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

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We describe here the structure of the hemocyanin from the Chilean gastropod *Concholepas concholepas* (CCH), emphasizing some attributes that make it interesting among molluscan hemocyanins. CCH exhibits a predominant didecameric structure as revealed by electron microscopy and a size of 8 MDa by gel filtration, and, in contrast with other mollusc 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 noncovalently linked, named CCH-A and CCH-B, with molecular masses of 405 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 yield two polypeptides, CCH-A1 (300 kDa) and CCH-A2 (108 kDa), whereas 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.

Hemocyanins, the oxygen carrier glycoproteins found in the hemolymph of many species of arthropods and molluscs, 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).

Molluscan hemocyanins assemble into hollow cylindrical layered molecules formed by 10 subunits. Each subunit, ranging from 350 to 450 kDa, includes seven or eight globular folded domains known as functional units (FUs),[1] which are arranged into pearl-like chains and covalently bound by a short flexible linker region of 10–15 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 3.5–4.5 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 to 9 MDa (3–10).

Profuse experimental studies, using different dissociation and association conditions of the native mollusc 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 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 mollusc 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 homodecameric 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 mollusc keyhole limpet (*M. crenulata*) known as KLH, have led to a growing interest in hemocyanin structure and function. In recent years, we have focused our research on the hemocyanin obtained from the mollusc *Concholepas concholepas* (CCH).

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[1] The abbreviations used are: FU, functional unit; KLH, keyhole limpet hemocyanin; CCH, *C. concholepas* hemocyanin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FPLC, fast pressure liquid chromatography; mAb, monoclonal antibody; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; IEF, isoelectric focusing; RtH, *R. thomasiana* hemocyanin.

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 phenomenon. 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—$AgNO_3$, ammonium sulfate, formaldehyde, *para*-nitrophenyl phosphate, and uranyl acetate were from Merck. The Seitz unit was from Kingston. The 0.2-μm filter unit was from Corning. Formvar and Parlodion were from Pelco International. Ampholytes (Bio-Lyte), the Mini IEF Cell Model 111 system, and prestained markers were from Bio-Rad. Agarose type VII, Amido Black, aprotinin, bovine serum albumin (BSA), EDTA, iodoacetamide, leupeptin, pancreatic elastase, phenylmethylsulfonyl fluoride, pepstatin, and thyroglobulin were from Sigma. The Mono-Q 5/5 column, Sepharose 4B, and blue dextran 2000 were from Amersham Biosciences. The 0.22-μm filter was from Millipore Corp. Anti-mouse IgG-horseradish peroxidase conjugate or alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), bicinchoninic acid protein kits, Casein SuperBlock, Coomassie Blue, CL-XPosure film (Blue X-Ray film), nitro blue tetrazolium (NBT)-BCIP systems, NBT, nitrocellulose membrane, polystyrene plates, prestained markers, SDS-PAGE reagents, Super Signal West Pico chemiluminescent substrate kit, Tween 20, and an Unblot in-gel chemiluminescent detection kit were from Pierce. All chemicals were analytical grade reagents, and the solutions were prepared with Milli-Q water.

Hemocyanin Sources—Live specimens of *C. concholepas* (Loco) were harvested from the Pacific Ocean, Bay of Quintay (33° 8′ south 71° 48′ west) V Region of Chile. Keyhole limpet hemocyanin was purchased from Pierce, and *C. concholepas* hemocyanin was from BIOSONDA (Blue Carrier).

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 mollusc 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 phosphate-buffered saline (PBS), 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, prefiltered in a Seitz unit, and sterilized through a 0.22-μm filter unit, and then stored at 4 °C. Protein concentration was determined using Coomassie Blue kit according to the manufacturer's instructions. This hemocyanin preparation was shown to be highly pure by gel chromatography and 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 phenylmethylsulfonyl fluoride, and 5 mM EDTA with or without 5 mM $CaCl_2$ and 5 mM $MgCl_2$.

