Identification, Molecular Cloning, and Phylogenetic Analysis of a Non-respiratory Pseudo-hemocyanin of Homarus americanus*

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Copper-containing hemocyanins serve to transport oxygen in many arthropod species. Here I describe the identification and cDNA cloning of a structurally closely related non-respiratory pseudo-hemocyanin (PHc) of the American lobster, Homarus americanus. This protein has lost the ability to bind copper and, therefore, oxygen because a histidine residue in copper-binding site A is replaced by tyrosine. Like many arthropod hemocyanins, PHc forms a hexamer. It consists of two different subunit types of 660 and 661 amino acids, respectively, that share a 94.4% sequence identity. Whereas Homarus hemocyanin is produced in the hepatopancreas, PHc is synthesized by the ovaries and the heart tissue. Because different levels of PHc were observed in distinct individuals, I propose an association of the synthesis of this protein with the molting or reproduction cycle, similar to the hexamers, insect storage proteins that are also related to the hemocyanins. However, phylogenetic analyses show that PHc derived independently from crustacean hemocyanins. Therefore, Homarus PHc is a member of a new class within the growing hemocyanin protein superfamily.

The body fluid of many arthropod species contains large copper proteins that serve to transport oxygen and are referred to as hemocyanins (1, 2). The principal structure of a hexamer of six similar or identical subunits in the 75 kDa range is conserved within all hemocyanins, although in many species, these hexamers associate to quaternary structures of up to 8 × 6 subunits (3). Each subunit carries one oxygen molecule by virtue of two copper ions that are coordinated by six histidine residues (4, 5).

Arthropod hemocyanins are members of a growing protein superfamily that also comprises the arthropod tyrosinases (prophenoloxidases), the insect hexamers, and the dipteran hexamerin receptors (6–10). Hexamers share about 20–25% sequence identity with the hemocyanins (7, 10) and have similar tertiary and quaternary structures (11, 12) but do not bind oxygen. The loss of the oxygen transport function is accompanied by the replacement of the coordinating histidine residues in the copper-binding center by other amino acids. Hexamerin-type proteins have been discovered in the hemolymph or storage tissue in all insects investigated (13). They are generally assumed to act as storage proteins that provide energy and amino acids for nonfeeding periods.

Occasionally, in the hemolymph of some decapodan Crustacea, similar proteins were observed that resemble the hemocyanins and hexamers in their specific appearance but do not bind oxygen (14, 15). In view of the recently proposed sister group relationship of the Insecta and Crustacea (e.g. 16, 17), these proteins were considered as possible ancestors of the hexamers (7, 10, 12, 13). However, here I report two sequences of such a protein and show that, although they likely represent storage proteins as well, they evolved independently from crustacean hemocyanins. Because this protein closely resembles the crustacean hemocyanins but lacks copper and the oxygen-binding function, here it is termed pseudo-hemocyanin (PHc).1

EXPERIMENTAL PROCEDURES

Animals—Living adult Homarus americanus (Crustacea, Malacostraca, Decapoda, Homaridae) were purchased from a local seafood dealer, anesthetized, and immediately dissected. The hemolymph was withdrawn from the abdominal sinus by a syringe and centrifuged for 10 min at 10,000 × g to remove hemocytes, tissue contamination, and clotted material. The supernatant was used as purified hemolymph. Tissues were either immediately used for RNA preparation or frozen in liquid nitrogen and stored at −80°C for no longer than 4 weeks.

Purification of PHc and Preparation of Antibodies—About 0.5–1 ml (approximately 50–200 mg of total protein) of freshly collected hemolymph was applied to a Biogel A 5 m column (1.5 × 100 cm) as described by Markl et al. (14). Elution was performed with 100 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and 5 mM CaCl2. The eluted material was analyzed by SDS-PAGE for the presence of PHc and purity. The oxygen binding capacity of the different fractions was analyzed spectrometrically by reading the absorption at 280 and 340 nm. The second large peak (16 S) contains the PHc fraction. Electron microscopy of the different fractions was performed by negative staining with uranyl acetate (18). Polyclonal antibodies against the PHc were raised in guinea pigs and checked for specificity by Western blotting.

