Hemocyanin of the Molluskan *Concholepas concholepas* Exhibits an Unusual Heterodecameric Array of Subunits*

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**Running title:** Structure of the *C. concholepas* hemocyanin

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SUMMARY

We describe here the structure of the hemocyanin from the Chilean gastropod *Concholepas concholepas* (CCH), emphasizing some attributes that make it interesting among molluskan hemocyanins. CCH exhibits a predominant didecameric structure as revealed by electron microscopy, a size of 8 MDa by gel filtration, and in contrast with other mollusk hemocyanins, its stabilization does not require additional Ca$^{2+}$ and/or Mg$^{2+}$ in the medium. Polyacrylamide gel electrophoresis studies, analyses by a MonoQ FPLC column, and Western blots with specific monoclonal antibodies showed that CCH is made by two subunits non-covalently linked, named CCH-A and CCH-B, with molecular masses of 405 kDa and 350 kDa, respectively. Interestingly, one of the subunits undergoes changes within the macromolecule: we demonstrated that CCH-A has an autocleavage site that under reducing conditions is cleaved to yields two polypeptides, CCH-A1 (300 kDa) and CCH-A2 (108 kDa), while CCH-B remains unchanged. The CCH-A nick occurs at 4°C, increases at 37°C and is not inhibited by the addition of protease inhibitors and/or divalent cations. Since the CCH structure is a heterodimer, we investigated whether subunits would be either intermingled forming heterodecamers or assembled as two homogeneous decamers. Light scattering and electron microscope studies of the *in vitro* reassociation of purified CCH subunits, demonstrated that the sole addition of Mg$^{2+}$ is needed for its reassembly into the native decameric molecule; no homodecamer reorganization was found with either CCH-A or CCH-B subunits alone. Our evidence showed that *C. concholepas* hemocyanin is an unusual example of heterodecameric organization.
INTRODUCTION

Hemocyanins, the oxygen carrier glycoproteins found in the hemolymph of many species of arthropods and mollusks, are model proteins due to their complex systems of self-assembly and their sophisticated quaternary structure. Hemocyanins of these two classes of organisms differ considerably in primary sequence, size, and subunit organization, suggesting a divergent evolution that maintains certain superficial similarities in their general structural design and function (1, 2).

Molluskan hemocyanins assemble into hollow cylindrical layered molecules formed by 10 subunits. Each subunit, ranging from 350 kDa to 450 kDa, includes 7 to 8 globular folded domains known as functional units (FUs), which are arranged into pearl-like chains, and covalently bound by a short flexible linker region of 10 to 15 amino acid residues. FUs vary in size from 45 to 55 kDa and each of them is capable of reversibly binding one oxygen molecule through a pair of copper atoms. Decamers reach molecular masses of 3.5 MDa to 4.5 MDa, and in bivalves and gastropods, they can self-associate as stable dimers. This association occurs due to an asymmetrical face-to-face interaction between decamers, resulting in huge structures, which range from 8 MDa to 9MDa (3-10).

Profuse experimental studies, using different dissociation and association conditions of the native mollusk hemocyanin, e.g., removal of divalent cations (11-14), pH changes and the addition of denaturing agents (15-16), have helped to elucidate its subunit composition and structure. In turn, the uses of immunochemical methods combined with partial proteolytic digestion of the subunits, supported by

1 The abbreviations used are: FUs, functional units; KLH, Keyhole limpet hemocyanin; CCH, Concholepas concholepas hemocyanin; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; FPLC, fast pressure liquid chromatography; IgG, immunoglobulin of G class; HRP, horseradish peroxidase; Mabs, monoclonal antibodies; ALP, alkaline phosphatase; NBT, nitro-blue-tetrazolium; BCIP, 5-bromo-4-chloro-indolyl-phosphate; IEF, isoelectric focusing; PVDF, polyvinylidene difluoride; RtH, Rapana thomasaiana hemocyanin.
sequence studies, have allowed researchers to establish structural relationships among different FUs and their location in the polypeptide chain (17-22). Thus, it has been concluded that mollusk hemocyanins varied, both in subunit composition and arrangement (1). Homogeneous decamer and didecamer structures consisting of only one kind of subunit are found in the bivalves of the genus Yoldia (23). In contrast, gastropod hemocyanins of *Megathura crenulata* (17, 24), *Haliotis tuberculata* (25, 26) and *Rapana thomasiana* (27) display two distinct homo-decameric forms, attributed to the presence of two different subunits. Interestingly, didecameric association occurs exclusively among equivalent decamers, generating two homogeneous hemocyanin isoforms, and hybrid molecules have not been described. Although *Murex fulvescens* hemocyanin has two different subunits, like the hemocyanin from the gastropod species mentioned above, its functional protein is a heterogeneous didecamer, as evidenced by oxygen consumption kinetics data (28).

The versatile properties of hemocyanins in biomedical and biotechnological applications (29-32), specifically, that from the mollusk Keyhole limpet (*M. crenulata*) known as KLH, have led to a growing interest in hemocyanins structure and function. In recent years, we have focused our research on the hemocyanin obtained from the mollusk *Concholepas concholepas* (*CCH*). Although this protein has been successfully used as a carrier in the antibody development (33-38), its structure is just beginning to be documented (39). To identify different epitopes on the molecule we developed murine monoclonal antibodies (Mabs) to whole CCH. It was found that it has two subunits (named CCH-A and CCH-B) that display common and specific epitopes (40).