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 cm^3). 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 BSA. To determine the exclusion volume of the column, blue dextran 2000 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) 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 HNO_3, evaporated, and dissolved in diluted HCl prior to analysis. Fixanal Ridel-Haën copper reference standard of 1.0 g of copper/liter 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 (0.5–1 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 1–5 min with 20 μl of 1–2% 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—The technique was performed as described by Laemmli (44) in a 3–12% gradient separating and 3% stacking gel. Protein samples were heated for 5 min at 100 °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 prestained markers. The gels were stained with Coomassie Blue or with $AgNO_3$ (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 4 °C (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 included in the kit. Then gels were washed in PBS-Tween solution and incubated for 1 h at room temperature with goat anti-mouse IgG-horseradish peroxidase 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 for 1 h at room temperature with goat anti-mouse IgG-alkaline phosphatase conjugate-diluted 1:12,000. The membranes were developed using NBT and BCIP. To stop the reaction, the membranes were washed with water.

Two-dimensional 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 bromphenol blue). The second dimension was run on a 3–20% 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 temper-

[2] B. Moltedo, D. Leiva, A. E. De Ioannes, and M. I. Becker, manuscript in preparation.

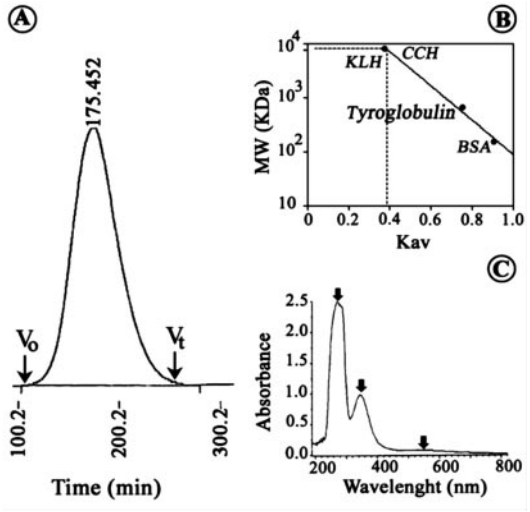

FIG. 1. **Purification of *C. concholepas* hemocyanin.** *A*, chromatography of purified CCH in 150 mM PBS (pH 7.2) at 4 °C on a Sepharose 4B column, 53 cm in height and 1 cm in diameter. The exclusion volume was determined using blue dextran. *B*, semilog plot of the proteins used as a standard to calibrate 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, 344, and 550 nm (*arrows*).

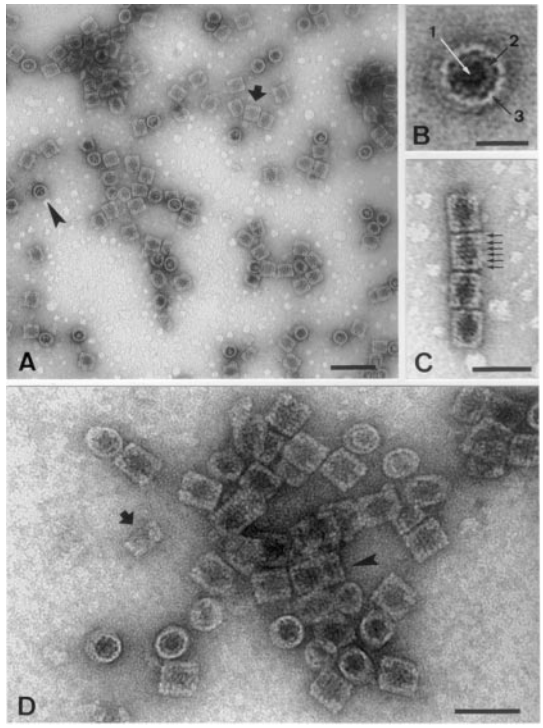

FIG. 2. **Electron microscopy of purified *C. concholepas* hemocyanin negatively stained.** *A*, low magnification micrographs of purified protein showing the top (*circles* of about 325 Å in diameter, *arrowhead*) and lateral (*rectangles* about 392 Å in height, *arrow*) views of hemocyanin molecules. *Bar*, 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 semiopaque ring (*arrow 2*) of 13 Å and, around it, a clear annular zone 24 Å in width (*arrow 3*). *Bar*, 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*). *Bar*, 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 multidecamer (*arrowhead*) forms. *Bar*, 50 nm.

ature, in both the first and second dimension. Two-dimensional gels were silver-stained for protein visualization.

