

## A Thermodynamic Study of the Interaction between Urease and Copper Ions

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### Abstract

A thermodynamic study of copper ions by jack bean urease (JBU) was carried out at two temperatures of 27 and 37°C in Tris buffer (30 mM; pH=7.0) using an isothermal titration calorimetry. There is a set of twelve identical and non-interacting binding sites for copper ions. The intrinsic dissociation equilibrium constant and the molar enthalpy of binding are 285  $\mu\text{M}$  and  $-15.2$  kJ/mol at 27°C and 346  $\mu\text{M}$  and  $-14.6$  kJ/mol at 37°C, respectively. The molar entropy of binding is  $17.2$  J K<sup>-1</sup> mol<sup>-1</sup> at 27°C and  $+19.1$  J K<sup>-1</sup> mol<sup>-1</sup> at 37°C. Hence, the binding process of copper ion to JBU is not only enthalpy driven but also it is entropy driven, which the role of entropy driven should be more effective by increasing the temperature.

**Keywords:** Urease; Copper ion; Isothermal titration calorimetry; Enthalpy of binding; Entropy of Binding

### Introduction

Jack bean urease (urea amidohydrolase; E.C. 3.5.1.5) is the first crystallized enzyme [1] and also is the first enzyme shown to contain nickel [2-3]. The crystal structure of urease has not been determined yet. The best resolution obtained at 3.5 Å only allowed to assign the octahedral crystals of this urease to a cubic spacegroup [4]. Jack bean urease has six identical subunits. Each subunit consist of a single kind of polypeptide chain containing 840 amino acid residue with relative molecular mass of 90770, excluding the two nickel ions per subunit[5]. Hence, a mass of the hexamer urease, including 12 nickel ions, being 545.340 kDa<sup>5</sup> (590 kDa by a sedimentation method [6]). The subunit of urease from microorganisms appear to be

smaller than jack bean urease in size and number [7-8].

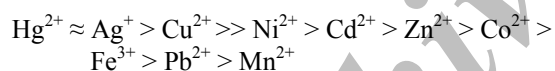
Urease catalyzes the hydrolysis of urea to carbonic acid and two molecules of ammonia[9]. The specificity of urease was believed to be absolute [10] until Fishbein et al. reported that N-hydroxyurea was a substrate [11-12]. N-hydroxyurea [11-13], (N,N')dihydroxyurea [14-15], semicarbazide [16], N-methylurea [17], formamid [18] and acetamide [17] are another examples of substrates for urease. The mechanisms of hydrolysis of urea by urease presently contemplated are those by Benini et al. [19] and Karplus et al. [20], which taking their origins from the mechanism proposed by Zerner's group [17]. Zerner et al. have proposed a model for jack bean urease catalysis in which one nickel coordinates the oxygen atom of urea, polarizing the carbonyl group, and a second nickel coordinates hydroxide ion, the

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catalytic nucleophile [17].

Ureases are inhibited by a number of compounds. The study of urease inhibitors may have medical or agronomic significance, as well as providing insight into the urease catalytic mechanism [21-22]. Substrate urea, product ammonium ions, and substrate analogues are weak inhibitors of urease [21, 23-24]. Thiols inhibit urease competitively in their thiolate anion form R-S<sup>-</sup> [22]. Amides and esters of phosphoric acid are also slow-binding inhibitors of urease, classified as the strongest inhibitors. Boric and boronic acids are rapidly binding urease inhibitors, comparatively weak. Fluoride ion was found to be a competitive inhibitor for jack bean urease.<sup>25</sup> There are some reports on the inhibition of urease by bismuth compounds, which are of medical importance since are widely used as bactericidal agents [26-29]. The inhibition of ureases by quinones has been mainly tested for their potential application with urea fertilizers [29-30].

