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Structural and Functional Characterization of Haemocyanin from the Anemone Hermit Crab *Dardanus calidus*

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Oxygen-binding to haemocyanin (Hc) is generally an exothermic process, with overall enthalpy of oxygenation varying from species to species. A number of crustacean Hcs showed a null or reduced enthalpy of oxygenation, among others, the anomuran *Pagurus bernhardus* and *Paralithodes camtscaticae* possess a completely temperature-independent oxygen-binding in a wide range of temperature and pH. Functional analysis performed on purified native, hexameric and dodecameric Hc forms of the anemone hermit crab *Dardanus calidus* allowed to calculate the enthalpy of oxygenation values that resulted equal to −36.2, −33.8 and −26.8 kJ/mol, respectively. Thus, the temperature sensitivity of oxygen binding of *D. calidus* Hc is in contrast with the temperature independence reported for *P. bernhardus* and *P. camtscaticae*, suggesting a high Hc functional heterogeneity within Anomura. Functional characterization also evidenced a strong oxygen affinity modulation by protons ($\Delta\log P_{50}/\Delta pH = -0.97$) and lactate [$\Delta\log P_{50}/\Delta\log(\text{lactate}) = -0.38$], and a significant decrease in cooperativity by physiological concentration of lactate (n_{50} from 2.8 to 1.7 at pH 7.5).

Key words: Bohr effect; enthalpy of oxygenation; lactate binding; oxygen affinity; subunit heterogeneity.

Abbreviations: ΔH, overall heat of oxygenation; Hc, haemocyanin; n50, slope of the Hill plot at 50% saturation; pI, isoelectric point; P50, partial pressure of oxygen required to saturate 50% of the binding sites.

Haemocyanins (Hcs) are giant oligomeric copper-containing proteins that are freely dissolved in the haemolymph of many arthropods and molluscs (*1*). Arthropod Hcs occur in the haemolymph as aggregates of the 16S unit, which is an hexamer of 5S functional subunits, *i.e.* as 24S, 37S, 48S, 53S and 62S units, depending on the species (*2*). In the haemolymph of most crustaceans, both hexamers (16S) and dodecamers (24S) are found.

Even though anomuran Hcs have not been extensively characterized from a structural and physiological point of view, a complete temperature-independence of oxygen-binding has been reported for the common hermit crab *Pagurus bernhardus* haemolymph (*3*) and for the king crab *Paralithodes camtscaticae* native Hc (*4*). This characteristic is not common among the majority of crustacean Hcs, that generally present an exothermic overall enthalpy of oxygenation (ΔH) of ~ -35 kJ/mol or larger (*5*, *6*). In order to get a further insight into the temperature-independence of oxygen binding found in the two anomuran Hcs previously cited, we undertook the characterization of the Hc system from the anemone hermit crab *Dardanus calidus*, belonging to *Diogenidae*, and in the present article we report the functional and structural characterization performed on the purified Hc mixture that contains the hexameric and dodecameric forms in the proportion present in the haemolymph, on purified hexameric and dodecameric Hcs, with particular interest in the modulating effects played by temperature, L-lactate, calcium and pH on oxygen-binding properties.

MATERIALS AND METHODS

Animal Collection and Haemolymph Sampling—Specimens of *D. calidus* (mean weight ± SE = 20.5 ± 2.7 g, $n = 19$) were collected on the south coast of Sardinia (Italy), transported to the laboratory at the Department of Applied Science in Biosystems, University of Cagliari, and immediately transferred to aquaria containing aerated filtered re-circulating natural sea water (20–22°C, salinity 38 ± 1‰). The level of pO_2, checked daily with an OxyGuard oxygen meter (Birkerod, Denmark), resulted equal to 150 ± 6 mmHg. Twenty percent of the water in the aquaria was renewed weekly. The animals were fed on molluscs *ad libitum* for at least 1 week before experiments.

All individuals investigated appeared to be in intermolt, as indicated by their hard exoskeletons. Haemolymph was withdrawn from living animals through the arthrodial membrane at leg base using a

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plastic syringe and allowed to coagulate for 30 min at 20°C, then the clot was broken with a tissue homogenizer and the sample centrifuged for 15 min at 4°C and 12,000 *g* to remove debris. An aliquot of the supernatants obtained was used to determine Hc concentration (expressed as mean ± SE) measuring the absorbance at 337 nm and using the extinction coefficient reported for *Palinurus vulgaris* Hc (*7*), while another aliquot was deproteinized (one volume of haemolymph plus two volumes of 10% trichloroacetic acid were incubated on ice for 5 min and centrifuged at 1,500 *g* for 10 min) to perform the assays for measuring lactate, urate and calcium concentrations. The rest of supernatants was ultracentrifuged at 100,000 *g* for 4 h at 4°C in 100 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.0; pellets were resuspended in 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.5 and the obtained solutions were dialysed overnight at 4°C against the same buffer and stored at 4°C until usage.

Haemolymph Lactate, Urate and Calcium Measurements—Lactate and urate assays were performed on deproteinized haemolymph supernatants obtained from clotted haemolymph samples, while calcium assay was performed on deproteinized haemolymph samples after 10-fold dilution. The results of the assays were expressed as mean ± SE.

Haemolymph L-lactate concentration was determined with the lactate dry-fast kit (Sentinel Diagnostics, Milan, Italy, measuring range 0.1–16.6 mM), measuring the absorbance at 550 nm of the dye product of two coupled enzymatic reactions catalysed by lactate oxidase and peroxidase in the presence of 4-aminoantipyrine and *N*-ethyl-*N*-(2-hydroxy-3-sulphopropyl)-3-methylaniline, that is directly proportional to the lactate concentration of the sample.

