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Evolution of novel functions: cryptocyanin helps build new exoskeleton in *Cancer magister*

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Summary

Hemocyanin, the blue blood protein of many arthropods and molluscs, reversibly binds oxygen at its highly conserved copper–oxygen-binding sites and supplies tissues with oxygen. Cryptocyanin, closely related structurally and phylogenetically to arthropod hemocyanin, lacks several of the six critical copper-binding histidines, however, and has lost the ability to bind oxygen. Despite this loss of function, cryptocyanin continues to be synthesized, an indication that it has been exploited to carry out new functions. Here, we show that cryptocyanin is present in extremely high concentrations in the hemolymph of the crab during the premolt portion of the molt cycle. Both proteins are specifically expressed in the same type of cell in the hepatopancreas and secreted into the hemolymph, but cryptocyanin plays a major role in forming the new exoskeleton, while hemocyanin functions in oxygen transport. A cessation in cryptocyanin, but not hemocyanin, synthesis after eyestalk ablation supports our hypothesis that cryptocyanin is closely regulated by molting hormones. The contrasts between the two gene products illustrate how a gene duplication of a copper–oxygen protein and its subsequent mutation may work in concert with the evolution of new regulatory mechanisms, leading to the assumption of new functions.

Key words: *Cancer magister*, cryptocyanin, hemocyanin, ecdysis, exoskeleton, Crustacea.

Introduction

The evolution of new proteins resulting from gene duplications and subsequent mutations is an important process in the adaptive ability of organisms. A key part of directing these new gene products towards useful functions is the development of alternate regulatory mechanisms. Even before the advent of contemporary genomics and proteomics, this process had been widely studied in the progressive expression of paralogous hemoglobin genes during embryonic, fetal and adult stages of vertebrate ontogeny, and the different oxygen-binding properties of the gene products have been characterized in detail (Bunn et al., 1977). The expansion of new protein structures and functions is now evident in the copper–oxygen-binding proteins, where two hemolymph proteins, hemocyanin and cryptocyanin, closely related members of the same gene family, play separate and significant roles in the developmental physiology of *Cancer magister*, the Dungeness crab.

Hemocyanin, the blue blood protein of arthropods and molluscs, reversibly binds oxygen at its highly conserved copper-binding sites and supplies tissues with oxygen (Van Holde and Miller, 1995). In *C. magister*, the hemocyanin circulates as two-hexamer 25S and one-hexamer 16S oligomers in the hemolymph, and the oligomers are composed of a total of six unique polypeptide subunits (Ellerton et al., 1970; Larson et al., 1981). The hemocyanin undergoes an ontogenic change in subunit composition during development from megalopa to adult crab, which is accompanied by corresponding changes in oxygen affinity (Terwilliger and Brown, 1993; Terwilliger and Ryan, 2001; Terwilliger and Terwilliger, 1982). Three of the subunits, Hc subunits 1, 2 and 3, are constitutive, while Hc subunits 4, 5 and 6 are progressively recruited and upregulated during development. Cryptocyanin has a similar sequence and hexameric quaternary structure and is phylogenetically closely related to hemocyanin (Burmester, 1999; Terwilliger et al., 1999). Cryptocyanin lacks several of the six critical copper-binding histidines, however, and has lost the ability to bind oxygen. Despite this loss of function, cryptocyanin continues to be synthesized in high concentrations. Its presence in the hemolymph of the crab, particularly at specific times in the molt cycle, indicates that it has been exploited to carry out new functions.

Ecdysis, or molting of the exoskeleton, is a challenging requirement for growth in the members of the Ecdysozoa. The process requires periodic and precise control to simultaneously form a new exoskeleton and remove the old one (Skinner, 1985). To understand how cryptocyanin is involved in the molting process, we investigated where and when this member of the hemocyanin gene family is synthesized and explored its

function. We compared the patterns of protein and mRNA expression of hemocyanin and cryptocyanin in cohorts of juvenile *C. magister* as the individual crabs progressed from ecdysis to postmolt, intermolt, premolt and the next ecdysis through multiple molt cycles. We also carried out eyestalk ablation experiments to assess the hormonal regulation of cryptocyanin production. The early juvenile stages of *C. magister* molt quickly and thus provide insights into the functions of these proteins over a brief time frame, in contrast to the annual molt cycle of the adult crab. The results provide a marked example of physiological evolution, where a gene duplication and subsequent mutation has resulted in two proteins with dynamically different patterns of regulation and distinct functions.

Materials and methods

Protein analyses

A cohort of *Cancer magister* (Dana) megalopas was collected near the mouth of Coos Bay, Oregon. They molted into first-instar juvenile crabs and were raised through the 7th instar in individual containers at the Oregon Institute of Marine Biology (OIMB) in running, unfiltered seawater at ambient temperature, salinity and daylight conditions. Their molt cycles were monitored and they were fed chopped fresh *Mytilus* daily.

