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The Hemocyanin of the Shamefaced Crab *Calappa granulata*: Structural-Functional Characterization

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Arthropod hemocyanins (Hcs) transport and store oxygen and are composed of six subunits, or multiples thereof depending on the species. *Calappa granulata* Hc is found as a mixture of dodecamers (95%) and hexamers (5%). Removal of calcium ions and alkaline pH induce an incomplete partially reversible dissociation of dodecameric Hc. Two-dimensional electrophoretic pattern of dissociated Hc indicated a large heterogeneity in Hc subunit: most differences are likely to be explained by post-translational modifications. Dodecameric Hc showed a large Bohr effect ($\Delta\log P_{50}/\Delta pH = -0.95$) and a normal cooperativity (h_{50} values = 2.7 ± 0.2) in the presence of 10 mM $CaCl_2$. The hexameric molecule displayed lower Bohr effect and cooperativity than the dodecamer. Lactate effect on the oxygen affinity ($\Delta\log P_{50} = 0.55$) and the increase of lactate concentrations in animals kept in emersion were related to the increased oxygen requirements that occur during hypoxia *in vivo*. Calcium affects oxygen affinity only at high concentrations: this Hc appeared to lack the calcium high-affinity binding sites found in other species. The effect of temperature on both oxygen affinity and cooperativity was measured in the absence and presence of 10 mM lactate, allowing calculation of the exothermic contribution of lactate binding ($\Delta H = -25$ kJ mol^{-1}).

Key words: calcium, hemocyanin, lactate, oxygen affinity, subunit heterogeneity.

Abbreviations: h_{50}, slope of the Hill plot at 50% saturation; Hc, hemocyanin; P_{50}, partial pressure of oxygen required to saturate 50% of the binding sites.

Hemocyanins (Hcs) are extracellular respiratory copper-containing proteins, which occur freely dissolved in the hemolymph, playing the role of oxygen transport and storage in some species of molluscs and arthropods (*1*). Although their functions are similar, the proteins differ in the tertiary and quaternary structure. Native arthropod Hcs are multimeric proteins in which each subunit has a molecular mass of ~75 kDa (5S) and contains one binuclear copper-containing binding site, which can reversibly bind molecular oxygen as peroxide in a μ–η^2:η^2 co-ordination mode between the two Cu(II) ions (*2*).

Different subunits are arranged as hexamers (16S corresponding to 1 × 6-mer or building block) or multiples of hexamers (24S, 36S, 48S and 62S corresponding to 2 × 6-mers, 4 × 6-mers, 6 × 6-mers and 8 × 6-mers, respectively). The native aggregation level appears to be characteristic for each species and may also be related to taxonomic groups (*2*, *3*).

Although most arthropod Hcs exhibit only a major aggregation state *in vivo*, it is usually possible to obtain dissociation into the lower levels of association by proper choice of non-physiological solution conditions, *i.e.*, increase of pH and removal of divalent cations by dialysis against EDTA. Previously, only *Penaeus monodon* Hc has been found to be stable against dissociation at high pH, independently of the presence of calcium ions (*4*). Dissociation of native Hc into lower aggregation states is often reversible, though to different extents depending on the species (*5*).

Subunits are usually heterogeneous and the number of monomers separated from the Hc of a particular species can depend on the technique employed: in decapod Hcs, three to ten different subunit types could be electrophoretically separated on high-resolution polyacrylamide gels, while three to six differently migrating polypeptide chains have been found in SDS-PAGE experiments (*6*). In the past, subunit heterogeneity has been described as an important manner of oxygen affinity modulation, which may become operative in response to changes in environmental conditions (*7*). Moreover, it has been reported that, in some crustacean Hcs, the subunit composition varies with sex, season, oxygen level, or salinity, which results in modulation of functional properties (*8*, *9*). Since within-species variation has been observed in so many species, the hypothesis that within-species variation, considered to be the rule in Brachyuran Hcs (*10*), now seems to apply to Crustaceans in general (*6*). It has also been reported that,

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in the formation of the dodecamer or larger structures, some additional subunits may be present (*11*, *12*) or are needed to connect the hexameric units (*13*).

Previous studies of functional properties of crustacean Hcs have shown that oxygen binding is highly cooperative and may be modulated by various effectors, specifically, pH, divalent cations such as Mg^{2+} and Ca^{2+}, metabolites such as urate and L-lactate, dopamine and related compounds; in this way Hcs are able to maintain efficient uptake and delivery of oxygen (*1*, *2*). The effect of pH on oxygen affinity widely differs among crustacean Hcs and seems to be independent of their phylogenetic relationship. As far as the effect of divalent cations on oxygen affinity is concerned, magnesium and, to a higher extent, calcium generally affect the oxygen binding as well as the stability of the associated molecules of many arthropodan and molluscan Hcs (*14*, *15*).

As regards the effect of organic acids, L-lactate, which is the anaerobic end product of glycolysis also in crustaceans (*16*), and urate, a byproduct of purine catabolism, have been reported to increase the Hc oxygen-affinity of a number of decapod crustaceans (*17–20*). The positive modulation of oxygen affinity brought about by these organic acids has been related to the increased oxygen requirements that occur *in vivo* during heavy muscular exercise (*21*) and hypoxia (*22–25*).

Calappa granulata is a crab with a granular white-rose carapace with purple regular spots (therefore it is also called "pomegranate crab") that lives in the Mediterranean Sea and Atlantic Ocean. Here we report the characterization of the Hc isolated from its hemolymph: analysis of the subunit heterogeneity was performed and the modulating effects of temperature, L-lactate, urate, calcium and pH on the oxygen transporting properties were investigated with the aim of contributing to the understanding of the linkage between structure, function and physiological adaptations in oxygen-transport proteins.

MATERIALS AND METHODS

Protease inhibitor cocktail tablets (Complete mini EDTA-free) were supplied by Roche Applied Science. Millex filters, used to avoid bacterial growth, were supplied by Millipore; Sephacryl S-300, prepacked Superose 6 HR 10/30 and HMW (high molecular weight) gel filtration kit were supplied by Amersham Biosciences. Molecular mass standards for SDS-PAGE were supplied by Sigma. Immobiline DryStrip gels were supplied by Amersham Pharmacia Biotech UK Ltd. All other reagents were of the highest purity commercially available.