Haemolymph urate concentration was determined with the uric acid liquid kit (Sentinel Diagnostics, Milan, Italy, measuring range 20–1,500 μM), measuring the absorbance at 546 nm of the dye product of two coupled enzymatic reactions catalysed by uricase and peroxidase in the presence of 4-aminoantipyrine and *N*-ethyl-*N*-(2-hydroxy-3-sulphopropyl)-3-methylaniline, that is directly proportional to the uric acid concentration of the sample.

Haemolymph Ca^{2+} concentration was determined with the calcium dry-fast kit (Sentinel Diagnostics, Milan, Italy, measuring range 0.25–5.0 mM), measuring the absorbance at 570 nm of the red complex formed by interaction between calcium ions and cresolphthalein complexone at pH 10.0, that is directly proportional to the calcium concentration of the sample.

Electrophoretic Analyses—Native PAGE was performed in 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 8.0, at 4°C, on 5% (w/v) gel at a protein concentration of 1 mg/ml, applying a constant current of 35 mA. After the exit of the bromophenol blue dye front from the gel, the run was continued for other 30 min and then the gel was stained with 0.2% (w/v) Coomassie brilliant blue R-250.

SDS–PAGE was performed under reducing conditions, according to the classical procedure (*8*) on discontinuous 7.5% (w/v) gels using the Sigma Marker High Range kit.

The first dimension of the 2D-PAGE was performed on an IPG-phor II (Amersham Biosciences), using a 7.0 cm Immobiline Dry Strip gel containing an immobilized 3.0–5.6 pH gradient, followed by the second dimension run on a 7.5% SDS–PAGE (*8*). Gels were stained with EZBlue Gel Staining Reagent (Sigma).

Hc Purification—Dialysed *D. calidus* haemolymph samples after ultracentrifugation were loaded on a DEAE-Sepharose CL-6B column (20 × 2.5 cm) equilibrated with 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.5. Hc elution was obtained applying a linear gradient of sodium chloride from 0 to 0.8 M in 120 min at a flow rate of 1 ml/min. Native PAGE analysis on the main peak fractions, showing only the presence of hexameric and dodecameric Hc forms, confirmed the high level of purification of the obtained sample, that will be indicated in the text hereafter as purified native *D. calidus* Hc. In order to separate the two Hc forms, purified native *D. calidus* Hc was loaded on a Sephacryl S-300 column (70 × 2.5 cm), equilibrated with 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 8.0 and elution was performed isocratically at a flow rate of 0.025 ml/min.

Oxygen-Binding Experiments—Oxygen dissociation curves were obtained recording at least 10 experimental points with a Varian Cary 50 spectrophotometer by the tonometric method (*9*) at 5–6 μM protein concentration (in terms of hexamers) on purified native and on purified hexameric and dodecameric *D. calidus* Hc forms, determining P_{50} and n_{50} values. Experiments were performed in 50 mM Tris–HCl, 10 mM $CaCl_2$, in the absence and presence of 5 mM L-lactate or 0.8 mM urate at 20°C, as a function of pH (7.0–9.2). One set of oxygen-binding experiments was performed as a function of lactate concentration at 15°C and pH 7.7 and another one as a function of calcium concentration at 20°C and pH 7.0. Each experiment was carried out in duplicate or triplicate and an average SD of ±3% for P_{50} values was calculated.

Curve fitting as a function of lactate concentration was carried out by using the following equation:

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

where $\log P_{50}^{obs}$ is the O_2 affinity observed at a given concentration [L] of L-lactate, $\log P_{50}{}^{0}$ is the O_2 affinity displayed in the absence of L-lactate, K_t and K_r are the association equilibrium constants for L-lactate to the unliganded and liganded Hc, respectively, and R is related to the number of L-lactate-binding sites per subunit (*i.e.* $R = 1$).

Overall oxygenation enthalpy of *D. calidus* Hc was calculated from oxygen-binding experiments performed as a function of temperature (15–25°C) in 50 mM Tris–HCl, 10 mM $CaCl_2$, in the absence and presence of 5 mM L-lactate at pH 7.5, using the integrated van't Hoff equation (*10*):

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

where T is the absolute temperature and R is the gas constant. Over the temperature range examined (15–25°C), van't Hoff plots were linear within the experimental error. Owing to the high temperature dependence of the pH values of Tris buffer, the pH meter calibration was performed at the specific temperature (15, 20, 25°C) of each experiment.

