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Thermodynamics of oxygenation-linked proton and lactate binding govern the temperature sensitivity of O_2 binding in crustacean (*Carcinus maenas*) hemocyanin

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SUMMARY

With the aim of understanding the molecular underpinnings of the enormous variation in the temperature sensitivity of hemocyanin–O_2 affinity encountered in crustaceans, we measured O_2 binding to *Carcinus maenas* hemocyanin at two temperatures, varying pH values and in the absence and presence of lactate ions in order to assess the contributions of oxygenation-linked binding of protons (the Bohr effect) and lactate ions to the overall enthalpies of oxygenation ($\Delta H'$). The hemocyanin binds maximally 0.35 lactate ions per functional subunit. Lactate (which accumulates under hypoxic conditions) increases O_2 affinity by preferentially raising the association equilibrium constant of the hemocyanin in the low-affinity Tense state (K_T), without significantly affecting that of the high-affinity Relaxed state (K_R). In the absence of lactate, the variation in the temperature sensitivity observed with decreasing pH tallies neatly with changes in the nature and magnitude of the Bohr effect. Accordingly, the normal, absent and reverse Bohr effects observed under alkaline, neutral and acid conditions, respectively, reflect endothermic proton dissociation, absence of proton binding and exothermic proton association, respectively, upon oxygen binding. Oxygenation-linked lactate binding is exothermic, highly pH dependent and peaks near pH 7.6, where it contributes approximately –30 kJ mol^{-1} to the overall heat of oxygenation. This predictably increases the temperature sensitivity of O_2 affinity, potentially hampering O_2 loading in warm, hypoxic habitats. The data demonstrate governing roles for lactate and proton ions in determining the temperature sensitivity of hemocyanin–O_2 affinity in crustaceans.

Key words: *Carcinus maenas*, crab, hemocyanin, lactate, oxygen binding, temperature effect.

INTRODUCTION

The oxygenation of gas-transporting proteins is a function of their intrinsic O_2 affinities, as well as their interactions with allosteric effectors. The latter interactions govern O_2 affinity, and may modulate O_2 loading at the respiratory surfaces and/or O_2 unloading and in the metabolizing tissues in response to specific environmental conditions and metabolic requirements. The interactions of hemoglobins (Hbs) and hemocyanins (Hcs) with protons and CO_2 (the Bohr effect, which decreases O_2 affinity and facilitates O_2 in the respiring tissues) are a well-known example.

Unlike the intensively investigated vertebrate Hbs, where protons, chloride and organic phosphate anions are major effectors that decrease Hb–O_2 affinity, crustacean Hcs commonly exhibit marked sensitivities to L-lactate and urate anions and to divalent cations that increase Hc–O_2 affinity (Mangum and Towle, 1977; Truchot, 1980; Mason et al., 1983). Lactate is an end product of anaerobic metabolism that enhances O_2 loading, so it may be a link in the negative feedback that favours aerobiosis in the face of decreasing O_2 availability (Truchot, 1980). In contrast to the tetrameric, intraerythrocytic vertebrate Hbs that consist of two α and two β chains and have a sedimentation constants of 4.4 S, crustacean Hcs commonly consist of 24 S dodecamers and/or 16 S hexamers which, respectively, comprise twelve and six 5 S polypeptide chains (Markl et al., 1979; Dainese et al., 1998; Molon et al., 2000; Podda et al., 2007). However, *Carcinus maenas* Hc consists of 24 S dodecamers only (Eriksson-Quensel and Svedberg, 1936; Markl et al., 1979).

A universal, although often neglected, factor governing O_2 affinity in ectothermic invertebrates is temperature. Based on the exothermic nature of their oxygenation reactions (Klotz and Klotz, 1955), the O_2 affinities of metal-containing, gas-binding proteins decrease with increasing temperature. Apart from the intrinsic heat of oxygenation (ΔH^{O_2}), the overall heat of oxygenation ($\Delta H'$) includes the heat of solution of oxygen (ΔH^{sol}, –13 kJ mol^{-1}) and heats of processes linked to oxygenation, such as the release or binding of protons [including the heat of ionization of buffers (ΔH^{H+})] and of other allosteric effectors ($\Delta H^{X,Y,etc}$).

