Binding of Urate and Caffeine to Hemocyanin of the Lobster *Homarus vulgaris* (E.) As Studied by Isothermal Titration Calorimetry†

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ABSTRACT: Hemocyanin serves as an oxygen carrier in the hemolymph of the European lobster *Homarus vulgaris*. The oxygen binding behavior of the pigment is modulated by metabolic effectors such as lactate and urate. Urate and caffeine binding to 12-meric hemocyanin (*H. vulgaris*) was studied using isothermal titration calorimetry (ITC). Binding isotherms were determined for fully oxygenated hemocyanin between pH 7.55 and 8.15. No pH dependence of the binding parameters could be found for either effector. Since the magnitude of the Bohr effect depends on the urate concentration, the absence of any pH dependence of urate and caffeine binding to oxygenated hemocyanin suggests two conformations of the pigment under deoxygenated conditions. Urate binds to two identical binding sites ($n = 2$) each with a microscopic binding constant K of 8500 M^{-1} and an enthalpy change $\Delta H°$ of −32.3 kcal mol^{-1}. Caffeine binds cooperatively to hemocyanin with two microscopic binding constants: $K_1 = 14\,100\ M^{-1}$ and $K_2 = 40\,400\ M^{-1}$. The corresponding enthalpy changes in binding are as follows: $\Delta H°_1 = -23.3$ kcal mol^{-1} and $\Delta H°_2 = -27.1$ kcal mol^{-1}. The comparison of urate and caffeine binding to the oxygenated pigment indicates the existence of two protein conformations for oxygen-saturated hemocyanin. Since effector binding is not influenced by protons, four different conformations are required to create a convincing explanation for caffeine and urate binding curves. This was predicted earlier on the basis of the analysis of oxygen binding to lobster hemocyanin, employing the nesting model.

The hemocyanins of arthropods are large, extracellular, copper-containing proteins that serve as oxygen carriers which consist of one, two, four, six, or eight basic hexameric assemblies. Their apparent molecular masses range from 0.45 × 10^6 to 3.9 × 10^6 Da (*1*−*6*). Each monomeric subunit carries a single binuclear copper-containing active site which can reversibly bind a dioxygen molecule (*7*). Hemocyanins of arthropods exhibit high cooperative oxygen binding and allosteric regulation, thus maintaining efficient uptake and delivery of oxygen.

The effect of pH on the oxygen affinity of hemocyanins has been extensively studied, and most arthropod hemocyanins exhibit normal Bohr effects; that is, the oxygen affinity decreases when the pH becomes more acidic (*8*−*10*). In contrast, all other modulators of arthropod hemocyanins increase the oxygen affinity. The modulators consist of a range of different chemical substances which probably bind to different areas in the hemocyanin molecule. Modulators are divalent cations such as Mg^{2+} and Ca^{2+} (*11*, *12*), the metabolites lactate (*13*) and urate (*14*−*17*), neuroactive compounds such as dopamine and related cardiac neuroamines (*18*, *19*), and the excretory products ammonium and trimethylamine (*20*).

The oxygen binding characteristics of the 12-meric hemocyanin from the European lobster *Homarus vulgaris* (E.) in the presence of urate and L-lactate have been analyzed in detail by several authors (*21*, *22*). The effects of both metabolites on oxygen affinity were found to be additive, and urate caused a significant decrease in the Bohr coefficient (*23*) which indicates a thermodynamical linkage between the two effectors, protons and urate. The urate analogue caffeine, also described as a potent effector of oxygen affinity (*24*), was found to have a higher binding constant for hemocyanin than urate in *H. vulgaris* (*21*).

