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Application of the extended solvation model for thermodynamic study of copper ion binding to Jack bean urease

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Abstract A Thermodynamic study on the interaction Jack bean urease, JBU, with Cu^{2+} ion was studied by isothermal titration calorimetry (ITC) at 300 and 310 K in 30 mM Tris buffer solution, pH 7.0. The heats of JBU + Cu^{2+} interactions are reported and analyzed in terms of the extended solvation theory. It was indicated that there are a set of 12 identical and non-cooperative sites for Cu^{2+} ion. The binding of Cu^{2+} ion with JBU is exothermic with dissociation equilibrium constants of 284.883 and 345.855 μM at 300 and 310 K, respectively.

Keywords Jack bean urease · Isothermal titration calorimetry · Inhibitor · Binding parameters

Introduction

Jack bean urease possesses nickel ions in the active site, whose task is to activate the substrate and water for the reaction. Ureases play an important role in the overall nitrogen metabolism in nature. Their key function is to provide organisms with nitrogen in the form of ammonia for growth [1–3]. Hydrolysis, the fundamental property of urea that greatly affects the management of urea as fertilizer, causes an abrupt overall pH increase, and this is the major cause of the negative side effects. The release of large amounts of ammonia into the atmosphere, as well as the plant damage induced by ammonia toxicity and the increase in soil pH, leads to significant environmental and economic problems [4–7]. The rapidly increasing importance of urea fertilizer in world agriculture (urea contains high nitrogen percentage, 46%) has stimulated research to find methods of reducing the problems associated with the use of this fertilizer. One approach to overcome the problems associated with the use of urea fertilizers is to find compounds that would inhibit the urease activity and thereby retard urea hydrolysis when applied to soils together with the fertilizer. Heavy metal ions inhibit both plant and bacterial ureases at the following approximate order of effectiveness: $Hg^{2+} \approx Ag^{+} > Cu^{2+} > Ni^{2+} > Cd^{2+} > Zn^{2+} > Co^{2+} > Fe^{3+} > Pb^{2+} > Mn^{2+}$, with Hg^{2+}, Ag^{+}, and Cu^{2+} ions practically known as the strongest inhibitors [7–11]. In practice, this inhibition is important for two reasons. One is that in view of heavy metal ion pollution, appropriate levels of urease activity in agricultural soils may be endangered. The other one is that this inhibition may be exploited in constructing urease inhibition-based sensing systems for in situ and real-time determination of trace levels of the ions, e.g., in environmental monitoring, food control, and biomedical analysis. With recent advances in the sensitivity and versatility of calorimeters, ITC has become an essential tool for the direct measurement of thermodynamic functions such as Gibbs-free energy (ΔG), enthalpy (ΔH), and entropy (ΔS) changes along with the dissociation equilibrium constant (K_d) from a single experiment. In this study, we have attempted to find the

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Table 1 The heats of JBU + Cu^{2+} interaction at 300 K, 310 K in 30 mM tris buffer solution of pH 7