The $\Delta H'$ values of crustacean Hc at physiological pH (7.6–7.8) vary tremendously (from –67 to +134 kJ mol^{-1}) (cf. Jokumsen and Weber, 1982; Burnett et al., 1988; Brix et al., 1989; Adamczewska and Morris, 1998; Chausson et al., 2004). The available literature has variously attributed adaptive advantages to both low and high temperature sensitivities, arguing that the former may stabilize tissue O_2 supply in the face of environmental thermal variations, and the latter may increase O_2 unloading in the respiring tissues, in parallel with temperature-induced increases in metabolic O_2 requirement. Extensive studies of vertebrate Hbs attribute adaptive reductions in temperature sensitivity to endothermic processes coupled to O_2 binding, such as changes in protein conformation (Wyman et al., 1977) or, more commonly, dissociation of proton, phosphate and chloride ions (Wyman, 1964; Weber et al., 1985; Fago et al., 1997b; Weber et al., 2003). However, no systematic studies appear to have been carried out on the interactive effects of ligand binding on the

enthalpies of arthropod Hcs, notwithstanding the enormous variation in their temperature sensitivities and the radically different allosteric control mechanisms encountered compared to Hbs. In contrast to vertebrate Hbs, where protons and organic phosphates preferentially decrease O_2 affinity of the Hb molecules in the low-affinity Tense state (K_T) without significantly affecting that of the Relaxed state (K_R), in crab *Callinectes sapidus* Hc, L-lactate ions increase both K_T and K_R, whereas protons decrease both K_T and K_R, and Ca^{2+} ions increase K_R (Johnson et al., 1988). Thus "*...the allosteric interactions of L-lactate and crustacean hemocyanins … provide an interesting contrast to the extremely well-studied allosteric interactions of the vertebrate hemoglobins*" (Graham, 1985).

Aiming to probe the contributions of the major endogenous allosteric effectors (protons and lactate) to the overall enthalpies of oxygenation, we measured the interactive effects of lactate, temperature and pH on the O_2 affinity of Hc in the blood of the shore crab *Carcinus maenas*, which is exposed to large variations in ambient temperature on a daily and seasonal basis.

MATERIALS AND METHODS

Animals

Specimens of shore crabs *Carcinus maenas* L., with carapace width of 5–7 cm, were collected in Aarhus Bay, Denmark, and maintained in well-aerated seawater (30–32 p.p.t., 12°C, P_{O_2} >135 mmHg; 12 h:12 h photoperiod) for at least 4 days without feeding. Prebranchial hemolymph samples (1–2 ml) were withdrawn from the branchial sinuses by piercing the arthropodial membrane at the bases of the walking legs using a syringe fitted with a 22-G hypodermic needle. Samples from individual crabs were kept separately on ice. Those that did not coagulate were pooled into a Petri dish and whipped with a glass spatula to separate coagulating fibrinogen that was removed by filtration. Hemolymph was frozen at –80°C in 120 μl aliquots that were freshly thawed for oxygen binding studies.

[Hc] and [L-lactate] measurements

Hc concentrations were estimated from peak absorbances near 335 nm, using an extinction coefficient of 17.5 mmol l^{-1} cm^{-1}, based on the $A_{1\%,1cm}$ value of 2.33 reported for *C. maenas* Hc (Nickerson and Van Holde, 1971) and a functional-unit mass of 75 kDa. Reagent grade L(+)-lactate, lithium salt ($C_3H_5O_3Li$) was purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). L-lactate concentration in freshly collected hemolymph samples of crabs kept in normoxic seawater) was measured according to Lowry and Passonneau's 'Method I' (Lowry and Passonneau, 2006).