Animal Collection and Sampling—Adult shamefaced crabs (*C. granulata*, $N = 50$),(wet mass 190–250 g), were purchased through local fish stores, placed in well aerated re-circulating natural seawater (35‰ salinity, 15°C) and fed on molluscs for at least one week before experiments. Only male crabs were used in order to eliminate inter-gender variation and the potential presence of yolk proteins in the hemolymph. Fresh hemolymph was withdrawn through the arthrodial membrane at the base of a walking leg by using a plastic 2.5-ml syringe.

Five animals were removed from their normal aqueous environment and kept in emersion through three days at 10°C before taking their hemolymph for lactate and urate measures. We were not able to keep alive any animal exposed to air at 10°C longer than a few days.

Complete protease inhibitor cocktail was added to the hemolymph, which was allowed to coagulate for 30 min at 20°C. Then the clot was broken with a tissue homogenizer and the sample centrifuged for 10 min at 4°C and $12{,}000 \times g$ to remove debris. The supernatant was pelleted by preparative ultracentrifugation for 4 h at 4°C and $100{,}000 \times g$ and the pellet was resuspended in 100 mM Tris-HCl buffer, pH 7.0 and 10 mM $CaCl_2$, then dialyzed overnight against the same buffer. Bacterial growth was prevented by filtering the solution through a Millex filter driven by a sterile syringe. In this way Hc solutions with a protein concentration of about 100 $mg \cdot ml^{-1}$ could be stored for months at 4°C without any degradation or damage of their functional properties.

Hemolymph L-lactate concentration was measured by using the Lactate Dry-Fast kit from Sentinel Ch. (Milan, Italy). This assay consists of a spectrophotometric measure at 550 nm of the dye product of two coupled enzymatic reactions catalyzed by lactate oxidase and peroxidase in the presence of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS). Absorbance is directly proportional to the lactate concentration of the sample.

Hemolymph urate concentration was determined by using the Uric Acid Liquid kit from Sentinel Ch. (Milan, Italy). This assay consists of a spectrophotometric measure at 546 nm of the dye product of two coupled enzymatic reactions catalyzed by uricase and peroxidase in the presence of *N*-ethyl-*N*-(2-hydroxy)-3-methylaniline sodium propane-sulfonate (4-aminoantipyrine/TOOS). Absorbance is directly proportional to the uric acid concentration of the sample.

Hemolymph calcium concentration was measured by using the Calcium Dry-Fast Kit from Sentinel Ch. (Milan, Italy). This consists of a spectrophotometric measure of a red complex formed by interaction between calcium ions and cresolphtalein complexone (CPC) at pH 10.0. Absorbance at 570 nm is directly proportional to the calcium concentration of the sample.

Hc Purification—Hc purification was performed by DEAE-Sepharose CL-6B chromatography on a 20×3 cm column, equilibrated with 50 mM Tris-HCl buffer, pH 7.5 and 10mM $CaCl_2$. Elution was carried out with a linear gradient from 0 to 0.8 M NaCl at flow rate of 1 $ml \cdot min^{-1}$.

In order to investigate the aggregation state of hemocyanin, an analytical prepacked Superose 6 HR 10/30 column was connected to a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden); elution was performed with 50 mM Tris-HCl buffer, pH 7.5 and 10 mM $CaCl_2$. The column was calibrated by using bovine serum albumin (67 kDa) and the Amersham Biosciences HMW gel filtration kit: aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (699 kDa), blue dextran (2000 kDa), and Hc solution was loaded into a Sephacryl S-300 preparative column (75×1.5 cm), with 50 mM Tris-HCl buffer, pH 7.5, and 10 mM $CaCl_2$. Elution from the columns was carried out at a flow rate of 0.1 $ml \cdot min^{-1}$.

Proteins were monitored by use of a spectrophotometer at 280 nm.

Hc concentrations were determined by measuring absorbance in a 1-cm cuvette at 280 and 335 nm, by using the

absorption coefficients of *Carcinus maenas* Hc ($\varepsilon^{1\%}$ = 14.2 and 2.33 respectively) (*26*).

Electrophoretic Experiments—PAGE was performed in 50 mM Tris-HCl buffer at pH 8.0 on 5% (w/v) gels at a protein concentration of 1 mg·ml^{-1} (*27*). The proportion of the bands of the stained gels was calculated by the Biosoft QuantiScan densitometric program.

SDS–PAGE was performed under reducing conditions according to the classical procedure (*28*) on 7.5% polyacrylamide gels. Sigma Marker High Range kit was used; it consists of glyceraldehydes-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa), glutamic dehydrogenase (55 kDa), albumin (bovine serum) (66 kDa), fructose-6-phosphate kinase (84 kDa), phosphorylase *b* (97 kDa), β-galactosidase (116 kDa) and myosin (205 kDa).

Gels were stained with 0.2% (w/v) Coomassie Brilliant Blue R 250.

Two-dimensional electrophoresis with Immobiline Dry strip gels containing a pre-formed 3.0–5.6 pH gradient immobilized in a homogeneous polyacrylamide gel was firstly performed by a Amersham Bioscience Ettan IPGPhor II, following the method supplied with the apparatus instructions. The second-dimension separation was carried out by SDS 7.5% PAGE (*28*). In order to obtain an ultrasensitive detection of proteins the EZBlue Gel Staining Reagent from Sigma was used.

Subunit Dissociation and Reassembly Experiments—Dissociation of dodecameric Hc into lower aggregation states was performed by prolonged dialysis of the samples against 50 mM Tris-HCl buffer, 10 mM EDTA, with a protein concentration of 5 mg·ml^{-1}at pH 7.0–9.2. During dialysis, the buffer solution was changed six times in 72 h. Addition of 2.0 M urea to the dialysis buffer solution was also done with the aim of obtaining complete dissociation of the protein into monomers.

Reassembly experiments were performed by dialysis against 50 mM Tris-HCl buffer, 10 mM $CaCl_2$, pH 7.0 at a protein concentration of 5 mg·ml^{-1}.

The aggregation state of Hc after dissociation and reassembly experiments was tested by the Superose 6 HR gel chromatography and the native PAGE above described.

O_2-Binding Properties—O_2-binding isotherms were recorded with a Varian Cary 50 spectrophotometer, using a modified Thunberg tube with the lower part sealed to an optical cuvette, as described by Giardina and Amiconi (*29*). Hc solutions were deoxygenated by vacuum in the tonometer, where oxygen was then introduced by addition of small, known amounts of air and, at the end of each equilibrium experiment, the tonometer was opened to the air. Each binding curve was characterized by at least 8–10 experimental points representing different O_2 saturations. The fractional O_2 saturation of Hc was easily calculated at each step from the initial and final ones (corresponding to the fully deoxygenated and fully oxygenated derivatives) by the absorbance change at 335 nm. Experiments on native dodecameric Hc were performed in 100 mM Tris-HCl buffer,10 mM $CaCl_2$ in the 6.9–9.2 pH range, in the absence and presence of various concentrations of calcium, L-lactate and urate at a protein concentration of 3–5 mg·ml^{-1}. Since Tris buffering could be ineffective at extreme values of the pH range used, accurate pH controls were performed both before and after each equilibrium experiment.

