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BINDING OF URATE AND CAFFEINE TO HAEMOCYANIN ANALYSED BY ISOTHERMAL TITRATION CALORIMETRY

MICHAEL A. MENZE[1,*], NADJA HELLMANN[2], HEINZ DECKER[2] AND MANFRED K. GRIESHABER[1]

[1]*Institut für Zoophysiologie, Heinrich-Heine Universität, Universitätsstraße 1, 40225 Düsseldorf, Germany* and
[2]*Institut für Molekulare Biophysik, Johannes-Gutenberg Universität, Jakob-Welder Weg 26, 55128 Mainz, Germany*

*Author for correspondence at address 1 (e-mail: mmenze@hotmail.com)

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Summary

Haemocyanin serves as an oxygen carrier in the haemolymph of decapod crustaceans. The oxygen-binding behaviour of the pigment is modulated by the two major anaerobic metabolites, L-lactate and urate. The binding of these two metabolites to haemocyanin has been investigated mainly indirectly by following the effector-induced changes in the oxygen-binding properties of the respiratory pigment. Only a few direct investigations of effector binding, employing ultracentrifugation techniques and equilibrium dialysis, have been carried out. No evidence for cooperative binding for either effector was detected using these methods. However, isothermal titration calorimetry (ITC) offers a useful tool to gain additional insight into the binding of effectors to these highly allosterically regulated macromolecules. By applying the ITC method to the fully oxygenated dodecameric haemocyanin of the lobster *Homarus vulgaris*, cooperativity in binding has been found for the urate analogue caffeine but not for urate itself: using urate and the urate analogue caffeine as ligands, two conformations of the oxygenated pigment were detected.

Key words: haemocyanin, isothermal titration calorimetry, oxygen binding, effector, caffeine, urate, cooperativity, crustacean, lobster, *Homarus vulgaris*.

Introduction

Two major anaerobic metabolites, L-lactate and urate, modulate the oxygen affinity of haemocyanin in some crustacean species (Truchot, 1980; Morris et al., 1985; Bridges and Morris, 1986; Morris and Bridges, 1994). L-Lactate, which accumulates as the main glycolytic end-product in anaerobic *Carcinus maenas*, *Cancer pagurus*, *Callinectes sapidus* and other crustaceans, lowers the P_{50} of the respiratory pigment independently of the proton concentration and reduces the cooperativity of oxygen binding (Truchot, 1980; Johnson et al., 1984). The lactate-binding site was found to be stereospecific, so a precise arrangement of both the hydrogen atom and the methyl group at the chiral carbon atom was required for full activity of the ligand (Graham et al., 1983; Graham, 1985).

Urate also increases the oxygen affinity of the haemocyanin of some crustaceans, as first described by Morris et al. (1985) in the freshwater crayfish *Austropotamobius pallipes*. Similar results were obtained in *Carcinus maenas*, *Homarus vulgaris* and several other crustaceaceans (Lallier and Truchot, 1989; Bridges, 1990; Nies et al., 1992). Urate accumulates in the haemolymph of these species (Lallier et al., 1987; Zeis, 1994) during hypoxic conditions, when uricase activity is diminished because its second substrate, oxygen, is scarce (Dykens, 1991). Several related purine bases and analogues such as caffeine were examined for their effectiveness in modulating the oxygen affinity of the haemocyanin of *A. pallipedes* (Morris et al., 1985, 1986; Morris, 1990). The specificity for a purine derivative structure was found to be low, but the purine ring system was essential for modulating haemocyanin oxygen-binding. The simultaneous presence of both *in vivo* factors, L-lactate and urate, had an additive effect on haemocyanin oxygen-binding in *H. vulgaris*; however, the increase in the oxygen affinity of the pigment was finite (Zeis et al., 1992).