O_2 binding measurements of native hemolymph

Hemolymph samples were buffered at varying pH by adding 1 mol l^{-1} Bis-Tris buffers to a final buffer concentration of 0.1 mol l^{-1}. O_2 equilibria of 4 μl hemolymph samples were recorded at 10 and 20°C using a modified gas diffusion chamber connected to Wösthoff gas mixing pumps (Bochum, Germany) that mix air and ultrapure (>99.998%) N_2 to increase O_2 tensions stepwise (Weber, 1981; Weber et al., 1987), while absorbance is continuously monitored at 365 nm. pH values were measured in triplicate in separate 110 μl sub-samples (to avoid KCl contamination from the calomel electrode) at the same temperatures as the O_2 equilibrium measurements, using a BMS 2 Mk 2 microelectrode coupled to a PHM 64 Research pH meter (Radiometer, Copenhagen, Denmark). For each O_2 binding curve at least four equilibrium steps between 20% and 80% saturation were recorded, and P_{50} and n_{50} values (O_2 tensions and Hill's cooperativity coefficients at 50% O_2 saturation, respectively) were interpolated from Hill plots, $\log[S/(1–S)]$ *vs* $\log P_{O_2}$, where S is the fractional O_2 saturation.

The effects and stoichiometry of L-lactate binding were investigated by recording Hc–O_2 equilibria at varying L-lactate concentrations, and at pH values close to 6.9 and 7.7, and thereafter interpolating the P_{50} and n_{50} values at these exact pH values (6.90 and 7.70) from regression analysis of $\log P_{50}$ *vs* pH.

For precise O_2 equilibrium measurements focusing on extreme, low and high O_2 saturations (extended Hill plots, see below), a subsample of the freshly prepared hemolymph was concentrated approximately twofold by centrifugation at 2500 ***g*** and 4°C for 1 h in Ultrafree-4 Millipore tubes with 10 000 Da molecular mass cut-off membranes.

The data were analysed in terms of the two-state MWC model, according to the equation:

$$S = [LK_TP(1+K_TP)^{(q-1)} + K_RP(1+K_RP)^{(q-1)}] / [L(1+K_TP)^q + (1+K_RP)^q] , \quad (1)$$

where S denotes saturation, L is the equilibrium constant between the tense (T) and relaxed (R) state in fully deoxygenated form, K_T and K_R are the O_2 association equilibrium constants for the low-affinity (T, tense) and high-affinity (R, relaxed) forms, respectively, P is the partial pressure of O_2, and q is the number of interacting binding sites (Monod et al., 1965). Curve-fitting to obtain K_T, K_R and the allosteric constant L, estimation of the standard errors and calculation of the derived parameters P_{50}, P_m (the median O_2 tension), n_{50}, n_{max} (the maximum cooperativity along the equilibrium curve) and ΔG (the free energy of cooperativity) were carried out as detailed earlier (Weber et al., 1995; Fago et al., 1997a; Bugge and Weber, 1999).

The overall heat of oxygenation at varying pH values (range 6.1–8.6) and in the presence and absence of 10 mmol l^{-1} L-lactate was evaluated from the difference in P_{50} values at 10 and 20°C, using the van't Hoff isochore (Wyman, 1964):

$$\Delta H' = 2.303R \cdot \Delta \log P_{50} / \Delta(1/T) , \quad (2)$$

where R is the gas constant and T the absolute temperature. The heat of effector binding was then interpolated as the difference between $\Delta H'$ values in the presence and absence of the effector (cf. Weber et al., 1985).

RESULTS AND DISCUSSION

Allosteric lactate interaction

Lactate concentration in hemolymph of crabs kept in aerated seawater was 0.034±0.023 mmol l^{-1} (N=5), confirming the low level previously reported for normoxic, resting *C. maenas* [0.1 mmol l^{-1} (Lallier and Truchot, 1989)].

