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Thermodynamics of effector binding to hemocyanin: influence of temperature

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Title: Temperature dependence of urate binding to the hemocyanin of the European lobster, *Homarus vulgaris* – an ITC study

Abstract: Hemocyanins are multible, allosteric regulated proteins and serve as oxygen carriers in many crustacean species. The oxygen binding behavior of the hemocyanin is modulated by two anaerobic metabolites, L-lactate and urate. Additionally, non-physiological effectors as caffeine, dimethylxanthines and methylxanthine could modulate the oxygen binding of hemocyanin. To understand the effect of the urate accumulation under hypoxic conditions, we investigate the binding behavior of urate to the hemocyanin via isothermal titration calorimetry at different temperatures. The binding of the urate at temperatures between 10 and 30°C could be explained by the "Two-Sets-of-Sites"-model with two allosteric and two non-allosteric binding places. The thermodynamic binding parameters support this model. The difference between the binding affinity of the non-physiological effector caffeine and the urate results from the hydrophobicity of the effector molecule.

Introduction: Hemocyanins are respiratory proteins found in many species of the two phyla Mollusca and Arthropoda. The smallest allosteric unit of the Crustacean hemocyanin is the hexamer and association states between hexamer and oligo-hexamer exists (van Holde and Miller, 1995; van Holde, 2001, Linzen et al. 1985). The association of the subunit occurred by divalent cations as Ca2+ and Mg2+ (Magnum, 1983). The multimeric structure of the hemocyanins enables a high cooperative oxygen binding and Hill-coefficients between 2 and 11 (Bonaventure and Bonaventura, 1980; Robert et al. 1987). The oxygen binding of the crustacean hemocyanin is modulated by a number of inorganic and organic molecules. Divalent cations and chloride (Truchot, 1975), protons (Magnum, 1983, Truchot, 1975), neurohormons (Morris and McMahon, 1989), and anaerobic end products such as urate (Morris et al. 1985) and L-lactate (Truchot, 1980) are modulators of the oxygen binding of the hemocyanin.

Morris et al. (1985) showed that urate increases the oxygen affinity of the hemocyanin of the freshwater crayfish *Austropotamobius pallipes* and the urate effect was confirmed later for other crustacean species (Lallier et al., 1987; Bridges, 1990). Urate accumulates under hypoxic conditions because of the oxygen-dependence of the uricase of crustacean (Dykens, 1991). Accessorily, other purine derivatives also influence the oxygen affinity, without physiological relevance. Zeis et al. (1992) analyze the oxygen and effector binding of the hemocyanin of the European lobster. Menze et al. (2001, 2005) investigate the pH-dependence of urate and caffeine binding to the hemocyanin of the European lobster via ITC studies and present a plausible allosteric model, but it seems, that urate accumulation is not essential during environmental hypoxia under chosen conditions.

Temperature is another environmental factor in the habitat of crustacean. High temperature could be induced low oxygen concentration in water (zitat). Decker et al. (2000) investigated the temperature influence of the aggregation state of the hemocyanin from *Astacus leptodactylus*. Zeis (1994) showed a temperature dependence of the oxygen binding of the hemocyanin of the European lobster in the presence of urate. To understand the binding of urate to the hemocyanin at different temperatures, titration experiments at different temperatures under normoxic and hypoxic conditions were determined. Menze et al. (2000, 2001, 2005) used caffeine as an analogue of urate and it serves as a useful model component. Caffeine binds much stronger and it is even more potent in influencing the oxygen affinity of the hemocyanin of the European lobster. To understand this different binding properties and influences of these two effectors, additionally, we used different xanthine derivatives in our investigations to characterize the specificity of the urate binding site.

Materials und Methods: *Purification of hemocyanin and control of the aggregation state:* Purification and characterization of the hemocyanin of the European lobster, *H. vulgaris*, was performed as described previously, with regards to its aggregation state and its molecular weight (Menze et. al., 2000).

ITC experiments: All calorimetric experiments were performed with the VP-ITC titration calorimeter (MicroCal Inc., Northhampton, MA). Design and operation of the instrument were described previously (Zitat). The protein solution was prepared by dialysis of purified hemocyanin against HEPES-buffer (100 mM HEPES, 150 mM NaCl, 20 mM CaCl2, 20 mM MgCl2; pH 8,0). Ligand solution was prepared by dissolving urate in this HEPES-buffer. We used the following ligands in the ITC experiment: urate, caffeine, paraxanthine, theophylline, theobromine, iso-caffeine, 1-, 3- and 7-methylxanthine and trimethyl-uricacid. The ITC experiments were carried out at temperatures between 10 and 30°C. The injection syringe rotated at 310 rpm and the time interval between the injections was about 250 s. The raw data were corrected by the subtraction of the heat of the addition of buffer to hemocyanin and the heat of dilution of the ligand into buffer. Additionally, the raw data were corrected for the injection signal of buffer into buffer. Titration curves were analyzed using the "Two-Sets-of-Sites"-model by the Origin software (MicroCal Inc., Northhampton, MA).

Modulator binding under hypoxic conditions: For measurement of the binding properties of the ligands under hypoxic conditions the method, described by Menze et al. 2005, was used. The temperature of the hypoxic ITC experiments were between 10 and 30°C and the equilibration temperature of the ligand and hemocyanin solution were three degree under the experimental temperature.

