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March 1, 2001

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

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Accepted 1 December 2000; published on WWW 12 February 2001

### 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 crustaceans (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  $0.87 \text{ mmol l}^{-1}$  for L-lactate and  $0.03 \text{ mmol l}^{-1}$  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 quaternary 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<sup>-1</sup> 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 pK<sub>a</sub> 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  $\Delta H^0$ , the reaction enthalpy (Wiseman et al., 1989; Freire et al., 1990; Ladbury and Chowdhry, 1996). The entropy  $\Delta S^0$  and free energy  $\Delta G^0$  of binding are obtained from:

$$\Delta G^0 = -RT \ln K = \Delta H^0 - T\Delta S^0, \quad (1)$$

where  $T$  is the absolute temperature (K) and  $R$  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:

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

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

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

where  $\Delta H^0_i$  is the molar enthalpy of binding to the  $i$ th site and  $K_i$  is the corresponding stoichiometric binding constant (1 mol<sup>-1</sup>). The general relationship between the stoichiometric and the microscopic binding constants for  $n$  identical interacting sites is given by:

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

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  $[L_T]$  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  $[L_T]$  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 ( $[M_t]$ ) used in the experiment:

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

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  $-70$  kJ mol<sup>-1</sup> injectate were assumed. Each point represents the heat ( $\Delta q$ )

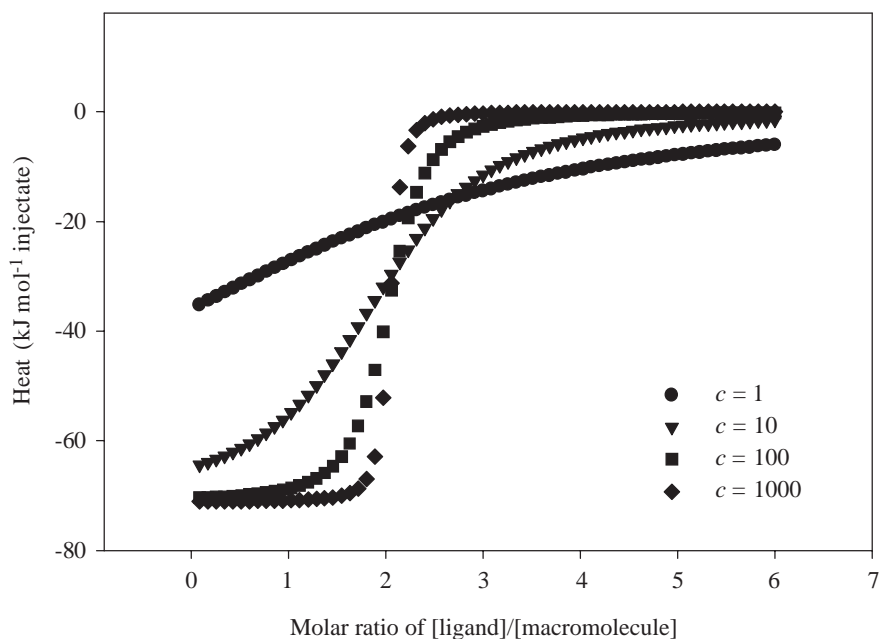


Fig. 1. Titration isotherms computed for a ligand/macromolecule system with an enthalpy change on binding ( $\Delta H^0$ ) of  $-70 \text{ kJ mol}^{-1}$  injectate and two non-interacting binding sites ( $n=2$ ) for the ligand. The isotherms were computed for measurements at different values of the parameter  $c$  (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):

$$\Delta H_{\text{app}}^0 = \Delta H_{\text{int}}^0 + n_{\text{H}} \Delta H_{\text{ionz}}^0, \quad (6)$$

where  $n_{\text{H}}$  represents the number of protons released or absorbed by the buffer system. In this case, the apparent enthalpy of binding ( $\Delta H_{\text{app}}^0$ ) includes both the intrinsic enthalpy of effector binding ( $\Delta H_{\text{int}}^0$ ) and the enthalpy of ionisation of the buffer system ( $\Delta H_{\text{ionz}}^0$ ).

#### 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  $100 \text{ mmol l}^{-1}$ , whereas urate is soluble only up to  $1 \text{ mmol l}^{-1}$ , 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  $100 \text{ mmol l}^{-1}$  Hepes buffer, pH 8.0, containing  $20 \text{ mmol l}^{-1}$   $\text{MgCl}_2$ ,  $20 \text{ mmol l}^{-1}$   $\text{CaCl}_2$  and  $150 \text{ mmol l}^{-1}$   $\text{NaCl}$ . 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  $5 \mu\text{l}$  injection of a  $1 \text{ mmol l}^{-1}$  caffeine or urate solution into a  $25 \mu\text{mol l}^{-1}$  haemocyanin and a  $31.4 \mu\text{mol l}^{-1}$  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  $\text{kJ mol}^{-1}$  caffeine or  $\text{kJ mol}^{-1}$  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