Isoelectric Focusing (IEF)—A Mini IEF Cell Model 111 (Bio-Rad) system was used. The preparation of the IEF gel was done according to the manufacturer's 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 had 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 0–70% buffer A containing 1 M NaCl for 30 min at 1 ml/min, and the final wash 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 PerkinElmer Life Sciences LS50 model spectrofluorometer. Scattered light was measured at 90° to the incident beam, with both entrance and exit monochromators set to 384 nm. The reassociation experiments were done at 20 °C, and buffer containing 100 mM Tris/HCl, pH 7.6, 50 mM $MgCl_2$ 10 mM $CaCl_2$ 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). Samples were hydrolyzed for 22 h at 110 °C in 6 N HCl containing 1% phenol. Waters PICO TAG equipment was employed, using Waters Millennium software, a pump model 510, and model 490 detectors. A Waters Novapak C18, 30-cm column was used. Mol % 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 polyvinylidene difluoride 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 (K_{av} = 3.8), indicating that the protein was homogeneous in size (Fig. 1*A*). The molecular mass was about 8 MDa by extrapolation in a semilog plot of molecular mass *versus* K_{av} (Fig. 1*B*), a value similar to that of other well documented gastropod hemocyanins (1). The absorption spectrum of CCH was similar to other hemocyanins and showed three peaks, at 268, 344, and 550 nm, that corresponded to aromatic residues, Cu^{2+}-O^{2+}, and Cu^{2+}-histidine coordination centers, respectively (Fig. 1*C*).

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 for at least 3 years, maintained intact their quaternary structure, as verified by electron microscopy (data not shown).

Structure—Fig. 2*A* shows an electron microscope preparation of purified CCH molecules negatively stained with uranyl. Circles of about 325 Å in diameter and rectangular specimens of about 392 Å in height were observed. At high magnification, the top views of the molecules (Fig. 2*B*), showed a dense core of about 153 Å in diameter (*arrow 1*), surrounded by a semiopaque 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*). Fig. 2*C* shows a side view of the molecule, displaying typical subunit structures arranged in layers of six parallel rows as indicated by the *arrows*. Didecamers were the most frequent forms observed, whereas decameric or multidecameric structures were scarce (Fig. 2*D*).

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 other mollusc hemocyanins (1).

Polypeptide Chains—The nonreducing SDS-PAGE and native gels analysis of the CCH indicated that the protein is composed of two different subunits (Fig. 3, *A* and *C*, 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 (LMRKDVDTLTD) 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 of five residues; CCH-A has an aspartic residue in the fourth position within the motif, whereas 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 nonreducing SDS-PAGE conditions, the protein displayed two main noncovalently bound polypeptides, CCH-A and CCH-B. Interestingly, aged CCH preparations, stored for a year at 4 °C, showed a change in their electrophoretic pattern, as compared with fresh protein (Fig. 3*A*, *lanes 1* and *2*). A shift in CCH-A subunit migration occurred, and a new polypeptide appeared over the CCH-B band (Fig. 3*A*, *lane 2*, *asterisk*); however, the migration of CCH-B subunits remained unaltered (Fig. 3*A*, *lane 2*). Under reducing conditions, fresh hemocyanin was also dissociated into two subunits, CCH-A with a molecular mass of 405 kDa and CCH-B with a molecular mass of 350 kDa. However, in addition to the main polypeptides, a faint band was observed below the CCH-B band (Fig. 3*B*, *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-A2), whereas CCH-B was stable (Fig. 3*B*, *lane 2*). After a year at 4 °C, CCH-A was fully converted into CCH-A1 and CCH-A2 (Fig. 3*B*, *lane 2*); however, the appearance of CCH as determined by electron microscopy remained unaltered. We concluded that in the fresh CCH sample, the polypeptide running just under CCH-B corresponds to CCH-A1, whereas 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 (Fig. 3*C*). The results show that the hemocyanin was also dissociated into two polypeptides, confirming that CCH-A and CCH-B subunits are held together by noncovalent interactions (Fig. 3*C*, *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 (Fig. 3*C*, *lane 2*). To determine whether this interpretation was correct, we used mAbs targeted to specific 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 (Fig. 4*C*), and overlaps with the Coomassie staining pattern in fresh or aged CCH (Fig. 4*A*, *lanes 1* and *2*, respectively). In contrast, when we applied anti-CCH-A 2D8 or 4E9 mAbs under the same conditions, the

