Thermodynamic study of magnesium ion binding to α-amylase

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The interaction of α-amylase (from *Bacillus amyloliquefaciens*) with Mg^{2+} ion was studied using UV spectrophotometric and isothermal titration calorimetric (ITC) methods at 27°C in 30 m*M* Tris buffer solution at *p*H = 7.0. The binding isotherm for metal-protein interaction was easily obtained by carrying out ITC experiment at two different concentrations (2 μ*M* and 50 μ*M*) of the protein. α-Amylase had eight identical and independent binding sites for Mg^{2+} ion, which showed non-cooperativity in the binding process. The binding of Mg^{2+} ion was exothermic ($\Delta H= -17.3$ kJ mol^{-1}) with association binding constant of 2.08 mM^{-1}. The binding slightly destabilized the enzyme against thermal denaturation, as evident from absorption studies.

Keywords: α-Amylase, magnesium ion, titration calorimetry, calorimetric method, metal binding, metal-protein interaction

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α-Amylase (α-1, 4 glucan-4-glucanohydrolase; EC 3.2.1.1) catalyzes the hydrolysis of α-1, 4 glycosidic linkages of starch components and glycogen[1,2]. It is widely distributed in plants, animals, and microorganisms and shows varying action patterns depending on the source[3]. It has been investigated extensively for various aspects like its structure and function[4-6], mechanism of secretion through cell membrane, and industrial application[7,8].

Calcium is required to maintain the structural integrity of α-amylase[9] and its removal leads to decreased thermostability and/or enzymatic activity[10], or increased susceptibility to proteolytic degradation[6]. So far, it has not been possible to obtain crystals of *Bacillus amyloliquefaciens* α-amylase (BAA) with bound calcium[6]. It has been reported from the measurements in the presence of EDTA and Ca^{2+} that BAA is stabilized by binding of Ca^{2+} ions[11]. Binding of excess Ca^{2+} on the surface of the α-amylase is exothermic, with mean association binding constant of 1.82 mM^{-1}; binding stabilizes the enzyme against surfactant and thermal denaturation and prevents the spontaneous decrease in its biological activity[12,13]. Similarly, the binding of cobalt ions is also exothermic with mean association binding constant of 8.33 mM^{-1}; the binding significantly increase the enzyme activity and decreases its thermal stability[13,14].

The affinity between divalent metal ions and the α-amylase varies considerably with the source of the enzyme[15]. There are some reports about the requirement of Mg^{2+} and Ca^{2+} ions for activation of BAA[2,16]. The Hg^{2+} and Cu^{2+}, however, inhibit BAA activity by 27.8 and 19.5%, respectively[17], whereas a lower inhibition (5.6%) is reported at a high concentration of Co^{2+} for α-amylase from some sources, such as poplar leaves[2,17]. There is no precise report available on the thermodynamics on the metal binding and its effect on the stability of BAA in the literature. In continuation of our previous work on the interaction between metal ions and α-amylase[12-14], in the present investigation, thermodynamics of binding of Mg^{2+} to BAA is studied using isothermal titration calorimetric and spectrophotometric techniques to find the number of binding sites, the strength of metal binding and the type of interaction between binding sites for Mg^{2+} ion. The effect of binding on the thermal stability of the enzyme is also studied.

Materials and Methods
Materials
Bacillus amyloliquefaciens α-amylase (BAA) and Tris-HCl were obtained from Sigma Chemical Co, USA. Magnesium nitrate was purchased from Merck Co. The enzyme sample was completely homogeneous. The 30 m*M* Tris-HCl solution (*p*H 7.0), made in double-distilled water was used as a buffer.

Methods
Isothermal titration microcalorimetric method
The isothermal titration microcalorimetric experiments were performed with the 4-channel commercial microcalorimetric system, thermal activity monitor 2277 (Thermometric, Sweden). Each

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channel was a twin heat-conduction calorimeter, where the heat-flow sensor was a semi-conducting thermopile (multi-junction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. The insertion vessel was made from stainless steel. Magnesium nitrate solution (20 mM) in Tris buffer was injected using a Hamilton syringe into the calorimetric stirred titration vessel, which contained 2 ml enzyme (2 μM or 50 μM). Thin (0.15 mm inner diam.) stainless steel hypodermic needles, permanently fixed to the syringe reached directly into the calorimetric vessel. Injection of Mg^{2+} ion solution into the perfusion vessel was repeated 20 times and each injection included 40 μl Mg^{2+}.