Hemolymph samples for analysis of cryptocyanin and hemocyanin levels were drawn three times a week from each crab, starting at day 0, 2nd instar. One µl of hemolymph was electrophoresed on non-dissociating, non-denaturing 5% polyacrylamide gels, pH 7.4 (pH 7.4 PAGE; Terwilliger and Terwilliger, 1982). The protein bands were quantified by video gel analysis and imaging software (Jandel Scientific, San Rafael, CA, USA). Standard curves were obtained by first lyophilizing purified cryptocyanin, two-hexamer 25S hemocyanin and one-hexamer 16S hemocyanin (Terwilliger, 1999; Terwilliger et al., 1999) and determining their extinction coefficients. Purified proteins were quantified spectrophotometrically, and a dilution series of each was run on pH 7.4 PAGE. Because the duration of the molt cycle increases and becomes progressively asynchronous with age of the crab, experimental results were graphed as a percentage of the molt cycle; increasing molt cycle duration is represented on the *x*-axis (Fig. 1). Additional cohorts have been raised and monitored through the 12th instar, with consistent patterns of hemocyanin and cryptocyanin fluctuations during the molt cycle.

Real-time quantitative PCR

C. magister megalopas were collected and raised in running seawater as above. Crabs were harvested during the 5th instar on specified days post-ecdysis until the cohort reached the 6th instar. RNA was isolated from hepatopancreas, epidermis and leg muscle tissues with the SNAP Total RNA Isolation Kit (Invitrogen, Carlsbad, CA, USA). Total RNA (1 µg) was reverse-transcribed using MuLV reverse transcriptase and oligo dT primer (Applied Biosystems, Foster City, CA, USA).

Cryptocyanin was amplified with non-degenerate primers based on the sequence of *C. magister* cryptocyanin (GenBank AF091261; Terwilliger et al., 1999); cryptocyanin (Cc) sense AGA CCC AGA TTG CCG AAG G and Cc antisense GCA CGC CTG GGG GAG TAT C. Hemocyanin primers were degenerate and based on *C. magister* N-terminal sequence for hemocyanin (Hc) subunits 1 and 2 (Durstewitz and Terwilliger, 1997a) and the crustacean hemocyanin Copper A site; Hc sense CAY MGN CAR CAR GCN GTB AAY MG and Hc antisense CGV CAN GTN ARY TGR TGR TGV ACC CA. For normalization, a set of degenerate primers for arthropod α tubulin was designed: tubulin sense ACK GCY GTB GAR CCS TAC and tubulin antisense TCV ARS GCR GCC ARR TCY TC.

Real-time quantitative PCR was carried out at Mount Desert Island Biological Laboratory using a Mx4000 Multiplex Quantitative PCR System and QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA). For each of the three target mRNAs, a reference cDNA from intermolt hepatopancreas was serially diluted to show the empirical relationship between threshold cycle (Ct) and template abundance. This relationship was then used as the basis for calculating relative mRNA expression in the test samples. Tubulin mRNA in hepatopancreas tissue was constant throughout the molt cycle, and mRNA levels for hemocyanin and cryptocyanin were normalized to that of tubulin mRNA.

Sequence of C. magister *hemocyanin subunits 1 and 2*

The sequences of hemocyanin subunits 1 and 2 of *C. magister* were determined in order to develop hemocyanin-specific *in situ* probes. *C. magister* hepatopancreas cDNA was prepared, and a 700 bp PCR product was amplified using the Hc subunits 1 and 2 sense and antisense primers as described above. Based on the sequence of this product, gene-specific primers were designed for amplification of the 3′ and 5′ ends using the SMART™ RACE cDNA Amplification Kit (BD Biosciences-Clontech, San Jose, CA, USA). Products were either directly sequenced or cloned into a pCR4-TOPO vector (TOPO TA cloning kit by Invitrogen). DNA was sequenced by the University of Oregon Sequencing Facility and the Oregon State University Center for Gene Research and Biotechnology. This resulted in the complete cDNA sequence of *C. magister* Hc subunit 1 (GenBank AY861676).

The sequence of *C. magister* Hc subunit 2 was obtained by using a set of degenerate hemocyanin primers designed against the conserved amino acid sequences MNEGEFVYA and QGDPHGKF. A 750 bp product was cloned, and a number of the resulting colonies were sequenced. One was very similar to *C. magister* Hc subunit 1 but different enough to allow the design of gene-specific primers for 3′ and 5′ RACE. This yielded the complete sequence of *C. magister* Hc subunit 2 (GenBank AY861677).

In situ *hybridization*

Gene-specific sense and antisense oligonucleotide probes were designed and synthesized by GeneDetect