The aim of this paper was to investigate the structural properties of *C. concholepas* hemocyanin protein. We found that, during the purification process, the CCH stabilization does not require additional divalent cations, and that during aging of the molecule, the CCH-A subunit undergoes a splitting process into two polypeptides. Furthermore, we analyzed whether the splitting was a result of a proteolytic cleavage or an autocleavage phenomena. We demonstrated that the autocleavage of CCH-A subunit generates CCH-A1 and CCH-A2 fragments, and occurs independently of the presence of Ca\(^{2+}\), Mg\(^{2+}\) or
protease inhibitors. Finally, since CCH contains two subunits, we analyzed whether they were intermingled or assembled as two homogeneous decamers in a manner similar to others hemocyanins. The results presented here led us to conclude that CCH has an unusual heterodecameric organization.
EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals—AgNO₃, Ammonium Sulfate, Formaldehyde, para-Nitrophenyl Phosphate, and Uranyl Acetate were from Merck, Germany. The Seitz unit was from Kingston, USA. The 0.2 µm Filter Unit was from Corning, USA. Formvar and Parlodion were from Pelco International, USA. Ampholytes (Bio-Lyte), Mini IEF Cell Model 111 system, and Pre-stained Markers were from BioRad Labs, USA. Agarose type VII, Amido Black, Aprotinin, Bovine Serum Albumin (BSA), Ethylenediaminetetraacetic acid (EDTA), Iodoacetamide, Leupeptin, Pancreatic Elastase, Phenylmethanesulfonyl fluoride (PMSF), Pepstatin, and Thyroglobulin were from Sigma, USA. The Mono-Q 5/5 Column, Sepharose 4B and Dextran blue 2000 were from Pharmacia, Sweden. The 0.22 µm Filter was from Millipore, USA. Anti-mouse IgG Horseradish Peroxidase (HRP) conjugate or Alkaline Phosphatase (ALP) conjugate, 5-bromo-4-chloro-indolyl-phosphate (BCIP), Bicinchoninic acid protein kits, Casein SuperBlock, Coomassie Blue, CL-XPosure film (Blue X Ray Film), NBT-BCIP systems, nitro-blue-tetrazolium (NBT), Nitrocellulose membrane, Polystyrene plates, Pre-stained Markers, SDS-PAGE reagents, Super Signal West Pico chemiluminescent substrate Kit, Tween-20, and Unblot In-Gel Chemiluminescent Detection Kit were from Pierce-Endogen, USA. All chemicals were analytical-grade reagents and the solutions were prepared with Mili-Q water.

Hemocyanin sources—Live specimens of Concholepas concholepas (Loco) were harvested from the Pacific Ocean, Bay of Quintay (33° 8' S 71° 48' W) V Region of Chile. Keyhole limpet hemocyanin was purchased from Pierce-Endogen, USA.

Hemocyanin purification—The general procedure described by Hercowitz et al. (41) was employed with modifications. Live C. concholepas specimens were transported to the laboratory in seawater. The hemolymph was collected by bleeding at 4°C through several diagonal slits made on the mantle and foot of the mollusk, and filtered through a glass mesh. Hemocytes and other cells were removed by...
centrifugation at 1,400 g over 20 min, at 4°C, and 0.1% sodium azide was added. The material was precipitated for 12 h at 4°C at 33% saturation with crystalline ammonium sulfate and centrifuged at 4°C for 1 h at 16,300 g in a Sorvall centrifuge; the clear supernatant was discarded. Precipitation and concentration procedures were repeated twice, and then the CCH pellet was suspended in PBS (phosphate buffer saline, 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, 0.1% sodium azide). This material was dialyzed at 4°C against PBS to remove ammonium sulfate. The CCH solution was clarified by centrifugation at 7,000 rpm, pre filtered in a Seitz unit and sterilized through a 0.22 µm filter unit, then stored at 4°C. Protein concentration was determined using Coomassie Blue or Bicinchoninic acid protein kits according to the manufacturer's instructions. This hemocyanin preparation was shown to be highly pure by gel chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In some experiments, purification was performed in the presence of 0.8 µM Aprotinin, 100 µM Iodoacetamide, 22 µM Leupeptin, 15 µM Pepstatin, 1 mM PMSF and 5 mM EDTA with or without 5 mM CaCl₂ and 5 mM MgCl₂.

**Gel Filtration Chromatography**—Gel filtration was performed at 4°C on a Sepharose 4B column, 53 cm in height and 1 cm in diameter (bed volume 41.6 cm³). The column was pre equilibrated in 150 mM PBS (pH 7.2) and then standardized with 3 mg of the following proteins: KLH, thyroglobulin and bovine serum albumin (BSA). To determine the exclusion volume of the column, Dextran blue 2,000 was used. Finally, 10 mg of the CCH was loaded in 5.3 ml of PBS at 4.62 ml/h. Chromatography was monitored at 280 nm. Experiments were also done with a CCH sample stored in a 50 mM PBS (pH 7.2) and chromatographed on MonoQ FPLC. The column was previously equilibrated with the same 50 mM PBS buffer. Elutions were performed with buffer during 5 min, followed by a linear gradient from 40 to 100 % 1 M NaCl for 30 min at 1 ml/min. MonoQ chromatography was monitored at 280 nm.

**Copper Content**—Copper was determined by atomic absorption spectroscopy in GBC equipment (Model 902, Australia) with a hollow copper cathode lamp of 324.8 nm (Photon). The analysis was performed at the Centro de Servicios Externos of the Pontificia Universidad Católica de Chile. Briefly,
purified CCH and KLH samples were hydrolyzed with HNO₃, evaporated and dissolved in diluted HCl prior to analysis. Fixanal Ridel-Haén copper reference standard of 1.0 g Copper/L was used.

**Electron microscopy**—The general procedure described by Fernández-Morán et al. (42) was used, with modifications (43). Twenty-µl aliquots of CCH samples (0.5 to 1 mg/ml) were applied during 1 min to Formvar or Parlodion coated copper grids, previously stabilized by vacuum evaporation on a carbon coat. They were stained for periods of 1 to 5 min with 20 µl of 1% to 2% aqueous uranyl acetate solution, previously filtered through a 0.22 µm filter. The grids were air-dried at room temperature and observed under a Phillips Tecnai 12 electron microscope at the Servicio de Microscopía Electrónica, Pontificia Universidad Católica de Chile.

**SDS-PAGE-Electrophoresis**—The technique was performed as described by Laemmlli (44) in a 3-12% gradient separating and 3% stacking gel. Protein samples were heated for 5 min at 100º C in the presence of SDS and β-Mercaptoethanol. Gels were run at 70 V during 12 h at room temperature. Molecular masses were estimated from band mobility with a calibration curve obtained from KLH-1 and KLH-2 polypeptides and pre-stained markers. The gels were stained with Coomassie Blue or with AgNO₃ (45).