Dose–response curves measured at two pH values (Fig. 1) show that lactate increases O_2 affinity, exerting the greatest effect in the 50–100 mmol l^{-1} concentration range, without markedly affecting the Bohr factor (depicted by the vertical distance between the two curves). Interpolated from the slopes of $\log P_{50}$ *vs* log[lactate] plots in this range, *C. maenas* Hc binds maximally 0.30 and 0.35 lactate ions per O_2 molecule at pH 7.0 and 7.7, respectively. Plotting $\log P_{50}$ values against free lactate concentrations (estimated assuming that each functional Hc subunit binds 0.3 lactate ions at half-saturation) does not tangibly alter these coefficients, which compare with values of 0.11–0.54 observed in decapod crustacean Hc under different experimental conditions [(cf. Zeis et al., 1992; Adamczewska and Morris, 1998) and studies cited therein].

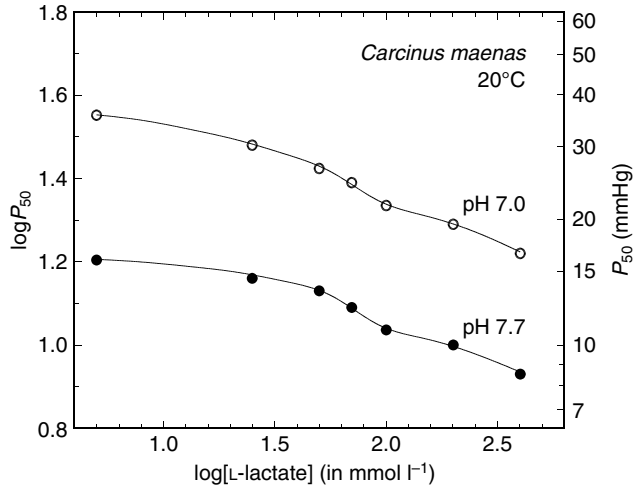

Fig. 1. (A) Effects of increasing lactate concentrations on hemolymph P_{50} values at pH 7.00 and 7.70 and 20°C. Hc subunit concentration, 0.85 mmol l^{-1}.

Extended Hill plots of precise O_2 equilibria of the hemolymph at pH 7.84 (Fig. 2) show slopes of unity in the lower and upper extremities of the curves, reflecting non-cooperative binding of the first and last O_2 molecules to the functional units. Fitting the MWC model to the data in the absence and in the presence of 10 and 50 mmol l^{-1} lactate, yields q-values (the number of interacting O_2-binding sites) of 4.5, 4.7 and 3.2, respectively (Table 1). Fitting the model with q fixed at 4 (the average integral value found when the model was fitted with q floating) shows P_{50} to be 7.7 mmHg, O_2 binding to be strongly cooperative (n_{50} and n_{max}=3.2), and O_2 association constants for the Hc in the deoxy (Tense) and the oxygenated (Relaxed) states (K_T and K_R) to be 0.01 and 1.61 mmHg^{-1}, respectively (Table 1). The identity between n_{50} and n_{max} values and the close agreement between P_{50} and P_m values (Table 1) indicate symmetry of the O_2 binding curves, justifying rigorous analysis of the allosteric effects in terms of P_{50} values (cf. Wyman, 1964).