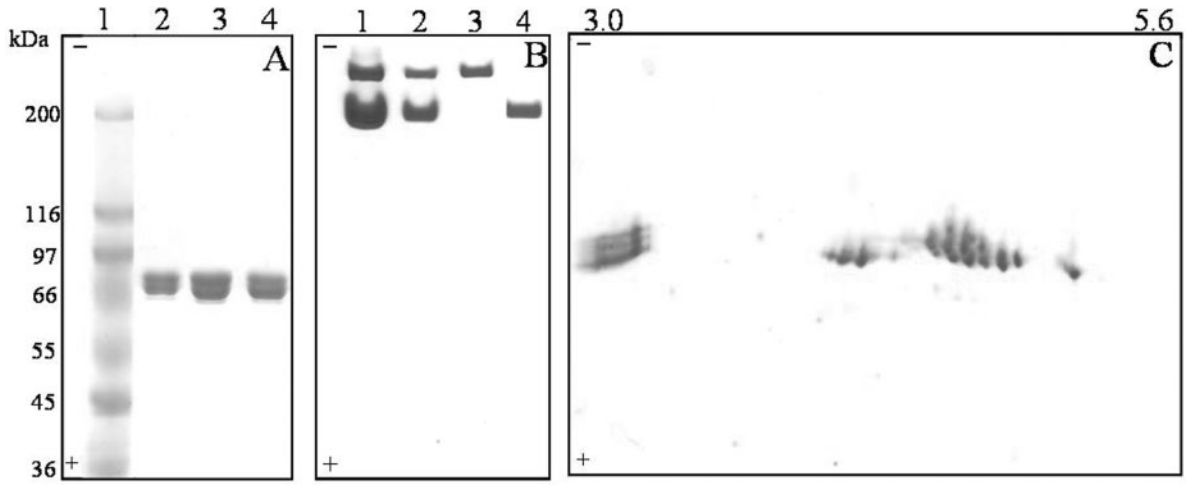

Fig. 1. **PAGE of purified *D. calidus* Hc and haemolymph proteins.** (A) SDS–PAGE on 7.5% polyacrylamide gel of: Molecular Weight standards (lane 1), purified native (lane 2), dodecameric (lane 3) and hexameric (lane 4) *D. calidus* Hc forms; (B) native PAGE on 5% polyacrylamide gel of: ultracentrifuged haemolymph (lane 1), purified native (lane 2), dodecameric (lane 3) and hexameric (lane 4) *D. calidus* Hc forms in 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 8.0. Both gels were stained with 0.2% Coomassie brilliant blue R-250. (C) 2D-PAGE of purified native *D. calidus* Hc on a 7.0 cm Immobiline Dry Strip gel containing an immobilized 3.0–5.6 pH gradient for the first dimension and on an homogeneous 7.5% SDS-PAGE for the second dimension.

Overall oxygenation enthalpy of *Palinurus elephas* and *Palinurus mauritanicus* purified Hc was measured under the same experimental conditions described for *D. calidus* Hc except for the absence of lactate and the presence of 0.5 mM urate.

RESULTS

Haemolymph Hc concentration of *D. calidus* resulted equal to 86.5 ± 4.3 mg/ml (192 ± 10 μM in terms of hexamers) while L-lactate and urate concentrations were equal to 4.4 ± 0.8 mM and 423 ± 66 μM, respectively. Therefore, in native haemolymph (where hexamers largely predominate) the molar lactate/Hc ratio was found to be ~20, thus indicating a 3-fold excess of lactate in terms of monomers, and the molar urate/hexamer ratio equal to ~2. Haemolymph calcium concentration resulted equal to 24.1 ± 1.9 mM, in agreement with those previously reported for other decapods (*11*, *12*).

Hc Purification and Electrophoresis Analyses—SDS–PAGE of purified native *D. calidus* Hc showed two major and one minor bands, with molecular weight values between 80 and 90 kDa (Fig. 1A), while native PAGE presented two bands (with a 80/20 ratio, Fig. 1B) that, on the basis of what has been shown on the native PAGE performed on *Calappa granulata* Hc (*13*), may be assigned to the hexameric (16S) and dodecameric (25S) Hc form, respectively, in order of their increasing mobility towards the anode. 2D-PAGE analysis of purified native *D. calidus* Hc, performed in the narrow pH range 3.0–5.6 in order to better discriminate between the different acidic polypeptide chains, showed a high number of polypeptide chains mostly differing in their pIs in the pH range 4.5–5.5 (Fig. 1C) and a series of not well-resolved spots at the acidic end of the first dimension strip (pH 3.0–3.5). Considering that all crustacean Hc subunits so far studied show a pI of ~5.0 and a very high (50–92%) sequence similarity (*14*), the presence of these very acidic spots requires further investigations.

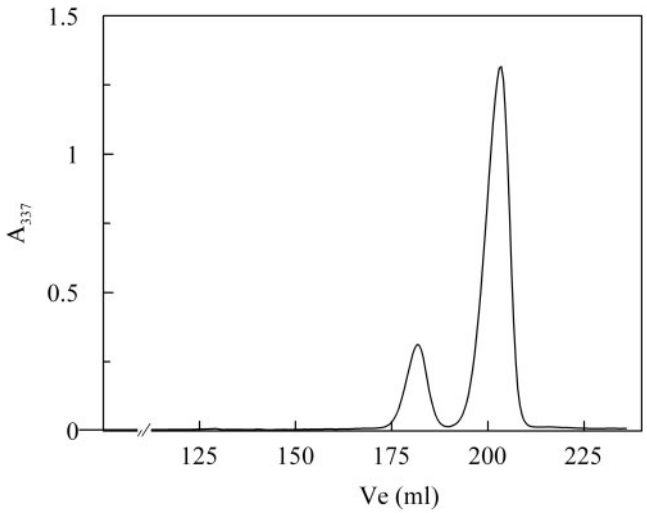

Fig. 2. **Gel filtration of purified native Hc**. Elution profile of purified native *D. calidus* Hc from a Sephacryl S-300 column (70×2.5 cm) in 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 8.0, at a flow rate of 0.025 ml/min.