**Native gel electrophoresis**—The procedure described by Swerdlow et al. (17) to characterize different subunits of KLH was applied. This system requires high pH buffer and EDTA to create alkaline dissociating conditions. Prior to electrophoresis, samples were dissociated by incubation in the sample buffer at 4ºC (140 mM Tris, 90 mM boric acid and 2.5 mM EDTA at pH 8.6). Separation was performed at room temperature during 24 h at 80 V. Finally, the gels were fixed and stained with Coomassie Blue.

**Identification of subunits with monoclonal antibodies**—To identify CCH subunits in native gels, the bands were developed *in situ* using the Unblot In-Gel Chemiluminescent detection Kit. Briefly, the gels were fixed, washed and incubated overnight at room temperature, either with hybridoma supernatant of monoclonal antibodies specific to CCH-A subunit ² or CCH-B subunit (40), diluted 1:1 with the buffer

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² B. Moltedo, D Leiva, A.E. De Ioannes and M.I. Becker, manuscript in preparation.
included in the kit. Then, gels were washed in PBS-Tween solution and incubated for 1h at room
temperature with goat anti-mouse IgG-HRP conjugate diluted 1:1000 in the same buffer. Finally, proteins
were visualized with Super Signal West Pico chemiluminescent substrate Kit. Both sides of the gel were
covered with cellophane sheets and exposed to CL-XPosure film.

In other experiments, CCH native electrophoresis gels were transferred to 0.2 µm pore nitrocellulose
membranes (46). The membranes were incubated overnight at 4°C with SuperBlock or 1% PBS-BSA, and
then incubated for 3 h with anti-CCH Mabs diluted 1:1 in SuperBlock. After washing with PBS-Tween
0.02%, the membranes were incubated during 1h at room temperature with goat anti-mouse IgG-ALP
conjugate diluted 1:12000. The membranes were developed using NBT and BCIP. To stop the reaction,
the membranes were washed with water.

Two-dimensional (2D) SDS-PAGE—The method was performed as described by Coligan et al. (47)
and Walker (48). The first dimension was run on a 3% SDS-PAGE tube gel; the sample buffer had no
reducing agents (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS and Bromophenol Blue). The second
dimension was run on a 3 to 20% polyacrylamide gradient system. The strip containing the first dimension
was incubated with a denaturing buffer containing 5% β-Mercaptoethanol in 62.5 mM Tris-HCl pH 6.8,
10% glycerol, 2.3% SDS, before application on the second dimension. Gels were run at 1,050 V/h, at
room temperature, in both the first and second dimension. 2D gels were silver-stained for protein
visualization.

Isoelectric focusing (IEF)—A Mini IEF Cell Model 111 BioRad system was used. The preparation of
the IEF gel was done according to the manufacturer instructions, with 4% acrylamide and 2% ampholytes.
Mixtures of proteins with known pI (range: 4.5-9.6) were used as standards. Gels were run and stained
with 0.05% Crocein Scarlet, according to the supplier’s instructions.

Isolation of CCH subunits—The general procedure described by Swerdlow et al. (17) was employed.
Purified CCH was dissociated into individuals subunits by dialysis against 5 volumes of 130 mM Glycine-
NaOH pH 9.6, 10 mM EDTA (buffer A) overnight at 4°C. Dissociated CCH was loaded on a MonoQ 5/5
FPLC column that has been equilibrated previously with buffer A at 1.0 ml/min. The elution was performed as follows: buffer A for 5 min, followed by a linear gradient of 0 to 70% buffer A containing 1 M NaCl for 30 min at 1 ml/min, and the final wash was performed at 70% buffer A containing 1 M NaCl for 5 min. MonoQ chromatography was monitored at 280 nm.

**Light scattering studies**—The experiments were done according to van Holde *et al.* (11) with minor modifications. CCH subunits purified by Mono-Q chromatography were used in a Perkin Elmer LS50 Model spectrofluorometer. Scattered light was measured at 90º to the incident beam with both entrance and exit monochromators set to 384 nm. The reassociation experiments were done at 20ºC and buffer containing 100 mM Tris/HCl pH 7.6, 50 mM MgCl₂, 10 mM CaCl₂ was added. Samples for electron microscopy analyses were taken and processed as described above.

**Amino acid composition**—The amino acid analysis was done at the Protein/DNA Technology Center, Rockefeller University, USA. Samples were hydrolyzed for 22 h at 110ºC in 6 N HCl containing 1% Phenol. Waters PICO TAG equipment was employed, using Waters Millenium software, 510 Pump, and 490 detectors. A Waters Novapak C18, 30-centimeter column was used. Mol percent values were obtained from 9.18 µg and 12.53 µg of hydrolyzed protein from isolated subunits CCH-A and CCH-B, respectively.

**Amino terminal sequence**—Soluble samples of CCH Mono-Q isolated subunits were subjected to SDS-PAGE and transferred to PVDF membranes (49). They were sequenced by Edman degradation at the Protein/DNA Technology Center, Rockefeller University.
RESULTS

General Properties of CCH

Purification and stability—Purification of CCH from hemolymph yielded a highly pure protein preparation, as assessed by gel filtration chromatography on a Sepharose 4B column. Hemocyanin eluted as a single and symmetrical peak (Kav=3.8) indicating that the protein was homogeneous in size (Figure 1A). The molecular mass was about 8 MDa by extrapolation in a semi-log plot of molecular mass versus Kav (Figure 1B); a value similar to that of other well documented gastropod hemocyanins (1). The absorption spectrum of CCH was similar to other hemocyanins and showed 3 peaks: at 268 nm, 344 nm and 550 nm, that correspond to: aromatic residues, Cu$^{2+}$-O$_2^{2+}$ and Cu$^{2+}$-Histidine coordination centers, respectively (Figure 1C).

A remarkable feature of CCH is that the presence of divalent cations is not needed in the buffers used for purification and storage. In contrast, the gastropod hemocyanins described previously require from 1 to 10 mM Ca$^{2+}$ or Mg$^{2+}$ to stabilize their huge structures (1). CCH samples, stored in PBS at 4°C during at least 3 years, maintained intact their quaternary structure, as verified by electron microscopy (data not shown).