Analysis of calorimetric data: Binding of all purine derivatives were analyzed by the Two-Sets-of-Sites model (equation 1). Here, Θ describes the saturation of the hemocyanin, [e] is the

$$\Theta = \frac{2K_1[e]}{1+K_1[e]} + \frac{2K_2[e]}{1+K_2[e]} \qquad (1)$$

effector concentration and K_i describes the binding constant of the respective binding site. If the binding affinity of the effector was very weak and an analysis based on this type of model was impossible, a simpler model of analysis was used, the non-interacting site model (equation 2). At this n describes the number of binding sites.

$$\Theta = n\frac{K[e]}{1+K[e]} \qquad (2)$$

Results: *Temperature dependent urate binding*: The calorimetric study of urate binding was determined at four different temperatures (10, 15, 25 and 30°C) under normoxic and hypoxic conditions. Because of the weak binding affinity of the physiological modulator urate, the artificial effector caffeine was used in the calorimetric binding experiment. Figure 1 A shows a comparison of the binding curves of urate and caffeine at 10°C under normoxic (PO2 = 150 Torr) and hypoxic (PO2 < 1 Torr) conditions. The binding curves illustrate a representative integrated data curve of the heat of binding during the calorimetric experiment. The caffeine-binding isotherm under normoxic conditions shows a similar sigmoid shape. Thus, an accurate analysis of the stoichiometry of binding is possible. The binding curve of urate and the binding curve under hypoxic conditions show a nonsigmoid shape. The binding under these conditions is very weak and without knowledge of a binding model the binding curves could only analysed by the non-interacting-site model. Menze et al. (2005) proposed a binding model with two allosteric and two non-allosteric binding sites. The analysis of the caffeine binding under normoxic condition with the Two-Sets-of-Sites model obtain a good approximation. The non-allosteric binding site should be unaffected by the oxygen concentration. Thus, the binding affinity of the non-allosteric binding sites should be identical under hypoxic and normoxic conditions. This presumption allow an analysis of the caffeine binding data under hypoxic condition with a good approximation with the Two-Sets-of-Sites model (figure 1 A).

The binding of urate is very weak under hypoxic conditions. If one assume, that the allosteric binding sites are affected by the oxygen concentration in the milieu and the allosteric conformation of the hemocyanin. The binding affinity to the allosteric binding sites under hypoxic conditions is very low. We presume, that the heat under hypoxic conditions depends mainly on the binding to the non-allosteric binding sites. So, an analysis with a two types of binding sites is possible under normoxic and hypoxic conditions, because of the detection of the binding affinity to the non-allosteric binding site under hypoxic conditions (figure 1 A).

Figure 1 B shows the binding isotherm of urate and caffeine under hypoxic and normoxic conditions at 30°C. Under hypoxic conditions no binding of urate was detected. An analysis of the binding of urate and caffeine under hypoxic conditions were not possible. Table 1 shows the results of the analysis of the binding curves of urate and caffeine under hypoxic and normoxic conditions at 10, 15, 25 and 30°C. The analysis of the urate binding curves was only possible at lower temperatures (10 and 15°C). The binding constant $K_{allo}{}^{O}{}_{2}$ decrease significantly with higher temperature for caffeine. Here the binding of caffeine to the

allosteric binding sites under normoxic conditions could be described with $K_{allo}^{O_2}$ (10°C) = 703,0 ± 14,18 mM^{-1} to $K_{allo}^{O_2}$ (30°C) = 88,68 ± 8,33 mM^{-1}. The binding of urate to the allosteric binding sites under normoxic conditions is much weaker with binding constant $K_{allo}^{O_2}$ (10°C) = 35,37 ± 13,33 mM^{-1}. K_{allo}^{hyp} is very low at all temperatures for caffeine $K_{allo}^{hypcaff}$ = 2,08 ± 0,96 mM^{-1} and urate $K_{allo}^{hypurate}$ = 0,55 ± 0,26 mM^{-1} as well as not detectable at 30°C for caffeine. The affinity of the non-allosteric binding site shows a weaker temperature sensitivity at low temperatures with $K_{nonallo}^{caff}$ (10°C) = 47,70 ± 5,21 M^{-1} and $K_{nonallo}^{caff}$ (15°C) = 45,37 ± 1,04 mM^{-1} and only at higher temperatures a significant decrease of affinity was measured. Urate could be characterized by a weaker but comparable temperature insensitive binding behavior to the non-allosteric binding sites with $K_{nonallo}^{urate}$ (10°C) 4,90 ± 1,10 mM^{-1} and $K_{nonallo}^{urate}$ (15°C) = 4,30 ± 0,80 mM^{-1}.

In figure 2 the enthalpy-entropy-compensation of the different binding sites were applied. For this study we used additionally caffeine-binding curves at different temperatures under normoxic conditions (data not shown). The enthalpies of caffeine binding to the allosteric binding sites range from $\Delta H_{0allo}{}^{O_2caff}$ (10°C) = -55,31 ± 0,12 kJ mol^{-1} to $\Delta H_{0allo}{}^{O_2caff}$ (30°C) = -75,25 ± 3,64 kJ mol^{-1}. The different types of binding sites show the typical linear dependence of enthalpy and entropy of binding. The enthalpy of the allosteric binding site for the under normoxic condition existing conformation lay in a lower energy level, than the enthalpy of the non-allosteric binding site under normoxic conditions with $\Delta H_{0nallo}{}^{O_2caff}$ (10°C) = -37,43 ± 3,87 kJ mol^{-1} to $\Delta H_{0nallo}{}^{O_2caff}$ (30°C) = -18,13 ± 2,38 kJ mol^{-1}. Additionally, the binding reaction of the allosteric binding sites is increasingly exothermic with higher temperature. The binding enthalpy of the non-allosteric binding sites show the converse effect. The heat capacity of the allosteric binding sites of caffeine binding under normoxic conditions decrease from ΔCp (10°C) = 0,48 to ΔCp (30°C) = - 2,10 kJ K^{-1} mol^{-1}. The entropie of caffeine binding to the allosteric binding sites is completely negative with $T\Delta S_{0allo}{}^{O_2caff}$ (10°C) = -23,63 ± 0,16 kJ mol^{-1} to $T\Delta S_{0allo}{}^{O_2caff}$ (30°C) = -46,58 ± 3,44 kJ mol^{-1}. In contrast the binding of caffeine to the non-allosteric binding sites could be characterized by $T\Delta S_{0nallo}{}^{O_2caff}$ (10°C) = -12,09 ± 4,09 kJ mol^{-1} to $T\Delta S_{0nallo}{}^{O_2caff}$ (30°C) = 6,69 ± 2,27 kJ mol^{-1}. Because of the few analyzable data and the high standard deviation of the enthalpy and entropy of the urate binding it seems impossible to calculate the heat capacity accurately.

