Short Communication

Hemocyanin in mollusks—A molecular survey and new data on hemocyanin genes in Solenogastres and Caudofoveata

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1. Introduction

The most common respiratory protein of mollusks is the blue, copper-containing hemocyanin (van Holde and Miller, 1995). It is not bound to hemocytes but suspended in the hemolymph. Its molecular mass ranges from $3500 \times 10^3$ to $8000 \times 10^3$ Da (dalton) or even more (Herskovits, 1988). These differences in molecular weight are due to the fact that the basic decamers that constitute the barrel-shaped protein may aggregate to didecamers or multi-decameric elongated particles (Herskovits, 1988). In cephalopods and chitons (Polyplacophora), there are exclusively decamers, whereas in protobranch bivalves and gastropods the predominantly observed aggregation state is didecamers (Herskovits, 1988; van Holde and Miller, 1995; Lieb and Markl, 2004; Bergmann et al., 2006, 2007; Gatsogiannis et al., 2007).

A typical hemocyanin monomer is composed of eight globular functional units (FU), which are arranged like pearls on a string and termed FU-a to FU-h. An exception are cephalopod hemocyanins that contain only seven FUs. According to molecular clock calculations, the single FUs evolved within the early Precambrian, thus they were present already before the extant molluscan classes derived (Lieb et al., 2000; Lieb and Markl, 2004). Further analyses of hemocyanin-genes from cephalopods, gastropods, a protobranch bivalve, and polyplacophorans (the latter is unpublished data) showed that all hemocyanin genes possess highly conserved phase 1 linker-introns, which separate the individual FU-exons from each other [(FU-a-intron-FU-b-intron-FU-c-intron-FU-d-intron...) Lieb et al., 2001; Altenhein et al., 2002; Bergmann et al., 2006, 2007]. Since these introns are present in all molluscan hemocyanin genes, they seem to be ancient and probably at first used to border a single FU-coding exon. They might have facilitated the duplication of this original unit, finally resulting in an eight FU-containing proto-hemocyanin monomer in the last common ancestor of mollusks, ca. 600–700 million years ago (Lieb et al., 2000).

To date, the complete cDNA sequence and also the constitution pathway of hemocyanin subunits are known for the “living fossil” cephalopod Nautilus pompilius (Bergmann et al., 2006; Gatsogiannis et al., 2007). Further complete coding sequences and data about gene architecture are published for the gastropods Haliotis tuberculata and Aplysia californica, the cephalopod Enteroctopus dofleini, and the protobranch bivalve Nucula nucleus (Lieb et al., 2000, 2001, 2004; Altenhein et al., 2002; Bergmann et al., 2006, 2007). Partial sequence data is accessible for Euprymna scolopes (Cephalopoda, GenBank Accession No. AY149460), and a number of gastropods, such as Helix pomatia, Rapana thomasiana, Concholepas concholepas (Drexel et al., 1987; De Ioannes et al., 2004; Perbandt et al., 2003) and some further species of Aplysia (Lieb and Markl, 2004; GenBank Accession Nos. AJ749651, AJ749650, AJ749652) and Haliotis (Streit et al., 2006; Clark et al., 2007). No hemocyanin data has been published or identified until now for monoplacophorans, non-protobranch bivalves, scaphopods, or the aplacophoran Solenogastres (Neomeniomorpha) and Caudofoveata (Chaetodermomorpha).

To investigate the general expression of hemocyanin in mollusks on a broad basis, we accomplished intense screening of WGS- and EST-libraries, where available, and attempted to amplify hemocyanin sequences in selected species of solenogasters, caudofoveates, and scaphopods.

2. Material and methods

Solenogastres, Caudofoveata, and Scaphopoda were collected in Raunefjord (Bergen, Norway) using a hyperbenthic sled (RP-sled) employed from RV Hans Brattstrøm (University of Bergen) in water depths between 150 m and 300 m. Species of Solenogastres collected were: Wirenia argentea Odhner, 1921; Genitoconia atriolonga
Salvini-Plawen, 1967, *Micromenina fodiens* (Schwabl, 1955); *Helluoherpia aegiri* Handl & Büchinger, 1996; *Dorothyenia sarsi* (Koren & Danielsen, 1877); *Nematomonma flavens* Pruvot, 1890; *Simrothiella margaritacea* (Koren & Danielsen, 1877). Caudofoveata species were: *Falcidens crossothus* Salvini-Plawen, 1968; *Scutopus ventralineatus* Salvini-Plawen, 1968; *Chaetodermia nitidulum* Lovén, 1844. Vouchers are deposited at the Invertebrate Collections, part of the Natural History Collections, at Bergen Museum/Universitetet i Bergen under deposit numbers ZMBN 79094 (*Falcidens crossothus*), ZMBN 79095 (*Scutopus ventralineatus*), and ZMBN 79096 (*Chaetodermia nitidulum*).

