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Thermodynamic studies on the interaction of calcium ions with alpha-amylase

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Abstract

The interaction of α-amylase from *Bacillus amyloliquefaciens* with divalent calcium ion was studied by equilibrium dialysis, isothermal titration microcalorimetry, UV spectrophotometry and temperature scanning spectrophotometry methods at 27°C in Tris buffer solution at pH 7.5. There is a set of 17 binding sites for calcium binding on the enzyme with weak positive cooperativeness in binding. The binding of calcium is exothermic ($\Delta H = -16$ kJ mol^{-1}) with mean dissociation binding constant of 0.55 mM. The binding of calcium caused the more stability of the enzyme against surfactant and thermal denaturation. Moreover, the binding of calcium prevents from the spontaneous decrease in biological activity of α-amylase.

Keywords: Biological activity; Calcium; α-Amylase; Isothermal titration calorimetry

1. Introduction

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]. α-Amylase is widely distributed in plants, animals, and microorganisms and shows varying action patterns depending on the source [3,4]. The enzyme has been investigated extensively from various aspects: its protein structure and function [5–7], its mechanism of secretion through cell membrane [8], and its industrial application [9,10].

Calcium is required to maintain the structural integrity of α-amylase [11]. Removal of calcium leads to decreased thermostability and/or decreased enzymatic activity [12], or increased susceptibility to proteolytic degradation [7]. So far, it has not been possible to obtain crystals of α-amylase from *Bacillus amyloliquefaciens* (BAA) with bound calcium [7] From measurements in the presence of EDTA and Ca^{2+}, it has been reported that BAA is stabilized by binding of calcium ions [13].

There are some reports about the requirement of Mg^{2+} and Ca^{2+} cations for stability and activation of BAA [2,14]. Recently, we reported that the BAA activity increased significantly with an increasing concentration of cobalt; however, the temperature of denaturation of the enzyme decreased [15]. The affinity between divalent metal ions and the α-amylase molecules varies considerably with the source of the enzyme [16]. The inhibition of BAA activity with Hg^{2+} and Cu^{2+} was reported as 27.8 and 19.5%, respectively [17] A lower inhibition (5.6%) at a high concentration of Co^{2+} was also reported for α-amylase

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from some sources [2,17]. The aim of the present investigation was to study the effects of calcium divalent cation, at low concentration, on the stability and thermal denaturation of α-amylase. The interaction of Ca^{2+} with enzyme was investigated by equilibrium dialysis and isothermal titration microcalorimetry techniques for elucidation of calcium binding sites.

2. Experimental

2.1. Material

α-Amylase from *Bacillus amyloliquefaciens* (BAA), dodecyl trimethylammonium bromide (DTAB) and Tris-HCl were obtained from Sigma Chemical Co. Dinitrosalicylic acid (DNS), calcium nitrate tetrahydrate and soluble starch were purchased from Merck Co. Visking membrane dialysis tubing (MW cut-off 10,000–14,000) was obtained from Scientific Instrument Center Ltd (SIC, Eastleigh, Hampshire, UK). All other materials and reagents were of analytical grades, and solutions were made in double-distilled water. Tris-HCl solution with 10 mM concentration, pH 7.5, was used as a buffer.

2.2. Methods

2.2.1. 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 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. Calcium solution (6 mM) was injected by use of a Hamilton syringe into the calorimetric stirred titration vessel, which contained 2 ml enzyme, 1.23 mg/ml, including Tris buffer (10 mM), pH 7.5. Thin (0.15-mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of calcium solution into the perfusion vessel was repeated 20 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 calcium solution was measured as described above, except that the enzyme was excluded. The enthalpy of dilution was subtracted from the enthalpy of enzyme–calcium interaction. The enthalpy of dilution of enzyme is negligible. The microcalorimeter was frequently calibrated electrically during the course of the study.

2.2.2. Equilibrium dialysis

Experiments were carried out at 300 K using an BAA solution with a concentration of 1.23 mg/ml, of which 2 ml aliquots were placed in dialysis bags and equilibrated with 2 ml of the calcium solution, covering the required concentrations range for over 96 h. Corrections for inequalities arising from Donnan effects were negligible at the ionic strength used. The free calcium concentrations in equilibrium with complexes of enzyme–calcium were assayed by the atomic absorption (Perkin–Elmer, Model 603) method. The molecular weight of BAA was taken to be 54,800.