Structure—Figure 2A shows an electron microscope preparation of purified CCH molecules negatively stained with uranyl. Circles of about 325Å in diameter and rectangular specimens of about 392Å in height were observed. At high magnification the circles, top views of the molecules (Figure 2B), showed a dense core of about 153Å in diameter (arrow 1), surrounded by a semi-opaque ring of about 13 Å in width (arrow 2), with a fine dense ring boundary, surrounded by a clear annular zone of about 24 Å in width (arrow 3). Figure 2C shows a side view of the molecule, displaying typical subunit structures arranged in
layers of 6 parallel rows as indicated by the arrows. Didecamers were the most frequent forms observed while decameric or multi-decameric structures were scarce (Figure 2D).

**Copper content**—CCH contains about 0.23% copper by weight, which corresponds to a molar ratio of 300 copper mol/mol of intact protein, like others mollusk hemocyanins (1).

**Polypeptide Chains**—The non-reducing SDS-PAGE and native gels analysis of the CCH indicated that the protein is composed of two different subunits (Figure 3A and 3C, respectively).

**Amino acid content**—Table I presents the amino acid analyses of both CCH subunits and shows that both proteins exhibited homology for 14 of 16 amino acids; significant differences were found in Asparagine (CCH-A > CCH-B) and Lysine (CCH-B > CCH-A).

**N-terminal sequence analysis**—The CCH-A subunit was sequenced up to residue 11 (LMRKDVDTLD) and CCH-B subunit, up to residue 7 (LXRKNVD); the hemocyanin motif is underlined (26). The sequence motifs between both subunits differ at least in one out of five residues: CCH-A has an Aspartic residue in the fourth position within the motif, while CCH B has an Asparagine residue.

**Isoelectric point**—The intact CCH molecule exhibited an isoelectric point near pH 6.0 in two separate experiments (data not shown).

**The autocleavage of CCH-A subunit**

**Description of the CCH structure and aging**—As stated above, when analyzing recently purified CCH under non-reducing SDS-PAGE conditions, the protein displayed two main non-covalently bound polypeptides, CCH-A and CCH-B. Interestingly, aged CCH preparations, stored for a year at 4ºC, showed a change in their electrophoretic pattern, as compared to fresh protein (Figure 3A, lanes 1 and 2). A shift in CCH-A subunit migration occurred and a new polypeptide appeared over the CCH-B band (Figure 3A, lane 2, asterisk); however, the migration of CCH-B subunits remained unaltered (Figure 3A, lane 2).
Under reducing conditions, fresh hemocyanin was also dissociated into two subunits, CCH-A with a Mr of 405 kDa and CCH-B with a Mr of 350 kDa. However, in addition to the main polypeptides a faint band was observed below CCH-B band (Figure 3B, lane 1). In aged CCH preparations, subunit CCH-A decayed completely and it was evident that CCH-A split with time into a polypeptide of 300 kDa (named CCH-A1) and a fragment of 108 kDa (named CCH A-2), while CCH-B was stable (Figure 3B, lane 2). After a year at 4ºC CCH-A was fully converted into CCH-A1 and CCH-A2 (Figure 3B, lane 2); however the appearance of CCH as determined by electron microscope remained unaltered. We concluded that in the fresh CCH sample, the polypeptide running just under CCH-B corresponds to CCH-A1, while and at this time, CCH-A2 was not visible yet, due to its smaller size.

To further investigate the structure of CCH, the fresh protein was dissociated at alkaline pH and analyzed by native PAGE (Figure 3C). The results show that the hemocyanin was also dissociated into two polypeptides, confirming that CCH-A and CCH-B subunits are held together by non-covalent interactions (Figure 3C, lane 1). Again, aged preparations showed an evident change in their pattern: the upper band disappeared and CCH-A and CCH-B subunits apparently comigrated (Figure 3C, lane 2). To determine whether this interpretation was correct, we used Mabs targeted to specifics subunits. We concluded that the band that remains unaltered corresponds to the CCH-B polypeptide, since it reacted with anti-CCH-B 2H10 or 1A4 Mabs, either using direct chemiluminescent staining on the gel (data not shown) or Western blot (Figure 4C), and overlaps with the Coomassie-staining pattern in fresh or aged CCH (Figure 4A, lanes 1 and 2 respectively). In contrast, when we applied anti-CCH-A 2D8 or 4E9 Mabs under the same conditions, the migration of the immunoreactive band changed in aged preparations (Figure 4B, line 2) as observed with Coomassie-staining (Figure 4A, line 2) indicating that aged CCH-A peptides do in fact change motility to comigrated with CCH-B.

**Analysis of the CCH-A fragments**—To determine the origin of fragments, CCH was analyzed by a bidimensional SDS-PAGE (Figure 5). When running the protein in the first dimension, with SDS but without reducing agent, both subunits were separated, confirming the results observed in Figure 3A. The
use of a reducing agent in the second dimension, showed that a fraction of CCH-A remains intact, while the fragments CCH-A1 and CCH-A2, originated from molecules of CCH-A appear. Subunits and fragments were identified by comparison with CCH control run only in the second dimension.

**Analysis of the factors contributing to the CCH-A split**—To further investigate the nature of the CCH-A cleavage phenomenon, we evaluated the effect of aging at different temperatures in the absence and presence of a mixture of protease inhibitors and divalent ions. The SDS-PAGE analyses confirm that the autolysis occurs irrespectively of the presence of protease inhibitors and additional divalent ions. Also, we exposed CCH samples to different pH (6, 7.2 and 8) and different temperatures (4º and 37°C) over nearly one year. The results obtained by the above method, showed that these treatments did not affect the cleavage. Besides, the increases of temperature accelerated the fragmentation process (data not shown).