$[Cu^{2+}]$/mM	[JBU]/μM	q/μJ (300 K)	q_{dilut}/μJ (300 K)	q/μJ (310 K)	q_{dilut}/μJ (310 K)	C/μJ K^{-1}
0.110	3.956	−335.4	−371.7	−281.3	−345.1	5.41
0.217	3.913	−538.5	−691.6	−462.3	−642.2	7.62
0.323	3.871	−672.1	−953.4	−586.8	−885.2	8.53
0.425	3.830	−765.8	−1176.0	−677.0	−1091.8	8.88
0.526	3.789	−834.8	−1367.1	−745.1	−1269.4	8.97
0.625	3.750	−887.7	−1522.5	−798.3	−1413.6	8.94
0.722	3.711	−929.4	−1658.3	−840.9	−1539.7	8.85
0.816	3.673	−963.1	−1775.2	−875.7	−1648.2	8.74
0.909	3.636	−990.9	−1873.4	−904.7	−1739.3	8.62
1.000	3.600	−1014.2	−1953.0	−929.2	−1813.4	8.50
1.089	3.564	−1034.0	−2026.5	−950.2	−1881.6	8.38
1.1765	3.529	−1051.0	−2090.9	−968.4	−1941.4	8.26
1.262	3.495	−1065.8	−2149.0	−984.3	−1995.3	8.15
1.346	3.461	−1078.8	−2196.6	−998.3	−2039.5	8.05
1.428	3.428	−1090.3	−2240.7	−1010.7	−2080.4	7.96
1.509	3.396	−1100.5	−2278.5	−1021.8	−2115.6	7.87
1.589	3.364	−1109.7	−2313.5	−1031.8	−2148.1	7.79
1.667	3.333	−1118.0	−2345.7	−1040.8	−2178.0	7.72
1.743	3.303	−1125.5	−2375.3	−1049.0	−2205.3	7.65
1.818	3.273	−1132.3	−2401.0	−1056.5	−2229.3	7.58
1.892	3.243	−1138.5	−2424.1	−1063.4	−2250.7	7.51
1.964	3.214	−1144.2	−2444.4	−1069.7	−2269.5	7.45
2.035	3.186	−1149.5	−2463.3	−1075.5	−2287.2	7.40
2.1053	3.158	−1154.4	−2480.8	−1080.9	−2303.4	7.35
2.174	3.130	−1158.9	−2496.9	−1085.9	−2318.3	7.30
2.241	3.103	−1163.1	−2511.1	−1090.5	−2331.4	7.26
2.308	3.077	−1167.0	−2523.5	−1094.8	−2343.1	7.22
2.373	3.051	−1170.6	−2534.0	−1098.8	−2352.8	7.18
2.437	3.025	−1174.0	−2543.8	−1102.6	−2362.0	7.14
2.500	3.000	−1177.2	−2552.2	−1106.2	−2369.7	7.10

Precision in q is ±0.1 μJ or better. q_{dilut} is the heat of dilution of $Cu(NO_3)_2$ in the buffer solution

binding parameters and conformational changes of JBU due to its binding with Cu^{2+} ion.

Experimental

Jack bean urease (JBU; MW = 545.340 kDa) and $Cu(NO_3)_2$ obtained from sigma chemical Co. The buffer solution used in the experiments was 30 mM Tris, pH 7.0, which was obtained from Merck. Experiments were carried out in 300 and 310 K. The experiments were performed with the 4-channel commercial microcalorimetric system, Thermal Activity Monitor 2277 (Thermometric, Sweden). Each channel is twin heat conduction calorimeter (multi-function 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. $Cu(NO_3)_2$ solution (10 mM) was injected into the calorimetric titration vessel, which contained 1.8 mL JBU, 4 μM, in Tris buffer 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 nitrate solution into the perfusion vessel was repeated 30 times and each injection included 20 μL cobalt nitrate 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 $Cu(NO_3)_2$ 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. The

heats of $Cu(NO_3)_2$ and protein solutions dilution were subtracted from the heats of $Cu(NO_3)_2$ solutions in JBU solutions. The determined heats for JBU + Cu^{2+} interactions were listed in Table 1 (in μJ). The microcalorimeter was frequently calibrated electrically during the course of the study.

Results and discussion

We have shown previously [12–20] that the heats of the macromolecules + ligands interactions in the aqueous solvent systems, q, can be reproduced by the following equation.

$$q = q_{\max} x'_B - \delta^{\theta}_A (x'_A L_A + x'_B L_B) - (\delta^{\theta}_B - \delta^{\theta}_A)(x'_A L_A + x'_B L_B) x'_B \quad (1)$$

δ^{θ}_A and δ^{θ}_B parameters are the indexes of JBU structural changes in the low and high Cu^{2+} concentrations, respectively. The positive values for δ^{θ}_A or δ^{θ}_B indicate that Cu^{2+} stabilizes the JBU structure and vice versa. If the binding of ligand at one site increases the affinity for ligand at another site, the macromolecule exhibits positive cooperativity. On the contrary, if the binding of ligand at one site lowers the affinity for ligand at another site, the enzyme exhibits negative cooperativity. If the ligand binds at each site independently, the binding is non-cooperative. $p > 1$ or $p < 1$ indicates positive or negative cooperativity of macromolecule for binding with ligand, respectively; $p = 1$ indicates that the binding is non-cooperative. x'_B can be expressed as follows:

$$x'_B = \frac{p x_B}{x_A + p x_B} \quad (2)$$

x_B is the fraction of the bounded Cu^{2+}, and $x_A = 1 - x_B$ is the fraction of unbounded Cu^{2+}. We can express x_B fractions, as the total Cu^{2+} concentrations divided by the maximum concentration of the Cu^{2+} upon saturation of all JBU as follows:

$$x_B = \frac{[Cu^{2+}]_T}{[Cu^{2+}]_{\max}} \quad x_A = 1 - x_B \quad (3)$$

$[Cu^{2+}]_T$ is the total concentration of Cu^{2+} and $[Cu^{2+}]_{\max}$ is the maximum concentration of the Cu^{2+} upon saturation of all JBU. In general, there will be "g" sites for binding of Cu^{2+} per JBU molecule. L_A and L_B are the relative contributions of unbounded and bounded Cu^{2+} in the heats of dilution with the exclusion of JBU, and can be calculated from the heats of dilution of $Cu(NO_3)_2$ in the buffer solution, q_{dilut}, as follows:

$$L_A = q_{\text{dilut}} + x_B \left(\frac{\partial q_{\text{dilut}}}{\partial x_B}\right), \quad L_B = q_{\text{dilut}} - x_A \left(\frac{\partial q_{\text{dilut}}}{\partial x_B}\right) \quad (4)$$

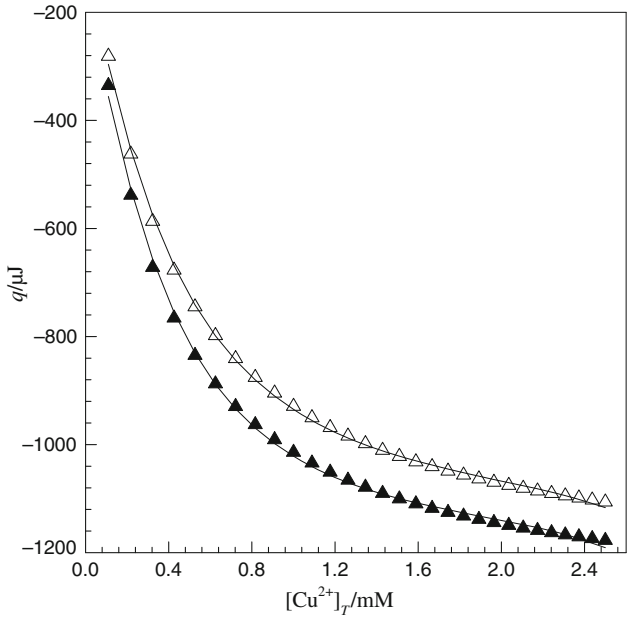

Fig. 1 Comparison between the experimental heats $T = 300$ K (*filled triangle*), and $T = 310$ K (*open triangle*) for JBU + Cu^{2+} interactions and the calculated data (*lines*) via Eq. 1. $[Cu^{2+}]_T$ are the total concentrations of $Cu(NO_3)_2$ solution in mM

The heats of JBU + Cu^{2+} interactions were fitted to Eq. 1 over a range of Cu^{2+} concentrations. In the fitting procedure, the only adjustable parameter (p) was changed until the best agreement between the experimental and calculated data was approached (Fig. 1). The optimized δ^{θ}_A and δ^{θ}_B values are recovered from the coefficients of the second and third terms of Eq. 1. The small standard errors and the high r^2 values (0.9999) support the method. The binding parameters for JBU + Cu^{2+} interactions recovered from Eq. 1 were listed in Table 2. The agreement between

Table 2 Binding parameters for JBU + Cu^{2+} interactions recovered from Eqs. 1 and 8

Parameters (K)	p	δ^{θ}_A	δ^{θ}_B	ΔH/kJ mol^{-1}	K_d/μM	g
$T = 300$	1	−0.078 ± 0.031	1.604 ± 0.013	−15.198 ± 0.013	284.883 ± 0.081	12
$T = 310$	1	−0.138 ± 0.021	1.282 ± 0.011	−14.597 ± 0.011	345.855 ± 0.023	12