Curve-fitting as a function of lactate concentration was carried out by using the following equation (*30*):

$$\log P_{50}^{\text{obs}} = \log P_{50}^{0} + R \log\{(1 + K_t\cdot[E])/(1 + K_r\cdot[E])\} \quad (1)$$

where $\log P_{50}^{\text{obs}}$ is the O_2 affinity observed at a given concentration [E] of the effector under investigation, $\log P_{50}^{0}$ is the O_2 affinity displayed in the absence of the effector, K_t and K_r are the association equilibrium constants for the effector to the unliganded and liganded Hc, respectively, and R is related to the number of effector binding sites per subunit (*i.e.*, $R = 0.5$). The use of P_{50} instead of P_m is justified by the fact that, owing to the highly symmetrical shape of the binding curves, P_m is almost equal to P_{50}.

Curve-fitting as a function of calcium concentrations was obtained by using a classic Hill binding equation (*31*). It should be noted that the Hc solutions used to test calcium effect on oxygen affinity were previously dialyzed at room temperature against 50 mM Tris-HCl buffer, 10 mM EDTA, pH 7.0.

The heat of oxygenation (ΔH, J·mol^{-1}) was calculated with the integrated van't Hoff equation:

$$\Delta H = -2.303\cdot R\cdot\Delta\log P_{50}\cdot[(T_1\cdot T_2)/(T_1 - T_2)] \quad (2)$$

where T is the absolute temperature and R = 8.31 J·K^{-1}·mol^{-1}. Moreover pH of each oxygen-binding experiment was adjusted as temperature change required, given that pH of Tris buffer is markedly temperature-dependent.

RESULTS

Hc concentration in the native hemolymph, measured in 50 specimens of the shamefaced crab (*C. granulata*), was equal to 25 ± 10 mg ml^{-1}, which corresponds to 30 μM (in terms of 12-mer); L-lactate and urate concentrations, measured in the same specimens, were equal to 3.3 ± 1.5 mM and 60 ± 22 μM, respectively. Therefore, the molar lactate/Hc ratio in the native hemolymph was found to be about 120, while the molar urate/Hc ratio was approximately 2.2. Calcium ion concentration in hemolymph was measured at 17.5 ± 8 mM, in agreement with those previously reported in decapods (*15*, *32*, *33*). Lactate and urate concentrations in the hemolymph of the animals increased to 12 ± 6 mM and 124 ± 32 μM, respectively, when they were kept in emersion at 10°C for three days.

Hc Purification—Hc was purified by DEAE-Sepharose CL-6B chromatography of the ultracentrifuged hemolymph (Fig. 1A). The purity of the preparation was assessed by the absorbance ratio A_{337}/A_{280} of 0.2, which corresponded to that expected for a solution containing 100% oxy-Hc. However, the presence of a minor protein band in the native PAGE (Fig. 1A) suggested two different aggregation states of Hc. Therefore, gel-filtration chromatography was carried out on a prepacked Superose 6 HR analytical column; the FPLC elution profile agreed with the electrophoretic data, because a minor peak eluted after the major one (Fig. 2); the latter was attributed to the dodecameric Hc ($M_r \approx 900$ kDa) and the former to the hexameric Hc ($M_r \approx 450$ kDa) on the basis of their molecular masses, which were estimated based on prior calibration of the column with protein standards (see "MATERIAL AND METHODS"). The dodecameric Hc prevailed (approximately 95%), as usually found in Brachyuran species (*2*)

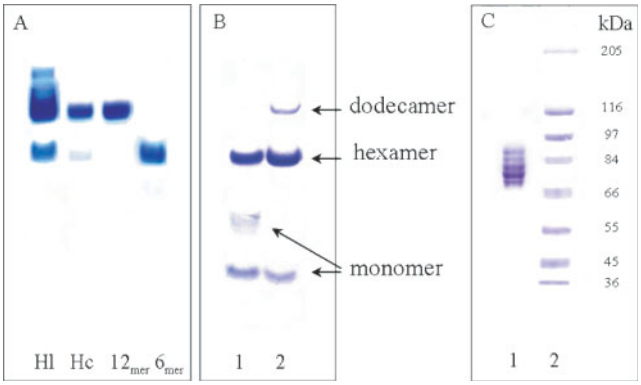

Fig. 1. **Electrophoretic analyses of *C. granulata* Hc. (A) native PAGE at different steps of purification; (B) native PAGE of dissociated and partially reassembled dodecameric Hc; (C) SDS of dodecameric Hc.** (A) PAGE at pH 8.0 of ultracentrifuged hemolymph (Hl), after DEAE-Sepharose CL-6B - chromatography (Hc), dodecameric (12-mer) and hexameric (6-mer) Hc separated by Sephacryl S-300 chromatography. (B) PAGE of dodecameric Hc after 72 h of alkaline dialysis with 10 mM EDTA and 2.0 M urea (lane 1), and after subsequent neutral dialysis with 10 mM $CaCl_2$ (lane 2). (C) SDS 7.5% PAGE of purified Hc under reducing conditions (lane 1); molecular mass standards (lane 2). All gels were stained with 0.2% Coomassie Brilliant Blue R 250.