The elution profile of purified native *D. calidus* Hc from a Sephacryl S-300 column equilibrated with 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.5, showed two well-resolved peaks (Fig. 2), which corresponded, in order of increasing elution time, to the dodecameric and hexameric Hcs, respectively. In contrast with previous results obtained on Hcs from other species, as in *P. camtschaticae* (*4*), the two aggregation forms of *D. calidus* Hc resulted to be stable (Fig. 1B), with no tendency to associate or dissociate for prolonged periods

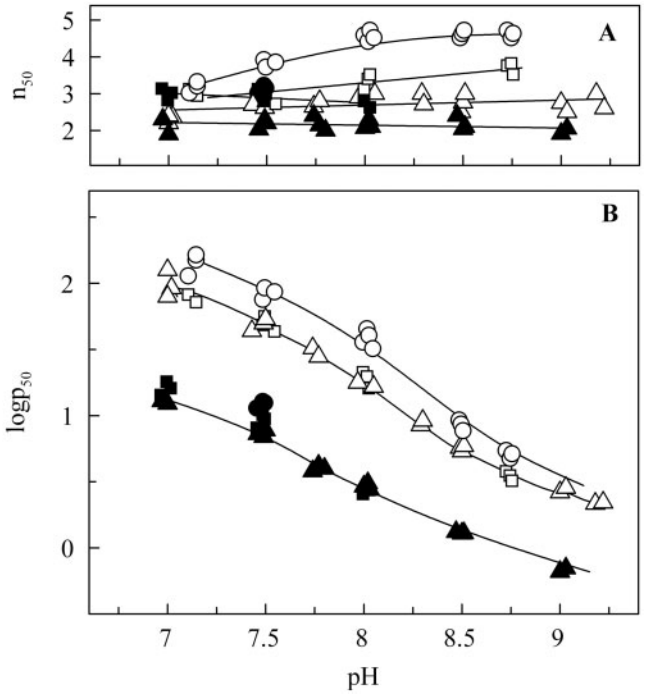

Fig. 3. **Hill coefficients and Bohr effect.** Effect of pH on cooperativity (A) and oxygen affinity (B) of purified native (triangles), dodecameric (circles) and hexameric (squares) *D. calidus* Hc forms in 50 mM Tris–HCl, 10 mM $CaCl_2$, 20°C, in the absence (open symbols) and presence (filled symbols) of 5 mM L-lactate, at a protein concentration of 2.5 mg/ml.

of time, and consequently they could be stored for at least four weeks at 4°C, without any modification of their functional properties.

Oxygen-Binding Properties—O_2-binding experiments carried out on purified native *D. calidus* Hc in 50 mM Tris–HCl, 10 mM $CaCl_2$, 20°C, as a function of pH (7.0–9.2) showed that protons largely modulate oxygen affinity ($\Delta\log P_{50}/\Delta pH = -0.97$), without modifying oxygen-binding cooperativity (n_{50} values = 2.7, being almost constant in the pH range examined, Fig. 3). The oxygen affinity of the hexameric Hc form did not differ from that of purified native *D. calidus* Hc in the pH range examined, while the cooperativity increased (n_{50} varied from 2.9 to 3.7 following the increase in pH). The dodecameric Hc form showed a slight decrease in oxygen affinity and a significant increase in cooperativity compared to both hexameric and native Hcs (n_{50} varying from 3.2 to 4.6 in the pH range considered, Fig. 3).

Effect of Organic Acids—Addition of urate at 2.5-fold higher concentration with respect to that measured in the haemolymph did not affect the oxygen affinity of purified native *D. calidus* Hc (Table 1). On the contrary, a large decrease of P_{50} was observed in the presence of 5 mM L-lactate, a concentration very close to that measured on fresh *D. calidus* haemolymph samples (Table 1). Therefore, to investigate the modulating effect of lactate on the O_2-binding properties of *D. calidus* Hc, a set of equilibrium experiments was

Table 1. **Oxygen-binding affinity and cooperativity of purified native, hexameric and dodecameric *D. calidus* Hc.**

	Native		Hexamer		Dodecamer	
	$\log P_{50}$	n_{50}	$\log P_{50}$	n_{50}	$\log P_{50}$	n_{50}
No effectors	1.70	2.62	1.74	3.0	1.93	3.84
+5 mM Lactate	0.86	2.03	0.98	2.97	1.09	3.13
+0.8 mM Urate	1.70	3.26	1.75	2.90	1.93	3.69

Experimental conditions: 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.5, at 20°C, with no effectors, in the presence of 5 mM L-lactate, or 0.8 mM urate. Each experiment was carried out in duplicate or triplicate and an average SD of ± 3% for P_{50} values was calculated.

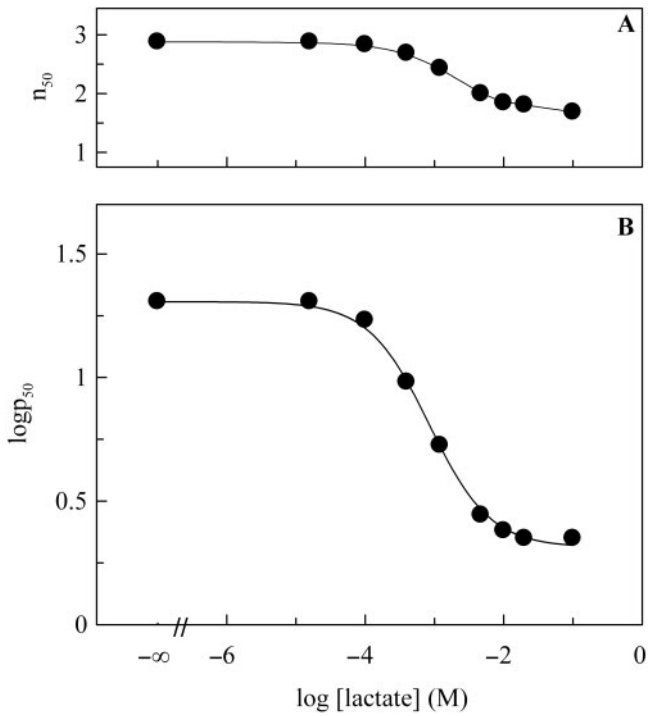

Fig. 4. **Effect of L-lactate concentration.** Cooperativity (A) and oxygen affinity (B) of purified native *D. calidus* Hc as a function of L-lactate concentration in 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.7, 15°C at a protein concentration of 2.5 mg/ml.