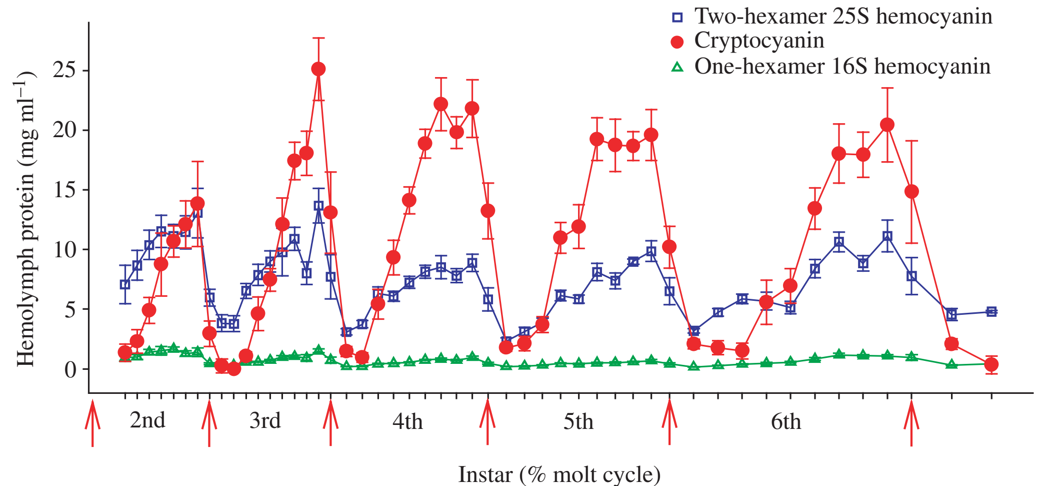

Fig. 1. Cryptocyanin and hemocyanin protein levels in the hemolymph during sequential molt cycles of *Cancer magister*. Instar times expressed as % molt cycle, adjusted to average duration for that instar stage (2nd instar molt cycle averaged 16 days, while 6th instar averaged 30 days). Hemolymph samples were run on non-denaturing, non-dissociating pH 7.4 PAGE. Protein concentrations were determined from image analysis of gels using standard curves of purified hemocyanin and cryptocyanin of *C. magister*. Data expressed as means ±1 S.E.M. (2nd instar, N=13; 3rd instar, N=23; 4th instar, N=23; 5th instar, N=16; 6th instar, N=5). Arrows indicate ecdysis.

(http://www.GeneDetect.com). The cryptocyanin-specific probe hybridized to nucleotides 1650–1697 of *C. magister* cryptocyanin (Terwilliger et al., 1999). The hemocyanin-specific probes were a mixture of Hc subunits 1–2 (described above) and Hc subunit 6 (Durstewitz and Terwilliger, 1997a) specific probes. The subunit 1–2 probe was generated against nucleotides 159–206, a region where the two subunits are identical, and the subunit 6 probe was designed against nucleotides 1759–1806 (GenBank U48881). A poly-dT probe was used as a control for mRNA quality. The probes were 3′ labeled with the DIG Oligonucleotide Tailing Kit (Roche, Indianapolis, IN, USA).

All solutions were prepared with DEPC-treated water. Freshly dissected tissues were fixed in R-F Fixative (Hasson, 1997) that included 1× PBS. The tissues were dehydrated, embedded in Paraplast-Plus and sectioned at 5 µm. Rehydrated slides were postfixed in 4% paraformaldehyde, acetylated and treated with proteinase K. Approximately 20 ng of probe was added to the prehybridization buffer for each slide, and hybridization was carried out overnight in a humid chamber at 37°C. Post-hybridization washes of 1× SSC and 0.5× SSC were done at 50°C. The DIG-labeled oligonucleotides were detected with an anti-DIG-alkaline phosphatase antibody. The slides were stained with NBT/BCIP staining solution containing 1 mmol l^{-1} levamisole.

To test for specificity of probe binding, both sense and antisense gene-specific oligonucleotide probes were used. As a control to ensure that the positively reacting antisense probes were specifically binding to RNA, RNase treatment of sections was done prior to hybridization with the antisense probe.

Immunohistochemistry

Crabs were fixed in Bouin's solution overnight at room temperature, embedded in Paraplast-Plus and sectioned at 10 µm. After rehydration, sections were blocked with 5% milk and incubated with either a hemocyanin- or cryptocyanin-specific monoclonal antibody (Terwilliger et al., 1999), which had been prepared at the University of Oregon Monoclonal Antibody Facility. Reaction with a biotinylated anti-mouse secondary antibody (Sigma, St Louis, MO, USA) was followed by detection with the peroxidase Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA), used according to the recommended procedures. The sections were then stained with the DAB Substrate Kit (Vector Laboratories). After staining, the sections were washed and lightly counterstained with hematoxylin.

Monoclonal antibody specificity was determined by western blot analysis against a range of purified proteins, including *C. magister* hemocyanin and cryptocyanin, plus crab hemolymph (Terwilliger, 1999; Terwilliger et al., 1999). Antibody specificity was tested on tissue sections by preadsorption of each of the monoclonal antibodies, anti-hemocyanin and anti-cryptocyanin, with both purified antigens, hemocyanin and cryptocyanin, before incubation with tissue sections (Beltz and Burd, 1989). Preadsorption of each antibody with its corresponding antigen eliminated immunoreactivity, while preadsorption with the alternate protein gave a positive reaction, confirming specificity of each antibody.

Eyestalk ablation

The eyestalks were surgically removed from juvenile 6th instar *C. magister* 2 days postmolt. The animals were maintained in running seawater and fed as previously described. Hemolymph samples were taken every other day and electrophoresed on pH 7.4 PAGE. Hemolymph proteins were quantified as described above.

Results

When hemolymph levels of cryptocyanin and hemocyanin were measured over sequential molt cycles during development from 2nd to 7th instar juvenile crabs, we found that concentrations of both proteins varied as a function of the molt cycle (Fig. 1). Cryptocyanin levels were extremely low in

hemolymph of crabs that had just undergone ecdysis (red arrows) and remained low until 20–30% into the new molt cycle. Cryptocyanin increased to high levels during the peak of premolt activity, reaching concentrations at least twice as high as those of hemocyanin, and then levels dropped precipitously, beginning just before the next ecdysis. Hemocyanin levels also decreased at ecdysis, but to a lesser extent than those of cryptocyanin, and started increasing immediately postmolt. Cryptocyanin disappeared from the hemolymph during the entire months-long intermolt stage of the adult *C. magister*, while hemocyanin was invariably present.