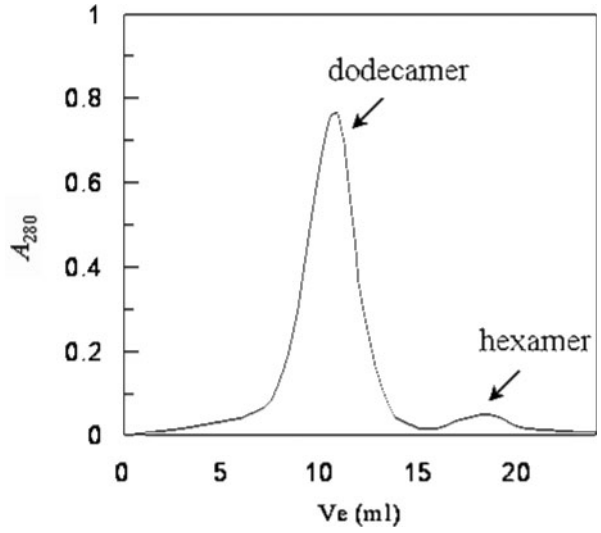

Fig. 2. **Gel filtration of native Hc.** Experimental conditions: prepacked Superose 6 HR column was connected to a FPLC system and elution was performed with 50 mM Tris-HCl buffer, 10 mM $CaCl_2$, pH 7.5, at a flow rate of 0.1 ml·min^{-1}. Absorbance was monitored at 280 nm.

and it was separated from the hexameric form by a size exclusion preparative Sephacryl S-300 chromatography (Fig. 1A). We did not try to identify the other proteins of the ultracentrifuged hemolymph with slower migration than the dodecamer (Fig. 1A): they perhaps are tyrosinase and/or cryptocyanin, as found in the hemolymph of other crustaceans (*34*, *35*), that ultracentrifugation could not separate, depending on their high molecular weight. In contrast with previous results obtained on Hcs from other species (*36*), both the dodecameric and the hexameric forms were stable in their own aggregation state: each fraction, separated by gel filtration, could be stored at 4°C for two weeks, without any damage of its functional properties and, if rechromatographed under the same experimental conditions, eluted as a single peak. This finding excludes the possibility that the percentage of the two Hcs present in the hemolymph could be restored under native conditions from isolated forms.

SDS-PAGE of Hc samples, obtained both from individual and pooled crabs, allowed separation of at least six types of subunit with molecular mass values of between about 71 and 88 kDa (Fig. 1C) with no differences between SDS-PAGE pattern of hexamers and dodecamers. However, percentages of the subunits were found to differ among specimens.

Two-dimensional electrophoresis performed on a pooled Hc sample is reported in Fig. 3. The high degree of heterogeneity of subunits was still observed when the experiments were carried out on individual crabs, and in addition the electrophoretic patterns were not reproducible from one animal to another.

Subunit Dissociation and Reassembly Experiments—Overnight dialysis at pH 7.0 of dodecameric Hc in the presence of EDTA showed that the molecule is stable in this aggregation state even without calcium (data not shown), whereas dissociation experiments showed that only an incomplete dissociation could be reached: hexamers are still present even when dialysis at pH 9.2 was prolonged through 72 h, (Fig. 1B, lane 1). Longer dialysis did not change this result. Previously, only *Penaeus monodon* Hc has been found to be stable against dissociation at high pH, independently of the presence of calcium ions (*4*). Dissociation into monomers was also attempted by dialysis in the presence of 2.0 M urea; moreover, under these drastic experimental conditions the protein gradually lost its capability to bind oxygen, as shown by a decrease of the intensity of the 337-nm absorption band.

Reassembly experiments into dodecamers of the hexamers and monomers (obtained from dodecamer dissociation) was extended through seven days of dialysis against 50 mM Tris-HCl buffer, 10 mM $CaCl_2$, at pH 7.0 and 4°C. Only about 10% of the dodecamer was restored and a significant amount of monomers remained (Fig. 1B, lane 2). The aggregation state of Hc in the dissociation-reassembly experiments described above was tested by analytical gel filtration on Superose 6 HR.

O_2-Binding Properties—O_2-binding properties of *C. granulata* Hc were studied by equilibrium experiments carried out in 100 mM Tris-HCl buffer, 10 mM $CaCl_2$ in the 6.9–9.2 pH range (Fig. 4). Under these experimental conditions, a strong Bohr effect ($\Delta\log P_{50}/\Delta pH \approx -0.95$) was displayed by the dodecameric Hc, whereas cooperativity of its oxygen-binding was almost constant in the 7.2–9.0 pH range (h_{50} values = 2.7 ± 0.2), even though it decreased slightly at the extreme values of the examined pH range.

On the basis of further electrophoretic analysis performed after overnight dialysis against 50 mM Tris-HCl buffer, 10 mM $CaCl_2$ at different pH values (*i.e.*, 7.0, 8.5 and 9.2) (data not shown), we may assert that Hc is stable in the dodecameric aggregation state in the whole pH range of the equilibrium experiments.

Another set of O_2-binding experiments was performed by using the hexameric Hc, isolated by Sephacryl S-300 chromatography. The native hexameric protein showed both a lower Bohr effect ($\Delta\log P_{50}/\Delta pH \approx -0.3$) and a lower level of

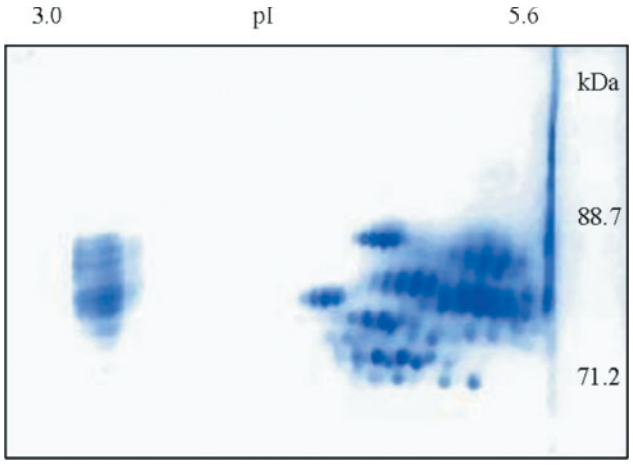

Fig. 3. **Two-dimensional electrophoresis.** Two-dimensional electrophoresis of a pooled Hc sample was carried out by using Immobiline DryStrip 7 cm gels containing a pre-formed 3.0–5.6 pH gradient immobilized in a homogeneous polyacrylamide gel. The second dimension separation was carried out by SDS 7.5% PAGE.