$p = 1$ indicates that the binding is non-cooperative. The positive δ^{θ}_A and δ^{θ}_B values prove that the JBU + Cu^{2+} complexes are stable, indicating that Cu^{2+} most likely promote JBU aggregation or inhibit the ureolytic activity by inducing protein polymerization along with blockage of thiol groups

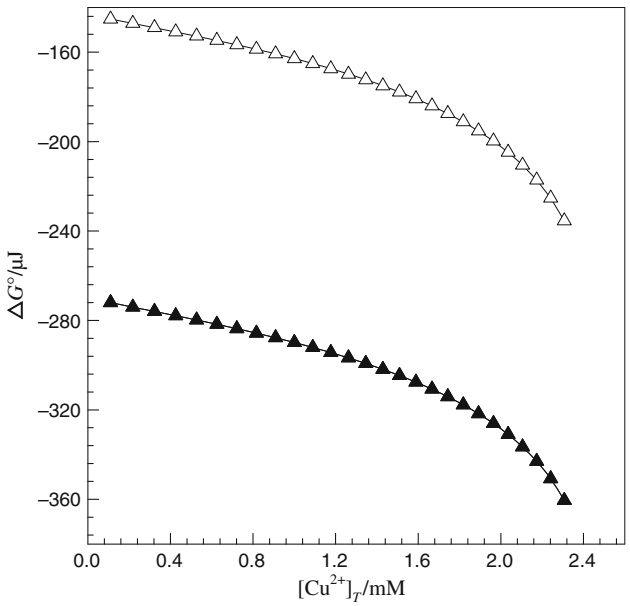

Fig. 2 Comparison between the experimental Gibbs energy values at $T = 300$ K (*filled triangle*), and $T = 310$ K (*open triangle*) for JBU + Cu^{2+} and calculated values (*lines*) via Eq. 7. The linearity of ΔG° against Cu^{2+} concentrations indicates that the structural effects compensate each other in the free energy which supports the extended solvation model. $[Cu^{2+}]_T$ are the total concentrations of $Cu(NO_3)_2$ solution in mM

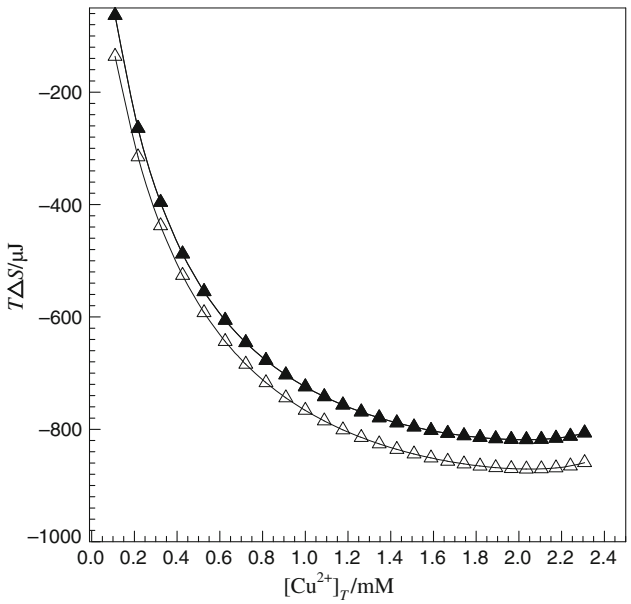

Fig. 3 Comparison between the experimental values at $T = 300$ K (*filled triangle*), and $T = 310$ K (*open triangle*) for JBU + Cu^{2+} interaction and calculated values (*lines*). $[Cu^{2+}]_T$ are the total concentrations of $Cu(NO_3)_2$ solution in mM

the calculated and experimental results (Fig. 1) is remarkable, and gives considerable support to the use of Eq. 1.