Gel Electrophoresis and Western Blotting—SDS-PAGE was performed on a 7.5% gel according to standard procedures (19). For Western blotting, the proteins were transferred to nitrocellulose at 0.8 mA/cm2. Nonspecific binding sites were blocked by 5% non-fat dry milk in TBST. Incubation with the anti-PHc-IgGs (diluted 1:10,000 in 5% non-fat dry milk and TBST) was carried out for 2 h at room temperature. The filters were washed three times for 20 min each in TBST and subsequently incubated for 1 h with goat anti-guinea pig Fab fragments conjugated with alkaline phosphatase (Dianova) diluted in 5% non-fat dry milk and Tris-buffered saline. The membranes were washed as described above, and the detection was carried out using nitroblue tetrazolium and bromo-chloro-indolyl-phosphate. N-terminal sequencing was performed by a commercial service (H. Heid, Deutsches Krebsforschungszentrum, Heidelberg, Germany) using protein samples separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane as described previously. The PHc and hemocyanin bands were excised and submitted to Edman degradation.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AJ132141 (PHc-1) and AJ132142 (PHc-2).

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† The abbreviations used are: PHc, pseudo-hemocyanin; NRP, non-respiratory protein; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBST, 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, and 0.25% Tween-20; SSC, saline/sodium citrate; kb, kilobase pairs.
Cloning and Sequencing of the PHc cDNAs—Total RNA was either isolated from the complete thorax of a single lobster without the legs and the cuticle or prepared from defined tissues by the method of Scheller and Karlson (20). Poly(A)⁺ RNA was purified from total RNA using the Poly(A)Tract kit (Promega). About 5 µg of poly(A)⁺ RNA were used for the construction of a directionally cloned cDNA expression library applying the Lambda ZAP cDNA synthesis kit (Stratagene). The library was amplified once and screened with α-PHc antibodies. Positive phage clones were converted to plasmid vectors using the material provided by Stratagene in the cDNA synthesis kit. The PHc cDNAs inserted in the pBK-CMV vector were sequenced on both strands by the commercial SeqLab (Göttingen) sequencing service.

Northern Blotting—Equal amounts of RNA from different tissues were denatured and subjected to electrophoresis in a 1% agarose gel containing 1% formaldehyde. After electrophoresis, gels were rinsed in 20 × SSC and transferred to a nitrocellulose membrane in 20 × SSC. Digoxigenin-UTP-labeled antisense RNA probes were transcribed from the complete cDNA clones using the Roche Molecular Biochemicals RNA in vitro transcription kit. The filters were pre-hybridized in 50% formamide, 5 × SSC, 0.1% N-laurylsarcosine, 0.02% SDS, and 1% blocking agent (Roche Molecular Biochemicals) for 1 h and then hybridized in the same solution containing the labeled probe overnight at 60 °C. Immunodetection was carried out using the Roche Molecular Biochemicals digoxigenin detection kit as described by the manufacturer.

Sequence Analysis and Phylogenetic Studies—The programs provided with Sequence Analysis Software Package 8.0 from the Genetics Computer Group, Wisconsin were used for sequence analysis and manipulation. For phylogenetic inference, the deduced PHc protein sequence was aligned by hand to the previously published alignment of hemocyanins and hexamerins (12), including other sequences that became available only recently. The complete alignment is available from the author upon request. Distances between the pairs of protein sequences were calculated and corrected for multiple changes according to Dayhoff’s empirical PAM 001 matrix by using the PROTDIST program of the PHYLIP 3.5c package (21). Phylogenetic inference was carried out using either the neighbor joining method (22) or the maximum parsimony method implemented in the PROTPARS program of the PHYLIP 3.5c software package (23). The robustness of the trees was tested by bootstrap analysis (24) with 100 replications (SEQBOOT program). Majority role consensus trees were obtained using the CONSENSE program.