Urate binding specificity: To understand the specificity of the urate binding we used different xanthine derivatives in the ITC-experiment under normoxic and hypoxic conditions. All

applied xanthine derivatives compete for the urate binding sites (Zeis, 1994). The binding curves of the dimethylxanthines could be analyzed with the Two-Set-of-Sites model under normoxic and hypoxic conditions at 20°C. The analysis of the other xanthinderivatives occurred under normoxic conditions at 20°C. Table 2 shows the thermodynamic binding parameter of the applied xanthine derivatives and caffeine under normoxic and partial hypoxic conditions. The binding of theobromine revealed the highest binding affinity with the binding constant K_{allo}^{Theob} = 219,3 ± 38,5 mM^{-1}. The binding of all dimethylxanthines is strongly exothermic. The methylxanthines could be characterized with a much weaker binding affinity. The binding of 1-methylxanthine could only be analyzed by the non-interacting-site model with K_{1M} = 18,71 ± 9,94 mM^{-1} and ΔH_0^{1M} = - 115,05 ± 16,73 kJ mol^{-1}. 7-methylxanthines show the highest affinity of the methylxanthines with $K_{allo}{}_{2}^{7M}$ = 150,67 ± 7,51 mM^{-1}. The binding affinity of the non-allosteric bindings sites are low and for the respective methylxanthines with $K_{nonallo}^{7M}$ = 15,30 ± 1,53 mM^{-1} and $K_{nonallo}^{3M}$ = 12,13 ± 1,78 mM^{-1} in the same range. The non-allosteric binding constants of the dimethylxanthines are also low, but there is a significant difference between the different dimethylxanthines with $K_{nonallo}^{theop}$ = 17,50 ± 2,19 mM^{-1} and $K_{nonallo}^{theob}$ = 29,20 ± 2,81 mM^{-1}. Furthermore, the non-allosteric binding constant of theophylline seems to be in the same range as $K_{nonallo}$ 7-methylxanthine. Isocaffeine could be characterized by a non-allosteric binding constant of $K_{nonallo}^{icaff}$ = 53,33 ± 4,39 mM^{-1}, which is higher than $K_{nonallo}^{caff}$ = 32,64 ± 3,75 mM^{-1} at 20°C.

To obtain the heat capacity of the binding of the dimethylxanthines of the under normoxic conditions existing binding sites, ITC-binding experiments at different temperatures (10, 15, 25 and 30°C) under normoxic conditions were performed. At all different temperatures, the dimethylxanthines could be characterized by a much stronger binding affinity to the allosteric binding sites. Theobromine binds with $K_{allo}{}_{2}^{theob}$ (10°C) = 436,3 ± 159,1 mM^{-1} with the highest affinity to the allosteric binding sites. With increasing temperature all dimethylxanthines had a significant weaker affinity to the allosteric binding sites. At this, theophylline could characterized by the weakest affinity with $K_{allo}{}_{2}^{theop}$ (10°C) = 186,5 ± 88,4 mM^{-1} to $K_{allo}{}_{2}^{theop}$ (30°C) = 40,8 ± 9,67 mM^{-1}. The binding enthalpy of the allosteric binding sites varied less at low temperatures, but with increasing temperatures $\Delta H_{0allo}{}_{2}$ became much more negative. The resulting change in the heat capacity in dependence of temperature could be characterized by a negative heat capacity change at higher temperatures with $\Delta Cp_{allo}{}_{2}^{theob}$ = -0,12 to -0,66 kJ K^{-1} mol^{-1}, $\Delta C_{pallo}{}_{2}^{parax}$ = 0,71 to -2,13 kJ K^{-1} mol^{-1} and $\Delta C_{pallo}{}_{2}^{theop}$ = -0,61 to -3,42 kJ K^{-1} mol^{-1}. The free Gibbs energy of the binding of the dimethylxanthines was nearly temperature independent, e. g. varying from $\Delta G_{0allo}{}_{2}^{theop}$ (10°C) = -28,24 ± 0,87 kJ

mol^{-1} to $\Delta G_{0allo}{}^{O_2{}^{theop}}$ (30°C) = -26,70 ± 0,60 kJ mol^{-1}. The free Gibbs energies of the other dimethylxanthines are approximately in the same magnitude.

Figure 3 shows the enthalpy-entropy-compensation of the xanthine derivatives at 20°C. The compensations of the two types of binding sites occurred at different energy levels. Again the non-allosteric binding sites lay in a higher energy level as the allosteric binding site. The enthalpy-entropy-compensation takes place in a linear dependence of temperature. The enthalpies of binding to the allosteric binding sites under normoxic conditions vary at this between $\Delta h_{allo}{}^{O_2{}^{3M}}$ = -77,29 ± 8,32 kJ mol^{-1} and $\Delta h_{allo}{}^{O_2{}^{parax}}$ = -55,39 ± 5,45 kJ mol^{-1}. The binding to the non-allosteric sites occurred in a higher energy level. Only the binding of isocaffeine to the non-allosteric binding sites seems to be entropically favoured with $T\Delta S_{0nallo}{}^{O2icaf}$ = 13,59 ± 7,58 kJ mol^{-1}.

Discussion: The physiological modulator urate enhances the oxygen affinity of the hemocyanin of the European lobster (Zeis et al. 1992, Nies et al., 1992). The artificial effector caffeine binds much stronger to the hemocyanin and the oxygen modulation of the hemocyanin is much more pronounced in the presence of caffeine (Menze et al. 2005). At first the temperature dependence of the oxygen half-saturation of the hemocyanin of the European lobster in ringer solution in the presence and absence of urate were pointed out by Zeis et al. (1994).