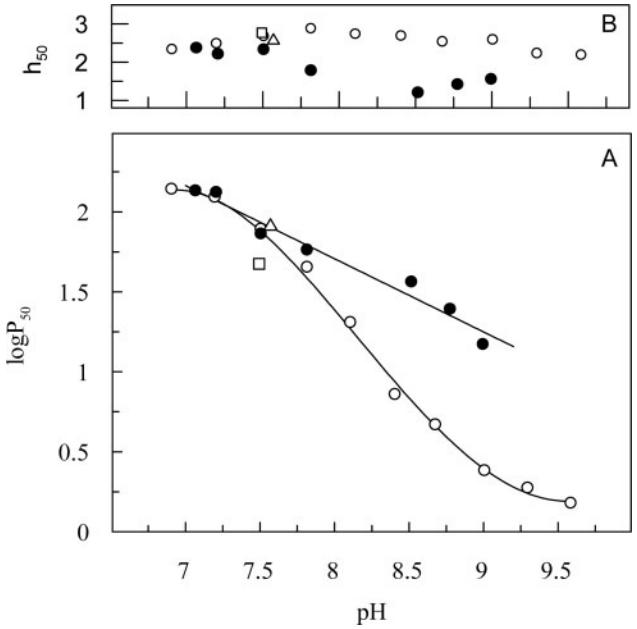

Fig. 4. **Bohr effect (A) and Hill coefficients (B).** Effect of pH on the oxygen affinity (in terms of log P_{50}) (A) and cooperativity (in terms of h_{50}) (B) of dodecameric (open circles) and hexameric (closed circles) native Hc. Conditions: 100 mM Tris-HCl buffer, 10 mM $CaCl_2$, at 20°C and 3–5 mg·ml^{-1} protein concentration; plus 5 mM lactate (square); plus 1.0 mM urate (triangle). Oxygen pressures are expressed in torr units (1 torr = 0.133 kPa). Experiments were performed in triplicate. An average SD of ±5% for P_{50} values was calculated.

cooperativity ($h_{50} = 1.5 \pm 0.3$) than the dodecameric protein. Moreover, the oxygen affinity of the hexamer was similar to that of the dodecamer in the 7.0–7.5 pH range, but as pH increased, it became lower than that of dodecamer.

It must be pointed out that the experiments were carried out on individual crabs and were reproducible from one animal to another: no functional differences were found in spite of the large structural heterogeneity displayed by SDS and two-dimensional electrophoretic analysis (data not shown).

Effect of Organic Acids—Addition of urate either at physiological or at 1 mM concentration (its limited solubility did not allow higher concentrations) did not affect the oxygen affinity of *C. granulata* Hc at all. In contrast, a small decrease of log P_{50} was observed in the presence of 5 mM lactate. Therefore, to gain further insight into the modulating effect of lactate on the O_2-binding properties of *C. granulata* Hc, a set of equilibrium experiments was carried out at pH 7.5 by increasing lactate concentration. The titration curve revealed that O_2 affinity was greatly enhanced ($\Delta\log P_{50}$, *i.e.* the difference between log P_{50} values measured in the absence and presence of saturating amounts of lactate, was equal to –0.55) (Fig. 5). Cooperativity appeared unchanged relative to that measured without lactate, but at lactate concentrations higher than 5 mM, h_{50} values were reduced from 2.75 ± 0.1 to 2.15 ± 0.15.

The curve can be fitted by applying least-square minimization procedures and using Eq. 1 with a R value of 0.5, thus suggesting a 6/12 ratio of binding-sites to Hc, *i.e.*, three molecules of lactate bound to each hexamer. The association constants of lactate to the deoxy- and oxy-Hc (K_t and K_r) were calculated ($K_t = 43.4$ M^{-1} and $K_r = 6.2 \times 10^{-2}$ M^{-1}), and lactate binding was found to be non-cooperative.

Effect of Temperature—The effect of temperature on the O_2 affinity was measured in the 12.5–22.5°C temperature range in the absence and presence of 10 mM lactate at pH 7.5 (Fig. 6), and the corresponding changes of enthalpy were calculated. In the absence and presence of physiological concentration of lactate, the ΔH values were –81 ± 3 and –106 ± 8 kJ mol^{-1}, respectively; this difference indicates that the lactate binding makes an exothermic contribution of about –25 kJ mol^{-1}. Moreover, there was a marked increase in oxygen-binding cooperativity with increasing temperature and decreasing oxygen affinity; this feature was observed both in the absence and presence of lactate, even though the increase of h_{50} value in the presence was higher ($\Delta h_{50} = 0.88$) than that in the absence ($\Delta h_{50} = 0.44$) of lactate (see panel B of Fig. 6) (Δh_{50} represents the difference between h_{50} values measured at 22.5 and 12.5°C).

Effect of Calcium—Addition of calcium at low concentrations (<10 mM) did not affect O_2 affinity and cooperativity of *C. granulata* Hc at all, in contrast with previously characterized crustacean Hcs, like those of *Panulirus interruptus* (*32*, *37*), *Panulirus japonicus* (*38*) and *Scyllarides latus* (*39*). In contrast, a large increase in O_2 affinity was observed at calcium concentrations higher than 10 mM, which is approximately the physiological one ($\Delta\log P_{50}$ = –1.87) (Fig. 7). As far as the cooperativity is concerned, it did not significantly vary ($h_{50} = 3.2 \pm 0.7$) in the examined concentration range (0–0.5 M). The calculated calcium coefficient ($\Delta\log P_{50}/\Delta\log [Ca^{2+}] = -0.94$) is consistent with a wide range of oxygen affinity that calcium concentration might produce. Moreover, calcium binding appeared to be non-cooperative, and the overall binding constant was equal to 4.27 M^{-1}.

DISCUSSION

In Crustaceans, 70–95% of the proteins in the hemolymph is the respiratory pigment Hc (*1–3*), which may be present

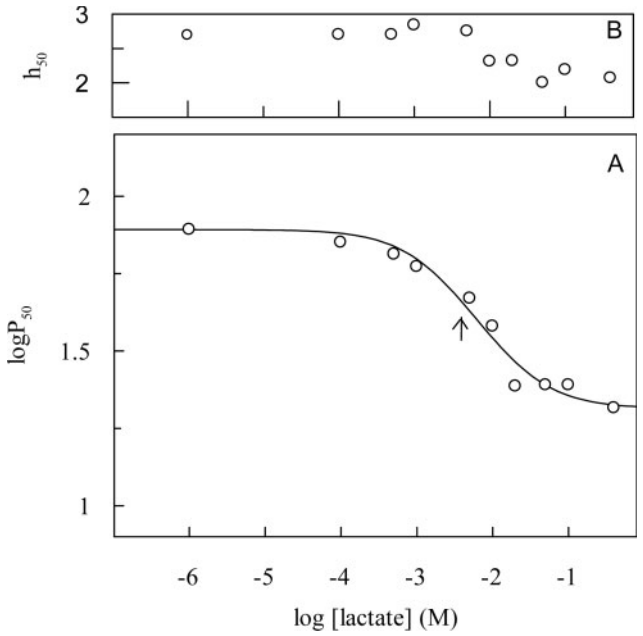

Fig. 5. **Effect of lactate concentration.** Oxygen affinity (in terms of log P_{50}) (A) and cooperativity (in terms of h_{50}) (B) of *C. granulata* Hc in 100 mM Tris-HCl buffer, 10 mM $CaCl_2$, pH 7.5, at 20°C and a protein concentration of 3–5 mg·ml^{-1}. Oxygen pressures are expressed in torr units (1 torr = 0.133 kPa); lactate concentrations are expressed in molar units. The curve was fitted by using Eq. 1. Arrow indicates the physiological average concentration of lactate. Experiments were performed in triplicate.