RESULTS

Purification and Analysis of PHc—The separation of the hemolymph proteins of adult H. americanus by gel filtration on a Biogel A5 m column yields two major peaks corresponding to the sedimentation coefficients in the analytical ultracentrifuge of about 16 S and 24 S (Fig. 1A; Ref. 14). The dodecameric (2 × 6) hemocyanin elutes at 24 S (14, 25). This peak displays an UV spectrum with a 280 nm:340 nm absorption ratio of about 5, which is typical for a native hemocyanin and indicates the formation of the copper-oxygen complex that causes increased absorption at a wavelength of 340 nm. The second major peak that elutes at 16 S has a 280 nm:340 nm ratio of >20 (Fig. 1B), indicating a protein that does not bind oxygen by a copper complex and that has therefore been referred to as non-respiratory protein (NRP) (14). However, to avoid possible confusion with chelicerate NRPs that are not related to hemocyanin (26), the H. americanus NRP has been renamed PHc here.

Electron microscopic examination of the 24 S fraction shows the presence of the dodecameric hemocyanin, consisting of two hexamers of 10 nm in diameter each (Fig. 2A) (25). The 16 S fraction (Fig. 2B) contains a protein with a diameter of about 12 nm that resembles a typical hexameric hemocyanin, with six subunits arranged as a trigonal antiprism (4). After separation on SDS-PAGE (Fig. 3A), the 24 S fraction shows three bands with apparent molecular masses of 76–80 kDa, and the 16 S fraction separates into two PHc subunits of about 85 and 86 kDa (14). Antibodies were raised against the 16 S fraction in guinea pigs. In Western blotting, these antibodies recognize only the PHc subunits, whereas the anti-hemocyanin antibodies (25) do not stain PHc (Fig. 3B). After transfer to a polyvinylidene difluoride membrane, 20–22 N-terminal amino acids of the PHc and hemocyanin subunits were determined by microsequencing (Fig. 3C). Because the first 12 amino acids of the two PHc subunits are identical, only the 86-kDa subunit was sequenced up to the 20th residue. Database research shows that this sequence clearly belongs to...
the hemocyanin superfamily.

Cloning and Sequencing of Homarus PHc cDNA—A cDNA library was constructed from poly(A)⁺ RNA isolated from thorax tissue of Homarus. The library was amplified once, and about 3 x 10⁵ clones were screened with the anti-PHc antibodies. 29 positive clones were identified. Three clones (clones 11, 31, and 42) with cDNA inserts between 2.3 and 2.5 kb were subjected to further analysis and sequenced (Fig. 4). Whereas clones 31 and 42 are identical (except in the length of the 5' region), clone 11 represents another PHc clone. The identity score on the nucleotide level within the coding region of the native polypeptides is 96%. However, there are fundamental differences in the 3' noncoding region, and only limited conservation is seen in the putative signal peptide. Restriction enzyme analysis (EcoRI/XhoI) of other positive clones allowed the assignment to either one of the clone types. Conceptual translation of the PHc clone shows that both sequences cover the actual N terminus of the native polypeptide, whereas they are not complete in the 5' sequences that very likely correspond to the signal peptide of the nascent protein. Clone 11 (referred to as PHc-1 in the remainder of the text) has 71 nucleotides of the putative signal peptide, clone 31 (PHc-2) has 65 nucleotides of the putative signal peptide, and clone 42 has 53 nucleotides of the putative signal peptide.

The deduced N termini of both PHcs are identical, match exactly to those that have been determined by microsequencing of the proteins, and confirm the identity of the clones. The inferred primary structures of the native PHc proteins yield polypeptides of 661 (PHc-1) and 660 (PHc-2) amino acids with deduced molecular masses of 77,313 and 76,970 Da, respectively. The PHc amino acid sequences share 623 common residues (94.4% identity; 97.6% similarity, considering conservative replacements). Computer analysis reveals the presence of four conserved potential N-glycosylation sites (NXT) in both PHc sequences.