We investigated the binding of urate and caffeine at temperature between 10 and 30°C by ITC. As recommended by Menze et al. 2005, we used the Two-Set-of-Sites model to analyze the ITC binding data. Figure 1 A shows the good approximation of the fit. The affinity of the effectors to the allosteric binding sites of the hemocyanin decreases significantly with increasing temperature significantly. At 30°C the heat dilution of the urate binding under hypoxic conditions was very weak and an analysis was impossible (figure 1 B). But the caffeine binding curve at 30°C under hypoxic conditions were analyzable and so urate probably binds to the hemocyanin but we were not able to detect the binding. The non-allosteric binding sites are not that temperature sensitive as the allosteric sites. Menze et al. (2005) obtained urate and caffeine binding curves at 20°C at pH 7,55. They proposed a allosteric model based on the well known Nested MWC model (Wyman, 1872; Robert et al. 1987). If we assume, that the by the applied Nested-MWC-model proposed rT-conformation is the under normoxic conditions available conformation, the binding data K_{rTcaff} = 361 ± 48 mM^{-1} matchs with the new temperature dependent data.

As indicate by the enthalpie-entropie compensation and the data in table x and y the binding of all effectors to the allosteric as well as the non-allosteric binding sites are enthallpically favoured. The free energy ΔG_0 of the allosteric binding sites drifts from $\Delta G_{0allo}{}^{O_2{}^{caff}}$ (10°C) = -31,68 ± 0,05 kJ mol^{-1} to $\Delta G_{0allo}{}^{O_2{}^{caff}}$ (30°C) = -28,7 ± 0,2 kJ mol^{-1}. The free energy of the non-allosteric binding sites is in a higher energy level. This was approved by the different energy levels of the enthalpy-entropy-compensation of the binding data of caffeine. The binding to both types of binding sites is enthalpy driven. The binding of urate has a much more entropically inappropriate with $T\Delta S_0allo{}^{O_2{}^{urate}}$ (10°C) = -56,75 kJ mol^{-1} than the caffeine binding with $T\Delta S_{0allo}{}^{O_2{}^{caff}}$ (10°C) = -23,63 kJ mol^{-1}, whereas the binding is supported by the enthalpy $\Delta H_{0allo}{}^{O_2{}^{urate}}$ (10°C) = -65,78 ± 4,94 kJ mol^{-1} and so the binding of urate to the allosteric binding sites of the hemocyanin is enthalpically compensated at the temperature of 10°C. A hydrophobic interaction could support the caffeine binding in this situation and results in a favourable entropy (Sturtevant, 1997; Calderone and Williams, 2001). In the binding process a release of water at the binding sites occurred and thus, arose the entropically fraction at the caffeine binding to the allosteric binding sites under normoxic conditions. On the other hand the charged urate formed hydrogen bonds at the binding sites (Cooper, 2005). Therefore, this resulted in a favoured free energy of caffeine binding of $\Delta G_{0allo}{}^{O_2{}^{caff}}$ (10°C) = -31,68 ± 0,05 kJ mol^{-1} compared to $\Delta G_{0allo}{}^{O_2{}^{urate}}$ (10°C) = - 25,51 ± 1,0 kJ mol^{-1}. In contrast, the binding of urate to the allosteric binding site under hypoxic conditions is entropiecally driven ($T\Delta S_{0nallo}{}^{hypurate}$ (10°C) = -4,33 kJ mol^{-1}), but the free energy is much lower ($\Delta G_{0nallo}{}^{hypurate}$ (10°C) = -9,69 kJ mol^{-1}) and the affinity of urate to the non-allosteric binding site much weaker.

This shown enthalpy-entropy-compensation is a simply consequence of the strong temperature dependence of ΔH_0 and ΔS_0. McPhail and Cooper (1997) proposed that a change in heat capacity in dependence of temperature could result in such an enthalpy-entropy-compensation. The binding of caffeine could be characterized by a negative change in heat capacity (from $\Delta C_{pallo}{}^{caff}$ (10°C) = 0,48 kJ K^{-1} mol^{-1} to $\Delta C_{pallo}{}^{caff}$ (30°C) = -2,10 kJ K^{-1} mol^{-1}). Negative heat capacity changes could depend on transfer of hydrophobic ligands from water to the binding site (Wadsö, 1972). Additionally, we investigate in the heat capacity changes of di-methylxanthines and we could characterize the heat capacity changes of the di-methylxanthines about 0,1 kJ K^{-1} mol^{-1} (10°C) to -3,10 kJ K^{-1} mol^{-1} (30°C). Thus, the heat capacity change of caffeine should be much stronger than of the dimethylxanthine because of its hydrophobicity, but this is not the case. As one sees above, there is no correlation between the hydrophobicity of the ligand and its change in heat capacity, because the higher the

proportion of methyl-groups at the effector the higher should be the negative heat capacity change. But studies of Clarke et al. (2001) and Cooper (2005) demonstrated, that the appreciation of heat capacity changes depend on hydrophobicity much more difficult.