The haemocyanin of arthropods consist of one, two, four, six or eight basic hexameric assemblies (Markl and Decker, 1992; Van Holde and Miller, 1992; Van Holde and Miller, 1995). Binding studies using ultracentrifugation techniques and equilibrium dialysis revealed two binding sites each for L-lactate and urate per dodecameric haemocyanin molecule of *H. vulgaris*, which suggest a 1:1 stoichiometry in binding between the effectors and the basic allosteric unit, the hexamer. The dissociation constants were $0.87\,\text{mmol}\,\text{l}^{-1}$ for L-lactate and $0.03\,\text{mmol}\,\text{l}^{-1}$ for urate. No evidence for cooperativity in ligand binding was detected using this method for either effector (Nies et al., 1992). Furthermore, the binding of urate led to a significant decrease in the cooperativity of oxygen binding and in the magnitude of the Bohr effect in *H. vulgaris* (Zeis et al., 1992; Nies et al., 1992).

A few other studies have examined the stoichiometry and

affinity of haemocyanin/effector interactions. In *Panulirus interruptus*, the California spiny lobster, which possesses a hexameric haemocyanin consisting of three different subunits (a, b and c), one lactate-binding site was found per hexamer. However, an artificial homo-hexamer of subunit b also contained one lactate-binding site per hexamer. The absence of a 1:1 ratio between the number of lactate-binding sites and the number of subunits suggests that the lactate-binding site occurs between subunits within the quartenary structure of the protein (Johnson et al., 1987). These findings support the concept that L-lactate acts as a classic allosteric effector, which means that the effector binds preferentially to oxyhaemocyanin at a site other than the oxygen-binding site and between subunits rather than to a specific site, stabilizing a particular allosteric conformation of the protein. However, approximately three L-lactate-binding sites were found per hexamer in *Callinectes sapidus* haemocyanin, with a dissociation constant of 3.2 mmol l^{-1} for each site (Johnson et al., 1984).

By performing urate competition experiments using several urate analogues, it was shown that the purine ring system is essential for interaction with the urate-binding site. The urate analogue caffeine was found to bind with a higher affinity to the urate-binding site than the naturally occurring effector and to be more effective in modulating the cooperativity and affinity of haemocyanin oxygen-binding (Zeis, 1994).

Additional insights into haemocyanin/effector interactions were gained using isothermal titration calorimetry. Binding of oxygen to the haemocyanin of *H. vulgaris* was known to be linked both to the proton concentration and to the concentration of urate or caffeine. Furthermore, the magnitude of the Bohr effect was found to be dependent on the urate concentration (Zeis et al., 1992; Zeis, 1994). To evaluate whether there was any link between the effectors (urate or caffeine) and protons at the level of the haemocyanin conformation dominant under oxygenated conditions, the binding of effector to the fully oxygenated haemocyanin of *H. vulgaris* was investigated at different pH values. The same study investigated whether any groups with a pKa in the physiologically relevant pH range (7.55–8.15) were involved in the binding of the effectors urate or caffeine to the haemocyanin of *H. vulgaris* (Menze et al., 2000).

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) can be used to characterise the interaction between a biological macromolecule and a small ligand. This method lends itself well to the simultaneous determination of the binding constant (K), the stoichiometry of ligand/protein interaction (n) and ΔH^0, the reaction enthalpy (Wiseman et al., 1989; Freire et al., 1990; Ladbury and Chowdhry, 1996). The entropy ΔS^0 and free energy ΔG^0 of binding are obtained from:

$$\Delta G^0 = -\boldsymbol{R}T\ln K = \Delta H^0 - T\Delta S^0 , \quad (1)$$

where T is the absolute temperature (K) and $\boldsymbol{R}$ is the gas constant.

In an ITC experiment, heats of reaction are measured as temperature changes during repeated injections of a fixed amount of ligand into a solution of the macromolecule. The integrated heat signals have to be corrected for contributions from temperature differences between the cell and the syringe (injection of buffer into buffer) and for heats of dilution (titration of ligand into buffer and buffer into protein solution). The resulting titration curves can be analysed by using a multiple non-interacting site model or a multisite interactive model. For the simplest model of n identical binding sites, the heat q developed on adding ligand to the protein solution, raising the free ligand concentration to [L], is given by:

$$q = \frac{n[\mathrm{M_T}]V\Delta H^0 K[\mathrm{L}]}{1 + K[\mathrm{L}]} , \quad (2)$$