Inhibition of urease by heavy metal ions is important not only in view of heavy metal ion pollution, appropriate levels of urease activity in agricultural soils may be endangered, but also this inhibition may be exploited in constructing urease inhibition-based sensing systems [31-33] for in situ and real time determination of trace levels of the ions, e.g. in environmental monitoring, food control and biomedical analysis. Heavy metal ions inhibit both plant [31,34-38] and bacterial ureases[39-40] at the following order of effectiveness [36,38]:



Hg<sup>2+</sup>, Ag<sup>+</sup> and Cu<sup>2+</sup> ions nearly always listed as the strongest inhibitors [31-36,38,41]. The inhibition has been habitually ascribed to the reaction of the metal ions with the thiol groups of the enzyme [33,41-42]. However, both copper and silver ions coordinate to nitrogen- (histidine) and possibly oxygen- (aspartic and glutamic acids) containing functional groups in urease [43-44]. Notwithstanding heavy metal ion binding to urease is important, there is not a comprehensive binding study in this case. Here, we applied isothermal titration microcalorimetry as a powerful tool for studying of copper ion binding to jack bean urease, which all thermodynamic parameters for the binding process can be found.

## Materials and Methods

### Materials

Jack bean urease (JBU) was obtained from Sigma

Chemical Co. Copper nitrate was obtained from Merck. The buffer solution used in the experiments was 30 mM Tris using double-distilled water, pH=7.0, which was obtained from Merck. Experiments were carried out at two temperatures of 27 and 37 °C.

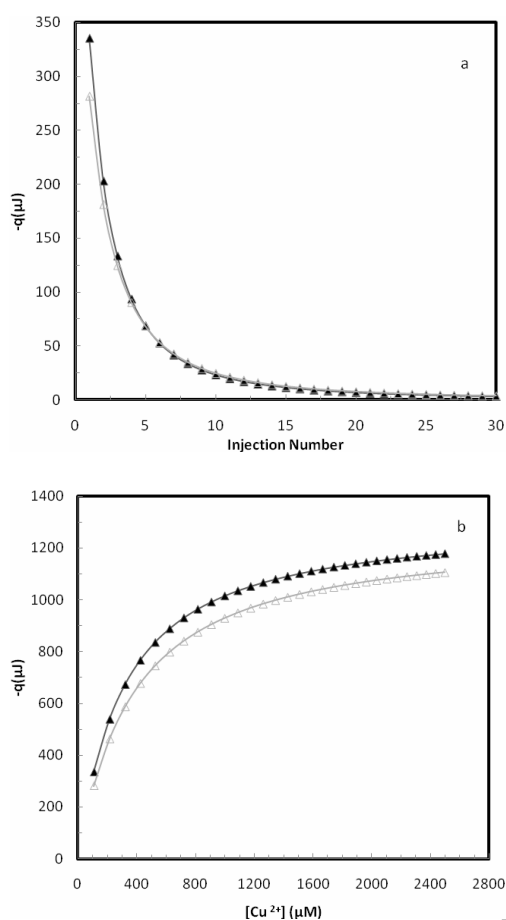
### Methods

The experiments were performed with the 4-channel commercial microcalorimetric system, Thermal Activity Monitor 2277 (Thermometric, Sweden). Each channel is twin heat conduction calorimeter (multijunction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. Both, sample and reference vessels were made from stainless steel. The limited sensitivity for the calorimeter is 0.1 μJ. Copper ion solution (10 mM) was injected into the calorimetric titration vessel, which contained 1.8 ml JBU, 4 μM (2.2 mg/ml), in Tris buffer (30 mM), pH=7.0, using a Hamilton syringe. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of copper ion solution into the perfusion vessel was repeated 30 times and each injection included 20 μl copper ion solution. The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of injection was calculated by the "Thermometric Digitam 3" software program. The heat of dilution of the copper ion solution was measured as described above except JBU was excluded. Also, the heat of dilution of the protein solution was measured as described above except that the buffer solution was injected to the protein solution in the sample cell [45]. The enthalpies of copper ion and protein solutions dilution were subtracted from the enthalpies of copper ion solutions in JBU solutions. The microcalorimeter was frequently calibrated electrically during the course of the study.