The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of each injection was calculated using the "Thermometric Digitam 3" software program. The heat of dilution of the metal ion solution was measured as described above, except that the enzyme was excluded. Also, the heat of dilution of the enzyme solution was measured as described above, except that the buffer solution was injected to the enzyme solution in the sample cell. The enthalpies of dilution for Mg^{2+} and enzyme solutions were subtracted from the enthalpy of BAA-Mg^{2+} interaction. The microcalorimeter was frequently calibrated electrically during the course of the study. The molecular weight of BAA was taken as 54,800[18].

Temperature-scanning spectroscopy

Absorbance profiles, which describe the thermal denaturation of BAA were obtained from a UV-visible spectrophotometer (CARY-100-Bio model) fitted with a temperature programmer, which controlled the rate of temperature change in melting experiments. The accuracy of temperature measurement was 0.1°C. The cuvette holder accommodated two samples — one as a reference buffer solution and the other for a sample experimental determination. Both reference and samples cells had identical Mg^{2+} concentration. The enzyme concentration in the sample cells for Mg^{2+} interaction was 1.2 mg/ml. The recording chart read the temperature, reference line (from reference cuvette) and the absorbance change at 280 nm for the sample in the cuvette.

Results and Discussion

The raw data obtained from isothermal titration calorimetry of BAA interaction with Mg^{2+} at two different concentrations (2 μM and 50 μM) of the enzyme are shown in Fig. 1. Fig. 1a shows the heat of each injection and Fig. 1b shows the total heat *vs* total concentration of Mg^{2+}, $[Mg^{2+}]_t$, in each step during the titration. The calorimetric data can be used to represent the heat of binding of Mg^{2+} ions per mole of BAA (ΔH) *versus* total concentration of Mg^{2+} ions (Fig. 2a), or *vs* total concentration of the enzyme (Figs. 2b & c).

The binding isotherm plot (Fig. 3a) and the Scatchard plot, $v/[Mg^{2+}]_f$ *vs* v (Fig. 3b) can be obtained using a simple method[19,20]. v is defined as the average moles of bound Mg^{2+} per mole of total BAA and $[Mg^{2+}]_f$ is the free concentration of Mg^{2+} ions. The method is based on the fact that at any constant value of ΔH, v and $[Mg^{2+}]_f$ are also constant at equilibrium. Thus, v as a function of $[Mg^{2+}]_f$ can be calculated from a minimum of two titrations. Also, from titration
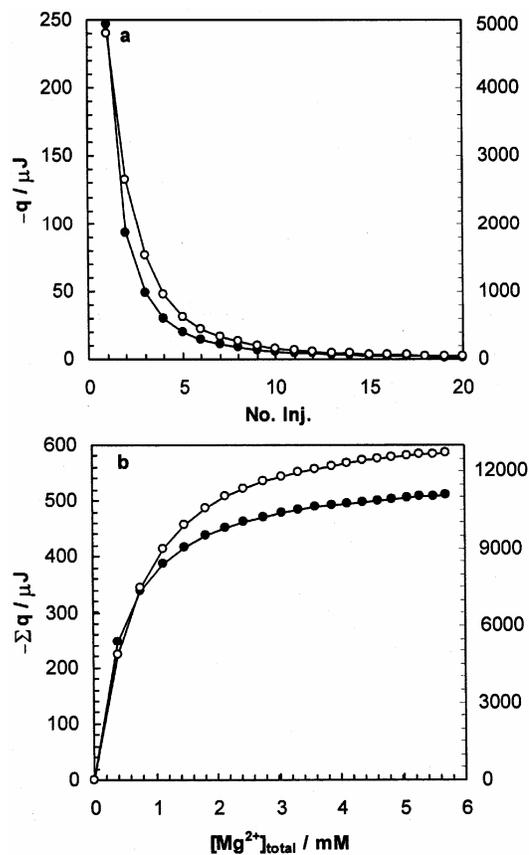