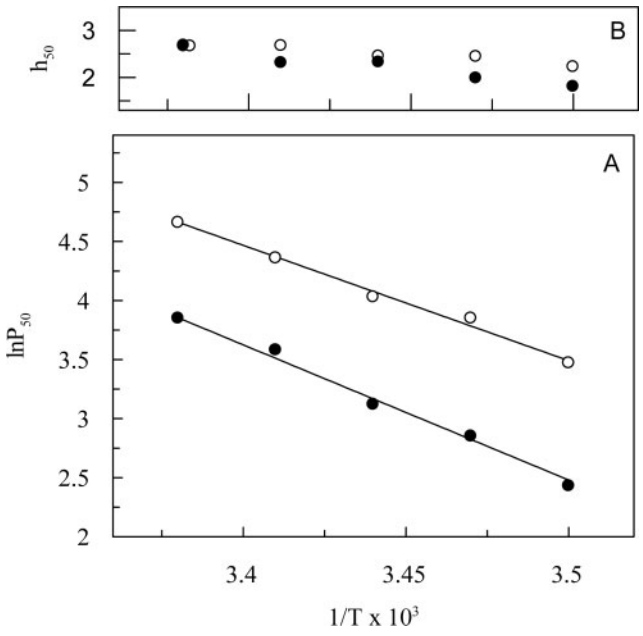

Fig. 6. **Effect of temperature on the oxygen affinity (A) and cooperativity (B).** van't Hoff isochores (ln P_{50} *vs.* 1/T degrees) were determined in 100 mM Tris-HCl buffer, 10 mM $CaCl_2$, pH 7.5, in the absence (open circles) and presence (closed circles) of 10 mM lactate at a protein concentration of 3–5 mg·ml^{-1}. Oxygen pressures are expressed in torr units (1 torr = 0.133 kPa). An average SD of ±5% for P_{50} values was calculated.

in different aggregation states: for instance, species belonging to the suborder of Palinura, such as *Palinurus elephas* and *Panulirus interruptus*, show only hexameric aggregation, while most species belonging to Astacura, like *Homarus americanus* and *Astacus leptodactylus*, and Brachyura, like *Carcinus maenas* and *Callinectes sapidus*, usually present a main dodecameric component and a small amount of hexameric Hc; moreover, the ratio between dodecamer and hexamer may depend on the adaptation temperature (*40*). In the shamefaced crab *C. granulata*, a Brachyuran crab, we also found these two Hc fractions; and chromatographic separation provided stable aggregates of the two structures. The dissociation of crustacean 2 × 6-mer Hcs is a process that is not fully reversible. Long ago, many researchers failed in attempts to reconstitute 2 × 6-meric structures, particularly in the case of Brachyuran crabs (*1*); but success came with the freshwater crayfish *Astacus leptodactylus*, whose native stoichiometry of 2 × 6-meric Hc was fully restored, while in the same study those of three other species were only partially reinstated (*5*). In the present study we have shown that only about 10% of the dodecameric Hc could be reassembled from its own dissociation products, whereas a higher amount of the hexameric form was restored.

O_2-Binding Properties—The oxygen-binding affinities of dodecameric and hexameric Hcs showed similarly low values at physiological pH, while that of the hexamer became lower than that of the dodecamer as pH increased. As far as the cooperativity is concerned, it must be pointed out that in all cases examined the hexamer shows a much lower degree of cooperativity than the dodecamer. This fact, nevertheless, should have no effect on the whole O_2-binding properties of the animal, since the hexameric Hc is present in the hemolymph in a very low percentage. For this reason, further functional properties were determined with isolated dodecameric Hc samples.

It must also be pointed out that the magnitude of the Bohr effect displayed by the Hc of *C. granulata* is in agreement with that of a number of other decapods, like *Callinectes sapidus*, *Homarus vulgaris* and *Cancer pagurus*, but quite higher than that shown by *Procambarus clarkii* and *Carcinus maenas* (Table 1).

Effect of Calcium—The importance of calcium ions as a factor controlling the *in vitro* and *in vivo* quaternary structure and also the O_2 affinity of decapod Hcs has long been known (*41–43*). Studies of Hcs from almost all species belonging to the suborder Palinura, such as *Panulirus interruptus* (*44*) and *Panulirus japonicus* (*38*), have shown that calcium may be active at low concentration by decreasing O_2 affinity. Moreover, in a recent study of Hc from *S. latus* (*39*), which also belongs to Palinura, the effect of calcium on the O_2 affinity was measured over a wide range of its concentration: calcium ions at low concentration strongly decreased oxygen affinity ($\Delta\log P_{50}$ = +1.5), whereas an opposite, large positive modulation was observed at high calcium concentrations ($\Delta\log P_{50}$ = –0.95) (see Fig. 7). In contrast, Hc of *C. granulata* showed no influence on O_2-affinity or cooperativity in the absence or presence of calcium at concentrations up to 10 mM. This functional behaviour is consistent with the absence of high-affinity calcium binding sites, recently found in the

Hc of *S. latus* and first described in *Panulirus interruptus*, where these cations appeared to be involved in maintaining the molecule in its associated state (*44*).

On the whole, the role of calcium ions *in vivo* could be to increase Hc-O_2 affinity at concentrations near the physiological level of approx. 10 mM; (*45*): in this respect, Hc of the crabs *Callinectes sapidus* (*46*) and *Cancer magister* (*33*) show an increase in O_2 affinity in the 0.3–50 mM and 4.0–32 mM calcium concentration range, respectively, thus indicating this effect to be physiologically operative. Hc of *C. granulata*, very close to these species from a phylogenetical point of view, appears to be modulated by calcium in a similar way, even though a larger increase of O_2 affinity ($\Delta\log P_{50} = -1.87$) than that reported for the other two crabs was found, because its effect was measured up to 0.5 M calcium. Like *Callinectes sapidus* and *Cancer magister*, *C. granulata* also belongs to the suborder Brachyura, so it can be suggested that the absence of any effect at low calcium levels on Hc O_2 affinity and cooperativity may be a characteristic property of Brachyuran Hcs. Moreover, in all these species of closely related crabs, calcium acts to raise the oxygen affinity of their Hc by interaction with low-affinity binding sites: this property could be utilised *in vivo* in particular conditions, such as during acidotic stress. For instance, in the Hc of the crab *Hemigrapsus nudus*, increased calcium levels have been reported to occur through a mobilization of $CaCO_3$ from the exoskeleton to produce compensating HCO_3^- against acidosis (*15*).