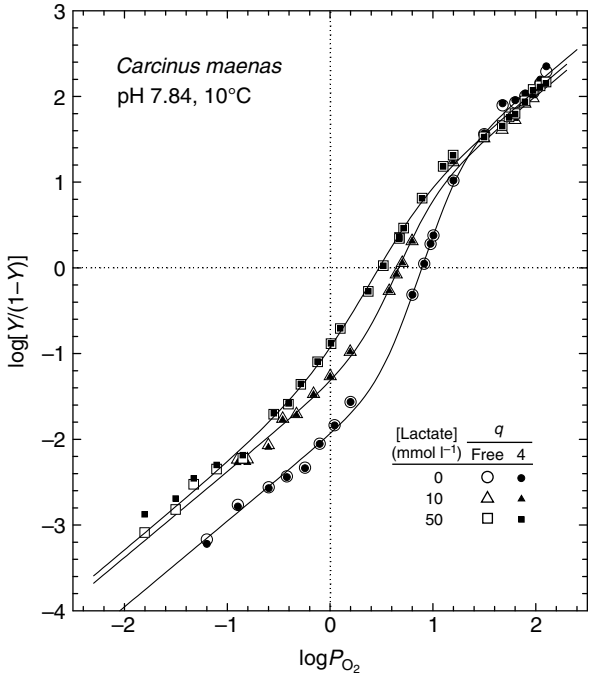

Fig. 2. Extended Hill plots of *C. maenas* Hc measured at 10°C in the absence (circles) and presence of 10 mmol l^{-1} (triangles) and 50 mmol l^{-1} (squares) L-lactate. Hc subunit concentration, 2.76 mmol l^{-1}. Open and closed symbols are data points where the MWC equation was fitted with q floating and fixed at 4, respectively, during the iteration (see Eqn 1). Mean pH=7.843±0.009.

Calculated as $\Delta G=RT\ln[<(L+1)(Lc^q+1)>/<(Lc+1)(L^{q-1}+1)>]$, where L is the allosteric constant and $c=K_T/K_R$ (Imai, 1982), the free energy of cooperativity equals 11.84 kJ mol^{-1} in the absence of lactate and q=4 (Table 1). As shown (Fig. 2, Table 1) lactate increases O_2 affinity (decreases P_{50}) by lowering L and raising K_T, without markedly markedly altering K_R. This indicates that the underlying molecular mechanism of the lactate effect is to shift the allosteric equilibrium towards the high affinity R state and to increase the O_2 affinity of the T state molecules, indicating that the T-state Hc becomes less stable in the presence of added lactate. The decreased K_R/K_T ratio decreases n_{50} values (slopes of the plots at log[Y/(1–Y)]=0) from 3.2 in the absence of lactate to 2.4 and 2.2 in the presence of 10 and 50 mmol l^{-1} lactate, respectively, and correspondingly lowers ΔG from 11.84 to 7.90 and 6.77, respectively.

Interactive effects of lactate and pH

Measurement of the O_2 affinity of *C. maenas* Hc at 10 and 20°C, in the absence and presence of lactate over a wide pH range where the Bohr effect (oxygenation-linked proton binding) changes from normal to absent and reverse (Fig. 3) provides an ideal opportunity to analyse the contributions of individual, oxygenation-linked processes to the overall enthalpy of oxygenation.

pH

As shown (Fig. 3) the Hc exhibits a strong normal Bohr effect φ (=$\Delta\log P_{50}/\Delta$pH)=–0.80 at alkaline pH range, which encompasses physiological values in decapods [7.44–7.84 (Mangum and Shick, 1972)], no Bohr effect (φ=0) at pH 6.9, and a distinct, reversed Bohr effect (φ=+0.46) at lower pH. The data show a drastic increase in the temperature effect with decreasing pH (Fig. 4), indicating that high temperatures will adversely affect O_2 loading under acidotic and hypoxic conditions.

Fig. 5 shows the relationship between $\Delta H'$ and pH in the absence and presence (Fig. 5 top, curves A and B, respectively) of lactate. In the absence of lactate (Fig. 5 top, curve A) the numerical value of $\Delta H'$ increases from ~–10 kJ mol^{-1} at pH 8 to plateaus of approx. –42 at pH 7.0 and ~60 at pH 6.5, revealing a clear inverse relationship with the Bohr factors (–0.8, 0 and +0.46) at these respective pH values. This indicates that (i) as with vertebrate Hb, oxygenation-linked proton dissociation is endothermic and thus counterbalances the heat released upon Hc oxygenation (ΔH^{O_2}) in the pH range where the Bohr effect is normal, (ii) the overall $\Delta H'$ value at pH 7.0 is devoid of contributions from proton binding (Δ^{H+}) and thus reflects the intrinsic heat of O_2 binding by the Hc, (iii) oxygenation-linked proton association (reverse Bohr effect) is exothermic and thus increases the temperature effect contributing about +18 (=–60 to –42) kJ mol^{-1} and (iv) in the absence of other effectors, proton concentrations play the dominating role in determining the temperature sensitivity of O_2 affinity in crustacean Hc.

