Developmental Changes in Hemocyanin Expression in the Dungeness Crab, Cancer magister

(Received for publication, June 17, 1996, and in revised form, November 18, 1996)

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The copper-based respiratory protein hemocyanin undergoes a developmental shift in subunit composition and function analogous to that seen in many hemoglobins. We studied hemocyanin gene expression in the Dungeness crab (Cancer magister) by Northern blot analysis. Animals were raised under controlled conditions, and total RNA was isolated from 13 developmental stages as well as from six tissue types in the adult animal. RNA was run on formaldehyde-agarose gels, blotted onto nylon membranes, and probed with 32P-labeled cDNA probes specific for C. magister adult hemocyanin. Results indicate that adult hemocyanin biosynthesis occurs in hepatopancreas tissue only. Analysis of developmental stages shows that expression of adult-type hemocyanin, as indicated by the appearance of hemocyanin subunit 6 mRNA, begins during the sixth juvenile instar.

Respiratory proteins function to combine reversibly with oxygen at the respiratory surfaces of an animal and to carry oxygen via the circulatory system to the tissues inside. In many Arthropoda and Mollusca, these oxygen transport proteins are hemocyanins, large multisubunit molecules that occur extracellularly in the hemolymph (1, 2). Arthropodan hemocyanins are composed of heterogeneous subunits with molecular masses of ~75 kDa. A single subunit contains two copper-binding sites, Cu1 and Cu2, each site complexing one copper atom by three histidine ligands (3). Both sites participate in the binding of one oxygen molecule. In deoxygenated Hc, the copper ions are in the Cu+ state. During oxygenation, a peroxide (O2−) bridge is formed between the Cu+ ions, oxidizing them to Cu2+ (4). The 75-kDa subunits self-assemble into hexamers or multiples of hexamers. In the hemolymph of the Dungeness crab (Cancer magister), 2-hexamer 25 S Hc and 1-hexamer 16 S Hc occur (5).

The Hc of C. magister is particularly interesting because it changes in both subunit composition and function during development of the crab from megalopa and early juvenile instar stages to adult (6). Adult C. magister 25 S Hc is composed of six different types of subunits (7). One of these, subunit 6, is absent in both 25 S and 16 S megalopa Hc (6) and typically does not appear in hemolymph Hc until sometime during the sixth juvenile instar. In addition, the stoichiometry of two other Hc subunits, 4 and 5, changes during the transition from larval to adult crab.

The developmental shift in subunit composition results in a new population of adult Hc molecules that have a higher affinity for oxygen than does juvenile Hc (8, 9). The larval-adult shift in Hc is analogous, then, to the fetal-adult shift in red blood cell hemoglobins in humans and other mammals (10) and to the developmental changes in extracellular hemoglobins in some invertebrates (11, 12). We hypothesized that the differences between juvenile Hc and adult Hc are due to an ontogenetically regulated change in Hc gene expression. To test this hypothesis, we first determined the site of Hc synthesis in adult C. magister. Because several different tissues have been proposed as possible sites of Hc synthesis in arthropods (13), it could potentially be made in multiple locations within the crab. We then investigated when during development adult-specific subunit 6 mRNA is first expressed.

EXPERIMENTAL PROCEDURES

Sample Collection and Total RNA Isolation—Adult male C. magister crabs were caught in the Coos Bay estuary (Oregon) by scuba diving or using baited crab rings. Crabs were quickly killed, and tissue samples (100 mg) were dissected, thoroughly rinsed with C. magister hemolymph buffer (50 mM Tris-HCl, 454 mM NaCl, 11.5 mM KCl, 13.5 mM CaCl2, 18 mM MgCl2, 23.5 mM NaN3, pH 7.6), and frozen in liquid nitrogen. Total RNA was isolated from tissues by the guanidinium isothiocyanate method using a RAPID total RNA isolation kit (5 Prime–3 Prime, Inc.). Total RNA yield was quantified by measuring absorbance at 260 nm.