**Heterodecameric array of CCH subunits**

**Isolation of CCH subunits, and analysis of its subunit organization**—To examine the subunit array of CCH, we subjected the protein to anion-exchange chromatography on a MonoQ FPLC column with a linear NaCl gradient for sample elution. When whole CCH was loaded on the column in native conditions, we obtained one symmetrical peak (Figure 6A); in contrast, two peaks were observed for CCH loaded under dissociating conditions (Figure 6B). Both peaks obtained from dissociated CCH were analyzed by electron microscopy after negative staining; only monomers were observed as compared to control CCH (Figure 6D and 6C, respectively). SDS-PAGE analysis of the fractions indicated that peak A contained CCH-A1 and CCH-A2 whereas peak B contained CCH-B (Figure 6E, lanes 1 and 2, respectively). The fact that we were able to separate both subunits only under dissociating conditions, strongly suggested a heterogeneous didecameric organization for CCH.
Light scattering studies: analysis of the subunits assembly—To confirm the heterodecameric subunit array of CCH, we monitored the reassociation of isolated CCH subunits by light scattering. Preliminary experiments, carried out with the whole protein, showed that under dissociating conditions (i.e. with high pH and without bivalent cations), there is no association as measured by change in light scattering. Studies conducted with the isolated subunits showed that when adding separately CCH-A (Figure 7A) or CCH-B (Figure 7B) in the presence of Tris buffer at neutral pH containing divalent ions, a slight and transient increase in light scattering was observed, indicating that subunits do not auto-associate per se. In contrast, an evident change in the slope of light scattering was observed only when the complementary subunit was added (Figure 7A and 7B).

The resulting association kinetics corresponds to a hyperbolic curve, which did not show saturation throughout the experiment (2,400 sec). This saturation was only observed in an 18 hour reassociation reaction. The first segment of the curve shows a second order kinetics for the formation of the didecamer, displaying a plateau after 18 hours. Moreover, the curve did not fit with a first order kinetics for the disappearance of the monomer (data not shown). Using the association model proposed by van Holde et al. (11) for *O. dobleini* hemocyanin, we conclude that the CCH association kinetics fits with a dimerization process that is followed by a second rate-limiting dimerization leading to very fast formation of the didecamer. To reach the above conclusions, we assumed that the didecameric structure was the final product and the effect of decamers was negligible, the later assumption was supported by electron microscopy. The disappearance constant $k_2$ for the dimer was $2.65 \pm 0.49$ (min$^{-1}$ (mg/ml)$^{-1}$) at a protein concentration of 15 mg/ml. This value is similar to that reported for *O. dobleini* under similar pH conditions, but using 20 times less protein, and $k_2$ had shown to be independent from the protein concentration (11).

These results were further supported by observations under transmission electron microscopy (Figure 7E-7G). The aspect of isolated subunits CCH-A and CCH-B at the beginning of the experiments are shown in Figure 7E and 7F respectively, while figure 7G shows the characteristic appearance of
didecamers of CCH, while figure 7G shows the characteristic appearance of didecamers of CCH molecules as seen after 48 h of the end of kinetics.

To further determine the conditions needed for the reassociation of the subunits, we analyzed their reassembly when separately adding Ca$^{2+}$ and Mg$^{2+}$. It was observed that only the addition of Mg$^{2+}$ was sufficient for this to occur; in both cases, the addition of Mg$^{2+}$ produced a change in the slope of association, which was not observed when adding Ca$^{2+}$ (Figure 7C and 7D). It is worth noting that these experiments, confirm our preliminary results indicating that, in the absence of divalent ions, the association reaction did not occur. Both experiments had similar association curves, which never reached a plateau.
**DISCUSSION**

At present, there is a growing interest in hemocyanins; from the scientific viewpoint, this attention is focused on their structure, evolution and diversity (1), while from the biomedical viewpoint, it deals with the relationship among their structural features and immunotherapeutic effects. There is also interest to know whether hemocyanins other than KLH have comparable immunological properties (29).

In this paper, we present structural characteristics of hemocyanin from *C. Concholepas* and demonstrate that it has special features, namely: it does not require additional divalent cations to stabilize its structure, the reassociation of isolated subunits depends on Mg\(^{2+}\), the CCH-A subunit undergoes autocleavage, and subunits assemble to form a heterodecamer. Although this hemocyanin differs from that of KLH, it has been successfully used as a carrier protein (33-38).

The function of divalent metal ions in hemocyanin organization is essential because they play at least two roles: on the one hand, in terms of tertiary or quaternary structure, they bridge residues or domains of the protein. In fact, molluscan hemocyanins require substantial levels of Ca\(^{2+}\) or/and Mg\(^{2+}\) in the medium to stabilize their huge structures (1). On the other hand, these ions mediate ligand-protein interactions, i.e., they modulate the O\(_2\)-hemocyanin cooperativity and association equilibrium constant (50, 51). The fact that CCH does not require additional divalent ions in the medium, suggests that these ions are bound with a high affinity constant. One interesting question to ask is whether the split of the CCH-A subunit, which generates fragments CCH-A1 and CCH-A2, could be attributed to a loss of divalent ions in CCH during the purification and storage procedures. However, the results on CCH aging clearly show that this phenomenon is temperature-sensitive, and occurs irrespectively of the addition of divalent cations.

We favor the idea of an autocleavage site in the CCH-A subunit, rather than an enzymatic one, because fragmentation occurs even in the presence of a wide protease inhibitors mixture during purification and storage. Furthermore the putative protease should be very specific, since, under reducing conditions of
PAGE-SDS, we observed only one cleavage site in the CCH-A subunit while CCH-B subunit remained unaltered (Figure 3B). These results indicate that there are two CCH-A subunit populations: one intact and another bearing the nick that yields the CCH-A1 and CCH-A2 fragments in reducing conditions. Unfortunately, we do not have a sample of CCH at time 0 of the process, i.e., a state where CCH-A and CCH-B are exclusively seen in the presence of the reducing agent. The electrophoretic analyses of CCH samples recently extracted from the *C. concholepas* without purification showed a pattern similar to that in Figure 3B, lane 1. Therefore, the autocleavage form of the protein should normally be present in the circulation of *C. Concholepas*. The biological significance of the CCH cleavage is unclear. We speculated that the nick-site might generate a signal (molecular clock) to remove aged molecules from the hemolymph. Indeed, an exciting prospect would be that the nick in CCH could generate polypeptides exhibiting an anti-fungal and anti-microbial function, as described in shrimp (52). However, arthropod and molluskan hemocyanin are very different.