These results have been indirectly obtained by analyzing the effector-induced changes in the half-saturation oxygen pressure (p_{50}) of the hemocyanin or by equilibrium dialysis. Until now, no studies on the binding behavior of urate and its dependence on pH have been published. However, in this study, direct insights into the interacting forces between urate and caffeine in the dodecameric hemocyanin of *H. vulgaris* were gained using isothermal titration calorimetry (ITC).[1] This method readily lends itself to the simultaneous determination of the binding constant (K), the stoichiometry of ligand−protein interaction (n), and $\Delta H°$, the reaction en-

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[1] Abbreviations: ITC, isothermal titration calorimetry; [Hc], concentration of dodecameric hemocyanin; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonicacid; TRIS, tris(hydroxymethyl)aminoethane; TRICINE, *N*-tris(hydroxymethyl)methylglycine.

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thalpy (*25*). Two major questions can be addressed by this study. Is there any thermodynamical linkage between the effectors urate and caffeine and protons on the level of the conformations dominating under oxygenated conditions? Are there any ionizable groups with a pK_a between pH 7.55 and 8.15 involved in the binding of urate or caffeine? To evaluate these two questions, caffeine and urate binding to the hemocyanin (*H. vulgaris*) was studied at different pH values and in buffers having different enthalpies of proton ionization.

METHODS

Purification of Hemocyanin. Male European lobsters [*H. vulgaris* (E.)] were obtained from a marine animal wholesaler (Hummer Petersen, Hamburg, Germany). The lobsters were maintained in 33‰ circulating seawater and at a temperature of 15 ± 1 °C. Hemolymph was drawn from the segment joint of the penultimate pereiopods into an ice-chilled syringe, and samples from three to five lobsters were pooled. The hemolymph samples were immediately centrifuged at 27000*g* for 1.5 h at 4 °C to remove cellular debris. The supernatant was dialyzed against TRIS buffer (20 mM TRIS-HCl buffer containing 40 mM NaCl, 20 mM $MgCl_2$, and 20 mM $CaCl_2$ adjusted to pH 8.0 at 20 °C) using a dialysis membrane with a 20 kDa molecular mass cutoff. To remove denatured proteins, the dialyzed sample was centrifuged again at 27000*g* for 1.5 h at 4 °C. The 24 S hemocyanin was then sedimented by ultracentrifugation for 3.5 h at 170000*g* and 4 °C. After centrifugation, the light reddish supernatant was discarded, leaving the hemocyanin in a dark blue pellet. It was resuspended in a sufficient amount of TRIS buffer and again centrifuged at 27000*g* for 1.5 h at 4 °C. Protein concentrations were determined by measuring the optical absorbance at 280 nm using an extinction coefficient of 1.42 $cm^2\ mg^{-1}$. Hemocyanin concentrations were determined by measuring the optical absorbance at 334 nm using an extinction coefficient of 0.269 $cm^2\ mg^{-1}$ for oxygen-saturated 12-meric hemocyanin and a molecular mass of 900 000 Da (*26*). About 80 mg of protein, in a volume of 2 mL of TRIS buffer, was loaded on a Resource Q anion-exchange column (Amersham Pharmacia Biotech, Freiburg, Germany) with a bed volume of 6 mL. The column was washed with 24 mL of TRIS buffer and developed at a flow rate of 6 mL min^{-1} using 120 mL of a linear gradient of 500 mM NaCl dissolved in TRIS buffer. The hemocyanin eluted at a NaCl concentration of about 350 mM. Appropriate fractions were pooled and concentrated by ultracentrifugation (5 h at 170000*g* and 4 °C). The pellet was again resuspended in a sufficient amount of buffer. This hemocyanin solution contained an average protein concentration of 80 μM and was homogeneous as judged from native gel electrophoresis.

Controlling the Aggregation State. The aggregation state of the hemocyanin, as well as the purity of the hemocyanin solution, was controlled by a Superose 6b (Amersham Pharmacia Biotech) size exclusion column with a 24 mL bed volume and equilibrated with TRIS buffer containing 500 mM NaCl. About 80 μg of the protein in a volume of 100 μL was applied and eluted at a flow rate of 0.2 mL min^{-1} with the same buffer. The aggregation state of the hemocyanin, which had an absorbance ratio A_{280}/A_{334} of 4.8−5.0, was found to consist of 95% dodecameric and 5% higher-aggregate forms. A possible influence of the different pH and buffer components used in the ITC experiments on the aggregation state of the hemocyanin was also checked using the same procedure except that the same buffers were used as in the calorimetry experiments. No influence of the pH and buffer compositions on the aggregation state of the hemocyanin was found.

