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A SIMPLE NOVEL METHOD FOR DETERMINATION OF AN INHIBITION CONSTANT BY ISOTHERMAL TITRATION MICROCALORIMETRY. THE EFFECT OF FLUORIDE ION ON UREASE

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A simple novel method was introduced for determination of an inhibitor binding constant (K_i) and enthalpy of binding by isothermal titration microcalorimetry technique. This method was applied to the binding of fluoride ion, as an inhibitor, with the active sites of jack bean urease at pH = 7.0 (Tris 30 mM) and T = 300°K. The dissociation equilibrium constant measured by this method was markedly consistent with the inhibition constant obtained from assay of enzyme activity in the presence of fluoride ion.

Keywords: Urease; Isothermal titration microcalorimetry; Inhibition constant; Enthalpy of binding

INTRODUCTION

Jack bean urease (urea amidohydrolase; E.C. 3.5.1.5) was the first enzyme to be crystallized[1] and also the first enzyme shown to contain nickel.[2,3] Jack bean urease has six identical subunits and each subunit consists of a single polypeptide chain containing 840 amino acid residues with $M_r = 90770$, excluding the two nickel ions per subunit.[4] Therefore, $M_r = 545{,}340$ for the hexamer urease molecule, including 12 nickel ions.[4] The subunit of urease from microorganisms appears to be smaller than jack bean urease in size and number.[5,6]

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Urease catalyzes the hydrolysis of urea to carbonic acid and two molecules of ammonia.[7] The specificity of urease was believed to be absolute[8] until Fishbein *et al.* reported that *N*-hydroxyurea was a substrate.[9,10] *N*-hydroxyurea,[9–11] (*N*,*N'*)-dihydroxyurea,[12,13] semicarbazide,[14] *N*-methylurea,[15] formamide[16] and acetamide[15] are other examples of substrates for urease.

Despite extensive investigation the detailed mechanism of ureolysis is not established and the structure of the metallocenter is unknown. 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 catalytic nucleophile.[15]

The study of urease inhibitors may have medical or agronomic significance, as well as providing insight into the urease catalytic mechanism.[17]

Here, isothermal titration microcalorimetry was applied as a powerful tool for studying ligand binding. A simple novel equation, very similar to the Michaelis–Menten equation, was introduced for determination of the binding constant and enthalpy of binding. The binding of fluoride ion, as an inhibitor, on the active sites of jack bean urease was also studied by this method. Moreover, the value of the dissociation constant for the enzyme–inhibitor complex has been determined by assay of enzyme activity and is consistent to that obtained by calorimetric measurement.

MATERIALS AND METHODS

Jack bean urease, L-glutamic dehydrogenase (GLDH), β-nicotinamide adenine dinucleotide, reduced form (NADH), α-ketoglutaric acid (α-KG), adenosine 5′-diphosphate (ADP), Tris-base and ethylendiaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. Urea and sodium fluoride were obtained from Aldrich. Solutions were made in double-distilled water. Tris-base solution (30 mM) pH = 7.0 was used as a buffer.

Assay Method

The hydrolysis of urea was measured by coupling ammonia production to a GLDH reaction:[18]

$$\text{Urea} + H_2O + 2H^+ \xrightarrow{\text{urease}} 2NH_4^+ + CO_2,$$

$$2NH_4^+ + 2\alpha\text{-KG} + 2\text{NADH} \xrightarrow{\text{GLDH}} 2\text{Glutamate} + 2\text{NAD}^+ + 2H_2O$$

The decrease in absorbance at 340 nm (A_{340}) is related to the decrease in NADH concentration and rate of reaction (V):[18]

$$V = \frac{(709.4/17.0)}{(\text{mg urease/ml reaction mixture})} \cdot \frac{A_{340}/\text{min}}{6.22}, \qquad (1)$$

where 709.4 and 17.0 are the M_r values for NADH and NH_3, respectively. The micromolar absorptivity of NADH is 6.22, when the radiation path length is 1 cm. The concentration of urease is taken as 0.025 mg per ml reaction mixture. The details of the assay method were as described in the Worthington enzyme manual.[18]

A recording Spectrophotometer, UV-3100 Shimadzu model, equipped with a thermostatted cell compartment, was used for recording the rate of absorbance changes.