At any rate, the presence of an internal nick-site in the sequence has been suggested for polypeptides as big as molluskan hemocyanins (1). There are other examples of polypeptide autocleavage, for instance in Glypican-1 Heparan Sulfate, which also has copper in its structure (53). Another example is that of tubulin paracrystals, which cleave into fragments by spontaneous splitting, depending on the temperature (54).

The presence of contaminant does not explain our observations since CCH-A autocleavage was observed in different independent batches of the protein, and the pattern of the cleavage was fully preserved, because the CCH-A fragments had always the same size, and fragments below 100 KDa were not observed. Furthermore, autocleavage occurs with time, and it is difficult to propose a protease with an active half-life of one year or more, something that has not been described yet.

The splitting of the CCH-A subunit, might be similar to that described for RtH2 subunit from *R. thomasiana* (27). In fact, *Concholepas* and *Rapana* are closely related neogastropods (*Muricidae, Rapaninae*). In both cases, the nick in the respective subunits was not observed in native PAGE, when the proteins were run under dissociating conditions (high pH and absence of divalent cations) and in the
absence of reducing agent. Moreover, the intact subunit CCH-A exhibits a slow migration in its electrophoretic pattern. As soon as the nick occurs in the CCH-A subunit, a shift in its electrophoresis migration is observed, but the release of fragments CCH-A1 and CCH-A2 requires the reduction of disulfide bonds. In contrast, RtH storage at 4°C during several months led to a pattern of at least five polypeptides in native PAGE, and RtH2 was fragmented without a reducing agent (27). The slow migration of intact CCH-A subunit could be explained as an incomplete SDS denaturation of the subunit under non-reducing conditions. A nick should make the molecule more unstable but CCH-A1 and CCH-A2 fragments would remain linked by an inter-FU disulfide bridge - an unusual feature shared with *Rapana* hemocyanin (27) - in addition to the contribution of hydrophobic and non-covalent interactions among subunits. The generation of an inter-FU disulfide bridge is a complex and expensive evolutionary process that may counterbalance the fragmentation of the subunit and the acquisition of improved functional features. In support of the later speculation, it is observed that aged and fresh CCH molecules can not be differentiated by electron microscopy.

The presence of two different subunits in CCH raises the question of how they assemble into the functional molecules. We cannot rule out *a priori*, an organization of CCH subunits as homogeneous decamers, so that the protein would correspond to a homogeneous dimer. However, the fact that we could separate both subunits only under extreme pH conditions strongly suggests a heterogeneous didecameric organization, instead of a homogeneous dimer organization as occurs in *M. crenulata* (7, 17, 24), *H. tuberculata* (55) and *R. thomasiana* (56). When CCH was run on a MonoQ FPLC column, a single protein peak was observed, whereas when dissociated CCH was run in the presence of EDTA and high pH, two protein peaks appeared, corresponding to subunits CCH-A and CCH-B. Nonetheless, these data do not explain whether the didecamers are formed by homogeneous or heterogeneous decamers. Hence, subunits CCH-A or CCH-B should be either intermingled or assembled as homogeneous dimer of decamers, formed by two homogeneous decamers.
To solve this problem, we monitored the reassociation of CCH by light scattering, a method widely used to study hemocyanin association mechanisms. The reassembly of complete molecules requires at least three factors: the presence of isolated subunits, a neutral pH, and the presence of Mg$^{2+}$ ions (1). In the case of CCH, reassociation of the whole molecule starts from both isolated subunits. The association kinetics was very slow and did not exhibit a plateau at 2,400 sec. The equilibrium condition was only achieved after incubating overnight at room temperature. Interestingly, CCH reassembly lacked first order kinetics, suggesting that the molecule requires the interaction of both subunits prior to the formation the didecamer. This behavior would indicate a more complex reassociation mechanism than the one described for hemocyanin of *O. dofleini* (11). The results obtained in the experiments of reassembly of CCH subunits in the presence of Mg$^{2+}$, suggest that CCH has a heteromultimer structure, since both subunits are mutually required and the association kinetics are similar to that observed in the whole molecule. Moreover, the possibility that CCH-A is unable self associate due to the nick seems unlikely, because, CCH-A is needed in addition to CCH-B to reconstitute the whole molecule. Furthermore, the CCH-B subunit was unable to self associate (Figure 7). However, for a better understanding of the reassociation process of CCH subunits, studies using stop flow light scattering and extended light scattering experiments are necessary.

The only example to date of a hemocyanin with a heterodidecameric structure is that of the mollusk *M. fulvescens*, which has an A$_{10}$B$_{10}$ organization (28), but the available data do not give an insight on the organization of its subunit in the decamer. Moreover, a cleavage phenomenon in the B subunit of *Murex* has also been described, although the present information does not allow us to conclude whether this cleavage is similar to CCH-A or to RtH2 subunits. *A priori*, it is not possible to discard that this feature would be a common characteristic of *Muricidae* hemocyanins. Thus, from the phylogenetic point of view, it is possible that the isoform pairs CCH-A~CCH-B of *Concholepas*, RtH1~RtH2 of *Rapana*, and A~B from *Murex*, would be similar to some degree. $^3$
Further studies are required to better understand the molecular basis of the specific association pattern of subunits from the *Concholepas concholepas* hemocyanin, additional immunochemical studies are necessary using Mabs against subunits and FUs (40, 57) in addition to sequence analysis and crystallographic data.

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3 J. Markl, personal communication.
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FIGURE LEGENDS

**FIG. 1. Purification** *C. concholepas* hemocyanin. **A.** Chromatography of purified CCH in 150 mM PBS (pH 7.2) at 4°C on a Sepharose 4B column, 53 cm in height and 1 cm in diameter. The exclusion volume was determined using Dextran blue. **B.** Semi-log plot of the proteins used as standard to calibrate the column: KLH (8 MDa), thyroglobulin (670 kDa) and BSA (60 kDa). CCH is noted. **C.** Absorption spectra of purified CCH, maximum peaks at 268 nm, 344 nm and 550 nm (arrows).