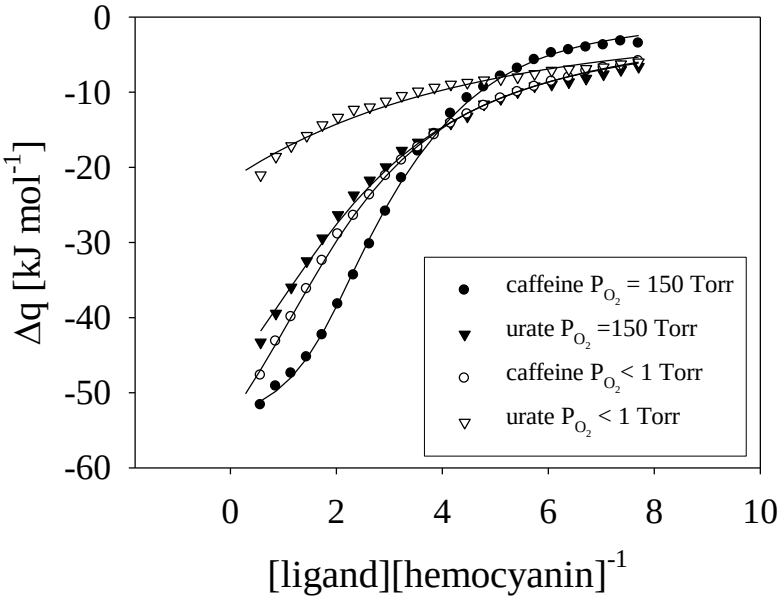
An additional explanation of negative heat capacity changes could be a temperature dependent and ligand induced change of conformation (Elguero et al. 1976, Eftink and Biltonen, 1980, Cooper, 2005). This seems to be the presumably explanation, because the conformationally changes occur in the temperature dependence of the heat capacity (Privalov and Privalov, 2000).

Urate binding specificity: The binding of different xanthine derivatives was investigated by ITC experiments under normoxic conditions. As Zeis (1994) already showed by replacement experiment, the basic structure of the 6-mono-oxopurine is essential for binding to the hemocyanin of the European lobster. To understand the weaker binding affinity of urate to the hemocyanin and to investigate the sterical structure of the effector in and to the binding site of the hemocyanin we arranged this ITC experiments. The binding of the xanthine derivatives could be characterized by allosteric binding constants to a conformation existing under normoxic conditions, if we assumed a conformational and allosteric change due to the negative heat capacity change of the dimethylxanthines and caffeine, and a binding to non-allosteric binding sites. The free energy of the xanthine derivatives to the allosteric binding sites at 20°C are between $\Delta G_{0allo}{}^{O_2{}^{caf}}$ (20°C) = -30,81 ± 0,23 kJ mol^{-1} and $\Delta G_{0allo}{}^{O_2{}^{TMH}}$ (20°C) = -28,27 ± 0,96 kJ mol^{-1}. At this 3-methylxanthine deviate with $\Delta G_{0allo}{}^{O_2{}^{3M}}$ (20°C) = -26,14 ± 0,40 kJ mol^{-1}, which is due to the weak binding affinity of the methylxanthine. The binding of the xanthine derivatives could be characterized by significantly different binding constants. This different binding affinity could depend on the different structure of the xanthine derivatives. Theobromine, which is characterized by methyl-groups at position 3 and 7 of the xanthine ring, binds with the strongest affinity to the allosteric binding sites ($K_{allo}{}^{O_2{}^{theob}}$ (20°C) = 219,3 ± 38,5 mM^{-1}). Theophyllin, which has methyl-groups at position 1 and 3, could be characterized with the weakest affinity ($K_{allo}{}^{O_2{}^{theop}}$ (20°C) = 124,9 ± 49,3 mM^{-1}) to the allosteric binding sites. During the ITC experiment, we had a constant pH of 8. The protonation of the xanthine structure occurs at high pH at first at position 3, following position 7 (Lister 1966; Elguero et al. 1976). Probably, theobromine would not ionized in the experiment. Theophylline is subject to ionization of position 3 and 7. The ionization seems to amplify the binding to the allosteric binding sites. In consideration of the binding data of the other methylxanthine, it is obvious that a methyl-group at position 7 of the xanthine ring promotes the binding to the allosteric binding sites, because of the high binding affinity of the

7-methylxanthine ($K_{allo}{}^{O_2{}^{7M}}$ (20°C) = 150,67 ± 7,51 mM^{-1}) to the hemocyanin. Trimethyl-uric-acid could be characterized by a much stronger binding to the allosteric binding sites ($K_{allo}{}^{O_2{}^{TMH}}$ (20°C) = 115,70 ± 48,51 mM^{-1}) as urate ($K_{allo}{}^{O_2{}^{urate}}$ (10°C) = 35,37 ± 13,33 mM^{-1}), probably, because of the higher portion of methyl groups in the structure. The number and position of methyl-groups and for this reason the hydrophobicity of the effector seems to support binding to the allosteric binding sites to the hemocyanin of the European lobster. This is the reason of weaker binding of urate, because of the high ionization of the urate molecule. Additionally, isocaffeine has a weaker binding affinity to the allosteric binding sites compared to caffeine. This is to be due to the change of the position of the methyl-group from position 9 instead of 7.

The binding of all xanthine derivatives to the non-allosteric binding sites is much weaker. There is a significant difference between the binding constants of theobromine ($K_{nallo}{}^{O_2{}^{theob}}$ (20°C) = 29,2 ± 2,81 mM^{-1}) , paraxanthine ($K_{nallo}{}^{O_2{}^{parax}}$ (20°C) = 24,7 ± 5,05 mM^{-1}) as well as caffeine($K_{nallo}{}^{O_2{}^{caff}}$ (20°C) = 32,6 ± 3,74 mM^{-1}) and the remaining xanthine derivatives, which at this approve the favourable binding of hydrophobic effectors. Additionally, isocaffeine could be characterized by a very high binding affinity to the non-allosteric binding sites ($K_{nallo}{}^{O_2{}^{icaf}}$ (20°C) = 53,3 ± 4,4 mM^{-1}). This could be due to the change of methyl-group position. In the case of the methyl-xanthine the binding affinities to the non-allosteric binding sites are all in the same range and there is no significant difference. The enthalpy-entropy-compensation of the xanthine derivatives approve the different energy levels of the two different binding sites as its was shown for caffeine at different temperatures. The binding of isocaffeine to the non-allosteric binding sites seemed to be entropically favoured, which emphasize the specific importance of the methyl-group at position 9.