2.2.3. Temperature-scanning spectroscopy

Absorbance profiles, which describe the thermal denaturation of BAA, were obtained from a Gilford Model 2400-S spectrophotometer fitted with a temperature programmer, which controls the speed of temperature change in melting experiments. The cuvette holder can accommodate four samples: one acts as a reference buffer solution and the others are for each experimental determination. All reference and samples cells had identical concentrations of calcium. The concentration of enzyme in the sample cells was 0.5 mg/ml. The recording chart reads the temperature, reference line (from the reference cuvette) and the absorbance change at 280 nm for each of the three samples in the cuvette.

2.2.4. Spectrophotometric study of denaturation

DTAB denaturation curves were obtained by measuring the maximum absorbance (280 nm) of the solutions containing 1.2 mg/ml BAA using a Shimadzu Model UV-3100 spectrophotometer and 1-cm cuvettes thermostated to maintain the temperature at 27.0±0.1°C. All measurements were made after

BAA and DTAB has been incubated for over 5 min, after which time the absorbance did not change.

2.2.5. Assay of α-amylase activity

BAA was determined by the method of Bernfeld [18]. The assay system contained 1.0 ml of enzyme solution (with concentration of 0.1%, w/v) and 1.0 ml of 1% soluble starch (pH 7.5 in Tris buffer) solution. This reaction mixture was incubated at 27°C for 3 min. The reaction was terminated by the addition of 2.0 ml of DNS (3,4 dinitro salicylic acid) reagent solution (including calcium nitrate tetrahydrate and NaOH). Color due to the reducing sugar liberated was developed by heating the reactants in a boiling water bath for 5 min, and then rapidly cooling in running tap water. After addition of 20 ml of double distilled water, the extinction value was determined at 540 nm. The blank was prepared in the same manner without enzyme.

One unit of α-amylase activity was defined as the amount of enzyme required to release a reducing group in 3 min from 1% soluble starch corresponding to 1 mg maltose hydrate.

3. Results and discussion

The binding isotherm has been plotted as the average number of bound calcium ions to one macromolecule of BAA, v vs. log $[Ca^{2+}]_f$, where $[Ca^{2+}]_f$ is the free concentration of calcium ions, as shown in Fig. 1a. In this case, the Scatchard plot is not linear, as shown in Fig. 1b. Therefore, the binding of calcium ion is cooperative [19–21]. The number of binding sites (g), the dissociation equilibrium constant (K_d) and the Hill coefficient (n) can be obtained by fitting of experimental data to the Hill equation [22]:

$$v = \frac{g(K_a[Ca^{2+}])^n}{1 + (K_a[Ca^{2+}])^n} \quad (1)$$

where K_a is the association equilibrium constant ($K_a = 1/K_d$). The number of binding sites for calcium ion is 17. The number of amino acid residues with negative charges on the surface of protein is slightly more than three times of this n value. Also, the dissociation equilibrium constant and the Hill coefficient are 0.50±0.05 mM and 1.1±0.05, respectively. Hence, calcium binding sites have a weak positive cooperatively (it may be considered as a noncooperative system).

Consider a solution containing a ligand (L), and a macromolecule (M_g) that contains g 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 ($M_g \rightarrow gM$) with the same set of dissociation equilibrium constant values. Thus, the reaction under consideration can be written:

$$M + L \Leftrightarrow ML \quad K_d = \frac{[M][L]}{[ML]} \quad (2)$$

and also

$$[L]_{total} = [L] + [ML] \quad (3)$$

$$[M]_{total} = [M] + [ML] = \left(\frac{K[ML]}{[L]}\right) + [ML] \quad (4)$$

Eq. (3) can be solved for [L], then substituted into the Eq. (4), which can then be rearranged to give the quadratic equation whose only real root is [23]:

$$[ML] = \frac{(B + K_d) - [(B + K_d)^2 - C]^{1/2}}{2} \quad (5)$$

where

$$B = [M]_{total} + [L]_{total} \quad C = 4[M]_{total}[L]_{total} \quad (6)$$

The sum of heat evolutions following the ith titration step, Q_i, can be expressed as

$$Q_i = \Delta H V_i [ML]_i \quad (7)$$

Here, V_i is the volume of the reaction solution and ΔH is the enthalpy of binding. Combining Eqs. (5) and (7) will lead to

$$\Delta H = \frac{1}{A_i}\{(B_i + K_d) - [(B_i + K_d)^2 - C_i]^{1/2}\} \quad (8)$$

where

$$A_i = \frac{V_i}{2Q_i} \quad (9)$$

Eq. (8) contains two unknowns, K_d and ΔH. A_i, B_i and C_i can be calculated in each injection, and then by fitting these known parameters to Eq. (8) using a computer program for nonlinear least-squares fitting [24] ΔH and K_d values may be obtained. The data