carried out on purified native *D. calidus* Hc at pH 7.7 and 15°C by increasing lactate concentration (Fig. 4). The titration curve revealed that oxygen affinity was greatly enhanced [$\Delta\log P_{50}/\Delta\log(\text{lactate}) = -0.38$], and cooperativity was significantly decreased as lactate concentration increased (n_{50} values are reduced from 2.9 to 1.7). The curve can be fitted by applying least-square minimization procedures and using Eq. 1 with an *R*-value of 1, thus suggesting a 6/6 ratio of binding-sites to Hc, *i.e.* six molecules of lactate bound to each hexamer. The association-binding constant of lactate to deoxy- and oxy-Hc (K_t and K_r, respectively) resulted equal to $370 \pm 3\ 6\ M^{-1}$ and $3.7 \pm 0.3 \times 10^3\ M^{-1}$.

Moreover, the lowest lactate concentration that exerts a saturating effect on oxygen affinity (5 mM) was used to

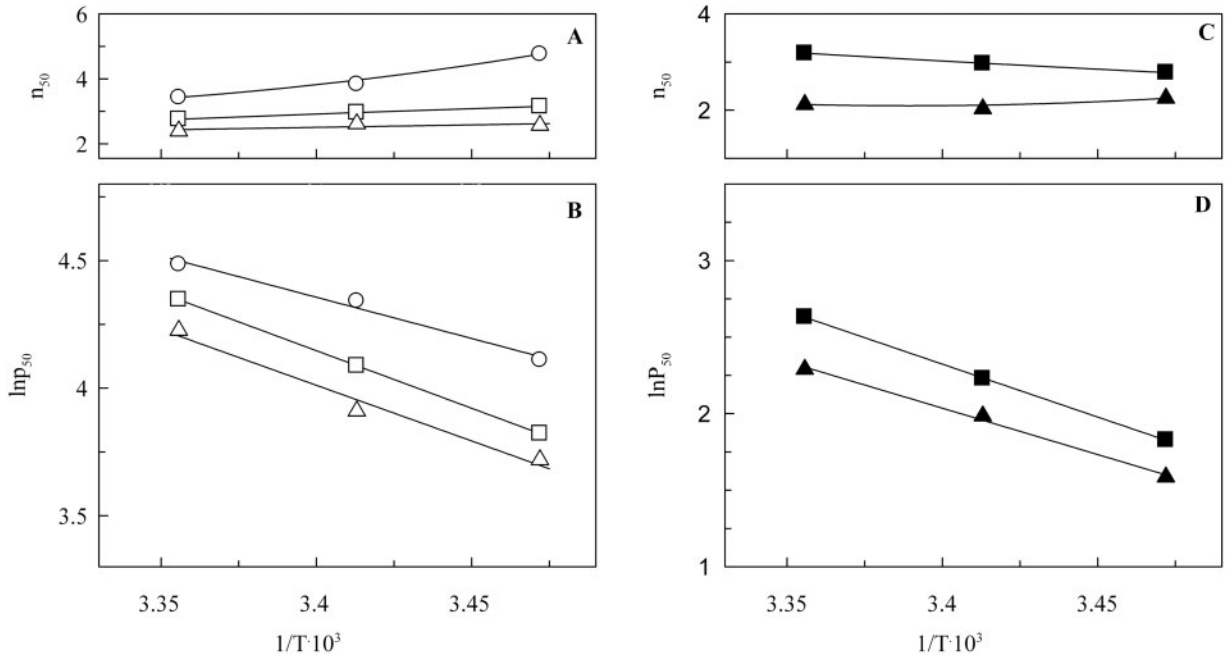

Fig. 5. **Effect of temperature on cooperativity (A, C) and oxygen affinity (B, D).** van't Hoff isochores of purified native (triangles), dodecameric (circles) and hexameric *D. calidus* Hc forms (squares) in 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.5 in the absence (open symbols) and presence (filled symbols) of 5 mM L-lactate, at a protein concentration of 2.5 mg/ml.

measure the lactate effect on purified native *D. calidus* Hc as a function of pH. As shown in Fig. 3, the effect of lactate on oxygen affinity is large and appeared to be slightly pH dependent, being higher at pH 7.0 ($\Delta \log P_{50} = 0.87$) than at pH 8.5 ($\Delta \log P_{50} = 0.75$). The cooperativity resulted decreased by the addition of L-lactate and almost pH independent ($n_{50} = 2.0 \pm 0.06$, Fig. 3). The effect of lactate on oxygen affinity and cooperativity of purified hexameric and dodecameric *D. calidus* Hc is comparable to that measured on native Hc (Fig. 3; Table 1).

Effect of Temperature—The effect of temperature on *D. calidus* Hc oxygen affinity has been measured in the temperature range 15–25°C, in 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.5, in the absence and presence of 5 mM lactate, and the corresponding vant' Hoff plots are reported in Fig. 5. The overall ΔH of oxygen-binding of purified native *D. calidus* Hc resulted equal to -36.2 ± 5.5 kJ/mol and -50.3 ± 3.5 kJ/mol in the absence and presence of 5 mM lactate, respectively, indicating an exothermic contribution of lactate binding equal to −14 kJ/mol. No significant variations of cooperativity were evident in the temperature range examined. Oxygen-binding enthalphy values were equal to -33.8 ± 2.0 kJ/mol and -57.4 ± 0.5 kJ/mol for hexameric *D. calidus* Hc in the absence and presence of 5 mM L-lactate, respectively, and to -26.8 ± 3.0 kJ/mol for dodecameric *D. calidus* Hc in the absence of lactate (Fig. 5).