A fraction of JBU molecule undergoing complexation with Cu^{2+}, Φ, can be expressed as follows:

$$\Phi = \frac{q}{q_{\max}} \tag{5}$$

$q_{\max}$ represents the heat value upon saturation of all JBU. The association equilibrium constant values, K_a, as a function of free concentration of Cu^{2+}, $[Cu^{2+}]_F$, can be calculated as follows:

$$K_a = \frac{\Phi}{(1-\Phi)[Cu^{2+}]_F} = \frac{\Phi}{(1-\Phi)[Cu^{2+}]_T(1-x_B)} \tag{6}$$

The standard Gibbs free energies as a function of Cu^{2+} concentrations can be obtained as follows:

$$\Delta G^\circ = -RTLnK_a \tag{7}$$

Standard Gibbs energies, ΔG°, at two temperatures of 300 and 310 K, calculated from Eq. 7, have shown graphically in Fig. 2. Calculated $T\Delta S^\circ$ values using ΔG° values at different temperatures are shown in Fig. 3.

For a set of identical and independent binding sites, we have previously [12–20] introduced the following equation:

$$\left[\frac{\Delta q}{q_{\max}}\right] M_0 = \left[\frac{\Delta q}{q}\right] L_0 \frac{1}{g} - \frac{K_d}{g} \tag{8}$$

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 ligand, respectively. q represents the heat of JBU + Cu^{2+} interaction at a certain L_0 and $\Delta q = q_{\max} - q$. $q_{\max}$ represents the heat value upon saturation of all biomacromolecule. If q and $q_{\max}$ are calculated per mole of biomacromolecule then the standard molar enthalpy of binding for each binding site (ΔH°) will be $\Delta H^\circ = \frac{q_{\max}}{g}$.

Therefore, the plot of $\left[\frac{\Delta q}{q_{\max}}\right] M_0$ versus $\left[\frac{\Delta q}{q}\right] L_0$ should be a linear plot with a slope of $\frac{1}{g}$ and a vertical-intercept of $\frac{K_d}{g}$. The linearity of the plot has been examined by different estimated values for $q_{\max}$ to reach the best value for the correlation coefficient. The best linear plot with the correlation coefficient value ($r^2 \approx 1$) was obtained (Figs. 4 and 5) using −1313.1 and −1261.2 μJ (equal to −182.378 and −175.167 kJ/mol) at 300 and 310 K, respectively. The number of binding sites (g) and the dissociation equilibrium constant (K_d), obtained from the slope and vertical-intercept plot, are listed in Table 2. The lack of a suitable value for $q_{\max}$ to obtain a linear plot of $\left[\frac{\Delta q}{q_{\max}}\right] M_0$ against $\left[\frac{\Delta q}{q}\right] L_0$ may be attributed to the existence of non-identical binding sites or the interaction between them. Binding parameters for JBU + Cu^{2+} interactions using the new model are listed in Table 2.

The inhibition of urease by heavy metal ions has been consistently ascribed to the reaction of the ions with the

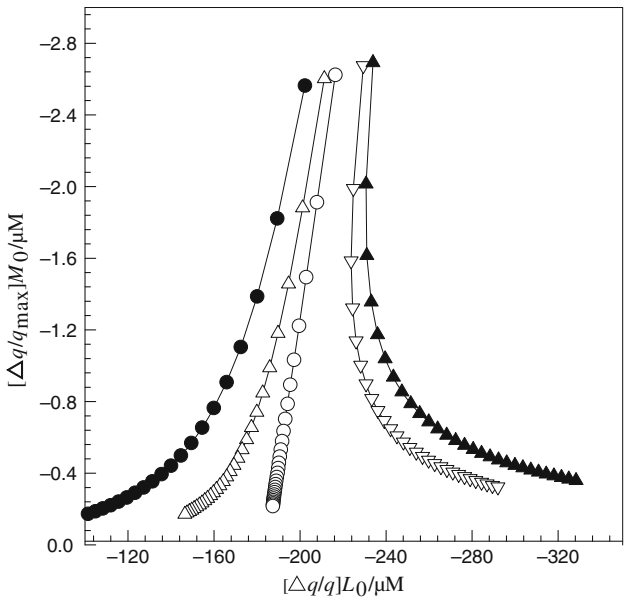

Fig. 4 The best linear plot of $\left[\frac{\Delta q}{q_{max}}\right]M_0$ vs. $\left[\frac{\Delta q}{q}\right]L_0$, according to Eq. 8 (*open circle*), using values of −1380 μJ (*filled circle*), −1420 μJ (*open triangle*), −1500 μJ (*down-pointing triangle*), −1520 μJ (*filled triangle*), and −1442.5 μJ (*open circle*), at 300 K for q_{max} to obtain the best correlation coefficient value ($r^2 = 1$)