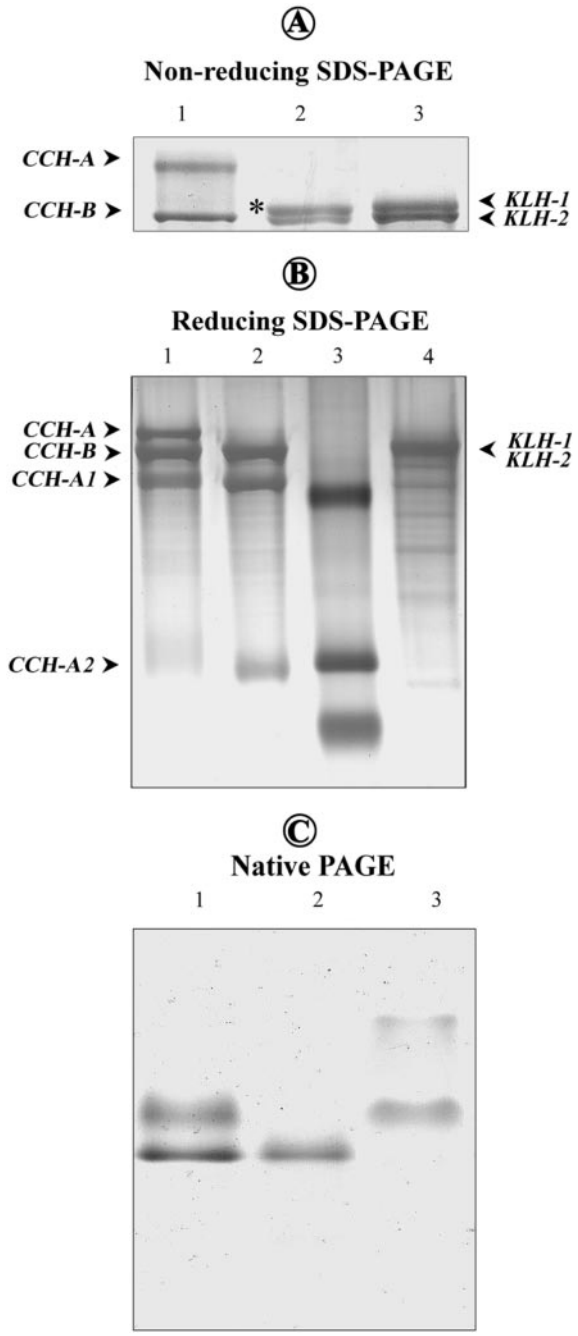

FIG. 3. **Electrophoretic studies of purified *C. concholepas* hemocyanin.** *A*, SDS-PAGE analysis under nonreducing conditions on a 3–7% polyacrylamide gradient gel, silver-stained. *Lanes* were loaded with 1–2 μg of each sample. *Lane 1*, fresh CCH, shows two main polypeptides, named CCH-A and CCH-B; *lane 2*, aged CCH (the *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 3–12% 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, whereas CCH-B does not change; *lane 3*, prestained 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 3–12% 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.