**FIG. 2. Electron microscopy of purified** *C. concholepas* hemocyanin negatively stained. **A.** Low magnification micrographs of purified protein showing top (circles, of about 325 Å in diameter, arrowhead) and lateral (rectangles, about 392 Å in height, arrow) views of hemocyanin molecules. The bar corresponds to 100 nm. **B.** High magnification top view of the molecule showing a dense core (arrow 1) of about 153 Å in diameter, surrounded by a semi-opaque ring (arrow 2) of 13 Å and, around it, a clear annular zone 24 Å in width (arrow 3). The bar corresponds to 20 nm. **C.** Side view of hemocyanin molecule showing a characteristic didecameric form with subunits arranged in layers built up by six parallel rows (arrows). The bar corresponds to 50 nm. **D.** A view of CCH showing that the most frequent structure was a didecamer, although it is possible to observe decamer (arrow) or multi-decamer (arrow head) forms. The bar corresponds to 50 nm.

**FIG. 3. Electrophoretic studies of purified** *C. concholepas* hemocyanin. **A.** SDS-PAGE analysis under non-reducing conditions on a 3 to 7% polyacrylamide gradient gel, silver-stained. Lanes were loaded with 1-2 µg of each sample: lane 1, fresh CCH, shows two main polypeptides, named CCH-A and CCH-B; lane 2, aged CCH, asterisk shows a change in CCH-A subunit migration; lane 3, control KLH; two bands are observed corresponding to KLH-1 and KLH-2 subunits. **B.** SDS-PAGE analysis under reducing
conditions on a 3 to 12% gradient polyacrylamide gel, silver-stained. Lanes were loaded with 1 µg of each sample. Lane 1, fresh CCH showing two main polypeptides: CCH-A and CCH-B and fragment CCH-A1; lane 2, aged CCH, showing that CCH-A is absent and polypeptides CCH-A1 and CCH-A2 are observed while CCH-B does not change; lane 3, pre-stained markers: Myosin (203 kDa), β-Galactosidase (118 kDa) and BSA (82 kDa); lane 4, KLH: KLH-1 (390 kDa) and KLH-2 (350 kDa). C. Native gel electrophoresis under dissociating conditions (140 mM Tris, 90 mM boric acid and 2.5 mM EDTA at pH 8.6) on a 3 to 12% gradient polyacrylamide gel; Coomassie blue staining. Lanes were loaded with 9 µg of each sample previously dissociated during 24 h in the sample buffer (140 mM Tris, 90 mM boric acid and 2.5 mM EDTA at pH 8.6). Lane 1, fresh CCH sample showing two polypeptides; lane 2, aged CCH, showing the comigration of polypeptides; lane 3, KLH used as control, showing the two subunits.

FIG. 4. Identification of *C. concholepas* hemocyanin subunits in native gels by specific monoclonal antibodies. Native gel electrophoresis under dissociating conditions (sample and running buffer contain 140 mM Tris, 90 mM boric acid and 2.5 mM EDTA at pH 8.6) on a 3 to 12% gradient polyacrylamide gel. Lanes were loaded with 5 µg of each sample previously dissociated in the sample buffer for 1 h. A. Coomassie blue staining, lane 1, fresh CCH sample showing two polypeptides; lane 2, aged CCH, showing the comigration of polypeptides; B, and C, correspond to the same gel as in (A) but transferred to nitrocellulose, which was incubated with 4E9 anti-CCH-A Mabs or 1A4 anti-CCH-B Mabs, respectively, followed by a goat anti-mouse IgG-ALP conjugate, and visualized with the BCIP plus NBT system. In fresh CCH the upper band corresponds to CCH-A and the lower band corresponds to CCH-B; and in aged protein, CCH-A change their electrophoretic pattern and comigrated through CCH-B, that remain unaltered as visualized by Coomassie blue (A).

FIG. 5. Bidimensional SDS-PAGE analysis of fresh *C. concholepas* hemocyanin. The first dimension was carried out in the absence of reducing agents (3% SDS-PAGE), while the second
dimension (3 to 20% gradient SDS-PAGE) was run under reducing conditions (sample load: 2µg). The polypeptides that are not disulfide-bound group are close to the diagonal axis (CCH-A and CCH-B), whereas polypeptides that migrate in the same vertical axis are bound by disulfide bridges, as CCH-A and its fragments CCH-A1 and CCH-A2. A fresh CCH sample was run under reducing conditions as a standard, to allow visualization of all CCH peptides by silver-stain.

Fig. 6. Analyses of the subunit structure of C. concholepas hemocyanin. A. Whole CCH submitted to anion-exchange chromatography on a MonoQ FPLC column, eluted with a linear NaCl gradient in PBS (pH 7.2), showed one symmetrical peak. B. CCH dissociated in Glycine-NaOH buffer (pH 9.6) plus 10 mM EDTA submitted to anion-exchange on a MonoQ HPLC column, eluted with a linear NaCl gradient showed two different peaks named A and B. C. Electron microscopy analysis of whole CCH obtained in (A) from MonoQ FPLC column using negative staining, shows the characteristic didecameric structure of CCH. The bar corresponds to 50 nm. D. Electron microscopy analysis of dissociated CCH from (B), negatively stained, shows a disorganized globular structure. The bar corresponds to 50 nm. E. SDS-PAGE analysis under reducing conditions of the fractions obtained in the MonoQ column. From left to right: lane 1 contained peak A corresponding to CCH-A1 and CCH-A2 bands, lane 2 contained fraction B corresponding to CCH-B, finally, lane 3 represents whole CCH used as control; lane 4: molecular weight standards: Myosin (203 kDa), β-Galactosidase (118 kDa), BSA (82 kDa), Ovalbumin (50.4), Carbonic anhydrase (33.4), Soybean trypsin inhibitor (26.7).