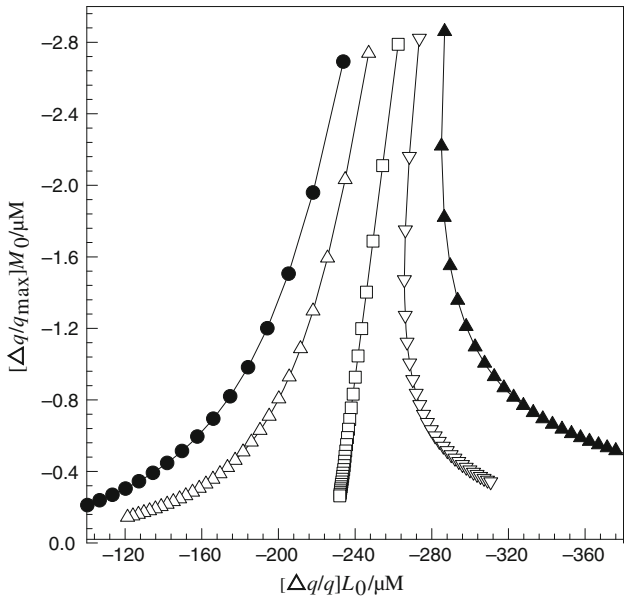

Fig. 5 The best linear plot of $\left[\frac{\Delta q}{q_{max}}\right]M_0$ vs. $\left[\frac{\Delta q}{q}\right]L_0$, according to Eq. 8 (*open square*), using values of −1300 μJ (*filled circle*), −1350 μJ (*open triangle*), −1450 μJ (*down-pointing triangle*), −1500 μJ (*filled triangle*), and −1408.4 μJ (*open square*), at 310 K for q_{max} to obtain the best correlation coefficient value ($r^2 = 1$)

thiol groups of the enzyme, resulting in the formation of mercaptides [21]. The most common mechanism of protein aggregation is believed to involve protein denaturation, via hydrophobic interfaces and often results in the loss of biological activity. The aggregation of Jack bean urease is related to the alterations of its biological properties, markedly the ureolytic and entomotoxic activities. δ_B^θ values for JBU + Cu^{2+} interaction are positive, indicating that Cu^{2+} presumably promote JBU aggregation or inhibit the ureolytic activity by inducing protein polymerization along with blockage of sulfhydryl groups. The positive δ_B^θ values show that the JBU + Cu^{2+} complexes are stable and strong which are in agreement with previous reports [1–11]. The improved stability as a consequence of structural changes introduced to the enzyme by Cu^{2+} ions is simply distinguishable by positive δ_B^θ values. The positive δ_B^θ values are indicative of specific Cu^{2+} binding sites exist in functionally relevant proteins and that occupancy of these sites stabilizes some conformational but still native state.

An increase in the standard Gibbs free energies of the JBU + Cu^{2+} interaction (Fig. 2), a large negative entropy of mixing (Fig. 4), and an increase in heat capacity over the temperature course of 300–310 K (Table 1) indicate that the hydrophobic property of JBU increases due to its interaction with Cu^{2+} ions. Cu^{2+} inactivates JBU at considerably higher concentrations, and the improved stability may be a consequence of structural changes introduced to the enzyme as indicated by more positive δ_B^θ values at the high Cu^{2+} concentration region. These interpretations are in agreement with the positive δ_B^θ values recovered from Eq. 1. The negative δ_A^θ values indicate that JBU + Cu^{2+} complexes are unstable and Cu^{2+} ions are weakly bounded to JBU in the low Cu^{2+} concentration domain. The large dissociation equilibrium constants for JBU + Cu^{2+} interactions are in agreement with this interpretation. It was found that the linear range of IBU inhibition by Cu^{2+} ions for this mixture was from 0.11 to 2.5 mM.

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