PHc Expression—The expression of PHc mRNA in different tissues was examined by Northern blotting (Fig. 5). A single strong PHc signal of about 2.5 kb was observed in the ovaries, whereas less PHc mRNA is present in the heart tissues. Except for the ovaries, no difference was observed between the sexes. No PHc expression was detected in the hepatopancreas, the site of hemocyanin synthesis in H. americanus (27), and in three other tissues analyzed (gills, connective tissue, and muscle). When comparing the level of PHc in the hemolymph of different individuals, significant differences in the amount of this protein were observed (Fig. 6). A total of eight adult lobsters (two female, four male, and two of undetermined sex) were analyzed.

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**Fig. 3.** Electrophoretic properties and N-terminal sequences of Homarus hemocyanin and PHc subunits. A, proteins of total hemolymph (HL), the 24 S peak (24), and the 16 S peak (16) were separated on SDS-PAGE and stained with Coomassie Blue. B, Western blotting analysis of total hemolymph using anti-hemocyanin antibodies (αHc) or anti-PHc antibodies (αPHc). C, total hemolymph was separated by SDS-PAGE, and the N-terminal sequences of the two PHcs (PHc-1 and PHc-2) and the three hemocyanin subunits (Hc1, Hc2, and Hc3) were determined.

**Fig. 4.** cDNA and deduced protein sequences of the pseudo-hemocyanins of H. americanus. DNA is numbered according to the nucleotide sequence of PHc-1; nucleotide substitutions in PHc-2 within the coding region are depicted by the letters in the top row, and bases missing in that clone are indicated by a dot (·). Amino acid numbering starts with the N terminus of the native polypeptide, and the sequences of the putative signal peptide are underlined. Amino acid changes in PHc-2 resulting from base exchanges are not shown. The stop codon (TAA) is indicated by three asterisks, and the polyadenylation site is double-underlined. Potential N-glycosylation sites are italic.
weighing around 500 g were investigated. Complete absence of PHc was detected in one male and in one individual of undefined sex, whereas hemocyanin subunits are present in all animals. Therefore, PHc is not a female-specific protein.

**Sequence Comparison and Phylogenetic Analysis—**Database searches show that PHc displays the highest similarity with crustacean hemocyanins (47–56% identity, 74–80% similarity) and the cryptocyanin of the Dungeness crab *Cancer magister* (15) (50% identity, 74% similarity), whereas lower similarity scores were observed with chelicerate hemocyanins, arthropod prophenoloxidases, and insect hexamers. Hemocyanins transport oxygen by two copper ions coordinated by six histidine residues, which are arranged in two copper-binding sites (1–5). These histidines are also present in the arthropod prophenoloxidases (8–10). However, whereas five of these residues are conserved within PHc as well, the first histidine in copper-binding site A is replaced in both PHc-1 and PHc-2 by a tyrosine (Fig. 7).

To infer the position of the PHc proteins within the superfamily of arthropod hemocyanins and hexamers, the amino acid sequences were included in a multiple sequence alignment that has been described in an earlier study (12). Two recently published sequences (15, 28) were added as well. The hemocyanins of the Chelicerata were considered to be the most ancient branch and were used here as the outgroup (12), although no differences in the arrangement of the clades were observed when using the arthropod prophenoloxidases as the outgroup (10). Both distance matrix methods and maximum parsimony show that the PHcs are associated with the cryptocyanin of *Cancer magister* (Fig. 8). There is strong statistical support that these three non-respiratory proteins are included within the clade of the crustacean hemocyanins (100% bootstrap value). However, whereas parsimony analysis indicates a moderately supported association of the PHcs and cryptocyanin with a clade comprising hemocyanin C of the spiny lobster *Panulirus interruptus*, hemocyanin 6 of *C. magister*, and a hemocyanin of the shrimp *Penaeus vannamei* (63% bootstrap support; Fig. 8A), distance matrix methods show PHc in the sister group position to all sequenced crustacean hemocyanins (75% bootstrap support; Fig. 8B).