Ovigerous female C. magister crabs were collected from the Pacific Ocean near Coos Bay; zoea larvae that hatched from the fertilized eggs on the females’ pleopods were harvested and frozen in liquid nitrogen. Megalopas were collected from surface waters of Coos Bay in late spring and maintained in running seawater aquaria at the Oregon Institute of Marine Biology as described (14). As the young crabs grew and molted, samples of each developmental stage from megalopa to fifth instar were obtained by quick-freezing animals in liquid nitrogen. For RNA isolation of these early developmental stages, 100-mg aliquots of frozen whole animals were dropped into liquid nitrogen and ground to a fine powder. For the larger developmental stages, sixth instar and older, 100-mp samples of hepatopancreas tissue were dissected and prepared as described above for adult crabs. All samples were stored at −80 °C. Total RNA was isolated using the guanidinium isothiocyanate method as described above.

Purification and N-terminal Sequence Analysis of C. magister Hc Subunits—Fresh hemolymph was obtained from the sinuses at the base of a walking leg of adult male crabs using a 22-gauge needle and syringe. Hemolymph was allowed to agglutinate on ice for 30 min and then centrifuged at 12,000 × g for 10 min in a Sorvall RC2-B refrigerated centrifuge at 4 °C. The supernatant was applied to a Bio-Gel A-5m chromatography column (1.8 × 115 cm) equilibrated in column buffer (0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, 10 mM MgCl2, 10 mM CaCl2) to separate 2-hexamer 25 S Hc from 1-hexamer 16 S Hc. Individual subunits were purified by subjecting the 25 S Hc fraction to a two-step combination of alkaline polyacrylamide gel electrophoresis at pH 8.9 and SDS-polyacrylamide gel electrophoresis (6). The SDS gel was electroblotted onto an Immobilon-P polyvinylidene difluoride membrane (Millipore Corp.) in a Western blot procedure, stained with 0.1% Coomassie Blue, 50% methanol, 10% acetic acid (15) for 1 min, and
RESULTS

Designing Hc-specific Primers for PCR—Our first goals were to develop primers and to amplify Hc coding sequences by PCR. The amplified cDNAs could then be used as probes in Northern blots. Because the CuA-binding site in arthropod Hc domain 2 is highly conserved in all crustacean and chelicerate Hc subunits thus far sequenced (19), we predicted that it would be a conserved feature in all six Hc subunits. Accordingly, primer CuA I (5'-GAGA-CTT-TTT-TGG-CTT-CAT-CAT-CAA-CTT-AC-3'), a 32-bp degenerate oligonucleotide, was designed based on the amino acid sequence NH2-ELFFWVHHQLT-COOH within the CuA site of Hc subunit a of the spiny lobster Panulirus interruptus (20). Reverse translation of part of the unique N-terminal amino acid sequence of Hc subunit 6 (Fig. 1) allowed the synthesis of a second degenerate primer, 5'-TCT-GCA-GGC-GGA-TCG-GAC-GGC-CA-3', specific for the 5'-end of Hc subunit 6 cDNA. The third primer, CuA II, was an antisense primer based on the sequence of our 1914-bp PCR product (see below). This primer, 5'-CAC-CTG-GTT-AGT-GAA-GCC-CTC-ATG-3', was designed to be specific for a region just downstream of the CuA site in C. magister Hc subunit 6.

PCR Amplification of Hc Subunit 6 cDNA—The locations of the primers relative to Hc subunit 6 cDNA are shown in Fig. 2. The first PCR experiment, using the CuA I primer plus a universal oligo(dT) primer directed against the poly(A)+ tail of mRNA (a gift from Dr. Ry Meeks-Wagner), amplified several major fragments between 1200 and 2000 bp in size. These fall within the expected size range for a Hc cDNA fragment extending from the CuA site to the 3'-end, based on known Hc sequences (21). A 1914-bp PCR product was cloned into a BlueScript II SK+ phagemid vector (Stratagene) as described before (16). In the second PCR experiment, using the 5'-subunit 6 primer in combination with primer CuA II, we were able to amplify and clone a 783-bp fragment corresponding to the 5'-end of Hc subunit 6 cDNA. We refer to this fragment as the "5'-probe."