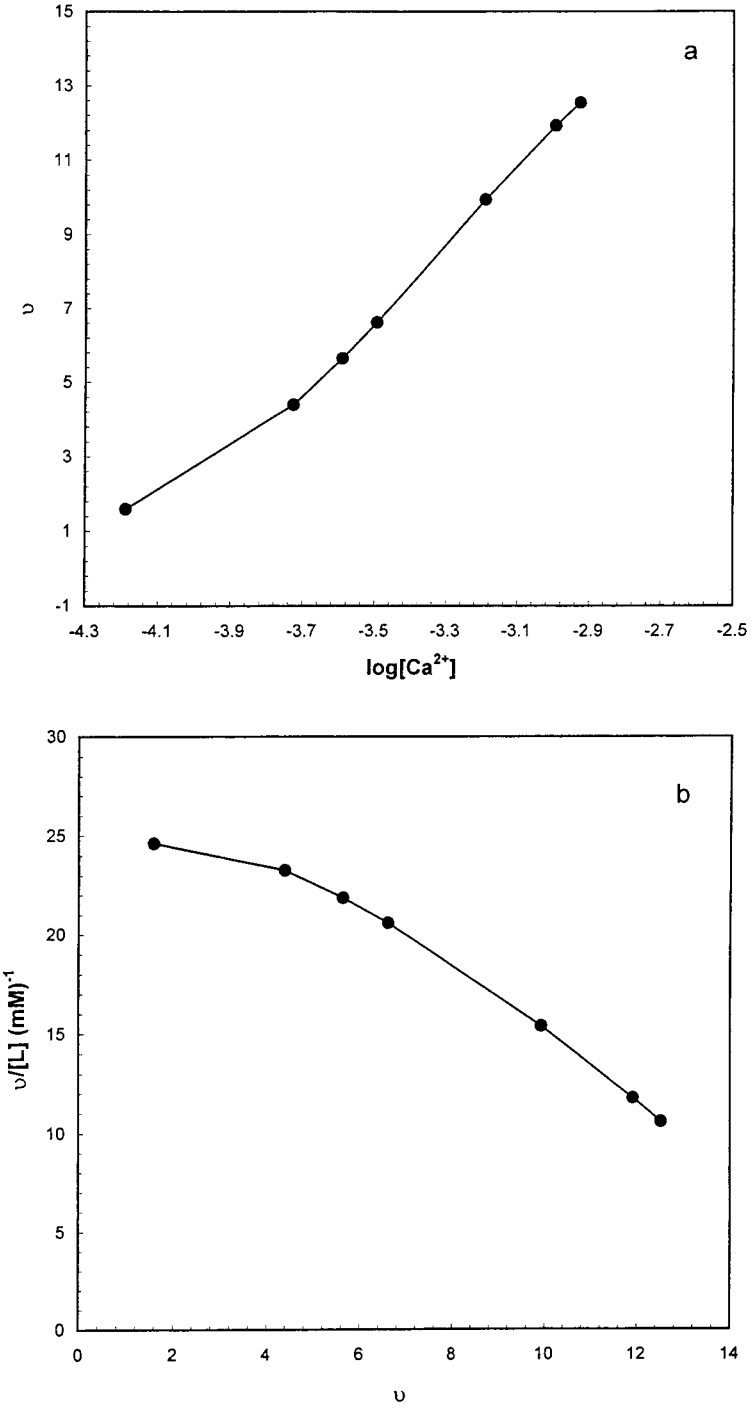

Fig. 1. Binding isotherm (a) and the Scatchard plot (b) for divalent calcium ion on interaction with BAA at pH 7.5 and 27°C.