where the total macromolecule concentration in the binding process is $[\mathrm{M_T}]$ with n binding sites. The binding process is characterised by a microscopic binding constant K ($\mathrm{l\,mol^{-1}}$) and an enthalpy ΔH^0 ($\mathrm{kJ\,mol^{-1}}$) in a total volume V (l). The equation for the multisite interactive model is given by:

$$\frac{q}{[\mathrm{M_T}]V} = \frac{(\Delta H^0{}_1)K[\mathrm{L}] + (\Delta H^0{}_1 + \Delta H^0{}_2)K_1K_2[\mathrm{L}]^2 + (\Delta H^0{}_1 + \Delta H^0{}_2 \ldots + \Delta H^0{}_\mathrm{i})K_1K_2 \ldots K_\mathrm{i}[\mathrm{L}]^\mathrm{i}}{1 + K_1[\mathrm{L}] + K_1K_2[\mathrm{L}]^2 + K_1K_2 \ldots K_\mathrm{i}[\mathrm{L}]^\mathrm{i}} , \quad (3)$$

where $\Delta H^0{}_\mathrm{i}$ is the molar enthalpy of binding to the ith site and K_i is the corresponding stoichiometric binding constant ($\mathrm{l\,mol^{-1}}$). The general relationship between the stoichiometric and the microscopic binding constants for n identical interacting sites is given by:

$$K_\mathrm{i} = \frac{n - (i-1)}{i} K, \quad (4)$$

where i is the successive binding step, K_i is the corresponding stoichiometric binding constant, K is the microscopic binding constant and n is the number of binding sites on the macromolecule. Equations 2 and 3 relate the measured heat q to the free ligand concentration [L]. In the most general case, the dependence of q on the total ligand concentration $[\mathrm{L_T}]$ cannot be solved numerically. Therefore, the numerical bisection method must be included into the fitting routine. Since, in the experiment, the total concentration of ligand $[\mathrm{L_T}]$ is achieved in a stepwise manner, the experimental curves correspond to the differential forms of equations 2 and 3. A detailed discussion of these equations is presented by Indyk and Fisher (1998).

The shape of an ITC binding curve is determined by the Wiseman value c (Wiseman et al., 1989). This value is the product of the number of ligand-binding sites on the macromolecule (n), the microscopic binding constant (K) and the concentration of macromolecule ($[\mathrm{M_t}]$) used in the experiment:

$$c = nK[\mathrm{M_t}] , \quad (5)$$

Fig. 1 shows the dependence of the shape of the curve of a binding isotherm on the value of c. Two non-interacting binding sites (n=2) with an enthalpy change on binding of $-70\,\mathrm{kJ\,mol^{-1}}$ injectate were assumed. Each point represents the heat (Δq)