TABLE I
Amino acid composition in mol % 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	ND[a]	ND
Glu	11.1	10.2
Gly	3.1	3.3
His	4.5	4.6
Ile	4.5	4.7
Leu	13.2	14.0
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	ND	ND
Tyr	5.9	5.9
Val	6.9	7.7

[a] ND, not determined.

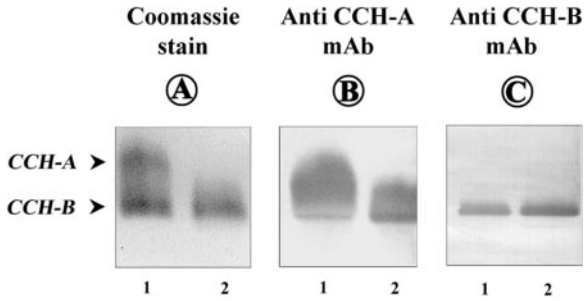

FIG. 4. **Identification of *C. concholepas* hemocyanin subunits in native gels by specific monoclonal antibodies.** Native gel electrophoresis under dissociating conditions (sample and running buffer contain 140 mM Tris, 90 mM boric acid, and 2.5 mM EDTA at pH 8.6) on a 3–12% gradient polyacrylamide 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-alkaline phosphatase 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 subunits changed their electrophoretic pattern and comigrated through CCH-B, which remained unaltered as visualized by Coomassie Blue (*A*).

migration of the immunoreactive band changed in aged preparations (Fig. 4*B*, *lane 2*) as observed with Coomassie staining (Fig. 4*A*, *lane 2*), indicating that aged CCH-A peptides do in fact change motility when migrated jointly with CCH-B.

Analysis of the CCH-A Fragments—To determine the origin of fragments, CCH was analyzed by a bidimensional SDS-PAGE (Fig. 5). When running the protein in the first dimension, with SDS but without reducing agent, both subunits were separated, confirming the results observed in Fig. 3*A*. The use of a reducing agent in the second dimension showed that a fraction of CCH-A remains intact, whereas the fragments CCH-A1 and CCH-A2, originating 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 autocleavage occurs irrespectively of the presence of protease

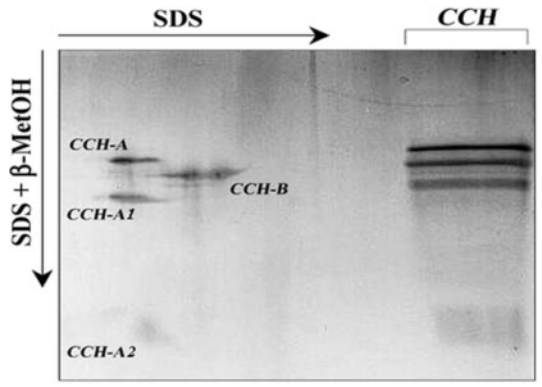

FIG. 5. **Bidimensional SDS-PAGE analysis of fresh *C. concholepas* hemocyanin.** The first dimension was carried out in the absence of reducing agents (3% SDS-PAGE), whereas the second dimension (3–20% gradient SDS-PAGE) was run under reducing conditions (sample load 2 μg). The polypeptides that are not disulfide-bound migrate close to the *diagonal axis* (CCH-A and CCH-B), whereas polypeptides that migrate in the same *vertical axis* are bound by disulfide bridges, such 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.

inhibitors and additional divalent ions. Also, we exposed CCH samples to different pH (6, 7.2, and 8) and different temperatures (4 and 37 °C) over nearly 1 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 (Fig. 6*A*); in contrast, two peaks were observed for CCH loaded under dissociating conditions (Fig. 6*B*). Both peaks obtained from dissociated CCH were analyzed by electron microscopy after negative staining; only monomers were observed as compared with control CCH (Fig. 6, *D* and *C*, respectively). SDS-PAGE analysis of the fractions indicated that peak A contained CCH-A1 and CCH-A2, whereas peak B contained CCH-B (Fig. 6*E*, *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 Subunit 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 (Fig. 7*A*) or CCH-B (Fig. 7*B*) 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 autoassociate *per se*. In contrast, an evident change in the slope of light scattering was observed only when the complementary subunit was added (Fig. 7, *A* and *B*).