Dideoxy sequencing of both clones confirmed they were Hc coding sequences. The 5'-end of the 1914-bp PCR product displayed 76% amino acid identity and 81% similarity to Hc subunit a from P. interruptus, indicating that it was a Hc cDNA. The 3'-end of the 783-bp 5'-probe overlapped by 133 bp and was identical in sequence to the CuA site within the 1914-bp cDNA (see Fig. 2). Digestion of the 1914-bp fragment with restriction enzyme EcoRV yielded two fragments, a 688-bp "CuA probe" and a 1226-bp "3'-probe" (Fig. 2).

Thus, using different combinations of primers, we were able to amplify three distinct regions of the Hc subunit 6 cDNA, the 5'-end, the CuA region, and the 3'-end. These cDNAs could now be used as probes to identify subunit 6 transcripts from various tissues and developmental stages.

Probe Specificity—The three probes obtained by PCR could be expected to hybridize equally well with Hc subunit 6 mRNA. They might differ in the extent of cross-reactivity with mRNAs corresponding to the other five Hc subunits (and, possibly, with other related proteins) because some regions of the Hc protein, and hence the cDNA, show a higher degree of sequence conservation than others.

To assay cross-reactivity of the probes with other Hc sub-

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GenBank accession number U48881 (Durstewitz, G., and Terwilliger, N. B. (1997) Mol. Biol. Evol.).
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units, three identical Northern blots were prepared using equal amounts of RNA from adult crab and several early juvenile stages. Autoradiograms of all three blots showed a 2.6-kb transcript in RNA from adult animals (Fig. 3). This is about the size expected for a full-length Hc mRNA. The CuA probe and 3'-probe, but not the 5'-probe, also hybridized to a lesser extent with juvenile transcripts of approximately the same size. We interpret these juvenile bands as cross-reactivity of the CuA probe and 3'-probe with mRNAs of Hc subunits other than subunit 6 since we know that juvenile stage Hc is composed of subunits 1–5 (6). The 3'-probe showed the greatest cross-reactivity, probably due to its long poly(A) tail. The 5'-probe displayed the least degree of subunit cross-reactivity, hybridizing only with adult mRNA, and was therefore chosen for the following experiments.

Northern Blot Analysis of Tissue-specific Hc Expression—Northern blots of total RNA from six different tissues of the Dungeness crab (heart, leg muscle, hypodermis, stomach, gill, and hepatopancreas) were probed with the 5'-probe (Fig. 4). Equal amounts of RNA were loaded as indicated by methylene blue total RNA stain. The autoradiogram showed a 2.6-kb transcript only in the RNA sample from hepatopancreas tissue.

Northern Blot Analysis of Developmental Changes in Hc Expression—No mRNA transcripts were detected in zoea, megalopa, or first through fifth juvenile instars when using the 5'-probe (Fig. 5). A 2.6-kb transcript was present in the sixth instar and older stages. All lanes contained equal amounts of total RNA as indicated by the methylene blue stain. The steady-state level of transcript increased with age of the crab. These results indicate that Hc subunit 6 biosynthesis begins during the sixth juvenile instar, and the steady-state level of mRNA continues to increase as the crab approaches maturity.

DISCUSSION

The results shown here, in which Hc mRNA was present only in hepatopancreas tissue and not in the other five tissues examined, identify the hepatopancreas as the source of Hc in the crab C. magister. In other crustaceans, tissues implicated in Hc synthesis based on mRNA in vitro translation and radioactive isotope incorporation include hepatopancreas tissue (22–25) and reticular connective tissue (23). Preliminary studies in our laboratory identified reticular connective tissue in C. magister as a site of synthesis for Hc, but for a closely related non-respiratory protein, termed cryptocyanin (26). Immunofluorescence studies convincingly showed the presence of Hc in crustacean stomach wall, reticular connective tissue (27, 28), and eyestalk cells (29). The evidence for biosynthesis was more indirect, however; and the studies left open the possibility that these were sites of Hc storage or degradation, contained Hc-filled cells transported from elsewhere, or even reflected a cross-reactivity of the antibody with cryptocyanin.

