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BINDING ISOTHERM DETERMINATION BY ISOTHERMAL TITRATION CALORIMETRY
Interaction between Cu^{2+} and myelin basic protein

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Abstract

A simple method for determination of binding isotherm in the protein–ligand interaction was introduced using isothermal titration calorimetric data. This general method was applied to the study of the interaction of myelin basic protein (MBP) from bovine central nervous system with divalent copper ion at 27°C in Tris buffer solution at pH=7.2. The binding isotherm for copper–MBP interaction is easily obtained by carrying out titration calorimetric experiment in two different concentrations of MBP. MBP has two binding sites for copper ion, which show positive cooperativity in its sites. The intrinsic association equilibrium constants are 0.083 and 1.740 μM^{-1} in the first and second binding sites, respectively. Hence, occupation of the first site has produced an appreciable enhancement 21 of the binding affinity of the second site. The molar enthalpies of binding are –13.5 and –14.8 kJ mol^{-1} in the first and second binding sites, respectively.

Keywords: calorimetric method, copper, isothermal titration calorimetry, myelin basic protein

Introduction

Calorimetry, the principal source of thermodynamic information, is a very general method due to the fact that practically all physical, chemical and biological processes are accompanied by heat exchange. So, calorimetry is one of the most powerful tools for expanding knowledge and understanding in many fields of science and technology [1–5]. A principal calorimetric technique that has contributed is isothermal titration calorimetry (ITC). The method of ITC is now widely used to obtain thermodynamics information about biochemical binding processes at constant temperature [4–5]. Experiments are performed by titration of a reactant into a sample solution containing the other reactant(s) necessary for reaction. After each addition, the heat released or absorbed as a result of the reaction is monitored by the isothermal titration calorimeter. The total concentration of titrant is the independent variable under experimental control. Thermodynamic analysis of the observed heat effects that permits quantitative

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characterization of the energetic processes associated with the binding reaction. ITC gives invaluable information about biomacromolecule-ligand interaction [6–13], allosteric transition [14], protein denaturation [15–19], enzyme inhibition [20–23], quality, safety and shelf-life of materials and material stability [24–27].

Different methods have been reported for data analysis of ligand binding study by ITC [28–33]. The principal of these methods is to fit the experimental data in an equation relating equilibrium constant, molar enthalpy of binding and reactants concentration. The Wiseman method [31] for data analysis has extensively used for ligand binding study by ITC and a computer program needs for using this method [34–41]. We have presented an equation with a useful linear graphical method in the ligand binding studies, to obtain equilibrium constant and enthalpy of binding by ITC data for noncooperative systems with one set of identical and independent binding sites [42, 43]. A graphical fitting simple method for determination of thermodynamic parameters has also been introduced, which has been applied in inhibitor binding on the enzymes [43, 44]. Determination of the binding isotherm for a set of identical and independent binding site has been reported recently [45]. Here, a new calorimetric data analysis is introduced to obtain the binding isotherm for a set of independent or interacting binding site, which is applied on the interaction between myelin basic protein and copper ion.

Materials and methods

Materials

Myelin basic protein (MBP) from bovine CNS and Tris-HCl were obtained from Sigma Chemical Co. Copper nitrate was purchased from Merck Co. All other materials and reagents were of analytical grades, and solutions were made in double-distilled water. Tris-HCl solution with 30 mM concentration, pH=7.2, was used as a buffer.

Methods

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 from stainless steel. Copper solution (100 μM) was injected by use of a Hamilton syringe into the calorimetric stirred titration vessel, which contained 1.8 mL MBP, 0.25 or 0.50 mg mL^{-1}, in Tris buffer (30 mM), pH=7.2. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of copper solution into the perfusion vessel was repeated 28 times, and each injection included 35 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 ‘Thermometric

Digitam 3' software program. The heat of dilution of the copper solution was measured as described above except MBP was excluded. Also, the heat of dilution of the protein solution was measured as described above except the buffer solution was injected to the protein solution in the sample cell. The enthalpies of copper and protein solutions dilution were subtracted from the enthalpy of MBP–copper interaction. The microcalorimeter was frequently calibrated electrically during the course of the study.

The molecular mass of MBP was taken to be 18.500 [46].