## Results and Discussion

The raw data obtained from ITC at two temperatures of 27 °C and 37 °C are shown in Figure 1. Figure 1a shows the heat of each injection and Figure 1b shows the cumulative heat at each total concentration of copper ion, [Cu<sup>2+</sup>]<sub>t</sub>. For a set of identical and independent binding sites, we have before shown different methods of ITC data analysis[45]. For a set of identical and independent binding sites, we have previously introduced the following equation[45-46]:

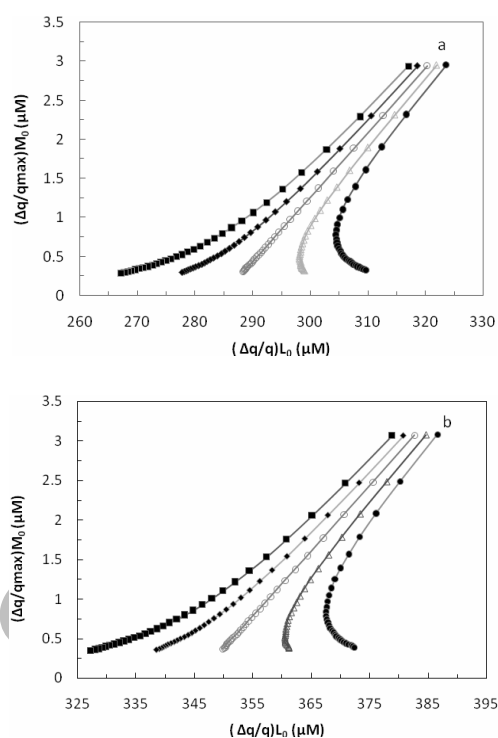
$$\frac{\Delta q}{q_{\max}} M_0 = \left(\frac{\Delta q}{q}\right) L_0 \frac{1}{g} - \frac{K_d}{g} \quad (1)$$



**Figure 1.** (a) The heat of copper ion binding to JBU for 30 automatic cumulative injections, each of 20  $\mu\text{l}$ , 10 mM of the copper nitrate solution, into sample cell containing 1.8 ml 4  $\mu\text{M}$  protein solution at 300 K ( $\blacktriangle$ ) and 310 K ( $\triangle$ ). (b) The total cumulative heat of binding vs. total concentration of copper ion calculated from Fig. 1a.

where  $g$  is the number of binding sites,  $K_d$  is the dissociation equilibrium constant,  $M_0$  and  $L_0$  are total concentrations of biomacromolecule and metal ion, respectively,  $\Delta q = q_{\text{max}} - q$ ;  $q$  represents the heat value at a certain  $L_0$  and  $q_{\text{max}}$  represents the heat value upon saturation of all biomacromolecule. If  $q$  and  $q_{\text{max}}$  are calculated per mole of biomacromolecule then the standard molar enthalpy of binding for each binding site ( $\Delta H^\circ$ ) will be  $\Delta H^\circ = \frac{q_{\text{max}}}{g}$ . According to the equation

(1), a plot of  $(\Delta q/q_{\text{max}})M_0$  vs.  $(\Delta q/q)L_0$  should be a linear plot by a slope of  $1/g$  and the vertical-intercept of  $K_d/g$ , which  $g$  and  $K_d$  can be obtained. The related plot for the binding of  $\text{Cu}^{2+}$  ions by JBU is shown in Figure 2. The linearity of the plot has been examined by different estimated values for  $q_{\text{max}}$  to find the best value for the