Effect of Calcium—Addition of calcium at very low concentration (<0.1 mM) did not affect oxygen affinity and cooperativity of purified native *D. calidus* Hc (Fig. 6). As calcium concentration increased (in the range 0.1–10 mM) oxygen affinity slightly decreased

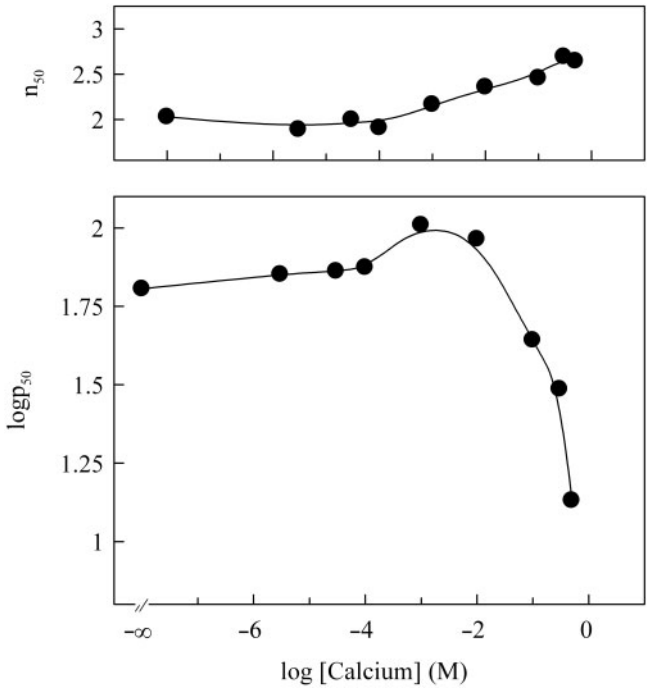

Fig. 6. **Effect of calcium concentration.** Cooperativity (A) and oxygen affinity (B) of purified native *D. calidus* Hc in 50 mM Tris–HCl, pH 7.0, 20°C at a protein concentration of 2.5 mg/ml.

and, at calcium concentrations higher than 10 mM, a large increase in O_2 affinity was observed ($\Delta \log P_{50} = -0.83$), with a calcium coefficient [$\Delta \log P_{50}$/$\Delta \log(Ca)$] equal to –0.32 (Fig. 6). As far as the

cooperativity is concerned, it increased from 2.0 to 2.7 as calcium concentration became higher than 10 mM.

DISCUSSION

Haemolymph of the anomuran hermit crabs *Pagurus striatus* and *P. berhnardus* contains only the dodecameric Hc form (*15*), while that of the king crab *P. camtschaticae* shows both dodecameric and hexameric forms, in the 80/20 ratio (*4*). In the haemolymph of *D. calidus*, a mixture of dodecameric and hexameric Hc forms has been found, with an inverted ratio compared to that found in *P. camtschaticae* (Fig. 1). Thus, Hc forms appear less homogeneously distributed in anomuran haemolymph with respect to other decapod infraorders. In fact in brachyuran haemolymph the dodecameric Hc form always prevails on the hexameric one, as in *Callinectes sapidus* (*16*) and *C. granulata* (*13*), in astacidean haemolymph only the dodecameric form is present, as in *Homarus vulgaris* and *Astacus* (*17*), in palinuran haemolymph only the hexameric form is present, as in *Panulirus interruptus* (*18*) and *Panulirus japonicus* (*19*).

A 2D-electrophoresis performed on purified native *D. calidus* Hc revealed the presence of a series of not well-resolved polypeptide chains that focalized at the lower end of the pH gradient used (~pH 3.0), together with a number of similar molecular weight polypeptide chains, differing for their pIs in the range 4.5–5.5 (Fig. 1C). The presence of very acidic polypeptide chains has been also evidenced in the 2D-PAGE analysis performed under the same experimental conditions on *C. granulata* Hc, which showed a much higher number of different polypeptide chains, differing not only for their pIs, but also for their molecular weight (*13*). The very acidic polypeptide chains were not present in the 2D-PAGE of the centipede *Scutigera coleoptrata* Hc (pH range 3.5–10) which showed 10 distinct protein spots with molecular weights of 75–80 kDa and pIs in the pH range 5.0–6.5 (*20*). Even if only a reduced number of 2D-PAGE analysis have been reported up to now on arthropodan Hcs, it appears that this technique may represent a valuable method for analysing Hc heterogeneity.

Oxygen affinity of purified native *D. calidus* Hc appeared quite strongly modulated by protons ($\Delta\log P_{50}/\Delta pH = -0.97$, Fig. 3), in agreement with data reported for the haemolymph of a number of brachyuran (*21*) and anomuran as *Pagurus impressus*, *Pagurus longicarpus and Pagurus pollicaris* [$\Delta\log P_{50}/\Delta pH = -0.98$, -1.06 and -0.88, respectively, (*22*)], but differently from data reported for the anomuran *Clibanarius vittatus* [$\Delta\log P_{50}/\Delta pH = -0.54$, (*22*)], *P. camtschaticae* [$\Delta\log P_{50}/\Delta pH = -0.57$, (*4*)] and *P. bernhardus* [$\Delta\log P_{50}/\Delta pH = -1.55$, (*3*)].