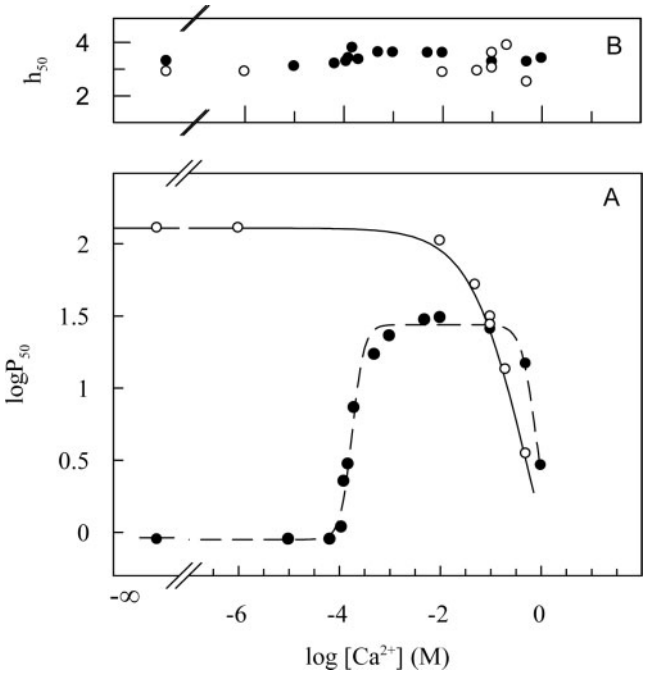

Fig. 7. **Effect of calcium concentration.** Oxygen affinity (in terms of log P_{50}) (A) and cooperativity (in terms of h_{50}) (B) of *C. granulata* Hc (open circles) in 100 mM Tris-HCl buffer, pH 7.0, at 20°C and a protein concentration of 3–5 mg·ml^{-1}. Oxygen pressures are expressed in torr units (1 torr = 0.133 kPa); calcium concentrations are expressed in molar units. Experiments were performed in triplicate. Data of *Scyllarides latus* Hc (closed circles) in the same experimental conditions were taken from Ref. *39*.

Unlike Brachyuran Hcs, those of *Panulirus interruptus* and, to a larger extent, *S. latus* show a decrease of O_2 affinity as low concentrations of calcium increased, and also an increase of the affinity as high calcium concentrations increased (*39*), thus suggesting this double opposite calcium effect to be a characteristic feature of Palinuran Hcs.

Effect of Temperature—As far the effect of temperature on Hc oxygen-affinity is concerned, the actual ranges of temperature that correspond to those in the animal's natural habitat have to be considered.

A study of the functional characteristics of cephalopods Hc (*47*) suggested that temperature exerts a profound influence on ecological range of the various species. In particular, taking 50–55 mm Hg as an upper limit of P_{50} value for safeguarding postbranchial saturation, temperatures needed by Hc of each species to reach that P_{50} value were found also to be the upper temperature limit of their habitat. Even though cephalopods belong to a different phylum from the crabs (Mollusca instead of Arthropoda) and are therefore phylogenetically distant, that hypothesis could also apply to the functional properties found in *C. granulata* Hc.

C. granulata lives in the Mediterranean Sae and in the part of the Atlantic Ocean between the Gulf of Biscaglia and Mauritania (*48*); P_{50} value below the upper limit needed to satisfy postbranchial saturation was measured at temperature that correspond to the Atlantic waters of Mauritanian coasts (*49*): this area indeed corresponds to the lowest latitude of *C. granulata* habitat. Moreover, taking into account the previous suggestion (*47*), the high temperature dependence on Hc oxygen-affinity could also predict a lower environmental-temperature limit for this species, given that an extremely high O_2 affinity would impair the unloading of O_2. O_2 affinity of *C. granulata* Hc also agrees with a regular oxygen release at the

Table 1. **Magnitude of Bohr and lactate effects in Hcs of some decapod crustaceans.** The first five species show a correlation between the amplitude of Bohr and lactate effect, the others do not (see "DISCUSSION," Effect of Organic Acids). Lactate coefficient of *C. granulata* Hc was calculated from the equilibrium experiments of Fig. 4.

Species	Subunit number	pH	$\Delta\log P_{50}/\Delta$log pH	$\Delta\log P_{50}/\Delta$log [lactate]	References
Procambarus clarkii	12		–0.42	0	(*51*)
Callinectes sapidus	12	7.5	–1.19	–0.26	(*52*, *55*)
Cancer magister	12	7.8	–1.22	–0.25	(*22*)
Calappa granulata	12	7.5	–0.95	–0.22	This study
Cancer pagurus	12	7.9	–1.0	–0.21	(*50*)
Homarus vulgaris	12	8.0	–1.2	–0.11	(*25*)
Carcinus maenas	12	7.4	–0.62	–0.096	(*50*)
Penaeus japonicus	12	7.6	–1.5	–0.077	(*20*)

temperature of Atlantic waters near the Gulf of Biscaglia (*49*), that correspond to the habitat of *C. granulata* with highest latitude.

If we consider the Mediterranean Sea, the other habitat of this species, its temperature falls in the middle range of those of the Atlantic habitat of this crab, allowing this species to normally uptake and unload oxygen in this area.

The results here reported are in agreement with the suggestion proposed in the case of cephalopods, even though buffered solutions of purified *C. granulata* Hc were used, instead of whole hemolymph, where a number of ions and metabolites in addition to lactate and calcium may affect its oxygen affinity.

The marked increase in cooperativity with increasing temperature, and with decreasing affinity (see Fig. 6), may counterbalance the adverse influence not only of unloading of O_2, but also of loading of O_2. Therefore, this increase in cooperativity may be an intrinsic feature of this Hc, directed to optimizing the utilization of O_2.