**Figure 2.** (a) The best linear plot of  $(\frac{\Delta q}{q_{\text{max}}})M_0$  vs.  $(\frac{\Delta q}{q})L_0$ , according to Eq. (1), using values of  $-1303 \mu\text{J}$  ( $\blacksquare$ ),  $-1308 \mu\text{J}$  ( $\blacklozenge$ ),  $-1313 \mu\text{J}$  ( $\circ$ ),  $-1318 \mu\text{J}$  ( $\triangle$ ),  $-1323 \mu\text{J}$  ( $\bullet$ ) at 300 K for  $q_{\text{max}}$  to obtain the best correlation coefficient value ( $R^2=0.999$ ) for a linear plot. (b) The best linear plot of  $(\frac{\Delta q}{q_{\text{max}}})M_0$  vs.  $(\frac{\Delta q}{q})L_0$ , according to Eq. (1), using values of  $-1251 \mu\text{J}$  ( $\blacksquare$ ),  $-1256 \mu\text{J}$  ( $\blacklozenge$ ),  $-1261 \mu\text{J}$  ( $\circ$ ),  $-1266 \mu\text{J}$  ( $\triangle$ ),  $-1271 \mu\text{J}$  ( $\bullet$ ), at 310 K for  $q_{\text{max}}$  to obtain the best correlation coefficient value ( $R^2=0.999$ ) for a linear plot.

correlation coefficient (near to one). The best linear plot with the correlation coefficient ( $R^2$ ) value (near to one) was obtained using  $-1313 \mu\text{J}$  and  $-1261 \mu\text{J}$  (equal to  $-182.4 \text{ kJ/mol}$  and  $-175.1 \text{ kJ/mol}$ ) at  $27^\circ\text{C}$  and  $37^\circ\text{C}$ , respectively, for  $q_{\text{max}}$  (Fig. 2). The lack of a suitable value for  $q_{\text{max}}$  to obtain a linear plot of  $(\frac{\Delta q}{q_{\text{max}}})M_0$  vs.

$(\frac{\Delta q}{q})L_0$  may be related to the existence of non-identical binding sites or the interaction between them. The value of  $g$  is 12, obtained from the slope and values of  $K_d$ , obtained from the vertical-intercept plot for a set of identical and independent binding sites are  $285 \mu\text{M}$  and  $346 \mu\text{M}$  at  $27^\circ\text{C}$  and  $37^\circ\text{C}$ , respectively. Dividing the  $q_{\text{max}}$  amount of  $-182.4 \text{ kJ/mol}$  and  $-175.1 \text{ kJ/mol}$  by

$g=12$ , therefore, gives  $\Delta H^\circ = -15.2$  kJ/mol and  $-14.6$  kJ/mol at  $27^\circ\text{C}$  and  $37^\circ\text{C}$ , respectively.

The molar enthalpy of each binding site ( $\Delta H^\circ$ ) and its dissociation equilibrium constant ( $K_d$ ) in a set of biomacromolecule binding sites can also be obtained via a simple graphical nonlinear fitting method using the following equation [45, 47-48].

$$\Delta H^\circ = 1/A \left\{ (B+K) - [(B+K_d)^2 - C]^{1/2} \right\} \quad (2)$$

A, B and C are constants in each injection, which have been defined as follows:

$$A = V/2q \quad B = gM_0 + L_0 \quad C = 4gM_0L_0 \quad (3)$$

where V is the volume of the reaction solution in the calorimetric sample cell in each injection step. Equation (2) contains two unknown parameters,  $K_d$  and  $\Delta H^\circ$ . A series of reasonable values for  $K_d$  is inserted into equation (2) and corresponding amounts for  $\Delta H^\circ$  are calculated and the graph  $\Delta H^\circ$  versus  $K_d$  is constructed. Curves of all titration steps will intersect in one point, which represents true amounts for  $\Delta H^\circ$  and  $K_d$ . The plots of  $\Delta H^\circ$  versus  $K_d$ , according to Eq. (2) for all injections are shown in Figure 3. The intrinsic dissociation equilibrium constant and the molar enthalpy of binding were obtained  $285 \mu\text{M}$  and  $-15.2$  kJ/mol at  $27^\circ\text{C}$  and  $346 \mu\text{M}$  and  $-14.6$  kJ/mol at  $37^\circ\text{C}$ , respectively (see Figure 3). These results are identical with results obtained by previous method described above.