To better understand the pattern of regulation of these two proteins, we analyzed the abundance of cryptocyanin and hemocyanin mRNA in three tissues of juvenile *C. magister* during a complete molt cycle using real-time quantitative PCR. In hepatopancreas tissue, expression levels of hemocyanin mRNA were relatively constant, with slight increases in postmolt and premolt crabs (Fig. 2A). The differences were approximately onefold, similar in magnitude to those reported for *Callinectes sapidus* hemocyanin mRNA (Brouwer et al., 2002). By contrast, cryptocyanin mRNA in the same *C. magister* crabs was expressed at levels more than 20-fold higher than hemocyanin (Fig. 2B). Cryptocyanin mRNA levels were low immediately postmolt and gradually increased. Cryptocyanin mRNA was consistently high during the mid third of the molt cycle and then decreased during the latter third. There was essentially no expression of either cryptocyanin or hemocyanin mRNA in epidermis or leg muscle over the molt cycle (data not shown).

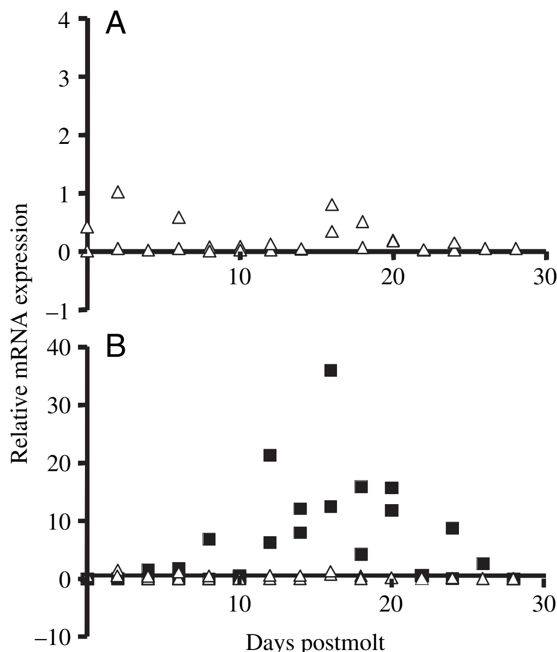

Fig. 2. Cryptocyanin and hemocyanin mRNA expression in *C. magister* hepatopancreas during one molt cycle. Fifth-instar juvenile cohort, ecdysis on day 0 and day 28. Real-time quantitative PCR data from three target mRNAs: cryptocyanin, hemocyanin and tubulin. mRNA levels of cryptocyanin and hemocyanin were normalized to that of tubulin mRNA, which was constant throughout the molt cycle. (A) Hemocyanin mRNA/tubulin mRNA (△); (B) cryptocyanin mRNA/tubulin mRNA (■), hemocyanin mRNA/tubulin mRNA (△). Note that the *y*-axis scale in B is 10-fold greater than in A. Two crabs sampled per time point.

To identify which cells in the hepatopancreas tubules or surrounding connective tissue were synthesizing hemocyanin or cryptocyanin, we carried out *in situ* hybridization studies. We determined the cDNA sequences of hemocyanin subunits 1 and 2, both constitutive subunits that are expressed in hemocyanins from megalopas, juveniles and adult *C. magister*, and used those as well as the sequence of hemocyanin subunit 6, a developmentally expressed subunit (Durstewitz and Terwilliger, 1997b) to design the hemocyanin-specific probes. Since there are many areas of overlapping similarity between hemocyanin and cryptocyanin, the probe sequences were carefully chosen from unique regions of each cDNA. To ensure specificity of each probe, we tested it as a PCR primer along with a gene-specific primer for either hemocyanin or cryptocyanin. In each case, we obtained a single product only with the corresponding gene-specific primer. *In situ* results using the hemocyanin antisense probes localized hemocyanin mRNA to the tubules of the hepatopancreas (Fig. 3B). It was expressed in the basal portions of the R cells, but was not evident in B cells or F cells. Cryptocyanin mRNA expression was also found only in R cells in the tubules of the hepatopancreas (Fig. 3C). The cryptocyanin *in situ* reaction was much stronger than that of hemocyanin. No reactivity was observed when the tissues were incubated with hemocyanin or cryptocyanin sense probes (Fig. 3B,C) or when the tissues were preincubated with RNase prior to exposure to the antisense probes (data not shown). No other tissues, including muscle, connective tissue, reserve cells, and epidermis, showed any reaction to the hemocyanin or cryptocyanin antisense probes, indicating that both hemocyanin and cryptocyanin mRNA expression are specifically localized in the same cell type, the R cells of the hepatopancreas.