**DISCUSSION**

The hemocyanins of arthropods have been subjected to detailed functional, structural, and evolutionary studies, mainly in the Chelicerata and Crustacea (see Refs 1–3 and 7). Among the Crustacea, only the Malacostraca possess hemocyanins, whereas these proteins are apparently absent in other crustacean taxa (35). In the American lobster, *H. americanus*, decameric hemocyanin represents about 60–90% of the total hemolymph proteins (27). An additional hexameric protein with similar biophysical properties was identified in *Homarus* as well as in other decapodan species and termed NRP (14). However, the nature of these proteins has long remained obscure.

Here I report two sequences of the NRP of *H. americanus*.
The conservation of two different, expressed genes provides further evidence that these hemocyanin-like proteins are functional, although they do not act as respiratory proteins. The inability of PHc to bind copper ions and, therefore, form the copper-oxygen complex (Fig. 1D) can now be explained by the replacement of a single histidine with a tyrosine in copper-binding site A in both types of subunits, whereas the other copper-coordinating residues are conserved (Fig. 7). Otherwise, the PHcs closely resemble the crustacean hemocyanins in sequence and structure. The molecular masses deduced from conceptual translation (77.0 and 77.3 kDa) of the cDNA clones are significantly lower than those determined experimentally by SDS-PAGE (85 and 86 kDa; Fig. 2). However, four potential N-glycosylation sites are present in each of the PHc subunits (Fig. 4), indicating a heavily glycosylated protein. By contrast, the crustacean hemocyanins possess either none or only one glycosylation site per subunit (Fig. 7).

Although the mRNA for the PHcs accumulates in the ovaries of the adult female (Fig. 5), there is not sufficient evidence to deduce sex-specific differences of PHc levels in the hemolymph (Fig. 6). One can speculate that ovary PHc mRNA may code for some storage proteins that are transported into the developing eggs. Additional studies are required to elucidate the potential role of the PHcs in reproduction. Heart tissue appears to be the principle site of PHc synthesis in both sexes and is sufficient to produce high amounts of PHc. However, the PHcs are not constitutively expressed because a complete absence of this protein was observed in two of eight individuals, demonstrating that this protein is dispensable (Fig. 6). One may speculate that the appearance of the PHcs is either associated with nutritional conditions or, similar to the Cancer cryptocyanin (15), depends on the molting cycle. Unfortunately, due to the long molting cycle of H. americanus, a possible stage-specific analysis of the expression of the PHcs is currently beyond experimental control.

It has been hypothesized that the PHcs might represent the ancestors of the insect hexamers (7, 10, 12, 13), copper-free hemolymph proteins that resemble the hemocyanins in structure and sequence (6, 13) but had lost the ability to bind oxygen. However, the present phylogenetic analyses clearly show that the PHcs, as well as the cryptocyanin of C. magister (15), are not particularly related to the hexamers but are authentic descendants of the crustacean hemocyanins (Fig. 8). The trees suggest that PHc and cryptocyanin emerged from the hemocyanins early in decapodan evolution, before the Astacura (Homarus) and Brachyura (Cancer) diverged. Although other specific roles of PHc are possible, it likely acts as some kind of storage protein. It has been repeatedly observed that hemocyanin concentration decreases drastically during starvation or molting (36). This does not necessarily mean that hemocyanins actually act as storage proteins sensu stricto, but they are dispensable, at least in some species and under particular developmental or environmental conditions. The evolutionary advantage of more specialized storage proteins like the PHcs may be to uncouple the synthesis of oxygen transport proteins and of proteins that specifically accumulate amino acids and energy. The fact that hemocyanins were used twice in evolution for similar purposes may hint at some particular structural advantages, which may be either their stability in the hemolymph or their high molecular mass that allows the accumulation of many amino acids with low osmotic impact.

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