Oxygen affinity of hexameric *D. calidus* Hc did not differ from that of purified native *D. calidus* Hc in the pH range examined, while that of dodecameric Hc resulted slightly lower, similarly to data reported for *Carcinus aestuari* dodecameric Hc form (*23*).

Oxygen-binding cooperativity of purified native *D. calidus* Hc resulted in the range of crustacean Hcs [$n_{50} = 2.0$–4.5, (*21*)], being similar to that of *C. vittatus* ($n_{50} = 3.0$), but much lower than that of *P. impressus*, *P. longicarpus and P. pollicaris* [$n_{50} = 4.5$, 4.7, and 4.7, respectively, (*22*)]. Moreover, *D. calidus* cooperativity was almost pH independent, in contrast with that of *P. berhardus* that showed n_{50} values increasing from 2.5 to 3.7 in the pH range 7.0–8.0 (*3*).

Purified hexameric and dodecameric *D. calidus* Hc forms showed higher cooperativity compared to purified native Hc (n_{50} for dodecameric Hc reaching the value of 4.6) with a significant pH dependence (n_{50} increasing from pH 7.0 to 8.5). A higher cooperativity of the dodecameric form has also been described in *C. sapidus* Hc (*16*).

L-lactate concentrations higher than 10 μM increased oxygen affinity of purified native *D. calidus* Hc, and the effect increased up to 5 mM L-lactate, with a lactate coefficient [$\Delta\log P_{50}/\Delta\log(\text{lactate})$ at pH 7.5] equal to -0.38. The physiological L-lactate concentration measured in *D. calidus* haemolymph resulted equal to 4.4 ± 0.8 mM, a value higher than that found in the majority of crustaceans previously examined. The lack of previous measurements on other anomuran species makes difficult to fully evaluate this result, but it should be considered that anomurans live in a shell like Gastropods, to protect their soft body parts from predators, and that in these conditions may be exposed to environmental hypoxia. Moreover, it should be considered that before proceeding with the withdrawal of haemolymph in anomuran species it is necessary to force the animals out of the shell that they inhabit, with an additional stress with respect to other crustaceans. On the basis of these considerations the results obtained suggested that L-lactate may be considered as an important *in vivo* Hc modulator in this species. The existence of L-lactate effect on oxygen affinity of crustaceans Hcs was first demonstrated by Truchot (*24*), and afterwards this organic acid was shown to increase the O_2 affinity of a number of Hcs, but not of all crustacean (*25*). Among anomuran, the only data reported so far concerned the positive effect of lactate on the oxygen affinity of *Petrolisthes eriomerus* haemolymph (*11*). At pH 7.5, in the presence of increasing concentrations of L-lactate, purified native *D. calidus* Hc cooperativity largely decreased (from 2.8 down to 1.7). A similar effect, although much reduced, was first described in *Cancer magister* Hc (*26*), and later in *C. sapidus* Hc (*25*, *27*), but was not detected in a number of other crustacean Hcs, *i.e*. *Homarus vulgaris*, *Maja squinado* (*28*), and *Palaemon serratus* (*29*).

Oxygen affinity of arthropodan Hcs generally decreases with temperature increase, due to the exothermic character of the oxygenation reaction, with ΔH values ranging between 0 and –158 kJ/mol (*21*). The overall enthalpy of oxygenation obtained for purified native *D. calidus* Hc (ΔH equal to -36.2 and -50.3 kJ/mol in the absence and presence of 5 mM L-lactate, respectively), indicated a significant temperature sensitivity of oxygen binding and suggested that the temperature-independence measured for *P. bernhardus* and *P. camtscaticae* Hcs may not be considered a general feature of anomuran Hcs. In this regard, it must be pointed out that these two anomuran species live in different habitats: *P. camtscaticae* lives in the Arctic Sea of the north Pacific in very stable environmental

Table 2. **Effect of temperature on the oxygen affinity of some decapod haemocyanins.**