Fig. 1—(a): Heat of Mg^{2+} ion binding on BAA for 20 automatic cumulative injections, each of 40 μl of Mg^{2+} ion, 20 mM into the sample cell containing 2 ml BAA solution at two initial concentrations of 2 μM (•) and 50 μM (o); and (b): Heat of binding *vs* total concentration of Mg^{2+}, calculated from Fig. 1a. The left vertical axis is for (•) and the right vertical axis is for (o).
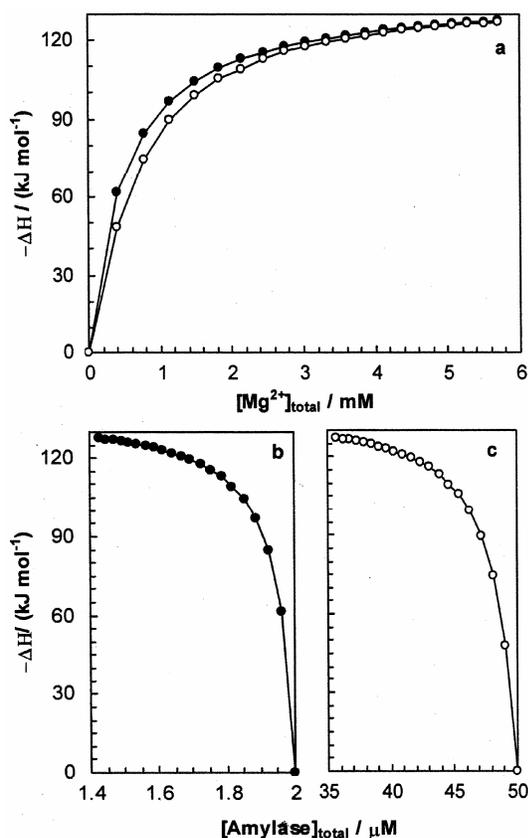
Fig. 2—(a): Heat of binding Mg^{2+} per mole of BAA (ΔH) *vs* total concentration of Mg^{2+}, calculated from Fig. 1b; and (b & c): Heat of binding of Mg^{2+} per mole of BAA (ΔH) *versus* total concentration of BAA [The initial concentration of BAA was 2 μM (•) and 50 μM (o)]

Fig. 3—Binding isotherm (a) and the Scatchard plot (b) of binding of Mg^{2+} ion by BAA at 27 °C. The best-fit curve of the experimental binding data was transformed to both binding isotherm and the Scatchard plot using equation $v/[Mg^{2+}]_f = K$ $(g-v)$ with g = 8 and $K = 2.08$ mM^{-1}

curves (Fig. 2), obtained at two different total concentrations (M_1 and M_2), the set of values of the total ligand concentrations (L_1 and L_2), for which ΔH is constant could be determined. This was done by drawing a horizontal line, defining a constant ΔH that intersects both titration curves (BAA concentrations M_1 and M_2), and determining the values of L_1 and L_2 at the points of intersection. The v can then be calculated from equation $v = (L_2-L_1)/(M_2-M_1)$. In this manner, a binding isotherm (Fig. 3a) or the Scatchard plot[21], $v/[Mg^{2+}]_f$ *vs* v (Fig. 3b) can be obtained.