The role of hemocyanin in transporting oxygen from the gills to the tissues has been well documented (Markl and Decker, 1992; Terwilliger and Brown, 1993; Terwilliger and Ryan, 2001; Van Holde and Miller, 1995). How does cryptocyanin, the protein whose presence was overlooked for years because of its resemblance to hemocyanin, contribute to the development and growth of the crab? We traced cryptocyanin and hemocyanin through the molt cycle from premolt megalopa to postmolt 2nd-instar juvenile crabs, using immunohistology with protein-specific monoclonal antibodies. The hemocyanin antibody reacted with contents of the blood vessels. During late premolt and immediately postmolt, cryptocyanin antibody reacted strongly with blood vessel contents and with the connective tissue, effectively outlining the organs, including hepatopancreas tubules, gills and stomach. In addition, cryptocyanin staining was evident in the elongated cells of the epidermis and in the newly secreted exoskeleton (Fig. 4A). Several hours after ecdysis, the new

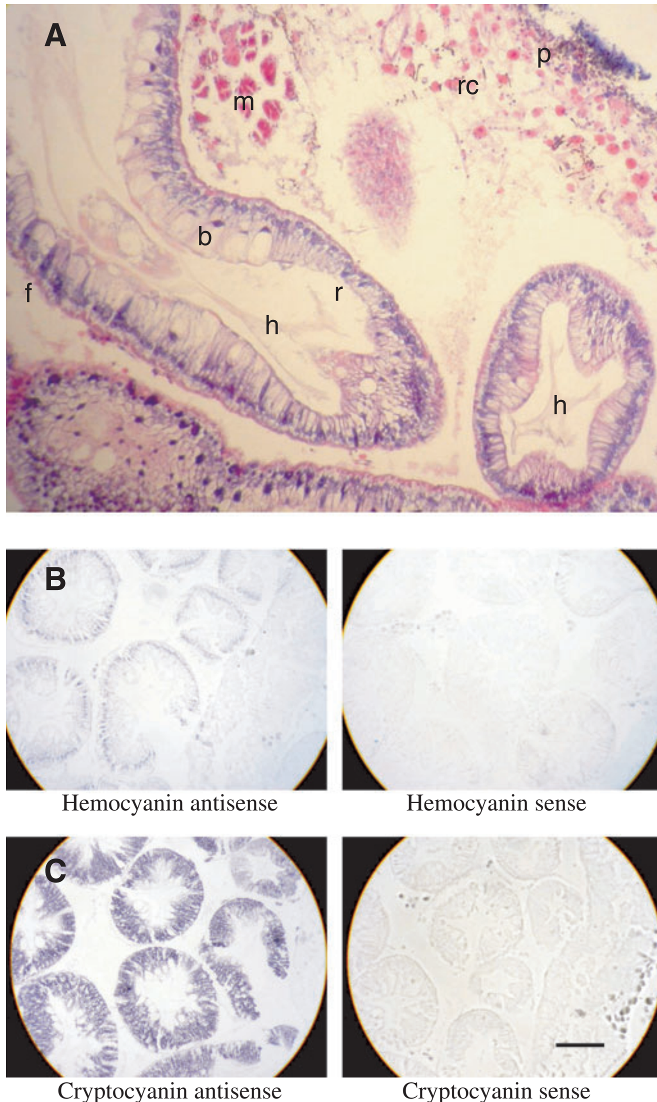

Fig. 3. (A) Hepatopancreas and adnexal tissue of premolt *C. magister* stained with hematoxylin and eosin. Abbreviations: h, hepatopancreas tubules; b, B cells; f, F cells; r, R cells; m, muscle; rc, reserve cells in connective tissue; p, pigment granules. (B,C) *In situ* hybridization. Cell-specific expression of cryptocyanin and hemocyanin mRNA in hepatopancreas tissue of premolt *C. magister*. (B) Hemocyanin-specific antisense probes reveal that hemocyanin mRNA is expressed in R cells of hepatopancreas tubules. Hemocyanin sense probes show no reactivity. (C) Cryptocyanin-specific antisense probe shows extremely strong reactivity in R cells of hepatopancreas. Same tissue as Fig. 2B, 10 μm distant. Cryptocyanin sense probe shows no reactivity. Scale bar: A=100 μm; B,C=300 μm.