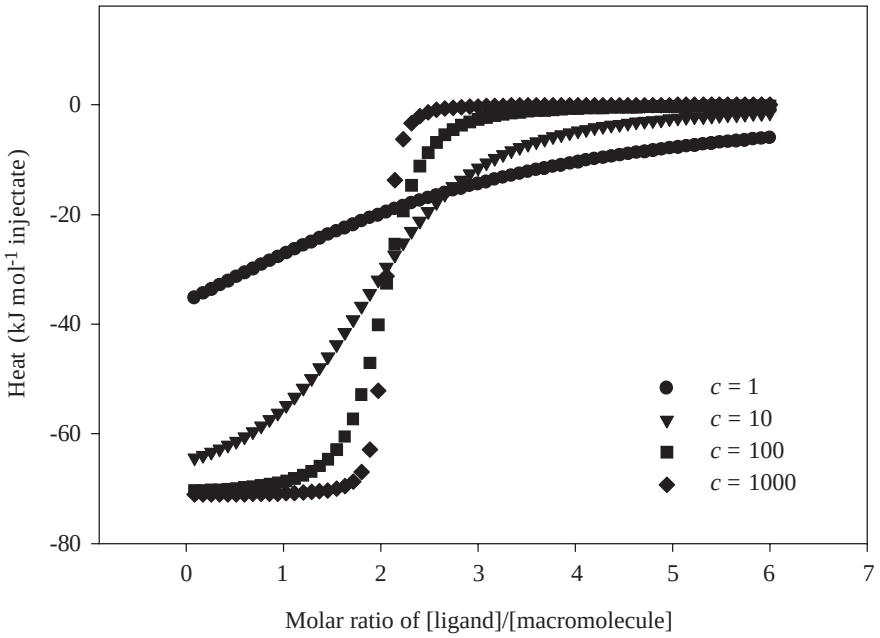

Fig. 1. Titration isotherms computed for a ligand/macromolecule system with an enthalpy change on binding (ΔH^0) of $-70\,kJ\,mol^{-1}$ injectate and two non-interacting binding sites (n=2) for the ligand. The isotherms were computed for measurements at different values of the parameter c (see equation 5) (Wiseman et al., 1989). See text for further explanation.

liberated per mole injectate for an incremental addition of the injectate *versus* the ratio of the accumulated total injectate concentration to the concentration of macromolecule. This form is the first derivative of a conventional binding curve, in which the total accumulated heat liberated is plotted against the total accumulated injectate concentration. If the binding process is investigated at a c value of approximately 1, an almost hyperbolic binding curve is obtained. With increasing values of c, the binding isotherms become sigmoidal. To obtain usable data, the c value of the binding curve should be between 1 and 1000 to avoid multiple minima during the fitting process (Wiseman et al., 1989) and, at the end of a titration experiment, the ratio of the concentration of ligand to the concentration of macromolecule in the cell should be at least twice the number of binding sites on the macromolecule.

Furthermore, if any proton exchange between the buffer medium and the macromolecule occurs during ligand binding, the measured enthalpy of binding depends on the ionisation enthalpy of the buffer used (Hinz, 1983; Fisher and Singh, 1995):

$$\Delta H^0_{app} = \Delta H^0_{int} + n_H \Delta H^0_{ionz}, \quad (6)$$

where n_H represents the number of protons released or absorbed by the buffer system. In this case, the apparent enthalpy of binding (ΔH^0_{app}) includes both the intrinsic enthalpy of effector binding (ΔH^0_{int}) and the enthalpy of ionisation of the buffer system (ΔH^0_{ionz}).

Titration of the dodecameric haemocyanin of *Homarus vulgaris* with caffeine and urate

Caffeine is a strong effector of the oxygen-binding behaviour of the dodecameric haemocyanin of the lobster *H. vulgaris* and binds with a higher affinity to the pigment than does the naturally occurring effector urate. At sufficiently high concentrations, caffeine is able to inhibit urate binding completely (Zeis et al., 1992; Nies et al., 1992). Furthermore, caffeine is soluble up to concentrations of $100\,mmol\,l^{-1}$, whereas urate is soluble only up to $1\,mmol\,l^{-1}$, which limits the range of c values obtainable for binding isotherms of the naturally occurring effector.

Fig. 2 shows raw data and integrated signals of a typical experimental titration of haemocyanin with urate and caffeine in $100\,mmol\,l^{-1}$ Hepes buffer, pH 8.0, containing $20\,mmol\,l^{-1}$ $MgCl_2$, $20\,mmol\,l^{-1}$ $CaCl_2$ and $150\,mmol\,l^{-1}$ NaCl. In Fig. 2A,C the power that has to be applied to keep the reference cell and the sample cell at the same temperature is plotted against the molar ratio of [effector] to [haemocyanin]. Each spike corresponds to a $5\,\mu l$ injection of a $1\,mmol\,l^{-1}$ caffeine or urate solution into a $25\,\mu mol\,l^{-1}$ haemocyanin and a $31.4\,\mu mol\,l^{-1}$ haemocyanin solution, respectively. Titration of the two ligands causes different exothermic reactions. As the ligand accumulates, the heat signal decreases because of the saturation of haemocyanin with the effector. Fig. 2B,C shows the integrated heat signals (in $kJ\,mol^{-1}$ caffeine or $kJ\,mol^{-1}$ urate) plotted against the molar ratio of the total caffeine or urate to the haemocyanin concentration. Under the experimental conditions chosen, an almost sigmoidal binding isotherm was obtained for the caffeine titration. Because of the smaller binding constant for urate, a hyperbolic binding curve for this effector was obtained despite the somewhat higher protein concentration.