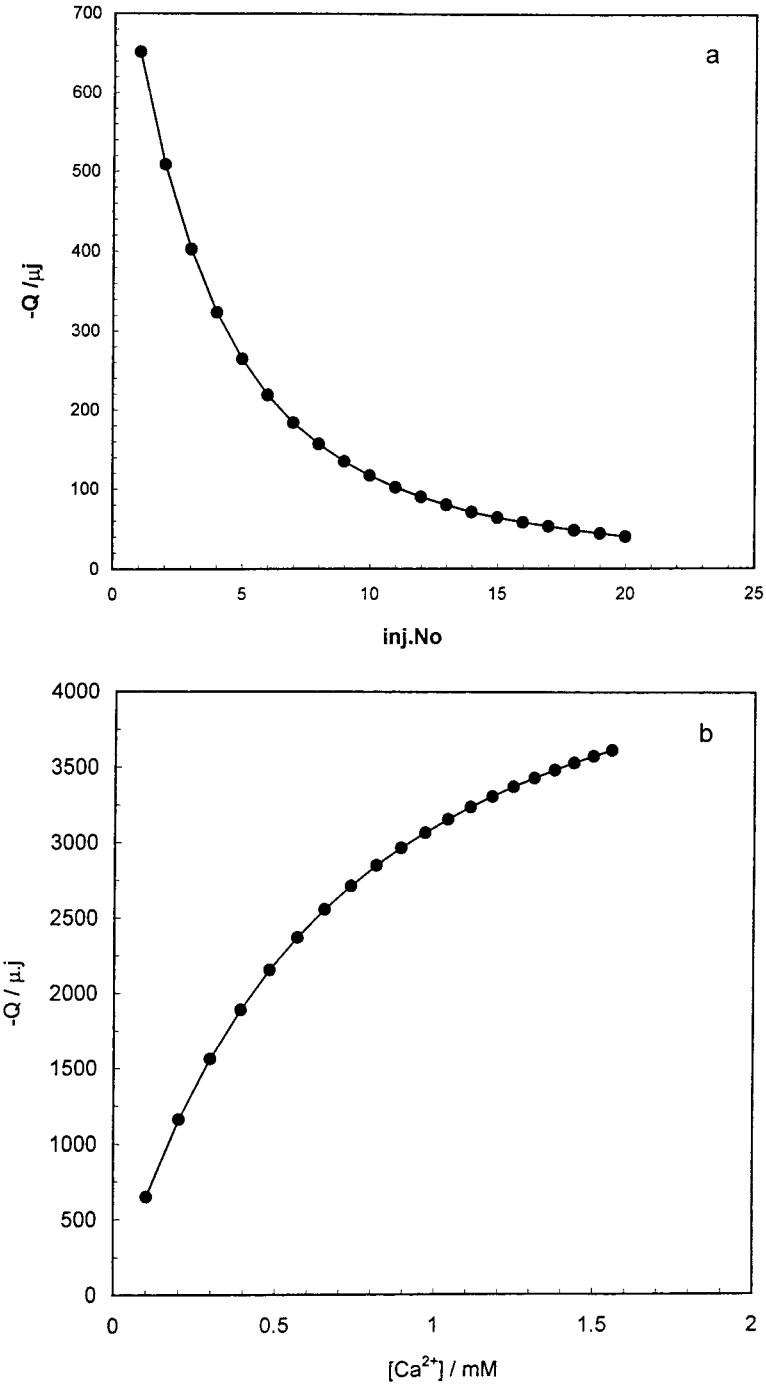

Fig. 2. (a) The heat of calcium binding on BAA for 20 automatic cumulative injections, each of 35 µl, of Ca^{2+} solution 6 mM, into the sample cell containing 2 ml BAA solution at a concentration of 1.233 mg/ml at pH 7.5 and 27°C. (b) The cumulative heat related to each total concentration of calcium, calculated per mole of binding site.