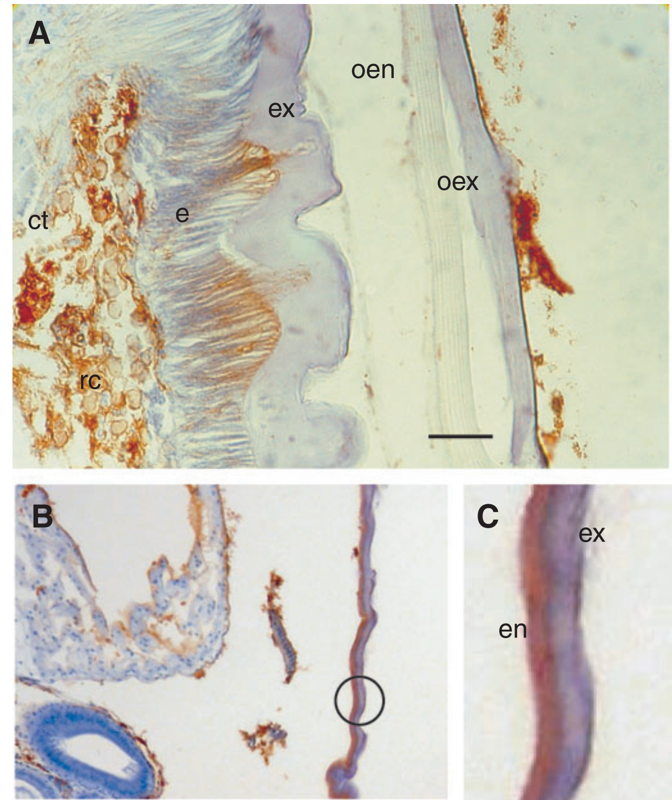

Fig. 4. Immunohistology of *C. magister*. Tissues oriented with external surface of crab on right; counterstained with hematoxylin. (A) Late premolt crab incubated with *C. magister* anti-cryptocyanin monoclonal antibody shows cryptocyanin staining brown in subepidermal connective tissue (ct), elongated epidermal cells (e) and newly secreted exocuticular layer (ex). Reserve cells (rc), old exocuticle (oex), old endocuticle (oen). (B) 18 h postmolt crab shows immunoreactivity to cryptocyanin in new endocuticle. Circle indicates area enlarged in C. (C) Area of new exoskeleton enlarged 10× from B. Newly formed endocuticle (en) is immunoreactive to cryptocyanin; new exocuticle (ex) is now immunonegative. Scale bar: A=70 μm; B=100 μm; C=10 μm.

exocuticle layer of the exoskeleton stained positively for cryptocyanin. When the new endocuticle layer was initially secreted ~18 h postmolt, it stained positively (Fig. 4B,C); by this time, the new exocuticle was no longer immunoreactive.

During premolt, large, rounded ‘protein cells’ or ‘reserve cells’ (Cuenot, 1893; Johnson, 1980; Travis, 1965) accumulated in the connective tissue, especially below the epidermis. In early premolt, these cells did not react with cryptocyanin antibody, but immediately before and during ecdysis, the reserve cells became positive, staining golden and then dark brown (Fig. 5). In the next few days postmolt, as hemolymph cryptocyanin decreased, the reserve cells were smaller, irregularly shaped and markedly fewer in number. By 3 days postmolt, no cryptocyanin was visible anywhere, nor were reserve cells apparent. At 6 days postmolt, cryptocyanin synthesis resumed, as evidenced by its reappearance in hepatopancreas cells and hemolymph. The protein in the hemolymph gradually increased in staining intensity until the next ecdysis at day 16 into 2nd instars.

The apparent hormonal regulation of cryptocyanin production and utilization was further explored through eyestalk ablation experiments on juvenile *C. magister*. Crustacean molt inhibiting hormone (MIH) is synthesized and stored in the X organ/sinus gland complex in the eyestalks of a crab, and removal of the eyestalks stimulates premature

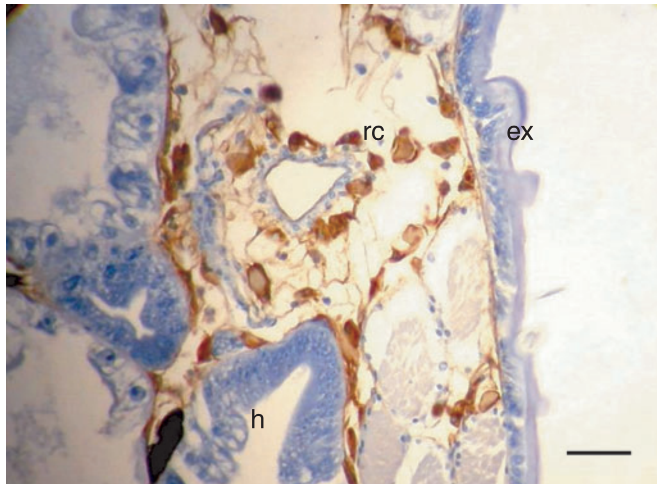

Fig. 5. Reserve cells (rc) show golden to dark brown immunoreactivity to cryptocyanin antibody in 30 min postmolt crab. Hepatopancreas (h), new exocuticle (ex). New endocuticular layer has not yet formed. Counterstained with hematoxylin. Scale bar=50 µm.

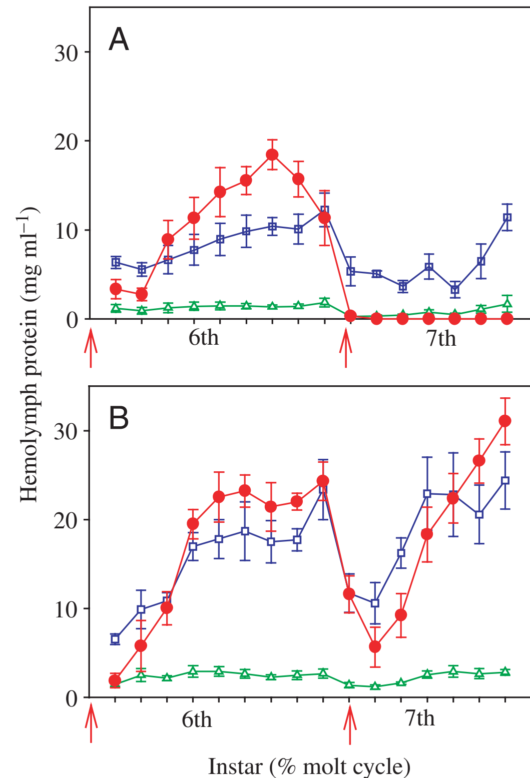

Fig. 6. Eyestalk ablation affects cryptocyanin patterns in hemolymph. Data expressed as in Fig. 1. Arrows indicate ecdysis. (A) Hemolymph proteins follow normal patterns in the initial post-ablate molt cycle. In the subsequent cycle, cryptocyanin does not reappear in the hemolymph, in contrast to hemocyanin. Eyestalks were ablated on day 2, 6th instar (*N*=7). Shortened molt cycle of ablated crabs averaged 21 days. (B) Hemolymph proteins in control crabs (*N*=6). Molt cycle averaged 32 days.