Isothermal Titration Microcalorimetry

The calorimetric experiments were performed with the 4-channel commercial microcalorimetric system, Thermal Activity Monitor 2277 (Thermometric, Sweden). Each channel is a twin heat-conduction calorimeter where the heat-flow sensor is a semiconducting thermopile (multi-junction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. The insertion vessel was made from stainless steel. Sodium fluoride solution (40 mM) was injected using a Hamilton syringe into the calorimetric stirred titration vessel, which contained 2 ml urease (0.0025 mM) in Tris buffer (30 mM), pH = 7.0. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of fluoride solution (0.025 ml) into the perfusion vessel was repeated 30 times. The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of each injection was calculated by the software "Theromometric Digitam 3 program". The heat of dilution of the fluoride solution was measured as described above except enzyme was excluded. The enthalpy of dilution was subtracted from the enthalpy of the urease–fluoride interaction. The enthalpy of dilution of urease was also negligible. The microcalorimeter was frequently calibrated electrically during the course of the study.

RESULTS AND DISCUSSION

Consider a solution containing a ligand (I), and a macromolecule (M_n) that contains n sites capable of binding the ligand. If the multiple binding sites on a macromolecule are identical and independent, the ligand binding sites can be reproduced by a model system of monovalent molecules ($M_n \rightarrow nM$) with the same set of dissociation equilibrium constant (K) values. Thus, the reaction under consideration can be written:

$$M + I \rightleftharpoons MI, \quad K = [M][I]/[MI]. \tag{2}$$

By titration of a solution containing "M" with a solution of ligand I, the equilibrium reaction is moved towards an increasing concentration of the MI complex. The heat value of the reaction depends on the concentration of MI complex ($Q \propto [MI]$). Moreover, the maximal value of heat that would be observed when all the M is present as MI, that is, $Q_{max} \propto [M]_{total}$, or $Q_{max} \propto [M] + [MI]$. Therefore, it can be concluded that:

$$\frac{Q}{Q_{max}} = \frac{[MI]}{[M] + [MI]}. \tag{3}$$

Because of the equilibrium assumption, [MI] can be expressed in terms of [I], [M], and K. Substituting for [MI]:

$$\frac{Q}{Q_{max}} = \frac{([M][I]/K)}{[M] + ([M][I]/K)}. \tag{4}$$

The heat equation for the simple unireactant system can be rearranged to yield the more familiar Henri–Michaelis–Menten equation:

$$\frac{Q}{Q_{max}} = \frac{[I]}{K + [I]} \tag{5}$$

or, in terms of the Lineweaver–Burk form,

$$\frac{1}{Q} = \frac{K}{Q_{max}}\frac{1}{[I]} + \frac{1}{Q_{max}}. \tag{6}$$

Thus, the plot of $1/Q$ versus $1/[I]$ gives the dissociation equilibrium constant (K) and the molar enthalpy of binding ($\Delta H = Q_{max}$). The value of Q_{max} will be equal to the molar enthalpy of binding, if the values of Q are calculated per mole of single-site protein (M).

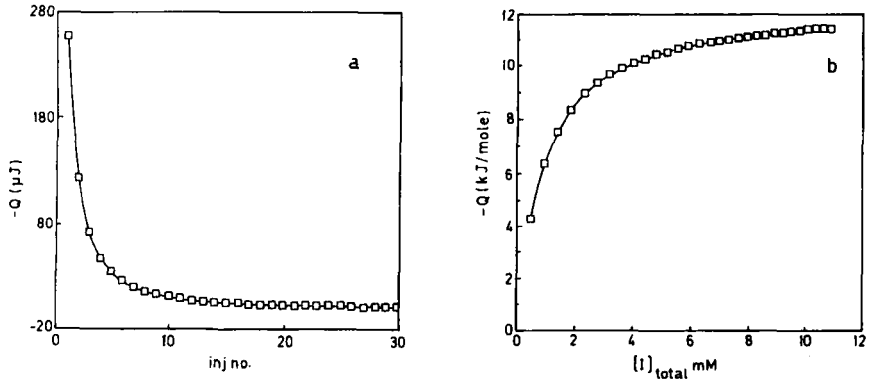

FIGURE 1 (a) The heat of fluoride binding on jack bean urease for 30 automatic cumulative injections, each of 25 μl, of sodium fluoride solution (40 mM) into the sample cell containing 2 ml urease solution at a concentration of 2.5 μM. (b) The heat of binding versus total concentration of fluoride ion, calculated from Figure 1(a).

The data obtained from isothermal titration microcalorimetry of jack bean urease with fluoride ion is shown in Figure 1. Figure 1(a) shows the heat of each injection and Figure 1(b) shows the heat related to each total concentration of fluoride ion. The total concentration of fluoride ion is much greater than the total concentration of binding sites on protein with one binding site, so it can be assumed that the total and free concentrations of ligand are approximately equal.