The shapes of the Scatchard plots are clearly characteristics of different types of cooperativity[21-24]. A linear plot, as shown in Fig. 3b describes a system with non-cooperativity. According to the Scatchard equation $v/[Mg^{2+}]_f = K(g-v)$, it might be possible to find binding parameters from intercepts and the slope of the Scatchard plot, where K and g are the association binding constant and the number of binding sites, respectively. The results obtained are: g = 8 and

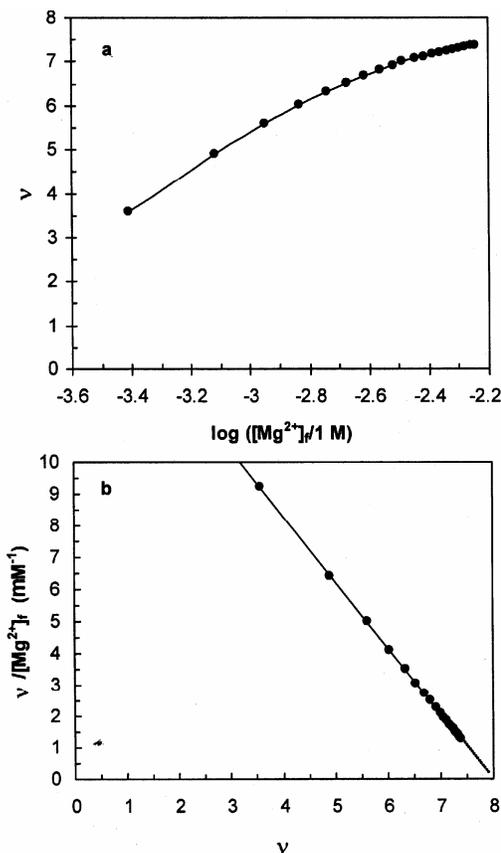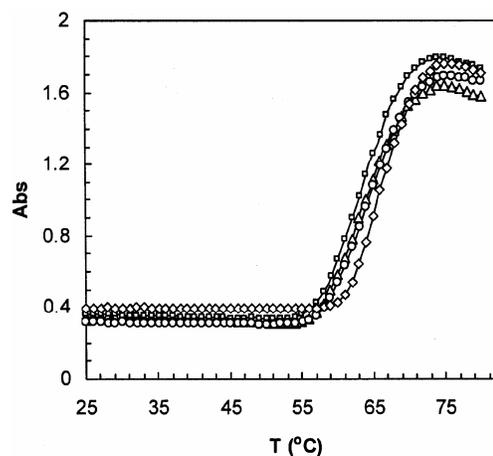Fig. 4—Change of absorbance for BAA (1.2 mg/ml) at $\lambda_{max}=280$ nm due to the increase of temperature at different fixed Mg^{2+} concentrations — 0 mM (◊), 2 mM (o), 4 mM (Δ) and 6 mM (□)

$K = 2.08$ mM^{-1}. The best-fit curve of the experimental binding data was then transformed to a Scatchard

plot[21,22] as shown in Fig. 3b. Moreover, the values of ΔH at different values of ν (obtained from Fig. 2) give the molar enthalpies of binding –17.3 in each binding sites. Results (ΔH and K values) obtained in the present study are in agreement with the results obtained earlier by calorimetric data analysis[25-26].

The thermal denaturation curves for BAA are shown in Fig. 4. In all cases, denaturation was followed, by measuring the absorbance at 280 nm at different concentrations. The denaturing temperature (T_m) of the enzyme decreased slightly with increasing the Mg^{2+} concentration. The values of T_m at different Mg^{2+} concentrations were obtained from the mid-point change of absorbance due to the increase of temperature, and were found to be 66, 65, 65 and 64°C in the presence of 0, 2, 4 and 6 mM Mg^{2+} concentration, respectively. A small decrease of 2°C for T_m at 6 mM is significant, since the accuracy of temperature measurement was 0.1°C . The binding of Mg^{2+} ions slightly destabilized BAA against thermal denaturation.

The Mg^{2+} binding on the BAA macromolecule occurs in a set of eight non-cooperative binding sites exothermically, with association binding constant of 2.08 mM^{-1}. The binding process is accompanied with slightly less enzyme thermal stability. The binding affinity of BAA to Mg^{2+} (2.08 mM^{-1}) is more than the Ca^{2+} (1.82 mM^{-1})[12,13]. Although, Mg^{2+} binding slightly decreased the thermal stability of BAA, the Ca^{2+} binding slightly increased the thermal stability[12,13]. This may be due to the presence of more number of binding sites for Ca^{2+} as compared to Mg^{2+} ion.

Acknowledgement

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