Finally, we demonstrated the strong temperature dependent binding of caffeine and the dimethylxanthine to the hemocyanin of the European lobster. This strong temperature dependent binding could result in a different influence on the oxygen binding. Accordingly, this should be the next step for the experimental procedure. The negative heat capacity of binding could result in a conformational change of the hemocyanin molecule induced by temperature. An allosteric model, which includes temperature dependence of caffeine/urate and oxygen binding could point out this behavior. Finally, we demonstrated, that the binding behaviour of effectors to the urate binding sites depend on the hydrophobicity of the effector molecule. This is the reason of the weak urate binding to the hemocyanin of the European lobster.



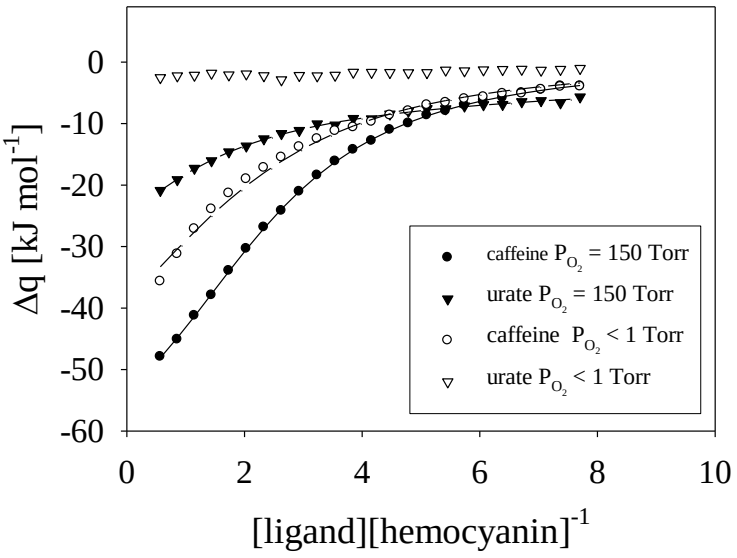


figure 1 B

figure 1: Titration of effector to hemocyanin of the European lobster at 10°C (figure xA) and 30°C (figure x B) under normoxic (oxygen concentration =150 Torr) and hypoxic (oxygen concentration < 1 Torr) conditions. The titration was performed by injecting 25 x 10 µl effector of 1 mM urate and caffeine into hemocyanin (25 µM) in HEPES buffer at pH 8,0 at 10°C (figure xA) and 30°C (figure x B). The lines correspond to the analysis based on two types of sites, with two binding sites of each type of site.

Table 1: binding constants $k_{O_2}^{\text{eff}}$ [mM^{-1}] and k_{hyp}^{eff} [mM^{-1}] of the two allosteric binding sites as well as $k_{nalloO_2}^{\text{eff}}$ and $k_{nallohyp}^{\text{eff}}$ of the two non-allosteric binding sites , and the corresponding binding enthalpies $\Delta H^0_{O_2{}^{\text{eff}}}$ [kJ mol^{-1}], $\Delta H^0_{hyp^{\text{eff}}}$ [kJ mol^{-1}], $\Delta H^0_{nalloO_2{}^{\text{eff}}}$ [kJ mol^{-1}] und $\Delta H^0_{nallohyp^{\text{eff}}}$ [kJ mol^{-1}] of caffeine and urate binding to the hemocyanin of the European lobster between 10 and 30°C (X ± SD).

	temperature [°C]			
parameters	10	15	25	30
caffeine				
$k_{O_2}^{\text{Koffein}}$ [mM^{-1}]	$703,00 \pm 14,18$	$468,67 \pm 140,15$	$177,25 \pm 22,32$	$88,68 \pm 8,33$
k_{hyp}^{Koffein} [mM^{-1}]	$2,08 \pm 0,96$	9,92	0,65	–
$k_{nalloO_2}^{\text{Koffein}}$ [mM^{-1}]	$47,70 \pm 5,21$	$45,37 \pm 1,04$	$26,55 \pm 3,55$	$17,9 \pm 3,55$
$k_{nallohyp}^{\text{Koffein}}$ [mM^{-1}]	$47,70 \pm 5,21$	$45,37 \pm 1,04$	$26,55 \pm 3,55$	$17,9 \pm 3,55$
$\Delta H^0_{O_2{}^{\text{Koffein}}}$ [kJ mol^{-1}]	$-55,31 \pm 0,12$	$-57,28 \pm 2,83$	$-62,99 \pm 5,46$	$-75,35 \pm 3,64$
$\Delta H^0_{hyp^{\text{Koffein}}}$ [kJ mol^{-1}]	$-69,57 \pm 4,46$	$-11,04 \pm 4,52$	$-101,47 \pm 28.22$	–
$\Delta H^0_{nalloO_2{}^{\text{Koffein}}}$ [kJ mol^{-1}]	$-37,43 \pm 3,87$	$-32,28 \pm 2,36$	$-28,84 \pm 4,61$	$-18,13 \pm 2,38$
$\Delta H^0_{nallohyp^{\text{Koffein}}}$ [kJ mol^{-1}]	$-74,63 \pm 18,53$	$-68,41 \pm 4,88$	$-58,57 \pm 5,33$	–
urate				
$k_{O_2}^{\text{Urat}}$ [mM^{-1}]	$35,37 \pm 13,33$	$50,37 \pm 9,82$	–	–
k_{hyp}^{Urat} [mM^{-1}]	$0,55 \pm 0,026$	$0,07 \pm 0,02$	–	–
$k_{nalloO_2}^{\text{Urat}}$ [mM^{-1}]	$4,90 \pm 1,10$	$4,30 \pm 0,80$	–	–
$k_{nallohyp}^{\text{Urat}}$ [mM^{-1}]	$4,90 \pm 1,10$	$4,30 \pm 0,80$	–	–
$\Delta H^0_{O_2{}^{\text{Urat}}}$ [kJ mol^{-1}]	$-65,78 \pm 4,98$	$-52,23 \pm 5,57$	–	–
$\Delta H^0_{hyp^{\text{Urat}}}$ [kJ mol^{-1}]	$-14,02 \pm 6,20$	$-9,42 \pm 2,81$	–	–
$\Delta H^0_{nalloO_2{}^{\text{Urat}}}$ [kJ mol^{-1}]	$-56,72 \pm 18,06$	$-66,40 \pm 12,17$	–	–
$\Delta H^0_{nallohyp^{\text{Urat}}}$ [kJ mol^{-1}]	$-91,01 \pm 7,78$	$-77,65 \pm 21,99$	–	–