Results and discussion

The raw data obtained from isothermal titration calorimetry of MBP interaction with copper ion in two different concentrations of the protein was shown in Fig. 1. Figure 1a is showing the heat of each injection and Fig. 1b is showing the heat of related to each total concentration of copper ion, $[Cu^{2+}]_t$. These raw calorimetric data

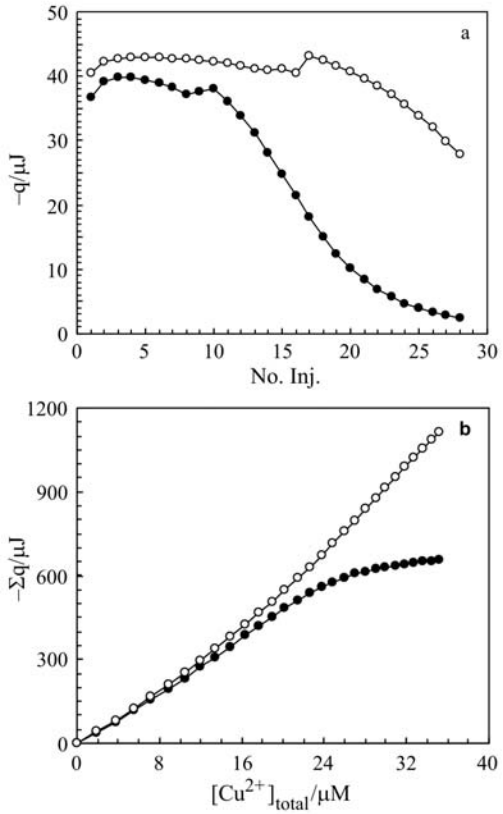

Fig. 1 a – The heat of copper binding on MBP for 28 automatic cumulative injections, each of 35 μL, of copper, 100 μM, into the sample cell containing 1.8 mL MBP solution at two initial concentrations of • – 0.25 mg mL^{-1} and ○ – 0.50 mg mL^{-1}. b – The heat of binding *vs.* total concentration of copper ion, calculated from Fig. 1a

can be used to show the heat of binding copper ions per mole of MBP (ΔH) *vs.* total concentration of copper ions, Fig. 2a, or *vs.* total concentration of the protein, Fig. 2b.

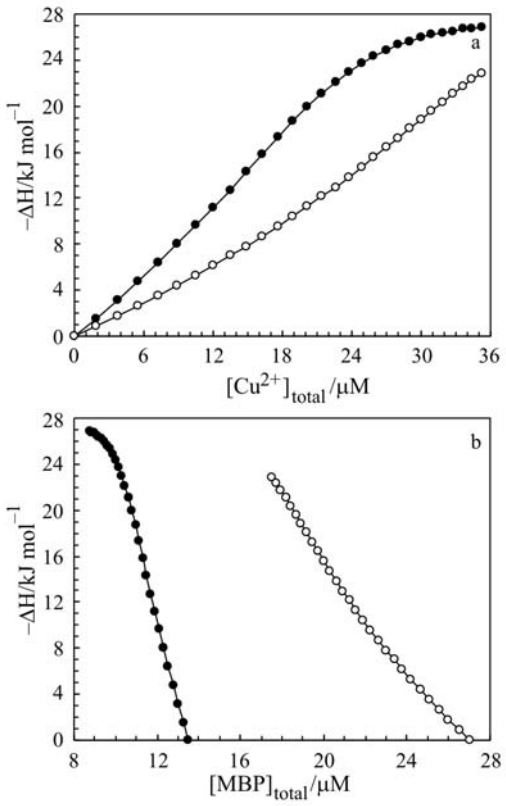

Fig. 2 a – The heat of binding copper ions per mole of MBP (ΔH) *vs.* total concentration of copper ions, calculated from Fig. 1b. b – The heat of binding copper ions per mole of MBP (ΔH) *vs.* total concentration of MBP. The initial concentration of MBP was • – 0.25 mg mL^{-1} and ○ – 0.50 mg mL^{-1}

In general, there will be 'g' sites for binding of copper ions per protein macromolecule and ν is defined as the average moles of bound copper ions per mole of total MBP. At any constant value of ΔH, the free concentration of copper ion (L^{free}) and ν are also constant at equilibrium on both two curves in Fig. 2. Copper ions exist in two forms of free and bound. Hence, $L^{free}=L-L^{bound}$, where L and L^{bound} are the total and bound concentration of copper ion, respectively. We do the titration calorimetric experiment in two concentrations of the protein, shown by **1** and **2**. Equality of L^{free} at any constant value of ΔH on both curves in Fig. 2 results the equation:

$$L_1-L_1^{bound}=L_2-L_2^{bound} \quad (1)$$

By applying $\nu=L^{bound}/M$, which M is the total concentration of the protein, and equality of ν at any constant value of ΔH on both curves in Fig. 2 it can be deduced Eq. (2) from Eq. (1).

$$L_1 - \nu M_1 = L_2 - \nu M_2 \tag{2}$$

This equation can be rearranged to give Eq. (3).