Specimens for DNA- and RNA-extraction were either fresh, or deep-frozen (shock frozen in liquid nitrogen and kept in −80 °C), or fixed in 100% ethanol (for DNA extraction only). Extractions were performed using the Qiagen DNAeasy blood/tissue kit and the RNAeasy mini kit. Reverse transcription was done with Superscript III (Invitrogen) for 1 h at 42 °C using 200 ng of RNA and a degenerated hemocyanin specific primer (Lieb et al., 2000). PCR were performed using standard three step conditions (step 1 94 °C for 10 s, step 2 53 °C for 30 s, step 3 72 °C for 2 min) for 35 times with an initial denaturation step of two minutes and a final extension phase of 10 min at 72 °C. Two hundred nano grams of genomic DNA, 500 ng of total RNA or 5 µl of cDNA were used as templates. Obtained amplicons were analyzed electrophoretically using 0.8–1.5% agarose gels (0.5× TBE). DNA-fragments were manually excised, purified using the Qiagen Gel extraction kit, and cloned into Strataclone (Stratagene) PCR cloning vector. Clones were checked according to the Strataclone manual by clone PCR using standard vector primers. Insert bearing plasmids were purified (Qiagen Miniprep kit) from overnight liquid cultures and sequenced on both strands.

Obtained sequences were processed using FinchTV (www.Genospia.com) and analyzed by using the 'Basic Local Alignment Search Tools' (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Further phylogenetic analyses were done using Prottest (Abascal et al., 2005), MrBayes (Ronquist and Huelsenbeck, 2003), RaxML (Stamatakis, 2006) and Mega (Tamura et al., 2007). The clock-like evolution of hemocyanin sequences was tested by the relative ratio test implemented in Mega. Divergence rates, calculated by the neighbor-joining method were then used to calculate the splitting times of the studied species. As calibration point we used the splitting of cephalopods and gastropods ca. 520 million years ago (Benton, M. J. 1993. The Fossil Record 2. Chapman & Hall, London, 845 pp.).

### 3. Results and discussion

#### 3.1. Solenogastres

Searching the databases revealed that there is no EST- or genomic data published for Solenogastres yet. To explore the presence of hemocyanin-coding genes, we thus performed DNA-extractions in five species (*Wrirena argenta*, *Genitoconia rosea*, *Micromenina fodiens*, *Helluoherpia aegiri*, *Dorothyenia sarsi*) and additional RNA-extractions in *Wrirena argenta*, *Genitoconia atrilonga*, *Micromenina fodiens*, *Nematomonma flavens*, *Simrothiella margaritacea*. For none of the chosen species did gel-electrophoretic analyses of genomic PCR show the hemocyanin-characteristic bands at 340 bp and 370 bp (see below; Lieb et al., 2001). That suggests that hemocyanin genes are either absent in Solenogastres or that they possess enourmously intron-rich gene architectures. In the latter case, however, we should have been able to use the RNA-extractions to amplify characteristic hemocyanin bands via RT-PCR, which was not the case. Our taxon sampling included species from four presumably not closely related families, whereby solenogaster phylogeny is not well enough resolved yet to be sure about the nature of the most basal taxon within the clade. The complete lack of hemocyanin coding genes in seven species, however, suggests that hemocyanin is not the universal respiratory protein in Solenogastres and that the ability to produce hemocyanin has been lost a long time ago. Alternatively, considering Solenogastres as the most basal branch in the molluscan phylogenetic tree (e.g., Haszprunar, 2000; Haszprunar et al., 2008), it could be argued that the lack of hemocyanin coding DNA is an ancestral trait. Solenogastres are known to bear different types of true blood cells, whereby a type containing granules with red coloration is common (e.g., Salvini-Plawen, 1978). These points to representatives of the very diverse hemoglobin family as respiratory proteins, but in the studied Solenogastres species we were not successful in identifying sequences assignable to any published hemoglobin.