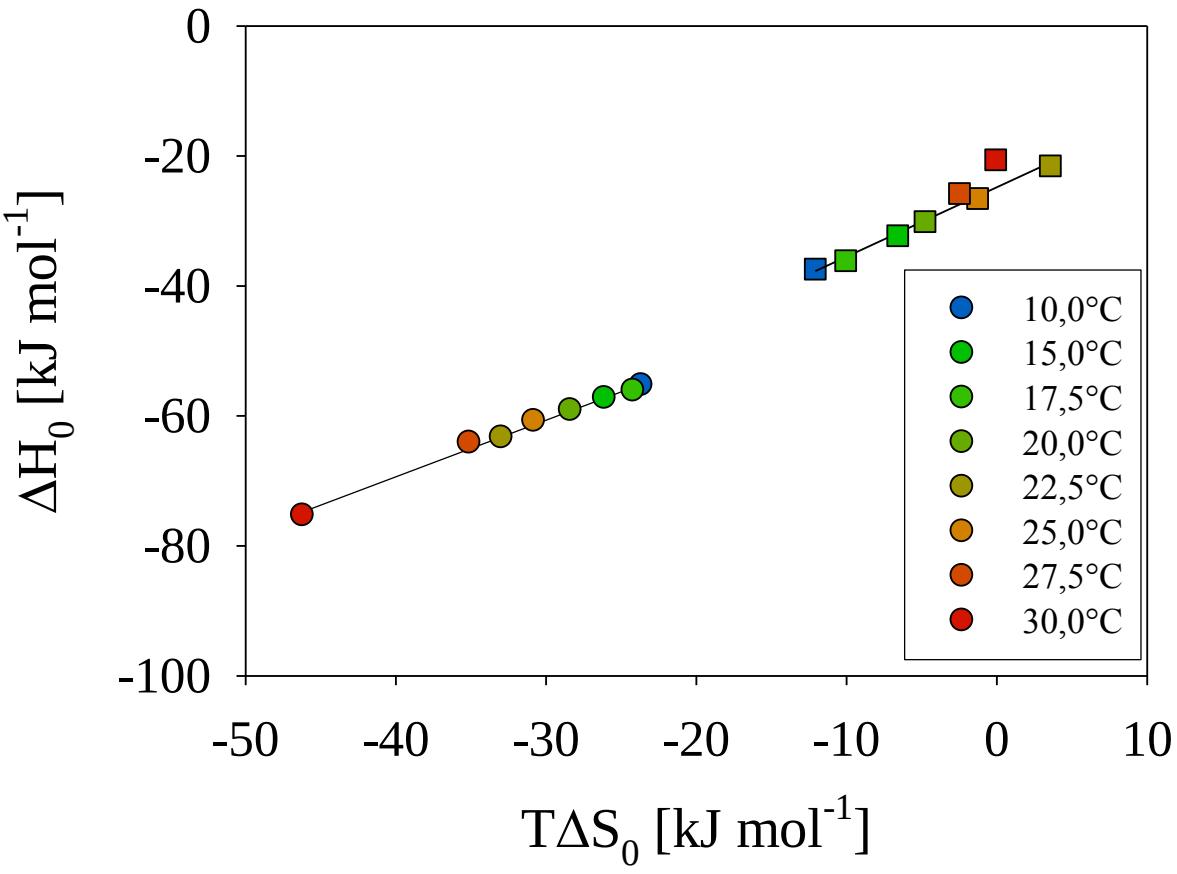


figure 2 : Enthalpy-entropy-compesnation of the binding of caffeine to the allosteric (circle) and non-allosteric (square) binding sites under normoxic conditions (oxygen concentration = 150 Torr) at different temperature. The enthalpy ΔH0 [kJ mol-1] and the entropy TΔS0 [kJ mol-1] were detected by the ITC experiment under normoxic condition at different temperatures.

Table 2: binding parameters $k^{eff}_{O_2}$ [mM^{-1}], k^{eff}_{hyp} [mM^{-1}], $k^{eff}_{nalloO_2}$ and $k^{eff}_{nallohyp}$ as well as $\Delta H^0_{O_2{}^{eff}}$ [kJ mol^{-1}], $\Delta H^0_{hyp^{eff}}$ [kJ mol^{-1}], $\Delta H^0_{nalloO_2{}^{eff}}$ [kJ mol^{-1}] and $\Delta H^0_{nallohyp^{eff}}$ [kJ mol^{-1}] of the binding of (A) theophyllne, theobromine and paraxanthine and (B) 3-methylxanthine, 7-methylxanthine, isocaffeine and 1,3,7-trimethyluricacid to the hemocyanine of the European lobster at 20°C (X ± SD).