$$\nu = \frac{L_2 - L_1}{M_2 - M_1} \tag{3}$$

Then L^{free} can be calculated by Eq. (4), which obtains from substitution of ν from Eq. (3) into the equation $L^{free} = L_1 - \nu M_1$

$$L^{free} = \frac{L_1 M_2 - L_2 M_1}{M_2 - M_1} \tag{4}$$

One can then calculate ν and L^{free} from Eqs (3) and (4), respectively. This procedure is repeated over the range of ΔH values that span the titration curves, thus yielding a full range of values of ν and L^{free}. In this way, one can obtain a binding isotherm as shown in Fig. 3a or the Scatchard plot [47], $\nu/[Cu^{2+}]_f$ *vs.* ν, as shown in Fig. 3b, where $[Cu^{2+}]_f$ is the free concentration of copper ion.

The shapes of the Scatchard plots are clearly characteristic of different types of cooperativity [48, 49]. A concave downward curve, as shown in Fig. 3b, describes a system with positive cooperativity. For obtaining approximated values of binding parameters, it might be possible to fit the binding data to the Hill equation [50],

$$\nu = \frac{g(K([Cu^{2+}]_f)^n}{1+(K([Cu^{2+}]_f)^n} \tag{5}$$

where K and n are the binding constant and Hill coefficient, respectively. The binding data for the binding of copper ions to MBP have been fitted to the Hill equation using a computer program for nonlinear least-square fitting [51]. The results are: g=2, K=0.38 μM^{-1} and n=1.6. The best-fit curve of the experimental binding data was then transformed to a Scatchard plot as shown in Fig. 3b. A simple method for calculating intrinsic association equilibrium constants for system with two cooperative sites (K_1 and K_2) has been introduced from the Scatchard plot [52]. It has been shown that, in the limit as ν approaches 0, $\nu/[Cu^{2+}]_f=2K_1$ and when ν=1, or at half-saturation, $\nu/[Cu^{2+}]_f=(K_1K_2)^{1/2}$. Thus, K_1 can be obtained from the ordinate intercept of a Scatchard plot and K_2 is derived from the value of $\nu/[Cu^{2+}]_f$ at half-saturation. The results obtained from Fig. 3b are K_1=0.083 μM^{-1} and K_2=1.740 μM^{-1}. So, occupation of the first site has produced an appreciable enhancement 21 of the binding affinity of the second site. Moreover, values of ΔH in different values of ν (obtained from Fig. 2) give the molar enthalpies of binding –13.5 and –14.8 kJ mol^{-1} in the first and second binding sites, respectively. Results obtained by this new method are agreement with our previous results [33].

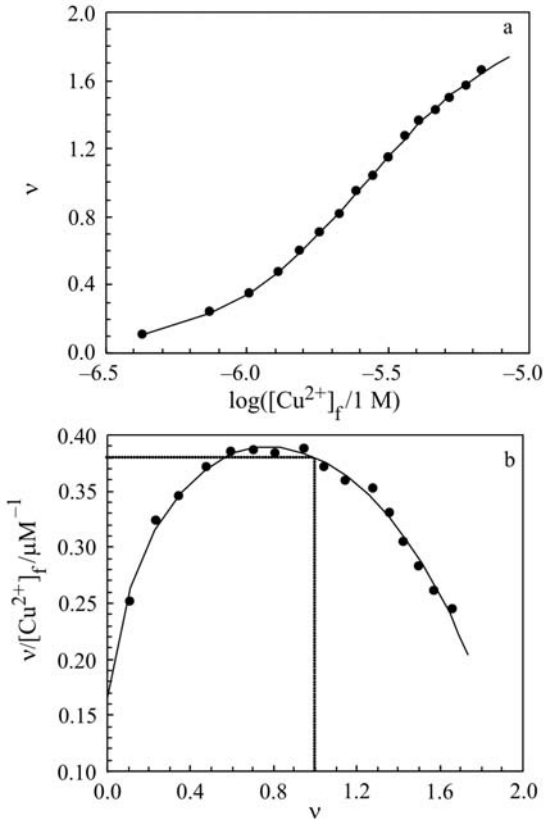

Fig. 3 a – The binding isotherm and b – the Scatchard plot of binding copper ion by MBP at 27°C. The best-fit curve of the experimental binding data was transformed to both binding isotherm and the Scatchard plot using Eq. (5) with g=2, K=0.38 μM^{-1} and n=1.6

The new calorimetric method described in this paper allows obtaining the binding isotherm for measurement of the complete set of thermodynamic parameters in protein ligand binding studies. The binding isotherm for ligand–protein interaction can be easily obtained by carrying out titration calorimetric experiment in two different concentrations of a protein.

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