Isothermal Titration Calorimetry (ITC). All calorimetric experiments were performed with a VP-ITC titration calorimeter (MicroCal Inc., Northampton, MA). Protein solutions were prepared by dialysis of a hemocyanin stock solution against 100 mM HEPES, TRIS, and TRICINE buffer at 4 °C adjusted to the pH values desired for the experiments. Each buffer contained 20 mM $CaCl_2$, 20 mM $MgCl_2$, and 150 mM NaCl. Ligand solutions were prepared by dissolving weighed amounts of urate or caffeine in the respective buffer. Urate concentrations were checked spectrophotometrically using an extinction coefficient E_{293} of 12.6 $cm^2\ \mu mol^{-1}$ (*27*). Heats of reaction were determined during repeated injections of a fixed amount of ligand into a solution of hemocyanin. The injection syringe was rotated at 310 rpm for the duration of each experiment, and the time interval between injections was about 400 s. The heat change accompanying the addition of buffer to hemocyanin and the heat of dilution of the ligands were subtracted from the raw data after correction for the injection signal of buffer into buffer. Titration curves were analyzed using Origin software (MicroCal Inc.). A multiple-noninteracting site model and a multisite interactive model were used to analyze the data. The integral form of the phenomenological equation for the single-site case, expressed in terms of the molar concentration of the unbound ligand [L], is given by eq 1:

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

where q is the heat developed on adding L to a macromolecule. The total macromolecule concentration in the binding process is $[M_T]$ with n binding sites. The binding process is characterized by a binding constant K (M^{-1}) and an enthalpy of ΔH° in a total volume V. The integral form of the equation for the multisite interactive model is given by eq 2:

$$\frac{q}{[M_T]V} = [(\Delta H^\circ_1)K_1[L] + (\Delta H^\circ_1 + \Delta H^\circ_2)K_1K_2[L]^2 + (\Delta H^\circ_1 + \Delta H^\circ_2 + \ldots \Delta H^\circ_i)K_1K_2\ldots K_i[L]^i]/(1 + K_1[L] + K_1K_2[L]^2 + K_1K_2\ldots K_i[L]^i) \quad (2)$$

where ΔH°_i is the molar enthalpy of ligand binding to the ith site and K_i is the corresponding binding constant (M^{-1}) for each individual binding site. In the most general case, no analytic form for q that is dependent on the total ligand concentration $[L_T]$ can be found. Therefore, the problem is usually solved numerically during the fitting routine, using computer programs such as Origin. On the basis of the numerical solution, the differential form of the binding function is obtained. In the work presented here, the Microcal Origin program (MicroCal Inc.) was used. A detailed discussion of this equations is presented by Indyk and Fisher (*28*).

RESULTS

Binding of Urate and Caffeine to Hemocyanin. The heat dissipation during isothermal titration of 12-meric hemocya-

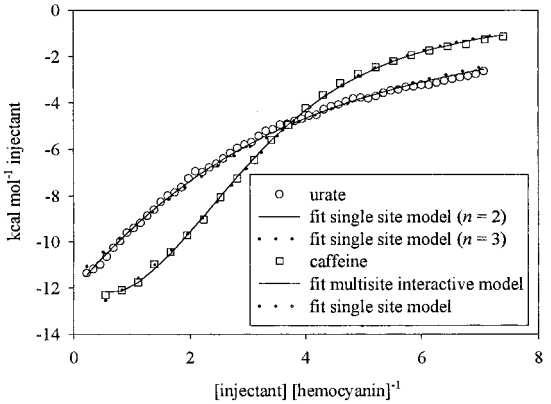

FIGURE 1: Titration of urate and caffeine into hemocyanin of *H. vulgaris*. The caffeine titration (squares) was performed by injecting 24 × 10 μL of 1 mM caffeine into 26 μM hemocyanin, and the urate binding isotherm (circles) was determined by making 54 × 5 μL injections of 1 mM urate into 33.3 μM hemocyanin. Both titrations were carried out in HEPES buffer at pH 8.0 and 20 °C. The influences of different models and stoichiometry on the quality of the obtained fits are visualized graphically (lines). See the text for further references.