Analysing urate and caffeine binding using ITC: indications for cooperative binding

The binding of urate to the haemocyanin of *H. vulgaris* was studied by Nies et al. (1992) using equilibrium dialysis. Caffeine binding was studied indirectly using urate

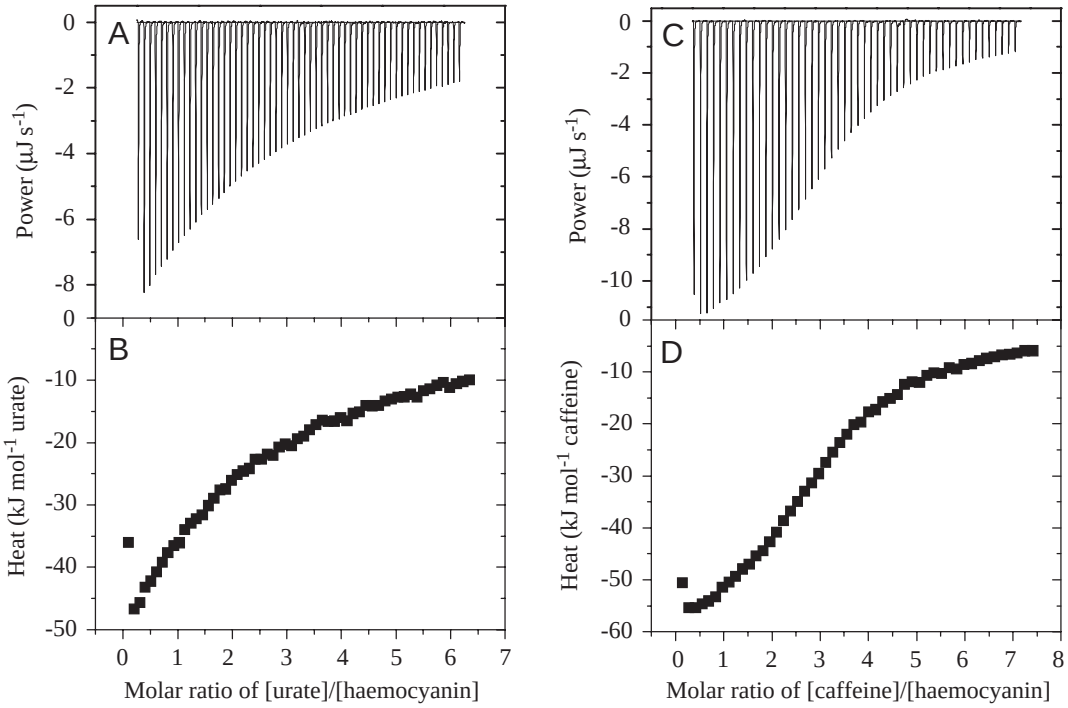

Fig. 2. Production of heat [kJ mol^{-1} injectate, which equals $\Delta q/\Delta[L_T](1/V)$, where q is heat, $\Delta[L_T]$ is the change in total ligand concentration and V is the total volume] during the titration of dodecameric haemocyanin (*Homarus vulgaris*) with urate (A,B) and caffeine (C,D). The titration was performed in 100 mmol l^{-1} Hepes buffer, pH 8.0, containing 20 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} $CaCl_2$ and 150 mmol l^{-1} NaCl. Raw data (A,C) and integrated data (B,D) are shown for 50 injections of 5 μl of 1 mmol l^{-1} caffeine into 25 μmol l^{-1} haemocyanin and for 50 injections of 5 μl of 1 mmol l^{-1} urate into 31.4 μmol l^{-1} haemocyanin. The titration experiment lasted for 266 min, including an elapsed time of 320 s between consecutive injections.

competition experiments. No indication of cooperativity in binding for either ligand was found in this study. However, as demonstrated by Menze et al. (2000), ITC binding isotherms for the two ligands could be conveniently explained only if cooperative binding was assumed for caffeine. This conclusion was deduced by analysing caffeine- and urate-binding isotherms using a non-interacting site model. By using this model, different numbers of binding sites n on the dodecameric haemocyanin (*H. vulgaris*) were obtained for urate (n=2) and caffeine (n=3.2). Since previous non-calorimetric binding studies (Nies et al., 1992) had revealed two binding sites for each of the two structurally similar ligands, a different number of binding sites seemed unlikely. Menze et al. (2000) has shown that a ligand/macromolecule system having two binding sites with different binding constants and slightly different enthalpies of binding can lead to a change in the number of binding sites measured if the binding isotherms are analysed using a non-interacting site model.