ecdysis (Chang et al., 1993; Skinner, 1985; Zeleny, 1905). Eyestalks were removed from 6th-instar juvenile crabs two days after they had molted from the 5th instar. The eyestalk-ablated juvenile crabs produced typical hemolymph patterns of hemocyanin and cryptocyanin and underwent premature ecdysis in the first post-ablate molt cycle (Fig. 6A). In the second post-ablate molt cycle (7th instar), hemolymph levels of hemocyanin in the ablated crabs returned to normal, but cryptocyanin did not reappear. Unablated 7th-instar control crabs underwent normal expression of cryptocyanin and hemocyanin (Fig. 6B). These results indicate that molting hormones have a major regulatory effect on cryptocyanin expression and catabolism.

Discussion

This study examines the physiological role of cryptocyanin in the Dungeness crab, *Cancer magister*, and finds that this protein is involved in the formation of the new exoskeleton. Hemocyanin and cryptocyanin are closely related hemolymph proteins that are in all probability the result of a gene duplication event (Burmester, 1999; Terwilliger et al., 1999). Our *in situ* and real-time PCR experiments demonstrate that cryptocyanin is synthesized in the same cell type within the hepatopancreas as hemocyanin. However, we find distinct differences in mRNA levels and hemolymph protein levels over the molt cycle, which indicate that cryptocyanin and hemocyanin have evolved separate regulatory control mechanisms that facilitate their different functions as structural protein or oxygen transport protein.

The amounts of cryptocyanin and hemocyanin in the hemolymph during the molt cycle differ both temporally and quantitatively. Although some dilution of total hemolymph protein occurs immediately after molting due to uptake of water (Mykles, 1980), the changes in concentration of cryptocyanin in the hemolymph from premolt to postmolt are much greater than those of hemocyanin. In addition, after ecdysis, cryptocyanin levels remain low longer than hemocyanin levels. Therefore, the differences between the two proteins are not due to a simple dilution effect. Early juvenile crabs molt at least 10 times in the first year and have brief intermolt periods, so the crabs undergo quick transitions from postmolt to premolt. Their postmolt levels of hemolymph cryptocyanin are low, while in the long intermolt period of the adult crab, cryptocyanin is not detectable. These patterns of protein levels in the hemolymph reflect the abundance and fluctuations of cryptocyanin and hemocyanin mRNA during the molt cycle as well.

Hemocyanin and cryptocyanin mRNA are both expressed in the hepatopancreas tissues of *C. magister* (Durstewitz and Terwilliger, 1997b; Terwilliger et al., 1999). Our preliminary histochemistry had localized cryptocyanin protein in connective tissue cells surrounding the hepatopancreas tubules and below the epidermis, as well as in the hemolymph. In the real-time PCR studies, therefore, we assayed hepatopancreas tissue, epidermis and, as a control, leg muscle. Cryptocyanin mRNA was not amplified in epidermis tissue, indicating that cryptocyanin is not synthesized in epidermal cells or underlying connective tissue. It

is clear that hepatopancreas cryptocyanin synthesis is markedly upregulated during early premolt and is downregulated as the crab approaches ecdysis, whereas hemocyanin seems to be synthesized at a low, relatively constant level. The decrease in cryptocyanin mRNA during late premolt preceded the decrease in hemolymph protein, consistent with translational regulation of cryptocyanin synthesis and/or a fairly long half-life of cryptocyanin protein. Therefore, we find major differences in tissue-specific abundance and timing between cryptocyanin and hemocyanin mRNA and protein during the molt cycle.

We had suspected that different cell types in the hepatopancreas tubules or surrounding connective tissue would be responsible for cryptocyanin and hemocyanin synthesis because of the differences in expression patterns. The crab hepatopancreas or digestive gland is composed of two major branches that originate laterally at the juncture between the cardiac and the pyloric stomach. Each branch further subdivides into three lobes, which continue branching into blind-ended tubules and fill the body space on either side of the stomach. The hepatopancreas tubules are loosely held together and enveloped in webby strands of cellular connective tissue (Fig. 3A). The tubule walls are composed of multiple cell types, R (resorptive), F (fibrillar), B (blister-like), M (midget) and E (embryonic), that have been characterized on the basis of morphology, position and histochemistry (Al-Mohanna and Nott, 1989; Jacobs, 1928). Multiple functions have been attributed to the hepatopancreas and its cell types, including synthesis and secretion of digestive enzymes, absorption of nutrients and storage of heavy metals. It is not certain whether the cell types represent stages of differentiation or unique cell lines (Gibson and Barker, 1979; Icely and Nott, 1992; Vogt, 1985).