nin of *H. vulgaris* with urate and caffeine is shown in Figure 1. Each point represents the heat Δq per mole of injectant liberated for an incremental addition of the injectants urate and caffeine, versus the ratio of the accumulated total injectant concentration $[L_T]$ divided by the concentration of hemocyanin $[M_T]$. This form of a plot is essentially the first derivative ($\Delta q\ \Delta[L_T]^{-1}$) of a convential binding curve in which the total accumulated signal is plotted versus the total accumulated injectant concentration. The graph depicts the typical enthalpic binding isotherms of caffeine and urate at pH 8.0, which is the in vivo pH of the hemolymph under normoxic conditions (C. R. Bridges, personal communication). The evaluation of the binding constant (K), the number of binding sites (n), and the enthalpy change ($\Delta H°$) for urate was difficult because the product of K, n, and the protein concentration $[M_T]$ was low. Under these conditions, n is highly correlated with $\Delta H°$ in the fitting process. This problem could in principle be solved if the protein concentration $[M_T]$ could be raised to such an extent that a high value of the product of K, n, and $[M_T]$ would result (*29*, *30*). Unfortunately, this is impossible because hemocyanin precipitates at the required concentrations. Furthermore, the solubility of urate in the buffers that are used is limited to about 1 mM. If n is not fixed to a certain value such as 2 or 3, the fitting process fails to converge. Therefore, the number of binding sites for urate was fixed in the fitting routine on the basis of a value determined in equilibrium dialysis experiments (*21*) where two binding sites for urate in the 12-meric hemocyanin were found. If the number of urate binding sites was fixed to 2 for a multiple-noninteracting site model, a binding constant K of 7500 ± 1500 M^{-1} and an enthalpy change $\Delta H°$ of −34.7 ± 0.4 kcal mol^{-1} were found for data obtained at pH 8.0 (Figure 1).

The characteristics of binding of caffeine to hemocyanin can be analyzed without preset values for the number of binding sites (n) when a multiple-noninteracting binding site model is used. The best fit of the caffeine binding curve (Figure 1) resulted in a value for n of 3.2 ± 0.04, a binding constant K of 71 100 ± 4200 M^{-1}, and an enthalpy change $\Delta H°$ of −14.9 ± 0.4 kcal mol^{-1}. Assuming a value of 2 or any other number of binding sites resulted in a significant reduction in the level of agreement between the data and the fitted curve.

Because the analysis of caffeine binding to hemocyanin indicated the presence of three binding sites, we also analyzed urate binding to the pigment assuming three binding sites for this metabolite. The fitted curve, however, shows a higher deviation from the measured data than when 2 binding sites are assumed (Figure 1). Since the difference between both fits can hardly be seen ad oculus in Figure 1, the degree of similarity between the fitted curve and the data measured was based on the χ^2 value. This value increases from 20 100 to 66 400 if the number of binding sites of urate binding is fixed at 3, indicating that 3 binding sites for urate in the dodecameric hemocyanin are less probable than 2.

When the similar structures of the two effectors and the fact that caffeine is able to inhibit urate binding completely (*21*) are considered, a different stoichiometry of binding of the two purines is unlikely. If we allowed for the occurrence of cooperativity by analyzing caffeine binding with a multisite interactive model, the isothermic titration of caffeine to the 12-meric hemocyanin can be analyzed assuming n = 2 (Figure 1). In this case, the χ^2 value drops from 30 300 to 11 400, indicating a better fit. The microscopic binding constants for caffeine and urate binding to hemocyanin are summarized in Tables 1 and 2.