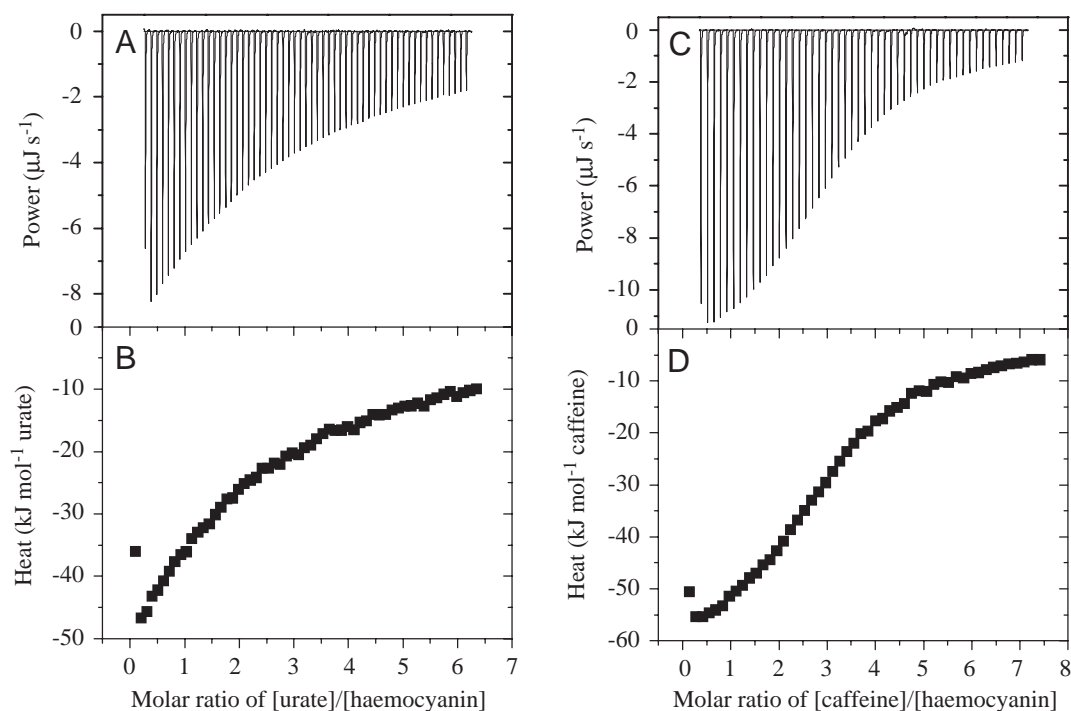


Fig. 2. Production of heat [ $\text{kJ mol}^{-1}$  injectate, which equals  $\Delta q/\Delta[L_T](1/V)$ , where  $q$  is heat,  $\Delta[L_T]$  is the change in total ligand concentration and  $V$  is the total volume] during the titration of dodecameric haemocyanin (*Homarus vulgaris*) with urate (A,B) and caffeine (C,D). The titration was performed in  $100 \text{ mmol l}^{-1}$  Hepes buffer, pH 8.0, containing  $20 \text{ mmol l}^{-1}$   $\text{MgCl}_2$ ,  $20 \text{ mmol l}^{-1}$   $\text{CaCl}_2$  and  $150 \text{ mmol l}^{-1}$   $\text{NaCl}$ . Raw data (A,C) and integrated data (B,D) are shown for 50 injections of  $5 \mu\text{l}$  of  $1 \text{ mmol l}^{-1}$  caffeine into  $25 \mu\text{mol l}^{-1}$  haemocyanin and for 50 injections of  $5 \mu\text{l}$  of  $1 \text{ mmol l}^{-1}$  urate into  $31.4 \mu\text{mol l}^{-1}$  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  $\Delta H^0$  and  $\Delta S^0$  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  $85001 \text{ mol}^{-1}$  and an enthalpy change ( $\Delta H^0$ ) of  $-135.1 \text{ kJ mol}^{-1}$  for binding to both sites. Caffeine bound cooperatively to haemocyanin with two microscopic binding constants of  $141001 \text{ mol}^{-1}$  ( $K_1$ ) and  $404001 \text{ mol}^{-1}$  ( $K_2$ ). The corresponding enthalpy changes for binding were  $\Delta H^0_1 = -97.5 \text{ kJ mol}^{-1}$  and  $\Delta H^0_2 = -113.4 \text{ kJ mol}^{-1}$  (Menze et al., 2000).

Urate binding exhibits a more unfavourable entropy change ( $\Delta S^0$ ) of  $-0.385 \text{ kJ mol}^{-1} \text{ K}^{-1}$  than caffeine binding, with  $\Delta S^0_1 = -0.252 \text{ kJ mol}^{-1} \text{ K}^{-1}$  and  $\Delta S^0_2 = -0.298 \text{ kJ mol}^{-1} \text{ K}^{-1}$ , leading to a higher free energy change for caffeine binding ( $\Delta G^0_1 = -23.3 \text{ kJ mol}^{-1}$ ,  $\Delta G^0_2 = -25.9 \text{ kJ mol}^{-1}$ ) than for urate binding ( $\Delta G^0 = -22.1 \text{ kJ mol}^{-1}$ ). As with caffeine binding, urate binding is enthalpy-driven at  $20^\circ\text{C}$ . 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  $\Delta H^0_{\text{app}}$  on the ionization enthalpy of three different buffer systems ( $\Delta H^0_{\text{ionz}}$  between 20.5 and 48.2 kJ mol<sup>-1</sup>) 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.

This work was supported by the Deutsche Forschungsgemeinschaft (Gr 456/20-1) and the State of Nordrhein-Westfalen (M.K.G.).

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