Our *in situ* results identify the R cells of the hepatopancreas as the site of synthesis of both cryptocyanin and hemocyanin. We suggest that the two proteins are secreted basally into the blood vessels located between and surrounding the hepatopancreas tubules in *C. magister*, an idea supported by ultrastructural evidence for pinocytotic trafficking at the basal surface of the R cell in *Penaeus semisulcatus* (Al-Mohanna and Nott, 1987). The cryptocyanin *in situ* reaction was much stronger than that of hemocyanin, consistent with the higher levels of cryptocyanin mRNA in the real-time PCR studies (Fig. 2B) and higher protein levels in the hemolymph (Fig. 1) of the premolt crab.

The rapid disappearance of cryptocyanin from the hemolymph at ecdysis stimulated us to see how the protein is utilized by the crab. We found that cryptocyanin plays a significant role in formation of the new exoskeleton. Secretion of a crab's new exoskeleton across the epidermis begins during premolt, shortly after apolysis of the old exoskeleton is initiated but before the old exoskeleton is shed (Skinner, 1962; Williams et al., 2003). Formation of the new exoskeleton occurs in layers, with first a thin epicuticle secreted into the extracellular space between epidermis and old exoskeleton, then a thicker exocuticle layer appearing beneath the new epicuticle during late premolt. Immediately after ecdysis, while the new exoskeleton is still soft, unfolding and expanding to a larger size than the old exoskeleton had been, the crab continues to secrete more exocuticle. This is the period when the fluid-inflated soft exoskeleton serves as a hydrostatic skeleton (deFur et al., 1985; Taylor and Kier, 2003). Within hours postmolt, secretion of the endocuticle layer begins beneath the new exocuticle. This layer continues to increase in thickness, while calcification and sclerotization of the new exoskeleton progress, until the crab enters intermolt.

Our immunohistochemical results provide morphological evidence that cryptocyanin is secreted into the hemolymph, becomes incorporated into the extracellular matrix around the internal organs and also moves across the epidermis into the extracellular matrix of the new exoskeleton in synchrony with the molt cycle. Thus, cryptocyanin, synthesized in the hepatopancreas, contributes to the structure of the exoskeleton, along with cuticular proteins (Coblentz et al., 1998; Compere et al., 2002) synthesized by the epidermis. When the postmolt portion of the exocuticle and then the endocuticle layers appear immediately after ecdysis, they are initially immunoreactive against cryptocyanin. The subsequent loss of immunoreactivity in the exocuticle that we observe could be the result of specific proteolytic cleavage, although it is more likely due to cross-linking during sclerotization, since proteins are known to cross-link during this process in both crustaceans and insects.

Insect hexamerins, additional members of the hemocyanin gene family, resemble cryptocyanin in several ways (Beintema et al., 1994; Telfer and Kunkel, 1991; Terwilliger, 1999). These hexameric hemolymph proteins also lack copper and therefore do not participate in oxygen transport or phenoloxidase activity. The concentrations of some hexamerins fluctuate with molting cycles, and this is regulated by ecdysteroids and juvenile hormones. In addition, while functional diversity of hexamerins is high, it has been demonstrated in several insect species that certain hexamerins are incorporated into the nascent cuticle (Konig et al., 1986; Webb and Riddiford, 1988).

Cryptocyanin that is not incorporated into the exoskeleton is removed from the hemolymph by the reserve cells soon after molting. Earlier studies had postulated that the reserve cells were the site of hemocyanin synthesis, based on electron microscopy of large crystalline inclusions in the cells (Ghiretti-Magaldi et al., 1977). Our *in situ* and our immunohistology studies indicate that reserve cells have neither hemocyanin nor cryptocyanin mRNA but do contain cryptocyanin at ecdysis. The crystalline structures identified by Ghiretti-Magaldi and others are probably cryptocyanin, not hemocyanin. Endocytosis of cryptocyanin by the reserve cells at ecdysis may be the result of a hormonally regulated appearance of cryptocyanin-specific receptors on the reserve cells, in a manner similar to the uptake of hexamerins in certain insects (Haunerland, 1996). We suggest that the reserve cells metabolize the cryptocyanin, whose amino acids may be used for energy and tissue growth in the newly molted crab, and then decrease in size and number until the next premolt.

Hormonal regulation of cryptocyanin production and utilization is supported by eyestalk ablation experiments on juvenile *C. magister*. The resumption of hemocyanin levels but not cryptocyanin in the hemolymph after the eyestalks were removed and the crabs underwent ecdysis point to a requirement

for ecdysteroid or X organ/sinus gland peptidergic regulation of cryptocyanin expression and catabolism.

Are there other roles for cryptocyanin in the molting crab? Recent reports indicate that the C-terminal domain of hemocyanin can be cleaved and can function as a bacteriocidal peptide in shrimp and crayfish (Destoumieux-Garzon et al., 2001; Lee et al., 2003). Cryptocyanin may have a similar function, especially in the soft postmolt exoskeleton. Cryptocyanin may have other functions as well, including calcium uptake and/or an osmotic role during ecdysial water uptake.

Hemocyanin and cryptocyanin are two closely related members of the same gene family that are synthesized in the same tissue-specific cells and are secreted into the hemolymph. Hemocyanin, carrying out its main role as oxygen transporter, is maintained at a relatively constant level in the hemolymph. Cryptocyanin, on the other hand, has evolved as a structural protein, and its dynamic regulation waxes and wanes with the molt cycle. The contrasts between the two gene products illustrate how a gene duplication of a copper–oxygen protein and its subsequent mutation may work in concert with the evolution of new regulatory mechanisms, leading to the assumption of new functions.

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