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Isothermal titration microcalorimetric method for studying the combined ligand binding with application to the binding of ethylurea and (N,N)dimethylurea on urease

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Abstract

A simple, novel method was introduced for determining equilibrium constants and enthalpies of binding of two different competitive ligands on a macromolecule by isothermal titration microcalorimetry technique. This method was applied to the simultaneous binding of ethylurea (I) and (N,N)dimethylurea (X), on jack-bean urease at pH 7.0 (tris-base; 30 mM) at 27°C. The dissociation equilibrium constants measured by this method were markedly consistent with inhibition constants obtained from assay of enzyme activities in the presence of I and X. © 1998 Elsevier Science B.V.

Keywords: Enthalpy of binding; Equilibrium constant; Isothermal titration microcalorimetry; Ligand binding; Urease

1. 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 kind of polypeptide chain containing 840 amino acids residue with relative molecular mass of 90770, excluding the two nickel ions per subunit [4]. Therefore, the relative molecular mass of the hexamer urease molecule should be 545 340, which includes 12 nickel ions [4]. The subunit of urease from microorganisms appear to be smaller than that of jack-bean urease in size and number [5,6].

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. [9,10] reported that N-hydroxyurea was a substrate. 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.

Additional information about the structure of jack-bean urease from denaturation of this enzyme [17,18]; Moreover, the combined inhibitory of ethylurea (I) and (N,N)dimethylurea (X) on jack-bean urease has been studied [19]. The values for dissociation constants of inhibitors (K_I and K_X for I and X, respectively) from enzyme–inhibitors complexes have been determined by using a simple, novel graphical method; K_I=26 and K_X=28 mM. In this paper, we have used isothermal titration microcalorimetry as a powerful tool for studying ligand binding. A simple, novel equation, very similar to the Michaelis–Menten equation, was introduced for the determination of

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equilibrium dissociation constants and enthalpies of binding. The values of equilibrium dissociation constants obtained by this method are compared with those reported by assay of enzyme activity in the presence of I and X.

2. Experimental

2.1. Materials

Jack-bean urease and tris-base were obtained from Sigma. Ethylurea and (N,N)dimethylurea were obtained from Aldrich.

The solutions were made in double-distilled water. Tris-base solution of 30 mM concentration, pH 7.0, was used as a buffer.

2.2. Method

The isothermal titration microcalorimetric 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 of stainless steel. I and X solution (500 mM, respect to each one) was injected by using a Hamilton syringe into the calorimetric stirred-titration vessel, which contained 2 ml urease, 0.5 mg/ml, including tris buffer (30 mM), pH 7.0. Thin (0.15 mm i.d.) stainless-steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of I and X solution into the perfusion vessel was repeated 20 times, and each injection included 30 μl reagent. 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 of Thermometric Digitam 3 program. The heat of dilution of the I and X solution was measured as described above, except that enzyme was excluded. The enthalpy of dilution was subtracted from the enthalpy of urease-I and urease-X interaction. The enthalpy of dilution of urease is also negligible. The microcalorimeter was frequently calibrated electrically during the course of the study.

3. Results and discussion

For a solution containing ligand I, and a macromolecule (M_n) containing n sites capable of binding the ligand, if the multiple binding sites on the 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 constants (K). Thus, the reaction can be written as

$$M + I \rightleftharpoons MI \quad K_I = [M][I]/[MI] \tag{1}$$

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

$$\frac{Q_I}{Q_{I,max}} = \frac{[MI]}{[M] + [MI]} \tag{2}$$

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

$$\frac{Q_I}{Q_{I,max}} = \frac{([M][I]/K_I)}{[M] + ([M][I]/K_I)} \tag{3}$$

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

$$\frac{Q_I}{Q_{I,max}} = \frac{[I]}{K_I + [I]} \tag{4}$$

By assuming that all of the macromolecule (M) is converted to the MI complex, the heat value of the reaction per mole of M can be calculated. However, this assumption is true if a large excess of ligand I is present, because there is equilibrium between M and MI. The absolute heat values of reaction vs. ligand concentration will be a rectangular curve. So, the molar enthalpy of binding can be obtained by extrapolation the heat of reaction to a large excess of ligand I; that is $\Delta H_I = Q_{I,max}$.

For the total heat of reaction (Q) due to the isothermal titration of macromolecule by solution

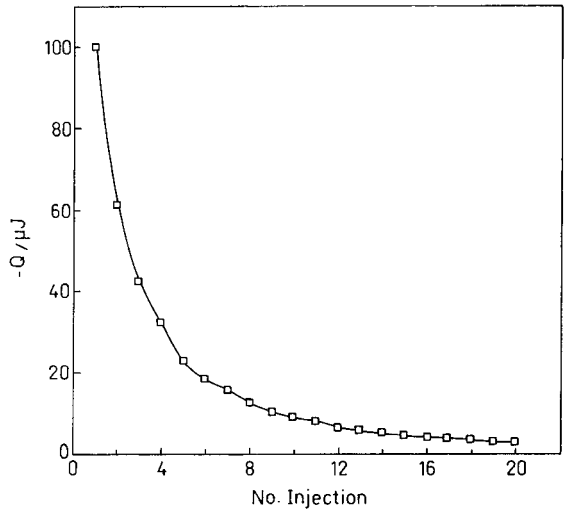

Fig. 1. The heat change of ethylurea (I) and (N,N)dimethylurea (X) binding to jack-bean urease for 20 cumulative, automatic injections, each of 30 μl, of mixed solution I and X, of 0.5 M each, into the sample cell containing 2 ml urease solution at a concentration of 0.5 mg/ml at pH 7.0 (Tris; 30 mM) and T=27°C.