(A)

	theophylline	paraxanthine	theobromine
$k^{eff}_{O_2}$[mM^{-1}]	$124,9 \pm 49,3$	$155,8 \pm 35,5$	$219,3 \pm 38,5$
k^{eff}_{hyp}[mM^{-1}]	≈2	$1,67 \pm 0,15$	$2,9 \pm 1,19$
$k^{eff}_{nalloO_2}$ [mM^{-1}]	$17,5 \pm 2,19$	$24,7 \pm 5,05$	$29,2 \pm 2,81$
$k^{eff}_{nallohyp}$ [mM^{-1}]	17,5	24,7	29,2
$\Delta H^0_{O_2{}^{eff}}$ [kJ mol^{-1}]	$-58,13 \pm 2,61$	$-55,39 \pm 5,45$	$-61,31 \pm 3,91$
$\Delta H^0_{hyp^{eff}}$ [kJ mol^{-1}]	$-86,86 \pm 12,81$	$-120,01 \pm 24,04$	$-96,57 \pm 67,71$
$\Delta H^0_{nalloO_2{}^{eff}}$ [kJ mol^{-1}]	$-77,84 \pm 10,28$	$-47,55 \pm 13,15$	$-50,49 \pm 12,87$
$\Delta H^0_{nallohyp^{eff}}$ [kJ mol^{-1}]	$-83,87 \pm 7,49$	$-53,92 \pm 8,36$	$-47,09 \pm 2,71$

(B)

	3-methylxanthine	7-methylxanthine	isocaffeine	1,3,7-TMU
$k^{eff}_{O_2}$ [mM^{-1}]	$46,28 \pm 7,89$	$150,67 \pm 7,51$	$272,00 \pm 5,29$	$115,70 \pm 48,51$
$k^{eff}_{nalloO_2}$ [mM^{-1}]	$12,13 \pm 1,78$	$15,30 \pm 1,73$	$53,33 \pm 4,39$	$8,86 \pm 0,30$
$\Delta H^0_{O_2{}^{eff}}$ [kJ mol^{-1}]	$-77,29 \pm 8,32$	$-55,57 \pm 2,66$	$-66,68 \pm 1,72$	$-68,86 \pm 7,06$
$\Delta H^0_{nalloO_2{}^{eff}}$ [kJ mol^{-1}]	$-49,99 \pm 15,63$	$-72,04 \pm 1,61$	$-12,92 \pm 7,37$	$-42,86 \pm 19,01$

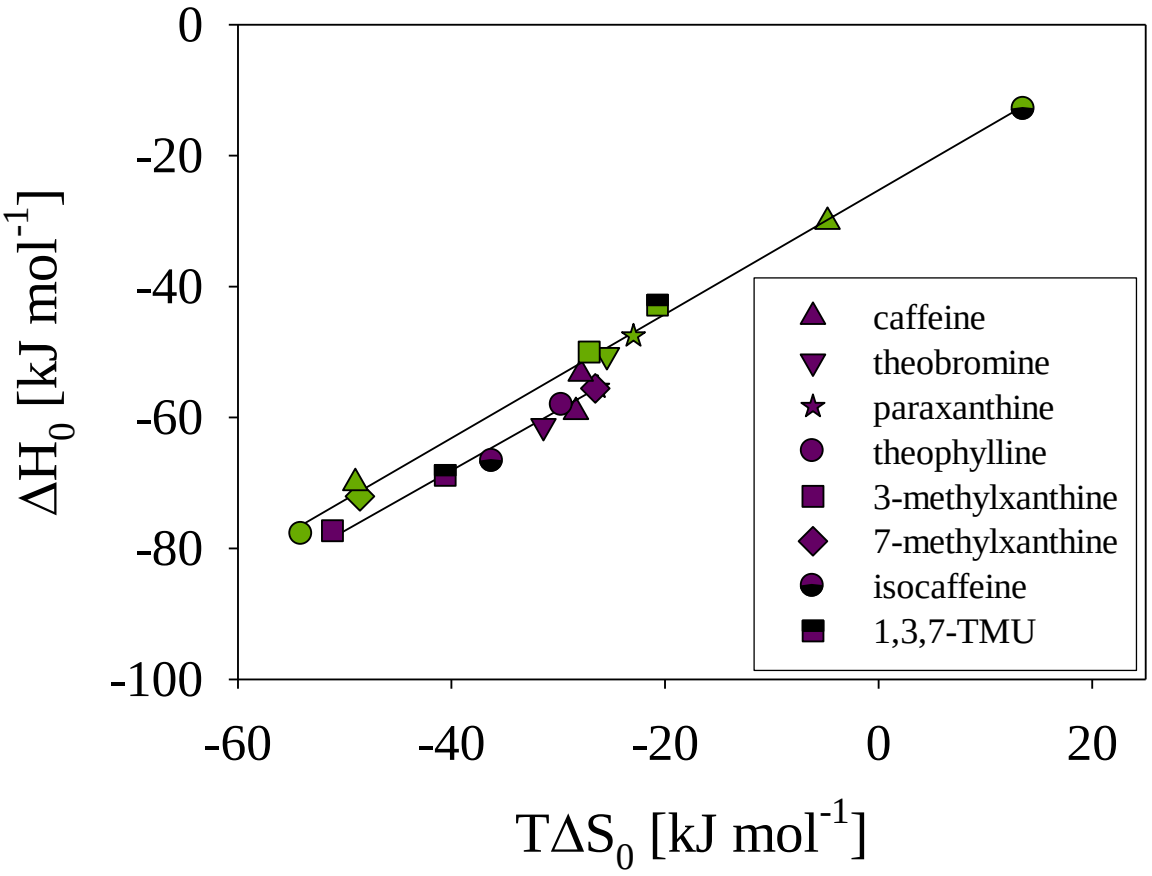


figure 3: Enthalpy-entropy-compensation of the binding of caffeine (symbol), theobromine (), paraxanthine (), theophylline (), 3-methylxanthine (), 7-methylxanthine (), isocaffeine () and 1,3,7 trimethyluricacid () to the allosteric (purple) and non-allosteric (green) binding sites under normoxic conditions (oxygen concentration = 150 Torr) at 20°C. The enthalpie ΔH0 [kJ mol-1] and the entropie TΔS0 [kJ mol-1] were detected by the ITC experiment under normoxic condition at 20°C.