The resulting association kinetics corresponds to a hyperbolic curve, which did not show saturation throughout the experiment (2,400 s). This saturation was only observed in an 18-h reassociation reaction. The first segment of the curve

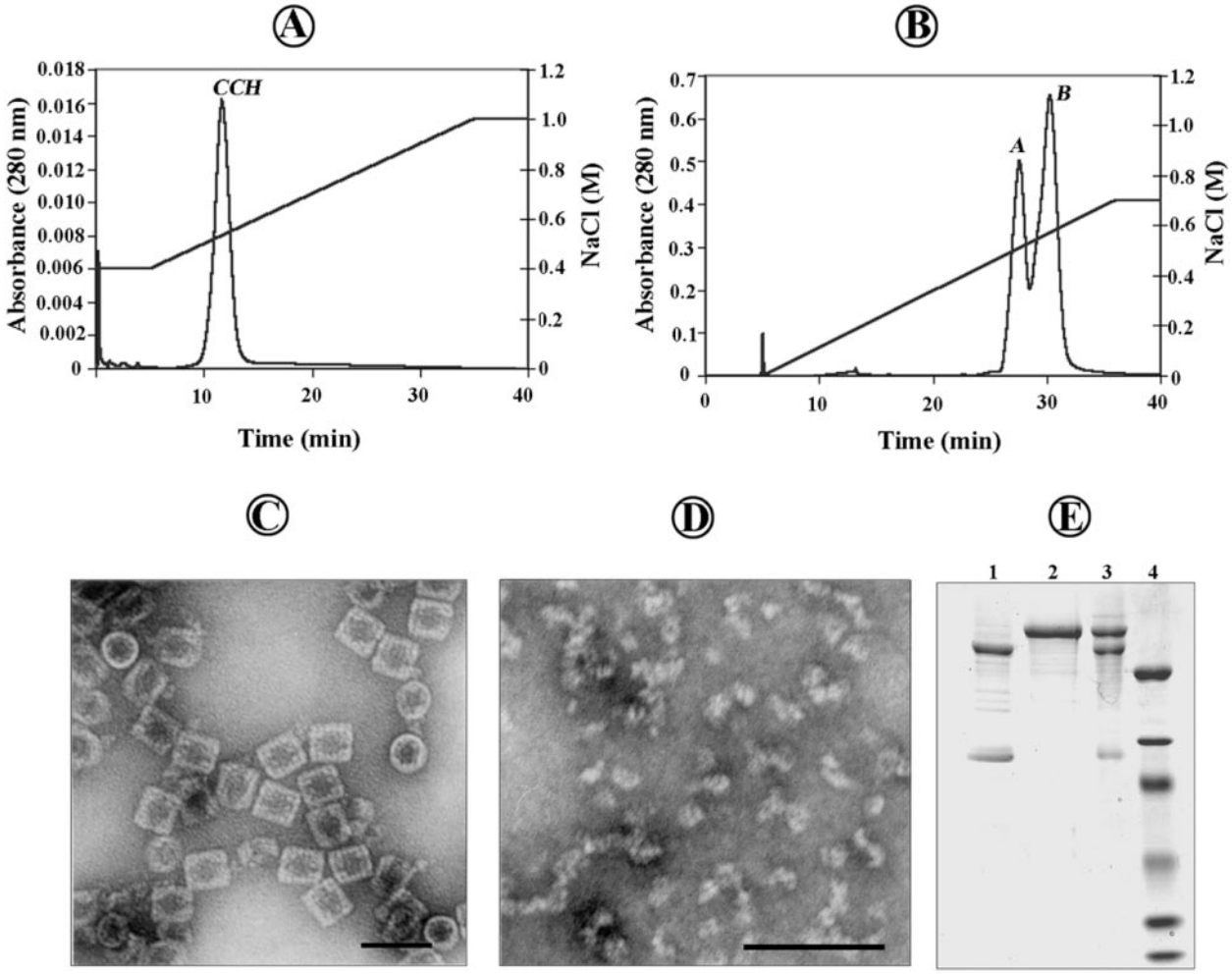

FIG. 6. **Analyses of the subunit structure of *C. concholepas* hemocyanin.** *A*, whole CCH submitted to anion exchange chromatography on a MonoQ FPLC column, eluted with a linear NaCl gradient in PBS (pH 7.2), showed one symmetrical peak. *B*, CCH dissociated in glycine-NaOH buffer (pH 9.6) plus 10 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 a 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. *Bar*, 50 nm. *E*, SDS-PAGE analysis under reducing conditions of the fractions obtained in the MonoQ column. *Lane 1*, contained peak A corresponding to CCH-A1 and CCH-A2 bands; *lane 2*, fraction B corresponding to CCH-B; *lane 3*, whole CCH used as control; *lane 4*, molecular mass standards: myosin (203 kDa), β-galactosidase (118 kDa), BSA (82 kDa), ovalbumin (50.4 kDa), carbonic anhidrase (33.4 kDa), and soybean trypsin inhibitor (26.7 kDa).

shows a second order kinetics for the formation of the didecamer, displaying a plateau after 18 h. 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 *Octopus dofleini* 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 ± 0.49 (min^{-1} $(mg/ml)^{-1}$) at a protein concentration of 15 mg/ml. This value is similar to that reported for *O. dofleini* under similar pH conditions, but using 20 times less protein, and has been shown that k_2 is independent from the protein concentration (11).

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

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+} (Fig. 7, *C* and *D*). 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), whereas from the biomedical viewpoint, it concerns the relationship among their structural features and immunotherapeutic effects. There is also interest in knowing whether hemocyanins other than KLH have comparable immunological properties (29).