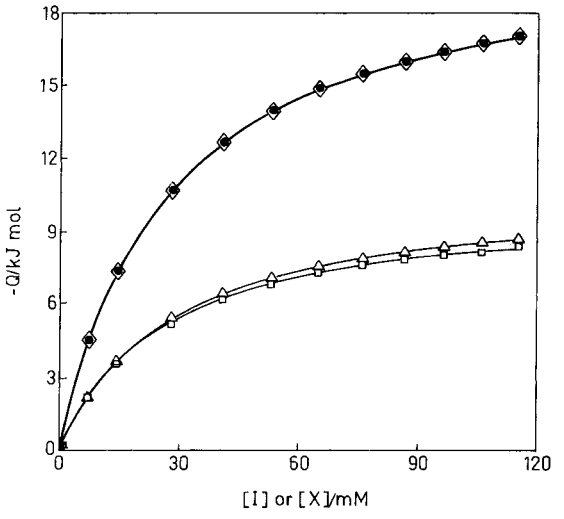

Fig. 2. The heat of binding for one mole of I and one mole of X to the binding sites on jack-bean urease, assuming (◇) unilateral reaction which is compared by (●) theoretical values according to Eq. (5) using results obtained by non-linear least-squares fitting. The theoretical curve has been resoluted for (□) I and (△) X, according to the first and second terms of Eq. (5), respectively.

containing both, I and X, it can be shown:

$$Q \equiv Q_I + Q_X = \frac{Q_{I,max}[I]}{K_I + [I]} + \frac{Q_{X,max}[X]}{K_X + [X]} \qquad (5)$$

By measuring the total heat of reaction at any fixed concentration of I and X, the dissociation equilibrium constants (K_I and K_X) and the molar enthalpies of binding ($\Delta H_I = Q_{I,max}$ and $\Delta H_X = Q_{X,max}$) for these ligands can be obtained.

The data obtained from isothermal titration microcalorimetry of jack-bean urease with ligands I and X is shown in Fig. 1, which shows the heat change on each injection. Fig. 2 shows the heat of binding relative to each total concentration of I and X. The total concentration of I and X is much more than the total concentration of binding sites on macromolecule with one binding site. So, it can be assumed that the total and free concentrations of ligand are approximately equal. The experimental values of heat at any fixed concentrations of I and X have also been fitted to Eq. (5) using a computer program for non-linear least-squares fitting [20]. The results are:

$$K_I = 26.3\,\text{mM} \qquad K_X = 28.4\,\text{mM}$$
$$\Delta H_I = -10.2\,\text{kJ mol}^{-1} \qquad \Delta H_X = -10.8\,\text{kJ mol}^{-1}$$

By using these values, the calculated total heat change and the contributions from each ligand are also plotted in Fig. 2. Experimental and theoretical values have good conformity with maximum errors of 0.1 mM and 0.1 kJ mol^{-1} for K and ΔH values, respectively. In addition, the dissociation equilibrium constants measured by this method were consistent with the inhibition constants obtained from assay of enzyme activity in the presence of I and X.

Acknowledgements

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References

[1] J.B. Sumner, J. Biol. Chem. 69 (1926) 435.
[2] N.E. Dixon, C. Gazzole, R.P. Blakeley, B. Zerner, J. Am. Chem. Soc. 97 (1975) 4131.
[3] G. Mamiya, K. Takishima, M. Masakuni, T. Kayumi, K. Ogawa, J. Protein Chem. 6 (1987) 55.
[4] K. Takishima, T. Suga, G. Mamia, Eur. J. Biochem. 175 (1988) 151.

[5] R.P. Hausinger, J. Biol. Chem. 261 (1986) 7866.
[6] S. Christians, R.P. Hausinger, Arch. Microbiol. 145 (1986) 51.
[7] H.L.T. Mobley, R.P. Hausinger, Microbiol. Rev. 53 (1989) 85.
[8] J.E. Varner, The Enzymes, Vol 4, 2nd edn., Academic Press, New York, 1960, pp. 247–256.
[9] W.N. Fishbein, T.S. Winter, J.D. Davidson, J. Biol. Chem. 240 (1965) 2402.
[10] W.N. Fishbein, P.P. Carbone, J. Biol. Chem. 240 (1966) 2407.
[11] R.L. Blakeley, J.A. Hinds, H.E. Kunze, E.C. Webb, B. Zerner, Biochem. 8 (1969) 1991.
[12] W.N. Fishbein, Anal. Chim. Acta 40 (1968) 269.
[13] W.N. Fishbein, J. Biol. Chem. 244 (1969) 1188.
[14] C. Gazzole, R.L. Blakeley, B. Zerner, Can. J. Biochem. 51 (1973) 1325.
[15] N.E. Dixon, P.W. Riddles, C. Gazzole, R.L. Blakeley, B. Zerner, Can. J. Biochem. 58 (1980) 1335.
[16] W.N. Fishbein, Biochem. Biophys. Acta 484 (1977) 43.
[17] A.A. Saboury, A.K. Bordbar, A.A. Moosavi-Movahedi, J. Chem. Thermodyn. 28 (1996) 1077.
[18] A.A. Saboury, A.K. Bordbar, A.A. Moosavi-Movahedi, Bull. Chem. Soc. Jpn. 69 (1996) 3031.
[19] A.A. Saboury, A.A. Moosavi-Movahedi, J. Enzyme Inhib. 11 (1997) 217.
[20] M.L. James, G.M. Smith, J.C. Wolford, Applied Numerical Methods for Digital Computer, Harper and Row, New York, 1985.