared sequences from functional units of Haliotis and Octopus hemocyanin. Multiple sequence alignment was done with Clustal software. The Haliotis hemocyanin sequences stem from our previous studies (12, 13) and the present study; the Octopus hemocyanin sequences were taken from Miller et al. (6). In the case of the similarity values, isofunctional exchanges are also considered. Note that each corresponding pair of the wall-forming functional units of the two hemocyanin isoforms from Haliotis that have now been sequenced (FU-d, FU-e, FU-f) shares 65–66% sequence identity. In contrast, the identity of the arc-forming functional units HtH1-g and HtH2-g is significantly higher (74%), and the identity of the collar-forming components HtH1-h and HtH2-h is somewhat lower (60%). In the phylogenetic tree this is illustrated by different branch lengths (see Fig. 5); it suggests that in the two hemocyanin isoforms of Haliotis, the molecular clocks of wall, arc, and collar run at different rates.

**Fig. 6.** Timescale of the evolution of the molluscan hemocyanins. A linearized tree was obtained on the basis of corrected protein distance data. The divergence times were estimated under the assumption that the Gastropoda and Cephalopoda diverged 520 million years ago (18). The bars represent the standard errors of the means. MYA, million years ago.
tively large and complex animals equipped with an efficient circulatory system, and such animals are usually thought to have evolved in the early Cambrian, about 540 million years ago (cf. Ref. 39). However, recent calculations based on 22 nuclear genes suggest that the early metazoan divergence was about 830 million years ago (40). Moreover, the Ediacaran fossil *Kimberella*, measuring up to 14 cm in size, which is found in late Precambrian strata, has recently been reconstructed as a mollusc-like animal with a soft shell (41). This indicates that rather complex mollusc-like metazoans did indeed exist long before the “Cambrian explosion.” The present phylogenetic tree suggests that an efficient hemolymph oxygen carrier was available for animals like *Kimberella* and supports the concept of a graduate evolution of the protostome phyla over hundreds of millions of years in the late Precambrian rather than their punctuate origin in the early Paleozoic.

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