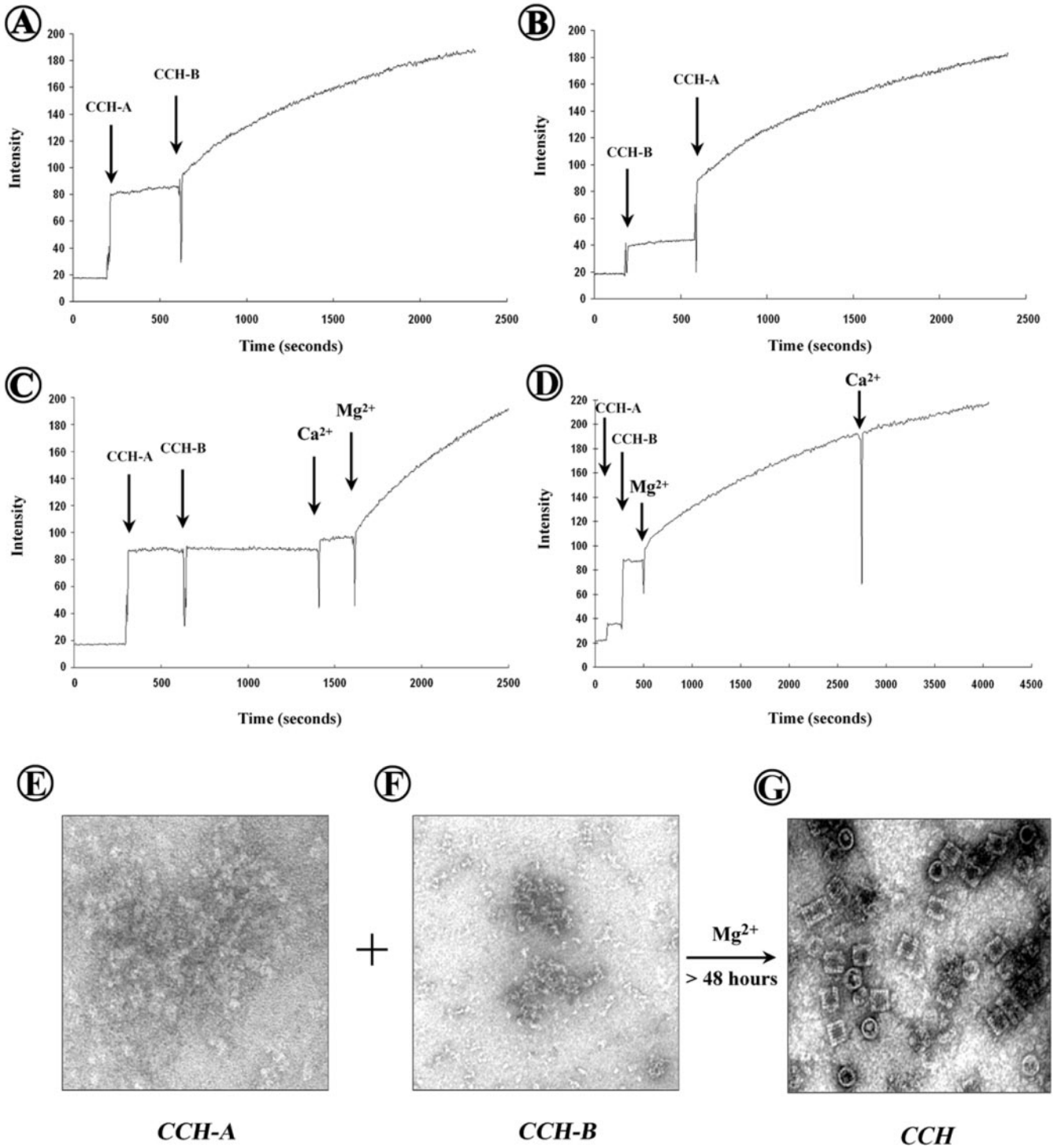

FIG. 7. **Analysis of the reassociation of *C. concholepas* hemocyanin subunits by light scattering and electron microscopy with negative staining.** *A*, association kinetics of the subunits first adding 25 μl of CCH-A (of a solution of 18 mg/ml) and then 35 μl of CCH-B (of a solution of 13 mg/ml). The initial medium contained 100 mM Tris/HCl, pH 7.6, 50 mM $MgCl_2$, 10 mM $CaCl_2$. *B*, similar to the former experiment, but, in this case, subunit CCH-B was first added followed by the addition of the 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 in *A* and the divalent cations, 4 μl of 2 M $CaCl_2$ and 11.1 μl of 1 M $MgCl_2$. *D*, similar to the former experiment except that the order of the ions was changed. In this case, Mg^{2+} was first added and then Ca^{2+}. *E* and *F*, 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.

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 one hand, in terms of tertiary or quaternary structure, they bridge residues or domains of the protein. In fact, molluscan hemo-

cyanins 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 inhibitor mixture during purification and storage. Furthermore, the putative protease should be very specific, since, under reducing conditions of SDS-PAGE, we observed only one cleavage site in the CCH-A subunit, whereas CCH-B subunit remained unaltered (Fig. 3*B*). 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 Fig. 3*B*, *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 antifungal and antimicrobial function, as described in shrimp (52). However, arthropod and molluscan 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 molluscan 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 always had 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 1 year or more, something that has not yet been described.

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* and *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 nonreducing 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 noncovalent 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 cannot 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. thomasian*a (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, subunit CCH-A or CCH-B should be either intermingled or assembled as a 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 s. 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 (Fig. 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 mollusc *M. fulvescens*, which has an $A_{10}B_{10}$ organization (28), but the available data do not give insight into the organization of its subunit in the decamer. Moreover, a cleavage phenomenon in the B subunit of M*urex* has also been described, although the present informa-

tion 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 the possibility 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 *C. concholepas* hemocyanin; additional immunochemical studies are necessary using mAbs against subunits and FUs (22, 40) in addition to sequence analysis and crystallographic data.

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