A similar pattern was observed by Fisher and Tally (1997, 1998), who studied the binding of ADP and NADPH to bovine liver glutamate dehydrogenase also using ITC. However, in contrast to previous binding studies using different methods, only ITC was successful in detecting substantial interactions between at least three of the individual binding sites of this hexameric protein. In this system, the three sequential binding constants for the formation of the binary and tertiary complexes were nearly identical, while the corresponding ΔH^0 and ΔS^0 values changed significantly in both magnitude and sign. These observations led to the new concept of isoergonic cooperativity in ligand binding (Fisher and Tally, 1997, 1998).

An analysis of caffeine and urate binding to the haemocyanin of *Homarus vulgaris* by ITC revealed two binding sites that displayed cooperative binding for caffeine but not for urate. The binding of urate was characterised by a microscopic binding constant (K) of 8500 l mol^{-1} and an enthalpy change (ΔH^0) of −135.1 kJ mol^{-1} for binding to both sites. Caffeine bound cooperatively to haemocyanin with two microscopic binding constants of 14 100 l mol^{-1} (K_1) and 40 400 l mol^{-1} (K_2). The corresponding enthalpy changes for binding were ΔH^0_1=−97.5 kJ mol^{-1} and ΔH^0_2=−113.4 kJ mol^{-1} (Menze et al., 2000).

Urate binding exhibits a more unfavourable entropy change (ΔS^0) of −0.385 kJ mol^{-1} K^{-1} than caffeine binding, with ΔS^0_1=−0.252 kJ mol^{-1} K^{-1} and ΔS^0_2=−0.298 kJ mol^{-1} K^{-1}, leading to a higher free energy change for caffeine binding (ΔG^0_1=−23.3 kJ mol^{-1}, ΔG^0_2=−25.9 kJ mol^{-1}) than for urate binding (ΔG^0=−22.1 kJ mol^{-1}). As with caffeine binding, urate binding is enthalpy-driven at 20 °C. Both binding processes are characterised by a favourable enthalpy change and an unfavourable entropy change with respect to the free energy

change of the system. However, for urate, the favourable enthalpy change is compensated by a larger entropy change, resulting in a smaller free energy change upon ligand binding. Therefore, the ratio between unfavourable entropy changes (release of ordered water molecules and increase in vibrational modes) and favourable entropy changes (loss of rotational, translational and conformational freedom) for caffeine binding is smaller than for urate binding. Since caffeine is an uncharged molecule with the same double ring structure as urate, the difference may originate from the amount of water ordered near the purine ring.

The difference between the binding constants for the two successive binding steps for caffeine is too small to be accurately detected by equilibrium dialysis. However, in the ITC experiments, the deviation from non-cooperative binding was amplified by at least two different binding enthalpies, which function as 'signals' in the experiments.

How can the fact that caffeine displays cooperative binding and urate does not be explained? Consider two protein conformations (*A* and *B*) for oxygenated haemocyanin, one of which is present at a much higher concentration (e.g. $[A] \gg [B]$). If two such conformations exist in equilibrium and differ in affinity for a certain ligand, the addition of this ligand will increase the relative amount of the conformation with the higher affinity. Thus, the dominant conformation *A* may be characterised by a higher affinity for urate than that of conformation *B*. With these assumptions, increasing the urate concentration would lead to only a slight change in the distribution of the two conformations since the high-affinity conformation *A* is already dominant. Under these conditions, cooperativity in urate binding would not be detectable. In contrast, for caffeine, we have to assume a higher affinity for conformation *B*. If caffeine is titrated, the conformational distribution would change significantly towards *B*, which would lead to the observed cooperativity in binding. The hypothesis of two haemocyanin conformations under oxygenated conditions is in accordance with an analysis of the pH-dependence of oxygen binding employing the nesting model for haemocyanin of the closely related lobster *H. americanus*. In this model, two conformations for the dodecameric oxygenated haemocyanin are required (Decker and Sterner, 1990).