The fetal-adult shift in mammalian hemoglobin expression has been studied extensively. Changes in hemoglobin patterns during ontogeny of many other vertebrates are also well documented (10). The amino acid substitutions that cause the observed changes in oxygen affinity can occur either at the active site of the subunit or, as in primate hemoglobins, in regions responsible for allosteric regulation by molecules like 2,3-bisphosphoglycerate. In some cases, different organic phosphate concentrations rather than different hemoglobins are present in fetal versus adult red blood cells. Invertebrate examples of ontogenetic changes in the composition of hemoglobin include the phantom midge Chironomus (11) and the brine shrimp Artemia salina (12). Our study now presents a case of developmentally regulated expression of the copper-based oxygen transport protein Hc. In C. magister, the shift in Hc sub-
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unit composition results in functional changes both in intrinsic oxygen affinity (8) and in Mg$^{2+}$ sensitivity (9). These changes may be an adaptation to changing ecological conditions during ontogenesis from a free-swimming planktonic larva to a benthic adult crab. The shift may also be part of a developmental pattern in which the changes in Hc function counterbalance a parallel ontogeny of ionic regulatory capabilities in C. magister (9, 14).

The onset and continuation of subunit 6 synthesis in this study are indicated by increasingly strong probe hybridization with a transcript of $\sim2.6$ kb starting during the sixth instar (Fig. 5). Use of either the CuA probe or the 3′-probe results in cross-reactions with other mRNAs, presumably those corresponding to Hc subunits 1–5, that are expressed during all developmental stages. This is evident from the less intense 2.6-kb signal that appears throughout the early stages (Fig. 3).

An alternative explanation for the presence of subunit 6 on SDS-polyacrylamide gels of adult Hc is that this new subunit could be the product of post-translational modification or proteolysis of an already existing polypeptide rather than marking the onset of expression of a different gene. The Northern blot procedure we used allows us to exclude this possibility for three reasons. First, any of our three Hc-specific cDNA probes should have hybridized strongly with a potential “pre-subunit 6” transcript being expressed in the early juvenile stages. No such signal was found. Second, the 5′-probe we used in these blots is clearly subunit 6-specific because its 5′-end was designed to match that subunit’s unique N-terminal sequence. Finally, the appearance of a new species of mRNA that hybridizes with our subunit 6-specific probe in the hepatopancreas of sixth instar crabs coincides with the appearance of a new Hc subunit in the animal’s hemolymph as shown by SDS-polyacrylamide gel electrophoresis. These data also exclude the possibility that subunit 6 might have been synthesized earlier and simply stored intracellularly until its eventual release into the hemolymph during the sixth instar stage.

The observed developmental differences in subunit stoichiometries require the initiation of expression of subunit 6, down-regulation of subunit 5 production, and an increase in subunit 4 synthesis (6). The proportions of each subunit in megalaopa Hc and early juvenile Hc are constant as are those in adult Hc, and, consistent with other studies on adult crustacean Hc (13), there appears to be only one type of 25 S molecule in these age groups. Intermediate stage juveniles having hemocyanins with subunit stoichiometries and oxygen affinities approaching those of the adult (9) probably have a mixture of both types of 25 S Hc.

The data presented here show that first, hemocyanin is synthesized in one tissue, the hepatopancreas, and second, expression of adult Hc subunit 6 begins during the sixth instar in the crab C. magister. The net effect of the initiation of synthesis of subunit 6 and the reversal in relative amounts of subunits 4 and 5 is an increase in oxygen affinity. These results lead to further questions about stage-specific regulation of synthesis of six different polypeptide chains and their coordinated assembly into developmentally appropriate multisubunit molecules. Future studies should enhance the understanding of the molecular mechanisms controlling these ontogenetic changes.

Acknowledgments—Our heartfelt thanks to Dr. Yi-Lin Yan for sharing ideas and technical expertise throughout this project. We also thank Clete Otoshi, Kristin O’Brien, Dr. Margaret Ryan, Dr. John Postlethwait, and Dr. Ry Meeks-Wagner for valuable contributions.

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