This contention is supported by numerous data in the literature. Particular examples are Hcs of (i) crab *Holthuisana transversa*, where an exceptionally low Bohr factor (–0.13) correlates with

Table 1. MWC and derived parameters for *Carcinus maenas* hemolymph with 0, 10 and 50 mmol l^{-1} L-lactate at 10°C

[L-lactate] (mmol l^{-1})	pH	P_{50} (mmHg)	P_m (mmHg)	n_{50}	n_{max}	logK_T	logSE	logK_R	logSE	log$L$	q*	ΔG (kJ mol^{-1})
A												
0	7.83	7.81	7.83	3.4	3.4	–1.9651	0.05169	0.1483	0.0879	4.6990	4.51	11.39
10	7.85	4.66	4.69	2.6	2.6	–1.3854	0.0839	0.0128	0.1010	3.1980	4.67	7.51
50	7.85	3.00	3.11	2.0	2.0	–1.4589	0.2894	0.0903	0.0455	1.8727	3.22	7.45
B												
0	7.83	7.69	7.70	3.2	3.2	–1.9861	0.0505	0.2055	0.0775	4.3670	4	11.84
10	7.85	4.63	4.67	2.4	2.4	–1.4364	0.0700	0.0495	0.0817	2.8739	4	7.90
50	7.85	3.00	3.10	2.2	2.2	–1.2314	0.0604	0.0708	0.0347	2.2671	4	6.77

*The MWC model was fitted to the data with q (the number of interacting O_2-binding sites) floating (A) or fixed at 4 (B).
SE, standard errors (of K_T and K_R values); for other abbreviations, see text.

highly exothermic oxygenation reactions ($\Delta H'$=–48 to –62 kJ mol^{-1}) observed over a range of operating conditions (Morris et al., 1988), (ii) hermit crab *Pagurus bernhardus*, where a massive Bohr effect [φ=–1.55 (Jokumsen and Weber, 1982)] tallies with a complete lack of temperature sensitivity at pH 7.2–7.8, whereas the temperature effect is large at low pH where the Bohr effect is zero (A. Jokumsen and R.E.W., unpublished), and (iii) krill *Meganyctiphanes norvegica*, and the hydrothermal vent crab *Segonzacia mesatlantica,* where even larger Bohr factors (φ=–1.9 and –2.9, respectively) are associated with overall endothermic heats of oxygenation [$\Delta H'$=+134 and +17 kJ mol^{-1} (Brix et al., 1989; Chausson et al., 2004)].

The differences between $\Delta H'$ in the presence and absence of lactate (depicted by curve B–A, Fig. 5 bottom) reflects the pH-dependent heats of reaction with lactate ions and of coupled reactions. As evident, lactate drastically raises the $\Delta H'$ values at alkaline (physiological) pH. Given that lactate increases O_2 affinity (Fig. 1) and thus undergoes oxygenation-linked binding (and deoxygenation-linked dissociation), the data show that lactate binding is exothermic, strongly pH-dependent, and peaks at a pH of approximately 7.6, where it contributes approximately –30 kJ mol^{-1} per O_2 molecule bound/released and decreases at higher and lower pH values. The exothermic, oxygenation-linked binding of lactate ions that increases the overall oxygenation enthalpy in crab Hc contrasts sharply with vertebrate Hbs, where endothermic, oxygenation-linked dissociation of organic effectors (2,3-diphosphoglycerate, DPG, and ATP) reduces the overall heat of oxygenation and thus the temperature sensitivity of O_2 affinity.