To compare all thermodynamic parameters in metal binding process for JBU, the change in standard Gibbs free energy ( $\Delta G^\circ$ ) should be calculated according to the equation (4), which its value can use in equation (5) for calculating the change in standard entropy ( $\Delta S^\circ$ ) of binding process.

$$\Delta G^\circ = -RT \ln K_a \quad (4)$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (5)$$

where  $K_a$  is the association binding constant (the inverse of the dissociation binding constant,  $K_d$ ). The  $K_a$  values are obtained  $3509$  and  $2891 \text{ M}^{-1}$  at  $27^\circ\text{C}$  and  $37^\circ\text{C}$ , respectively. Hence:

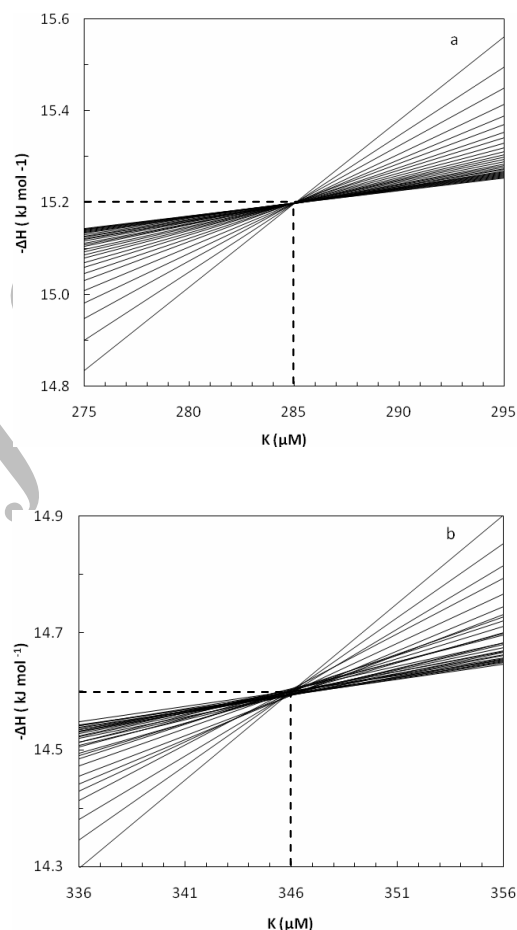
$$\Delta G^\circ = -20.4 \text{ kJ/mol} \quad \Delta S^\circ = +17.2 \text{ J/K mol (at } 27^\circ\text{C)}$$

$$\Delta G^\circ = -20.6 \text{ kJ/mol} \quad \Delta S^\circ = +19.1 \text{ J/K mol (at } 37^\circ\text{C)}$$

It means that the binding process is spontaneous resulted by not only enthalpic but also entropic driven.

All thermodynamic parameters for the interaction between JBU and copper ion at two temperatures of  $27$  and  $37^\circ\text{C}$  have been summarized in Table 1. There is a

set of twelve identical and non-interacting binding sites for copper ions to JBU. The binding process is exothermic at both temperatures. The binding process of is not only enthalpy driven but also it is entropy driven. The role of entropy driven in the binding process should be more effective by increasing the temperature. The molar entropy of binding means that the difference between the entropy of  $\text{Cu}^{2+}$ -JBU complex ( $S_{\text{Cu-JBU}}$ )



**Figure 3.**  $\Delta H$  versus  $K_d$  for all 30 injections at  $300 \text{ K}$  (a) and  $310 \text{ K}$  (b) using data in Fig.1b. The coordinates of the intersection point of the curves give the true values for  $\Delta H$  and  $K_d$ .

**Table1.** Thermodynamic parameters of binding for copper ions to JBU obtained by ITC

T (K)	g	K ( $\mu\text{M}$ )	$K_a$ ( $\text{M}^{-1}$ )	$\Delta H^\circ$ (kJ/mol)	$\Delta G^\circ$ (kJ/mol)	$\Delta S^\circ$ (J/mol K)
300	12	285	3509	-15.2	-20.4	17.2
310	12	346	2891	-14.6	-20.6	19.1

and the entropy of native JBU ( $S_{\text{JBU}}$ ):  $\Delta S = S_{\text{Cu-JBU}} - S_{\text{JBU}}$ . Hence, the disorder of the protein structure has been increased due to the binding of copper ions.

### Acknowledgement

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