Species	Habitat	ΔH (kJ/mol)	References
Infraorder Astacidea		−16.5 ± 10.7	
Nephrops norvegicus	Sublittoral	−5.0	(*37*)
Homarus gammarus	Sublittoral	−27.6	(*38*)
Homarus americanus	Sublittoral	−10.0	(*38*)
Homarus americanus/gammarus	Laboratory	−23.4	(*38*)
Infraorder Palinura		−53.4 ± 20.5	
Jasus edwardsii	Sublittoral	−31.1	(39)
Scyllarides latus	Sublittoral	−71.5	(*40*)
Panulirus interruptus	Sublittoral	−57.6	(*41*)
Palinurus elephas	Sublittoral	−65.0	This study
Palinurus mauritanicus	Mesopelagic	−64.3	This study
Infraorder Thalassinidea		−67.3 ± 15.8	
Upogebia deltaura	Sublittoral	−65.4	(*42*)
Upogebia stellata	Sublittoral	−82.1	(*42*)
Jaxea nocturna	Sublittoral	−79.6	(*42*)
Callianassa subterranea	Sublittoral	−78.2	(*42*)
Calocaris macandreae	Sublittoral	−56.8	(*42*)
Callianassa californiensis	Littoral	−42.0	(*43*)
Infraorder Anomura		−26.1 ± 18.9	
Galatea strigosa	Sublittoral	−18.0	(*37*)
Pagurus berhnardus	Littoral	0.0	(*3*)
Dardanus calidus	Sublittoral	−50.3	This study
Birgus latro	Terrestrial	−38.5	(*44*)
Coenobita clypeatus	Terrestrial	−42.8	(*44*)
Paralithodes camtschaticus	Sublittoral	0.0	(*4*)
Munida rugosa	Sublittoral	−25.5	(*45*)
Munida sarsi	Mesopelagic	−33.6	(*45*)
Infraorder Brachyura		−39.3 ± 36.0	
Goniopsis cruentata	Semiterrestrial	−47.0	(*46*)
Aratus pisoni	Semiterrestrial	−34.0	(*46*)
Cardisoma guanhumi	Semiterrestrial	−45.0	(*46*)
Gecarcinus ruricola	Semiterrestrial	−44.0	(*46*)
Gecarcoidea natalis	Semiterrestrial	−59.0	(*47*)
Ocypode saratan	Semiterrestrial	−18.0	(*48*)
Uca inversa	Semiterrestrial	−51.7	(*49*)
Metopograpsus messor	Semiterrestrial	−57.4	(*49*)
Holthuisana transversa	Semiterrestrial	−54.0	(*50*)
Cardisoma guanhumi	Semiterrestrial	−45.0	(*46*)
Leptograpsus variegatus	Intertidal	−67.4	(*51*)
Callinectes sapidus	Littoral	−55.0	(*52*)
Menippe mercenaria	Littoral	−54.0	(*35*)
Hemigrapsus nudus	Littoral	−30.0	(*5*)
Goneplax rhomboides	Littoral	−64.0	(*53*)
Cancer borealis	littoral/sublitt and mesopelagic	−130.0	(*35*)
Calappa granulata	Sublittoral	−118.5	(*13*)
Atelecyclus rotundatus	Sublittoral	−14.0	(*53*)
Corystes cassivelaunus	Sublittoral	−13.6	(*37*)
Liocarcinus depurator	Sublittoral	−7.0	(*53*)
Cancer magister	Sublittoral	−43.0	(*54*)
Carcinus maenas	Sublittoral	−10.0	(*55*)
Segonzacia mesatlantica	Hydrothermal vent	17.2	(*56*)
Cyanagraea praedator	Hydrothermal vent	1.2	(*34*)
Bythograea thermydron	Hydrothermal vent	35.0	(57)
Choracaris chacei	Hydrothermal vent	−14.0	(*58*)

Oxygenation enthalphy within each infraorder is expressed as mean value ± SD.

conditions [temperatures always comprised between 3°C and 10°C (*4*)], while *P. bernhardus* is an intertidal species that lives in European Atlantic Ocean under very variable environmental temperatures. The loss or the reduction of temperature effect on oxygen binding to respiratory proteins has been suggested to be an adaptation to rapid temperature variations, which could occur in intertidal habitat (*3*, *30*). Species living at more

constant environmental temperatures have shown either a pronounced temperature sensitivity, as the Antarctic isopod *Glyptonotus antarcticus*, living at very constant temperatures (between −2 and +2°C), that showed a ΔH equal to −158 kJ/mol (*31*), or reduced temperature sensitivities, as the anomuran *P. camtscaticae*, the pelagic mysid *Gnathopausia ingens*, living in deep-sea oxygen minimum layer characterized by a pO_2 as low as 6 mmHg and temperatures always comprised between 4°C and 6°C, that showed a ΔH equal to −6.7 kJ/mol (*32*), and the Antarctic krill *Euphasia superba*, that lives at very constant temperatures (as *G. antarcticus*), that showed a ΔH comprised between −15 and 0 kJ/mol (*33*). The large effect of temperature on the oxygen affinity of *G. antarcticus* has been related to the exceptional constancy of its environmental temperatures, that obviate for the need of an adaptive reduction in temperatures sensitivity that may be necessary in species exposed to wide variation in environmental temperatures in order to provide a sufficient loading/unloading of oxygen at higher temperatures (*31*).

A relative insensitivity to temperature variations of oxygen-binding to Hc was also observed for all the hydrothermal vent decapods studied so far and it has been interpreted as an advantage in an environment with large oxygen and temperature fluctuations (*34*).

The enthalpy of oxygenation of the anemone hermit crab *D. calidus* Hc is compared in Table 2 with other decapod ΔH values: small differences are observed between the species examined within the infraorder Thalassinidea (SD = 23%), that share a common habitat (littoral/sublittoral) while a much higher SD value (72%) was found for the species examined within the infraorder Anomura that highly differ in their habitat, as well as for the species examined within the infraorder Brachyura with a SD value of 92%. The most significant contributions to this high scattering are given by the endothermic oxygenation enthalpy values of different hydrothermal vent crab Hcs, *i.e*. *Cyanagraea praedator*, *Segonzacia mesatlantica* and *Bythograea thermydron* [1.2, 17.2 and 35 kJ/mol, respectively, (*34*)], and by the very high exothermic enthalpy values of *Cancer borealis* (*35*) and *C. granulata* Hcs (*13*), with respect to the mean ΔH values calculated for semi-terrestrial (−45.5 kJ/mol), littoral and sublittoral species (−35.8 kJ/mol).

Among the oxygen-binding proteins, crustacean Hcs have been showed to possess the largest extent of heterogeneity both at structural level, in terms of changes in subunit primary structure and/or in the ratios between subunits or oligomers, and at functional level in terms of their responses to different modulators affecting the oxygen-binding properties, as inorganic and organic ions, pH, sulphide, thiosulphide, neuro-hormones (*36*). Data reported in Table 2 evidenced that the influence of temperature on decapod Hc oxygen binding varies differently within the infraorders examined, strongly supporting that temperature should be included in the list of physiological modulators that contribute to the adaptive plasticity of Hcs.

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