The single previously reported value for the enthalpic contribution of lactate binding to Hc [–25 kJ mol^{-1} at pH 7.5 for Hc of the crab *Calappa granulata* (Olianas et al., 2006)] is in good agreement with our data. Remarkably, when measured directly by calorimetric methods, the enthalpy change for urate binding to *Homarus vulgaris* Hc is much higher [135 kJ mol^{-1} at pH 8.0 (Menze et al., 2001)]. The enthalpies of lactate binding to crab Hc are also considerably lower than for DPG binding to human Hb, where similar values (~46–55 kJ mol^{-1}) were obtained from van't Hoff plots and calorimetric methods (Benesch et al., 1969; Bunn et al., 1971; Nelson et al., 1974). Literature on mammalian Hbs illustrate how the tyranny of the van't Hoff equation may be bypassed by endothermic processes (e.g. conformational changes and proton and chloride binding) (Wyman et al., 1977; Ikeda-Saito et al., 1983; De Rosa et al., 2004) that reduce the temperature sensitivity of O_2 affinity. Arguably, a low $\Delta H'$ value is an advantageous trait for O_2 loading at high temperature when an effector (such as lactate) increases O_2 affinity and thereby raises the exothermic nature of oxygenation,

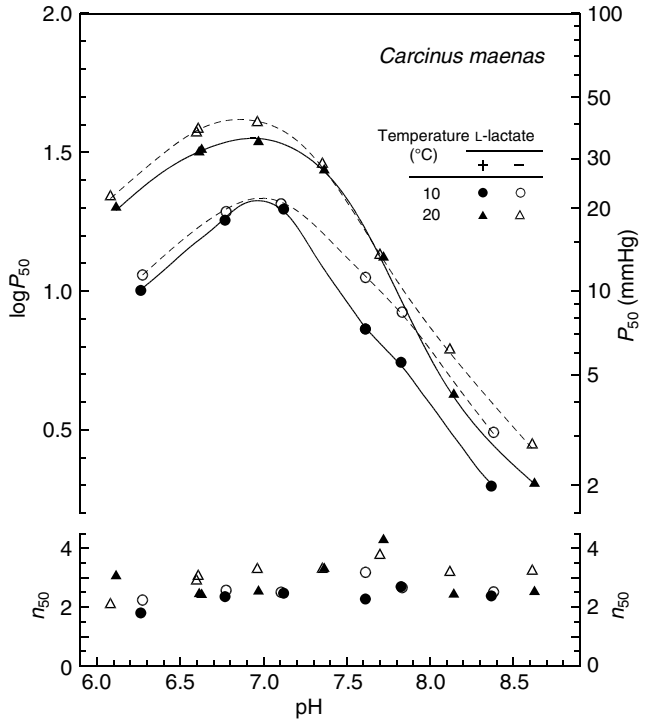

Fig. 3. P_{50} and n_{50} values of *C. maenas* Hc measured at 10°C (circles) and 20°C (triangles) in the absence of added lactate (open symbols) and presence (closed symbols) of 10 mmol l^{-1} L-lactate. Hc subunit concentration, 1.11 mmol l^{-1}.

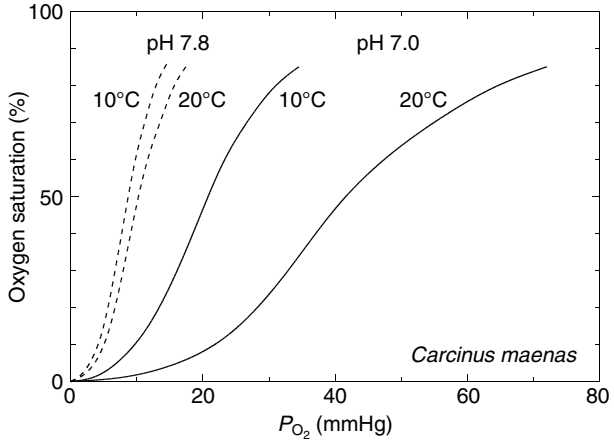

Fig. 4. O_2 equilibrium curves of *C. maenas* Hc at 10 and 20°C and pH 7.8 and 7.0, interpolated from data in Fig. 3.