Influence of pH on the Binding of Caffeine and Urate. From Figure 2, it is obvious that the binding isotherms measured at similar hemocyanin concentrations coincide. The average values of the two microscopic binding constants for caffeine binding to hemocyanin were determined to be 14 100 ± 5800 M^{-1} (K_1) and 40 400 ± 4500 M^{-1} (K_2) with similar enthalpy changes of binding: $\Delta H°_1 = -23.3 \pm 3.7$ kcal mol^{-1} and $\Delta H°_2 = -27.1 \pm 1.2$ kcal mol^{-1}. Urate binding to both sites can (n = 2) be described with a binding constant K of 8500 ± 1600 M^{-1} and an enthalpy change $\Delta H°$ of −32.3 ± 2.3 kcal mol^{-1}. The variations in the binding constants at any given pH are within the error of the analysis and cannot be attributed to the differences in the proton concentrations. Thus, protons do not influence caffeine and urate binding in the pH range of 7.55−8.15.

Dependence of $\Delta H°$ on the Buffer System. The lack of any pH dependence between pH 7.55 and 8.15 of caffeine binding to hemocyanin indicates that no amino groups with pK_as in this region are involved in the binding process, either directly at the binding sites or at any site where conformational changes as a result of effector binding occur. It seems, therefore, that no proton transfer is involved at all. To verify this result, caffeine binding was studied at pH 8.0 in three different buffer systems (TRIS, HEPES, and TRICINE) characterized by differing heats of proton dissociation (4.9−11.51 kcal mol^{-1}). A change in the pK_a of any of the amino groups involved in effector binding should result in proton transfer between the binding complex and the buffer molecules. In this case, the apparent enthalpy of binding ($\Delta H°_{app}$) includes both the intrinsic enthalpy of effector binding ($\Delta H°_{int}$) and the enthalpy of ionization of the buffer system ($\Delta H°_{ionz}$):

$$\Delta H°_{app} = \Delta H°_{int} + n_H \Delta H°_{ionz}$$

where n_H represents the number of protons released or

Table 1: Thermodynamic Parameters for the Binding of Caffeine and Urate to Hemocyanin of *H. vulgaris* in HEPES Buffer[a]

	caffeine				urate	
pH	K_1 (mM^{-1})	K_2 (mM^{-1})	$\Delta H°_1$ (kcal mol^{-1})	$\Delta H°_2$ (kcal mol^{-1})	K (mM^{-1})	$\Delta H°$ (kcal mol^{-1})
7.55	9.5 ± 5.1	45.6 ± 3.6	-26.2 ± 4.9	-26.1 ± 5.9	9.0 ± 1.9	-32.5 ± 1.5
7.85	20.6 ± 5.0	38.1 ± 1.7	-19.1 ± 2.5	-26.9 ± 3.3	9.8 ± 1.4	-29.6 ± 1.9
8.00	nd	nd	nd	nd	6.5 ± 0.7	-35.4 ± 1.0
8.15	12.2 ± 1.0	37.6 ± 3.5	-24.7 ± 2.8	-28.4 ± 3.0	8.8 ± 1.1	-32.0 ± 1.0
means	14.1 ± 5.8	40.4 ± 4.5	-23.3 ± 3.7	-27.1 ± 1.2	8.5 ± 1.6	-32.3 ± 2.3

[a] Values are averages from two to five replicates of the experiment. The protein concentrations were 16.5−55.8 μM (12-mers) in the urate experiments and 39.5−49.5 μM (hemocyanin) in the caffeine experiments. The ligand concentration in the syringe was 0.6, 1, 1.5, or 2.0 mM. Experiments were performed at 20 °C. The errors given are standard deviations of the mean. The errors given by the fitting routine for the individual parameters are in the same range.