#### 3.2. Caudofoveata

As in Solenogastres, EST or genomic data are not available for representatives of Caudofoveata yet. Thus we performed DNA- and RNA-extractions in three species: *Falcidens crosstothus*, *Chaetodermia nitidulum*, *Scutopus ventralineatus*. The gel-electrophoretic analyses of genomic PCR using degenerated but highly specific hemocyanin primers (see Lieb and Markl, 2004) resulted in the characteristic bands at 340 bp and 370 bp (see Fig 1B). RT-PCR assays using the same degenerated primers and a second primer pair resulted in the expected hemocyanin coding amplicon-lengths of 370 bp and 900 bp, respectively (see Fig 1A). Furthermore, we were able to clone and completely sequence three *Falcidens crosstothus* cDNA fragments encompassing 360 bp, 900 bp, and 1600 bp. Further sequence analyses using the BLAST algorithm revealed at E-values ranging from 9e−97 to 9e−88 that these cDNA fragments represent hemocyanin-coding mRNA. By multiple sequence alignments using the well characterized *Halitosis tuberculata* hemocyanin as reference (Lieb et al., 2000, 2001; Altenhein et al., 2002) we could assign the fragments to the respective functional units (Fig. 1C): (i) one short 370 bp encompassing cDNA coding for ~100 amino acids of a conserved region of FU-e, (ii) two 900 bp segments sharing an identity of 84% (similarity 90%) and most probably corresponding to FU-f and partially FU-g, and (iii) a 1600 bp long segment complementing FU-g to its entire length and protruding into FU-h. Due to 100% identities of overlapping regions, one of the 900 bp segments termed here *Falcidens crosstothus* Hemocyanin‘ (FcH1-f, Fig. 2) and the 1600 bp segment could be joined resulting in a coding region for half of FU-f, one complete FU-g and one third of FU-h. The second 900 bp long segment, FcH2-f, might be an allelic form of the FcH-f gene but most probably represents a second hemocyanin-isofrom, which in fact is very common (Herskovits, 1988; van Holde and Miller, 1995; Lieb and Markl, 2004; Bergmann et al., 2006, 2007) and also the case in *Halitosis tuberculata* (see Fig. 2). As the similarity between FcH1-f and FcH2-f is higher than the similarity of each of the genes to the respective *Halitosis* genes (HH1-f and HTH2-f, see Fig. 1C), these gene duplicates must have had arisen after the lines leading to Caudofoveata and Gastropoda split from each other. This has also been shown for other molluscan hemocyanins (Bergmann et al., 2006; Gielens et al., 2000).

#### 3.3. Bivalvia

Extensive gene libraries are accessible for pteriomorph bivalve species, such as *Argospecten*, *Mytilus*, and *Crassostrea*, but no hemocyanin (−like) sequences could be identified among the published data. Our attempts to amplify hemocyanin gene sequences in *Mytilus galloprovincialis* by PCR using degenerated primers (see above and Lieb et al., 2004) could not reveal hemocyanin (−like) sequence data, either. Thus, we assume that hemocyanin is not present in the
Pteriomorphia, one of the largest clades within Autolamellibranchiata. No data are available yet for other autolamellibranch bivalves. The presence of hemocyanin in protobranch bivalves, however, points to a secondary loss of hemocyanin genes in Pteriomorphia.

3.4. Scaphopoda

There are no EST- or genome data published that could contain “hidden” information on the presence of hemocyanin in scaphopods. Our attempts to amplify hemocyanin gene sequences were negative for two not further identified species. The lack of silent hemocyanin genes (pseudogenes) points to a trend that goes back a long way into scaphopod evolution. Based on these data, the presence of hemocyanin in Scaphopoda cannot be ruled out in general, but hemocyanin appears at least not to be their predominant respiratory protein.

3.5. Phylogenetic analyses and molecular clock

We used the 300 amino acids coding hemocyanin sequences for ML-analyses and Bayesian inferences and, based on the sim-
ilar mid-range evolutionary rate of known molluscan hemocya-
nins, we employed these data for new molecular clock calcula-
tions (Fig. 2). Probably due to the still quite restricted taxon
sampling we could not obtain well resolved and highly sup-
ported phylogenetic trees, but our trees show the tendency of
caudofoveate hemocyanin to cluster with the hemocyanin of
Polyplacophora and Cephalopoda, including the “living fossil”
species of the Nautilidae. The resulting tendential sistergroup
relationships of Cephalopoda with Polyplacophora and Caudof-
oveata and of Bivalvia with Gastropoda are in contrast to the
morphology-based Cyrtosoma-concept propagating a cephalo-
pod–gastropod clade (Salvini-Plawen, 1980; Haszprunar, 2000;
Haszprunar et al., 2008) but in accordance with other phyloge-
genies based on molecular data (e.g. Giribet et al., 2006; Dunn
et al., 2008). According to our molecular clock calculations,
Caudofoveata separated from the other molluscan classes ca.
415 mya, which is in good accordance to our previous molecular
clocks (Lieb et al., 2001; Bergmann et al., 2006, 2007).

4. Conclusions

The lack of hemocyanin coding DNA in Scaphopoda, pterio-
morph bivalves, and Solenogastres is here interpreted as a multiple
loss of the respective DNA sequences. Loss of hemocyanin expres-
sion and even complete reduction of the coding sequences is not
uncommon even within taxa the members of which usually ex-
press hemocyanin, such as Patello gastropoda within the gastro-
poles. Absence or presence of hemocyanin thus is not a valid
phylogenetic signal and, for example, cannot clarify the phyloge-
netic relationship between the aplacophoran Solenogastres and
Caudofoveata. Nevertheless, we want to point out that the com-
plete lack of hemocyanin coding sequences in Solenogastres is,
in addition to a number of fundamental differences in morphology
(for review see Todt et al., 2008), one more reason to reject a
class-level taxon Aplacophora in favor of two separate classes Sole-

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