The pH-dependence of urate and caffeine binding to haemocyanin from *H. vulgaris* was also investigated by Menze et al. (2000) using ITC. A possible dependence on pH might be expected since the magnitude of the Bohr effect was found to depend on the urate concentration (Zeis et al., 1992). No pH-dependence for binding for either ligand could be measured in the pH range investigated (7.55–8.15), since the binding isotherms measured at different pH values were found to coincide. Furthermore, no dependence of ΔH^0_{app} on the ionization enthalpy of three different buffer systems (ΔH^0_{ionz} between 20.5 and 48.2 kJ mol^{-1}) were found for caffeine binding to *H. vulgaris* haemocyanin (Menze et al., 2000). Thus, no change in the pKa of any amino acid was involved when caffeine binding occurred. The observed dependence of the magnitude of the Bohr effect on urate concentration could be explained if two haemocyanin conformations occurred under deoxygenated conditions. If these conformations differed in their affinity for protons and urate/caffeine, the observed behaviour might occur. Thus, *in toto*, four different conformations have to be assumed for the dodecameric haemocyanin of *H. vulgaris*. Four conformations were also predicted for the haemocyanins of other crustaceans such as *H. americanus* and *Carcinus maenas* on the basis of the analysis of the pH-dependence of oxygen binding employing the nesting model (Decker and Sterner, 1990; Dainese et al., 1998). To provide further insights into the allosteric regulation of haemocyanin, the binding of urate and caffeine to haemocyanin should be investigated under deoxygenated conditions.

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References

Bridges, C. R. (1990). Purines and their interaction with other factors controlling haemocyanin oxygen affinity. In *Invertebrate Dioxygen Carriers* (ed. G. Preaux and R. Lontie), pp. 402–405. Leuven: University Press.

Bridges, C. R. and Morris, S. (1986). Modulation of hemocyanin oxygen affinity by L-lactate a role for other cofactors. In *Invertebrate Oxygen Carriers* (ed. B. Linzen), pp. 341–352. Heidelberg: Springer-Verlag.

Dainese, E., Di Muro, P., Beltramini, M., Salvato, B. and Decker, H. (1998). Subunit composition and allosteric control in *Carcinus aestuarii* hemocyanin. *Eur. J. Biochem.* **256**, 350–358.

Decker, H. and Sterner, R. (1990). Nested allostery of arthropodan hemocyanin. The role of protons. *J. Mol. Biol.* **211**, 281–293.

Dykens, J. A. (1991). Purineolytic capacity and origin of hemolymph urate in *Carcinus maenas* during hypoxia. *Comp. Biochem. Physiol.* **98**B, 579–582.

Fisher, H. F. and Singh, N. (1995). Calorimetric methods for interpreting protein–ligand interactions. *Meth. Enzymol.* **259**, 194–220.

Fisher, H. F. and Tally, J. (1997). Isoergonic cooperativity in glutamate dehydrogenase complex: a novel form of allostery. *Biochemistry* **36**, 10807–10810.

Fisher, H. F. and Tally, J. (1998). Isoergonic cooperativity: a novel form of allostery. *Meth. Enzymol.* **295**, 331–348.

Freire, E., Mayorga, O. L. and Straume, M. (1990). Isothermal titration. *Analyt. Chem.* **62**, 950–959.

Graham, R. A. (1985). A model for L-lactate binding to *Cancer magister* hemocyanin. *Comp. Biochem. Physiol.* **81**B, 885–887.

Graham, R. A., Magnum, C. P., Terwilliger, R. C. and Terwilliger, N. B. (1983). The effects of organic acids on oxygen binding of hemocyanin from the crab *Cancer magister* hemocyanin. *Comp. Biochem. Physiol.* **74**A, 45–50.

Hinz, H. J. (1983). Thermodynamics of protein–ligand interactions: calorimetric approaches. *Annu. Rev. Biophys. Bioeng.* **12**, 285–317.