Fig. 7. Analysis of the reassociation of C. concholepas hemocyanin subunits by light scattering and electron microscopy with negative staining. A. Association kinetics of the subunits first adding 25 µl CCH-A (of a solution of 18 mg/ml), then 35 µl CCH-B (of a solution of 13 mg/ml). The initial medium contained 100 mM Tris/HCl pH 7.6, 50 mM MgCl₂ 10 mM CaCl₂. B. Similar to the former experiment but, in this case, subunit CCH-B was first added followed by addition of CCH-A subunit. C. Association
kinetics of the subunits in an initial medium containing 100 mM Tris/HCl pH 7.6, to which the following compounds were added: CCH-A and CCH-B, in the same volume as (A) and the divalent cations, 4 µl 2M CaCl$_2$ and 11.1 µl 1 M MgCl$_2$. D. Similar to the former experiment but where the order of the ions was changed. In this case Mg$^{2+}$ was first added, and then Ca$^{2+}$. E, and F, represents samples of the subunits CCH-A and CCH-B, respectively, isolated and viewed under the electron microscope at the beginning of the experiments. G. Appearance of the preparation of (D) 2 days after the end of kinetics.
### Table I

*Amino Acid Composition in Mole % of CCH-A and CCH-B*

| Amino Acid | CCH-A | CCH-B |
|------------|-------|-------|
| Ala        | 7.8   | 8.1   |
| Arg        | 5.0   | 5.9   |
| Asn        | 7.3   | 5.7   |
| (Cys)²     | ND⁴   | ND⁴   |
| Glu        | 11.1  | 10.2  |
| Gly        | 3.1   | 3.3   |
| His        | 4.5   | 4.6   |
| Ile        | 4.5   | 4.7   |
| Leu        | 13.2  | 14    |
| Lys        | 1.0   | 1.5   |
| Met        | 2.6   | 2.3   |
| Phe        | 11.1  | 10.5  |
| Pro        | 9.9   | 9.4   |
| Ser        | 1.6   | 1.5   |
| Thr        | 4.3   | 4.7   |
| Trp        | ND⁴   | ND⁴   |
| Tyr        | 5.9   | 5.9   |
| Val        | 6.9   | 7.7   |

¹Not determined
Figure 1

(A) Time (min)

(B) MW (KDa)

(C) Absorbance

Structure of the *C. concholepas* hemocyanin
Figure 2
Figure 3

A
Non-reducing SDS-PAGE

1 2 3

CCH-A ▶
CCH-B ▶
KLH-1 ▶
KLH-2 ▶

B
Reducing SDS-PAGE

1 2 3 4

CCH-A ▶
CCH-B ▶
CCH-A1 ▶
KLH-1 ▶
KLH-2 ▶

C
Native PAGE

1 2 3

CCH-A ▶
CCH-B ▶
Figure 4

Coomassie stain  

\[ CCH-A \]  
\[ CCH-B \]  

1 2 1 2 1 2

Anti CCH-A Mabs  

Anti CCH-B Mabs
Figure 5
Figure 6

A

B

C

D

E

Structure of the *C. concholepas* hemocyanin
Figure 7

[A] Intensity vs. Time (seconds) for CCH-A and CCH-B.

[B] Intensity vs. Time (seconds) for CCH-A and CCH-B.

[C] Intensity vs. Time (seconds) for CCH-A, CCH-B, Mg²⁺, and Ca²⁺.

[D] Intensity vs. Time (seconds) for CCH-A, CCH-B, Mg²⁺, and Ca²⁺.

[E] Micrographs of CCH-A and CCH-B.

[F] Micrograph of CCH resulting from the addition of Mg²⁺ after 48 hours.

Structure of the *C. concholepas* hemocyanin
Hemocyanin of the molluskan concholepas concholepas exhibits an unusual heterodecameric array of subunits
Pablo De Ioannes, Bruno Moltedo, Harold Oliva, Rodrigo Pacheco, Fernando Faunes, Alfredo E. De Ioannes and María Inés Becker

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VOLUME 282 (2007) PAGES 20475–20483

The ubiquitin ligase SCF(βTrCP) regulates the degradation of the growth hormone receptor.

Peter van Kerkhof, Joyce Putters, and Ger J. Strous

The following corrections were noted by the authors. On Page 20475, right column, bottom line, interferon-α/β should be interferon-α/β. On Page 20482, left column, line 8, DpSGFXpS should be DpSGXXpS; left column, line 20, DSGFXS should be DSGXXS; left column, line 37, DSGFXS should be DSGXXS; right column, lines 14 and 15, interferon receptors should be interferon type I receptors; right column, line 16, DSGFXS should be DSGNYS; and right column, line 18, interferon-γ should be interferon-α/β, and DSGFXS should be DSGXXS.

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CREB modulates the functional output of nucleus accumbens neurons: a critical role of N-methyl-D-aspartate glutamate receptors.

Yanhua H. Huang, Ying Lin, Travis E. Brown, Ming-Hu Han, Daniel B. Saal, Rachael L. Neve, R. Suzanne Zukin, Barbara A. Sorg, Eric J. Nestler, Robert C. Malenka, and Yan Dong

In the last paragraph of the second column on Page 2758, we incorrectly referenced a previous work in the following sentence: “These results are consistent with a previous study in hippocampal neurons in which synaptic NMDARs were particularly sensitive to caCREB but not dnCREB (12).” In this previous work (Ref. 12), dnCREB was not directly studied.

VOLUME 279 (2004) PAGES 26134 –26142

Hemocyanin of the molluscan Concholepas concholepas exhibits an unusual heterodecameric array of subunits.

Pablo De Ioannes, Bruno Moltedo, Harold Oliva, Rodrigo Pacheco, Fernando Faunes, Alfredo E. De Ioannes, and Maria Inés Becker

On Page 26138 (right column, line 29 from the top), the sentence should read as follows: “SDS-PAGE analysis of the fractions indicated that peak B contained CCH-A1 and CCH-A2, whereas peak A contained CCH-B (Fig. 6E, lanes 1, and 2, respectively.” On Page 26139 (legend to Fig. 6, line 7), “peak A” should be “peak B,” and “fraction B” should be “fraction A.”

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This article has been withdrawn at the authors’ request.