Table 2: Thermodynamic Data Obtained from the Titration of Caffeine into 12-meric Hemocyanin (*H. vulgaris*) at pH 8.0 in HEPES, TRICINE, and TRIS Buffers[a]

buffer	$\Delta H°_{ionz}$ (kcal mol^{-1})	K_1 (mM^{-1})	K_2 (mM^{-1})	$\Delta H°_1$ (kcal mol^{-1})	$\Delta H°_2$ (kcal mol^{-1})
HEPES	4.90	13.4 ± 1.9	73.6 ± 4.8	-27.9 ± 2.3	-20.2 ± 2.7
HEPES	4.90	12.8 ± 1.9	69.7 ± 4.6	-28.7 ± 2.5	-20.4 ± 3.1
TRICINE	7.76	13.7 ± 2.3	80.4 ± 6.3	-28.5 ± 2.8	-20.2 ± 3.3
TRICINE	7.76	12.5 ± 2.2	76.4 ± 6.1	-31.2 ± 3.2	-17.3 ± 3.8
TRIS	11.51	18.1 ± 3.9	55.7 ± 4.6	-25.8 ± 2.3	-26.1 ± 3.0
TRIS	11.51	16.4 ± 2.8	55.3 ± 3.9	-24.2 ± 2.6	-27.4 ± 3.4
mean		14.5 ± 2.2	68.5 ± 10.7	-27.7 ± 2.4	-21.9 ± 3.9

[a] The protein concentrations were 25.7−26.6 μM (12-mers). The concentration of the injectant (caffeine) was 1.0 mM. Experiments were performed at 20 °C. Each set of values are results of a single experiment. The errors given are those calculated on the basis of the least-squares analysis.

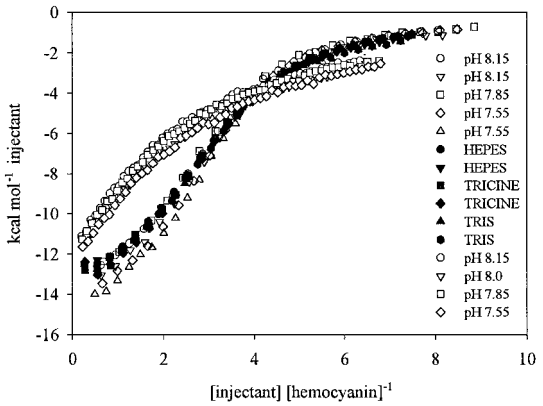

FIGURE 2: Binding of caffeine and urate to hemocyanin (*H. vulgaris*) at different pH values and in three buffer systems. The titrations were performed by injecting 24 × 10 μL of caffeine (2 or 1.5 mM) into 39.5−49.5 μM hemocyanin at three different pH values (white symbols). The urate binding isotherms were determined by injecting 55 × 5 μL of 1 mM urate (light gray symbols) into 31.5−36.8 μM hemocyanin. The dark gray symbols represent measurements in three different buffer systems at pH 8.0 after injection of 25 × 10 μL of 1 mM caffeine into 25.7−26.6 μM hemocyanin.

absorbed by the buffer system (*31*, *32*). We found no dependence of $\Delta H°_{app}$ on the ionization enthalpy of the buffer (Table 2). However, a weak dependence on protein concentration in the binding parameters was found. The values for the second binding constant (K_2) were somewhat higher at lower protein concentrations [$K_2 = 68.5 \pm 10.7$ mM^{-1} (Table 2) when [Hc] = 25.7−26.6 μM] than those found at a higher protein concentration [$K_2 = 40.4 \pm 4.5$ mM^{-1} (Table 1) when [Hc] > 39.5 μM].