Effect of Organic Acids—The fact that L-lactate affects the oxygen affinity of crustaceans Hcs was first demonstrated by Truchot (*50*), and this organic acid was later shown to increase the O_2 affinity of a number of Hcs (*18*). Not all Hcs are sensitive to lactate, however: for instance, those of *Procambarus clarkii* (*51*), *S. latus* (*39*) and *Palinurus elephas* (own unpublished data) are not affected.

The present results demonstrate that a physiological concentration of L-lactate (see arrow in Fig. 5) does increase O_2 affinity of *C. granulata* Hc ($\Delta\log P_{50} = -0.2$), even though a larger effect was shown at higher L-lactate concentrations; this behaviour indicates that lactate may be an important *in vivo* modulator in this species. Moreover, an increased lactate concentration (12 ± 6 mM instead of 3.3 ± 1.5 mM) was found in the hemolymph of some specimens forced to leave their aqueous environment; this fact indicates that oxygen uptake can be improved by higher lactate concentrations, which produce a further increase of oxygen affinity ($\Delta\log P_{50} = -0.3$) (see the second part of the titration curve in Fig. 5).

It has been reported that lactate is the main anaerobic end-product in crustaceans, so it might participate in the adjustment of hemolymph oxygen transport in situations such as environmental hypoxia or muscular exercise (*16*, *52*). That crustaceans are often tolerant of hypoxic exposure may be explained by the possibility of increasing O_2 affinity and so allowing a sufficient gas exchange at gill level. This latter, in acute hypoxia, is a result of hyperventilation-induced alkalosis. In chronic hypoxic exposure, increased effectiveness of O_2-uptake across the gills is maintained as a result of build up of metabolites such as lactate and/or urate which also function to increase the Hc-O_2 affinity (*53*). The difference between the roles of these two organic acids during hypoxia needs to be pointed out. In fact lactate accumulates in the hemolymph during severe hypoxic situations (*54*) and exercise (*52*), as anaerobic end-product of glucose metabolism; in contrast, under moderate hypoxia, urate concentrations in the hemolymph increase due to a decrease in uricate activity lacking the second substrate. Therefore, it has been suggested that a shortage of oxygen leads to an initial increase in urate concentration followed by an increase in lactate concentration as hypoxia becomes more severe (*25*). Interestingly, it was suggested that lactate sensitivity is a primitive and insensitivity a derived feature (*23*).

Taking into account the fact that the effect of lactate opposes the normal Bohr shift in maintaining a high O_2 affinity during heavy exercise, it was suggested that the magnitude of the lactate effect would correlate with the importance of the Bohr shift (*51*). This hypothesis was validated by the crayfish *Procambarus clarkii* Hc, which showed practically no lactate effect and very small Bohr effect (*51*), and by the crabs *Cancer pagurus* (*50*), *Callinectes sapidus* (*54*) and *Cancer magister* (*22*) Hcs, where large Bohr shifts ($\Delta\log P_{50}/\Delta$pH between –1.22 and –1.0) correlate with strong lactate effects ($\Delta\log P_{50}/\Delta\log$ [lactate] between –0.257 and –0.21). Table 1 details values for the lactate and Bohr coefficients of the Hc from a number of crustaceans. Our estimate of the lactate coefficient in *C. granulata* ($\Delta\log P_{50}/\Delta\log$ [lactate] = –0.25) correlates well with the large Bohr shift, –0.95, in this species and may confirm the previous proposal.

On the other hand, further studies have shown that urate, a byproduct of purine catabolism, may also contribute to increasing Hc O_2-affinity (*20*, *25*); species with urate-sensitive Hc may lack the correlation of large lactate effects with large Bohr shifts: for instance, a positive urate modulation was also observed in both *Homarus vulgaris* (*25*) and *Penaeus japonicus* (*20*) Hcs, which showed a large Bohr shift ($\Delta\log P_{50}/\Delta$pH = –1.2 and –2.01), but a slight lactate effect ($\Delta\log P_{50}/\Delta\log$ [lactate] = –0.11 and –0.077). *C. granulata* Hc, which in contrast is insensitive to urate, presents the correlation described above.

Binding of lactate shows no cooperativity, suggesting that the binding of the first lactate molecule only affects the structure of the part of the protein molecule adjacent to the first lactate-binding site; the conformational change does not affect the second lactate-binding site. Moreover, lactate appears to have higher affinity for the oxy- than the deoxy-conformation, as reported in other hexameric (*46*) and dodecameric Hcs (*56*, *57*).

Note that the effect of lactate in increasing O_2 affinity is dependent on the removal of excess hydrogen ions, which could be released simultaneously from anaerobic tissues. If the H^+ are not fully buffered, the effect of lactate may be reduced or negated (*58*).

The number of lactate-binding sites found in a number of species ranges from 1 to 3 per hexamer, implicating interactions between monomers. In agreement with those results, here we report a 6/12 ratio of binding-sites to Hc, *i.e.*, three molecules of lactate bound to each hexamer. Furthermore, the enthalpy change of oxygenation of *C. granulata* Hc showed an exothermic contribution of lactate binding equal to –25 kJ mol^{-1} of oxygen, which was calculated by the difference between the enthalpy change of oxygenation measured in the absence (-81 ± 3 kJ mol^{-1}) and presence (-106 ± 8 kJ mol^{-1}) of physiological lactate concentrations.

Subunit Heterogeneity—In the past, specific Hc subunits have been indicated to be involved in the formation of aggregation states of crustacean Hcs above that of the hexamer, as in the case of *Cancer pagurus*, *Maja squinado*, *Astacus leptodactylus* (*11*) and *Callinectes sapidus* (*55*). However, this does not seem to be the case of *C. granulata* Hc, because the low percentage of reassembly into dodecamer was not improved if proteins used for reassembly were

obtained only from Hc in the dodecameric aggregation state.

The electrophoretic data on dissociated *C. granulata* Hc show a very high degree of heterogeneity, which was further confirmed by two-dimensional electrophoresis. This approach provided a good resolution of the small differences in charge and molecular mass that actually occur among Hc subunits. This method had previously resolved into ten distinct protein spots the Hc subunits of the centipede *Scutigera coleoptrata*, improving knowledge of their heterogeneity (*59*). All the data reported in Fig. 1C and Fig. 3 point to a different amino acid sequence of each subunit and/or post-translational modifications, such as phosphorylation or neutral and acid glycosylations. Thus, genetic polymorphism will not account for this heterogeneity.

Despite the large structural subunit heterogeneity evidenced in *C. granulata* Hc, functional experiments were reproducible from one animal to another.

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