Indyk, L. and Fisher, F. H. (1998). Theoretical aspects of isothermal titration calorimetry. *Meth. Enzymol.* **295**, 350–364.

Johnson, B. A., Bonaventura, C. and Bonaventura, J. (1984). Allosteric modulation of *Callinectes sapidus* hemocyanin by binding of L-lactate. *Biochemistry* **23**, 872–878.

Johnson, B. A., Bonaventura, C. and Bonaventura, J. (1987). Determination of L-lactate binding stoichiometry and differences in allosteric interactions of structurally distinct homohexamers from *Panulirus interruptus* hemocyanin. *Biochim. Biophys. Acta* **916**, 376–380.

Ladbury, J. E. and Chowdhry, B. Z. (1996). Sensing the heat: the application of isothermal titration calorimetry to thermodynamic studies of biomolecular interactions. *Chem. Biol.* **3**, 791–801.

Lallier, F., Boitel, F. and Truchot, J. P. (1987). The effect of ambient oxygen and temperature on haemolymph L-lactate and urate concentrations in the shore crab *Carcinus maenas*. *Comp. Biochem. Physiol.* **86**A, 255–260.

Lallier, F. and Truchot, J. P. (1989). Haemolymph oxygen transport during environmental hypoxia in the shore crab, *Carcinus maenas*. *Respir. Physiol.* **77**, 323–336.

Markl, J. and Decker, H. (1992). Molecular structure of arthropod hemocyanins. *Adv. Comp. Env. Physiol.* **13**, 325–376.

Menze, M. A., Hellmann, N., Decker, H. and Grieshaber, M. K. (2000). Binding of urate and caffeine to haemocyanin of the lobster *Homarus vulgaris* (E.) studied by isothermal titration calorimetry. *Biochemistry* **39**, 10806–10811.

Morris, S. (1990). Organic ions as modulators of respiratory pigment function during stress. *Physiol. Zool.* **63**, 253–287.

Morris, S. and Bridges, C. R. (1994). Properties of respiratory pigments in bimodal breathing animals: air and water breathing by fish and Crustacea. *Am. Zool.* **34**, 216–228.

Morris, S., Bridges, C. R. and Grieshaber, M. K. (1985). A new role for uric acid: modulator of haemocyanin oxygen affinity in crustaceans. *J. Exp. Zool.* **235**, 135–139.

Morris, S., Bridges, C. R. and Grieshaber, M. K. (1986). The potentiating effect of purine bases and some of their derivatives on the oxygen affinity of haemocyanin from the crayfish *Austropotamobius pallipes*. *J. Comp. Physiol.* B **156**, 431–440.

Nies, A., Zeis, B., Bridges, C. R. and Grieshaber, M. K. (1992). Allosteric modulation of haemocyanin oxygen affinity by L-lactate and urate in the lobster *Homarus vulgaris*. II. Characterization of specific effector binding sites. *J. Exp. Biol.* **168**, 111–124.

Truchot, J. P. (1980). Lactate increases the oxygen affinity of crab hemocyanin. *J. Exp. Zool.* **214**, 205–208.

Van Holde, K. E. and Miller, K. I. (1992). Haemocyanins. *Q. Rev. Biophys.* **15**, 1–129.

Van Holde, K. E. and Miller, K. I. (1995). Hemocyanins. *Adv. Protein Chem.* **47**, 1–81.

Wiseman, T., Williston, S., Brandts, J. F. and Lin, L. N. (1989). Rapid measurement of binding constants and heats of binding by using a new titration calorimeter. *Analyt. Biochem.* **179**, 131–137.

Zeis, B. (1994). Einfluss von Urat auf die funktionellen Eigenschaften von Haemocyanin. Dissertation, University Duesseldorf, Germany.

Zeis, B., Nies, A., Bridges, C. R. and Grieshaber, M. K. (1992). Allosteric modulation of haemocyanin oxygen-affinity by L-lactate and urate in the lobster *Homarus vulgaris*. I. Specific additive effects on haemocyanin oxygen affinity. *J. Exp. Biol.* **168**, 93–110.