DISCUSSION

Urate increases the oxygen affinity of hemocyanin in various crustacea (*14*, *24*). In the case of *H. vulgaris* (E.), the half-saturation oxygen pressure (p_{50}) of the (2 × 6)-meric hemocyanin is lowered and the cooperativity is decreased. The chemical analogue caffeine affects cooperativity and affinity even more strongly than urate (*33*). To understand the mechanisms of allosteric interaction, effector binding has to be characterized in terms of the binding constant and stoichiometry. Calorimetric analysis of the binding process can give further insight into allosteric regulated systems. This was shown by Fisher and Tally (*34*), who employed the concept of isoergonic cooperativity to substrate binding in liver glutamate dehydrogenase.

In dialysis experiments, the number of binding sites for urate was found to be 2 for the (2 × 6)-meric hemocyanin of *H. vulgaris* and a binding constant K of 37 000 M^{-1} was determined. If it is assumed that there are 2 binding sites for caffeine, the binding constant was found to be 110 000 M^{-1} (*21*). These results have been obtained by equilibrium dialysis without further analysis of the entropic and enthalpic contributions of the binding processes. Furthermore, caffeine binding was studied only indirectly by using urate competition experiments.

To gain direct insight into the thermodynamics of the interactions between urate and caffeine in the dodecameric hemocyanin of *H. vulgaris*, we reinvestigated the binding characteristics using isothermal titration calorimetry (ITC). To gain further insight into the allosteric regulation of effector binding to hemocyanin, we investigated the influence of pH on the binding characteristics of the two effectors to prove a possible thermodynamical linkage between urate and protons and caffeine and protons in the oxygenated hemocyanin.

Analysis of Urate and Caffeine Binding to Hemocyanin of H. vulgaris. The binding of urate and caffeine to the crustacean hemocyanin was studied at 20 °C within the physiological pH range of 7.55−8.15 (*35*, *36*). Because the

product of K, n, and $[M_T]$ for urate binding was low, the titration curves were analyzed on the basis of a preset number of binding sites ($n = 2$) according to previous equilibrium dialysis experiments (*21*). The binding constant, as measured by ITC, was found to be lower than the constant obtained in the previous study ($K = 8500\ M^{-1}$ and $K = 37\,000\ M^{-1}$, respectively). The latter was determined in a physiological saline solution, using equilibrium dialysis at 15 °C, and this could explain why a higher value for the binding constant was found. However, an influence on the number of urate and caffeine binding sites in the 12-meric hemocyanin, by temperature or buffer composition, seems unlikely, which tends to justify the use of preset values in the analysis.

Unexpectedly, caffeine binding seems to involve 3.2 binding sites (n) with a binding constant K of $71\,100\ M^{-1}$ and an enthalpy change $\Delta H°$ of -14.9 kcal mol^{-1} based on a model with n identical binding sites. However, caffeine binding data could also be described assuming two cooperative binding sites. The microscopic binding constants K_1 ($14\,100\ M^{-1}$) and K_2 ($40\,400\ M^{-1}$) (by using 39.5−49.5 μM hemocyanin, Table 1) indicate positive cooperativity.

Our results indicate that caffeine displays cooperative binding but urate does not. How can this be rationalized? If two protein conformations (A and B) exist for oxygenated hemocyanin, one could be present in a higher concentration ([A] ≫ [B]). The dominating conformation A should be characterized by a higher affinity for urate compared to that of conformation B, while caffeine binds preferably to B. With these assumptions, there would be only a slight change in the distribution between the two conformations with increasing urate concentrations. Thus, cooperativity in urate binding would not be detectable. In contrast, if caffeine were added, the conformational distribution would change significantly toward B which could lead to the observed cooperativity in binding. The hypothesis of two hemocyanin conformations under oxygenated conditions is in accordance with an analysis of the pH dependence of oxygen binding employing the nesting model. In this model, two conformations for the (2 × 6)-meric oxygenated hemocyanin of the closely related *H. americanus* is required (*8*).

This study indicates the presence of two effector binding sites for urate in the (2 × 6)-meric hemocyanin (*H. vulgaris*). A possible binding site for the allosteric effector would be along the 3-fold symmetry axis of each hexamer. Binding of an effector at this site could arrest the conformational transition within the allosteric unit, the hexamer.