A plot of 1/Q versus 1/[I] is shown in Figure 2. This linear plot has been obtained by assuming $n = 12$, because a hexamer urease macromolecule has 12 identical active sites.[15] The values of K and ΔH obtained from the axis intercepts were:

$$K = 0.94\,\text{mM}, \quad \Delta H^\circ = -11.9\,\text{kJ/mole}.$$

The double reciprocal Lineweaver–Burk plots[19,20] for jack bean urease in different fixed concentrations of fluoride, at $pH = 7.0$ and $T = 300°K$ are shown in Figure 3. The value of V_{max} is unchanged by the fluoride but the apparent Michaelis constant (K'_m) value is increased which confirms competitive inhibition of urease by fluoride. The value of V_{max} was equal to 105 μmole min^{-1}. The values of K'_m at any fixed concentration of fluoride were obtained from Figure 3 and plotted versus the concentrations of fluoride in the inset of Figure 3 in a secondary plot to give

$$K_m = 2.69\,\text{mM}, \quad K = 0.94\,\text{mM}.$$

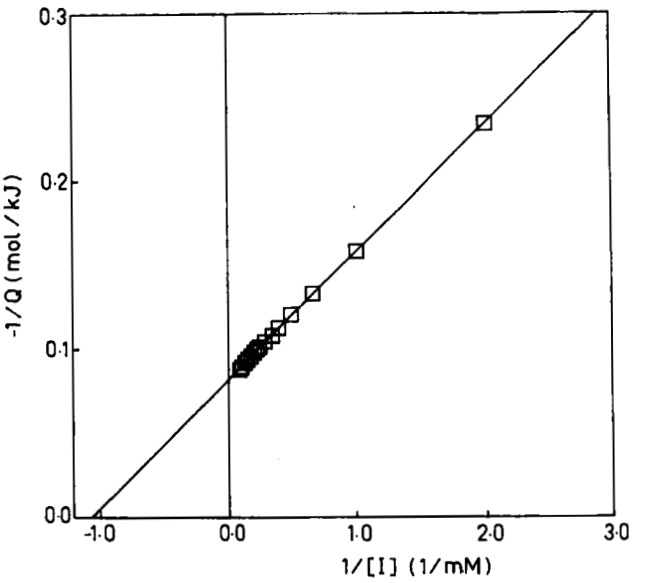

FIGURE 2 The inverse heat of fluoride binding on jack bean urease versus inverse total concentration of fluoride ion, according to Eq. (6), at pH = 7.0 and 300°K.

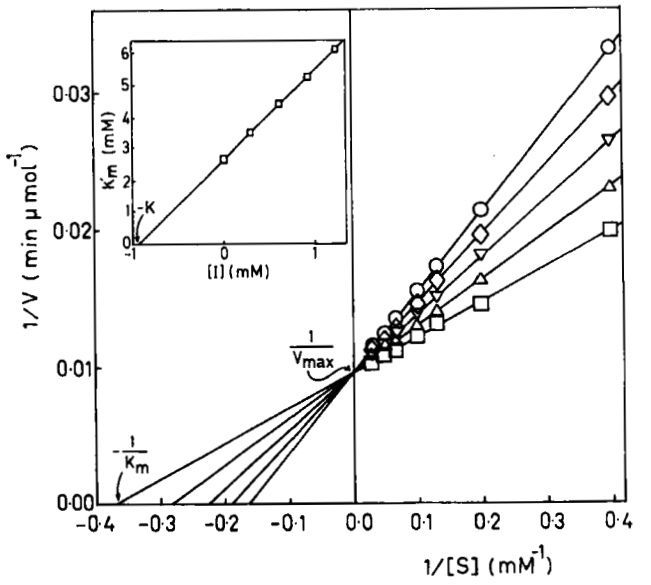

FIGURE 3 Double reciprocal Lineweaver–Burk plots for kinetics of jack bean urease at pH = 7.0 and T = 300°K in the presence of different fixed concentrations of sodium fluoride: 0 mM (□), 0.3 mM (△), 0.6 mM (▽), 0.9 mM (◇), and 1.2 mM (○). In the inset, a secondary plot of 1/[S]-axis intercepts versus [I] is shown.

The Michaelis–Menten constant (K_m) obtained from these experiments was identical to that from a previous report.[21]

The conformity of the dissociation binding constants (K) obtained from thermodynamic and kinetic studies supports our assumption that 12 independent and identical binding sites are present on the urease macromolecule.

Acknowledgement

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