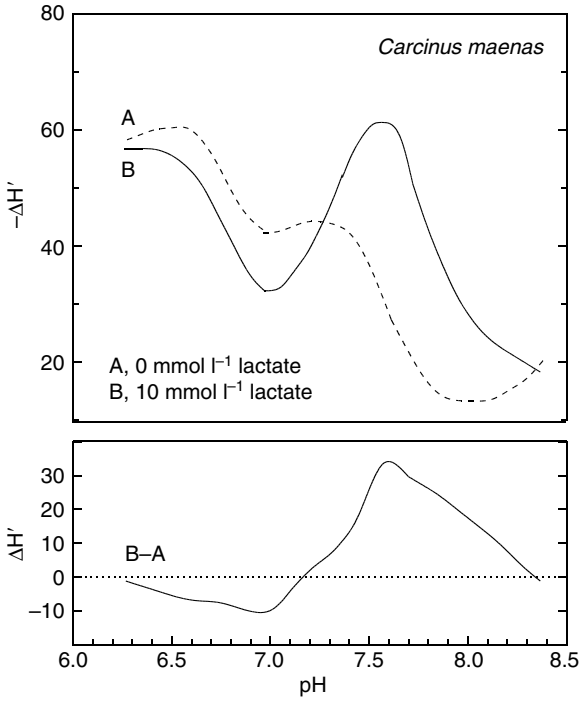

Fig. 5. (Top) Overall oxygenation enthalpies (ΔH′) derived using the van't Hoff isochore and P_{50} values at 10 and 20°C and different pH values (Fig. 3), in the absence of added lactate (A) and the presence of 10 mmol l^{-1} lactate (B). (Bottom) The interpolated, pH-dependent difference between these values (B–A).

just as low ΔH values resulting from ligand reactions coupled to oxygen binding are advantageous for maintaining a high O_2 affinity in vertebrate Hbs.

Although lactate that is produced under anaerobic conditions increases Hc–O_2 affinity and thus may favour O_2 loading and survival under hypoxic conditions (Truchot, 1980), our data show that a thermodynamic consequence of oxygenation-linked lactate binding is an increased temperature sensitivity in the physiological pH range (cf. Fig. 5), which may compromise O_2 loading at high temperature. While this specific 'lactate effect' (ΔH^{lact}) is maximal near 7.5, the overall temperature sensitivity (ΔH′) remains high at lower pH values (imparted by lactate accumulation), where the Bohr effect successively disappears and reverses (resulting in loss of O_2-linked endothermic proton dissociation and initiation of ectothermic proton binding, respectively). However, apart from hampering O_2 binding at high temperature, increased temperature sensitivity will promote O_2 loading in the gills at low temperature. In this regard it may be significant that increases in lactate levels may induce behavioural hypothermia, since lactate injection reduces the preferred temperature in *C. maenas* (De Wachter et al., 1997).

In conclusion, the large variations in the effects of temperature on O_2 affinity of crustacean Hcs (observed between different species and within the same individuals at different times) appear to be directly related to variations in the heats of reaction of processes coupled to the oxygenation reaction, notably proton and lactate binding, indicating that hemolymph levels of these ions are major factors controlling the temperature sensitivity of oxygen binding to crustacean hemocyanin.

In this light it would be interesting to investigate the enthalpic components in Hcs that lack a lactate effect [as in the hydrothermal vent crab *Syanagraea praedator* (Chausson et al., 2001)] or have an opposite lactate effect [as in the land crab *Gecarcoidea natalis*, where lactate decreases O_2 affinity (Adamczewska and Morris, 1998)] as well as the thermodynamic consequences of binding other Hc effectors, such as divalent cations, whose concentrations may vary greatly (with water salinity, ionoregulatory capacities and the moult cycle) and urate, which may bind to Hc with a higher affinity than L-lactate [40-fold higher in *Homarus vulgaris* Hc (Nies et al., 1992)]. In *Carcinus maenas*, whose Hc has about twice as many urate (and caffeine) binding sites than that of *H. vulgaris* (Hellmann et al., 2004), hemolymph urate concentrations increase drastically in response to hypoxic exposure (Lallier et al., 1987). In line with the effect of lactate at pH >7.2 reported here (Fig. 5), urate increases the temperature sensitivity of slipper lobster *Scyllarides latus* Hc, which has four urate binding sites per hexamer, at pH 7.5 (Sanna et al., 2004).

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