Remarkably, the hemocyanin concentration was found to have a weak influence on the caffeine binding parameters. The K_1 parameter of caffeine binding seems to be relatively unaffected by the hemocyanin concentration. However, the K_2 value shows a slight increase at lower hemocyanin concentrations from $40\,100\ M^{-1}$ at 39.5−49.5 μM hemocyanin to $68\,500\ M^{-1}$ at 25.7−26.5 μM (Tables 1 and 2). Although this is a small variation, it is greater than expected, in view of the deviations in K values seen within both concentration ranges. A decrease in the binding constant with increasing protein concentrations is also described for the binding of cAMP to RNAse (*29*). This trend might suggest the appearance of higher than dodecameric hemocyanin assemblies, with increasing protein concentrations, which differ in their binding characteristics for caffeine.

Influence of pH on Urate and Caffeine Binding to Hemocyanin of H. vulgaris. There is no pH dependence of urate and caffeine binding in the pH range between 7.55 and 8.15. This is apparent without any detailed analysis, since the binding isotherms measured at similar hemocyanin concentration coincide (Figure 2). This is supported by the result that caffeine binding is not linked to proton transfer between the buffer system and the binding complex. Otherwise, a dependence of the binding parameters on pH or a change of $\Delta H°_{app}$ with buffer composition would have been observed (Tables 1 and 2). Thus, the experimentally obtained binding enthalpy corresponds only to the enthalpy change due to binding of caffeine, and no change in the pK_a of any amino acid is involved when binding occurs. Since caffeine lacks any charged side groups and has a higher affinity than urate, we propose that binding is largely driven by hydrophobic interactions.

Physiological Considerations. Under hypoxic conditions, urate concentrations in the hemolymph of some decapod Crustacea increase (*16*, *37*) which is due to a decrease in uricase activity lacking the second substrate oxygen (*34*, *38*). Increasing concentrations of urate shift the p_{50}s of oxygen binding curves toward lower values. This might enable a sufficient oxygen loading of hemocyanin at the gills under hypoxic conditions. In this study, we found a binding constant K of about $8500\ M^{-1}$, corresponding to a half-saturation of hemocyanin at 120 μM urate. The total urate concentration in the hemolymph of *H. vulgaris* was determined to about 80 μM under normoxic conditions (*23*). It is important to realize that the circulating urate concentration is never truly zero and thus will have some role in determining the oxygen affinity of the pigment in quiescent, nonstressed animals. For a typical value of 35 μM for the hemocyanin concentration of lobster hemolymph (34.9 ± 4.9 μM; data not shown), the pigment is loaded to only 33% with urate so that the affinity has probably been optimized with respect to the physiological concentration of the effector. Under extreme hypoxia, the urate concentration increases to 160 μM (*33*). Under this condition, the hemocyanin would be saturated with urate to about 51%. Since the saturation level is changed only moderately when the urate concentration is raised from 80 to 160 μM, one would expect only a relatively small change in the p_{50} for oxygen binding. It is interesting to note, therefore, that only a slight shift in the p_{50}, from about 5.8 to 5.2 Torr, upon increasing the urate concentration from 120 to 170 μM is reported by Zeiss et al. (*23*).

The shift of p_{50} due to urate binding was investigated as a function of pH (*23*), and the Bohr effect was found to be reduced with increasing urate concentrations. This indicates that the allosteric interaction depends on pH. Precisely which hemocyanin conformations were susceptible to pH changes, with respect to urate binding affinity, were unknown. In our study, we found that binding to fully oxygenated hemocyanin is independent of pH. Consequently, we expect that pH regulates the urate binding on those conformations which are predominant in deoxygenated hemocyanin. The existence of two conformations in the deoxygenated state which differ in their affinity for protons and urate could explain such a behavior. This could also account for the change in the affinity of the first oxygen bound to hemocyanin, when the urate concentration is altered (*21*).

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