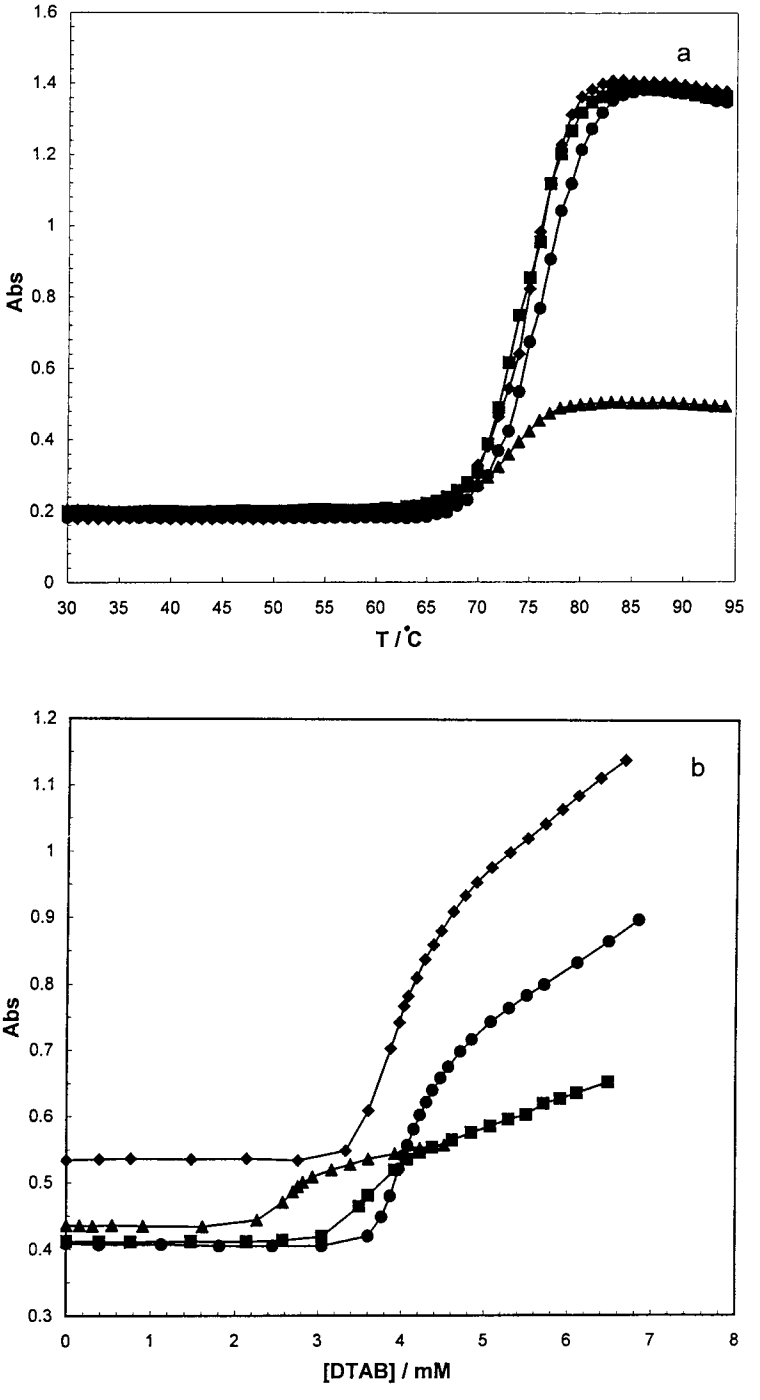

Fig. 3. The change of absorbance of BAA at λ_{max}=280 nm due to the increase of temperature (a) and concentration of DTAB (b) at different fixed concentrations of calcium ion: 0 mM (▲); 2 mM (■); 4 mM (◆); and 6 mM (●). The concentration of BAA was 0.5 mg/ml for thermal denaturation and 1.2 mg/ml for denaturation by DTAB at 27°C.

obtained from isothermal titration microcalorimetry of α-amylase interaction with calcium ion is shown in Fig. 2. Fig. 2a shows the heat of each injection and Fig. 2b shows the heat of related to each total concentration of calcium ion. The results are: K_d=0.55 mM DH=−16 kJ mol^{-1}.

The thermal denaturation curves for BAA are shown in Fig. 3a. In all cases, denaturation was followed by measuring the absorbance at 280 nm in different concentrations of calcium. The denaturing temperature (T_m) of the enzyme increases on increasing the concentration of calcium.

The values of T_m in different concentrations of calcium were obtained from the midpoint change of absorbance due to the increase of temperature. The values of T_m are 72.5, 74.4, 74.6 and 76.0±0.5°C in the absence, and in the presence, of 2, 4 and 6 mM calcium, respectively. Therefore, the existence of calcium led to more thermal stability. The essential feature of this study is the observed similarity between the effect of DTAB, as a denaturant cationic surfactant, and temperature on the process of denaturation. The profiles of denaturation of BAA by DTAB are shown in Fig. 3b. The concentration of DTAB in the midpoint of transition, $[DTAB]_{1/2}$, increases on increasing the calcium concentration. Hence, calcium binding to BAA also causes more stability of the enzyme against denaturation by surfactant.

The free energy of protein unfolding ΔG^0 was calculated as a function of DTAB concentration by assuming two-state mechanism and using the equations [25]

$$F_D = \frac{A_N - A_{obs}}{A_N - A_D} \tag{10}$$

$$\Delta G^0 = -RT \ln \frac{F_D}{1 - F_D} \tag{11}$$

where A_{obs} is the observed absorbance used to follow unfolding in the transition region, and A_N and A_D are the values of absorbance to the native and denatured conformations of the protein, respectively. Fig. 4 shows the free energy of unfolding, which is calculated from Eq. (11) based on the data on the Fig. 3b which varies linearly with DTAB concentration in the limited region. The simplest method of estimating the conformational stability in the absence of denaturant, ΔG^0 (H_2O), in different concentration of calcium, is to

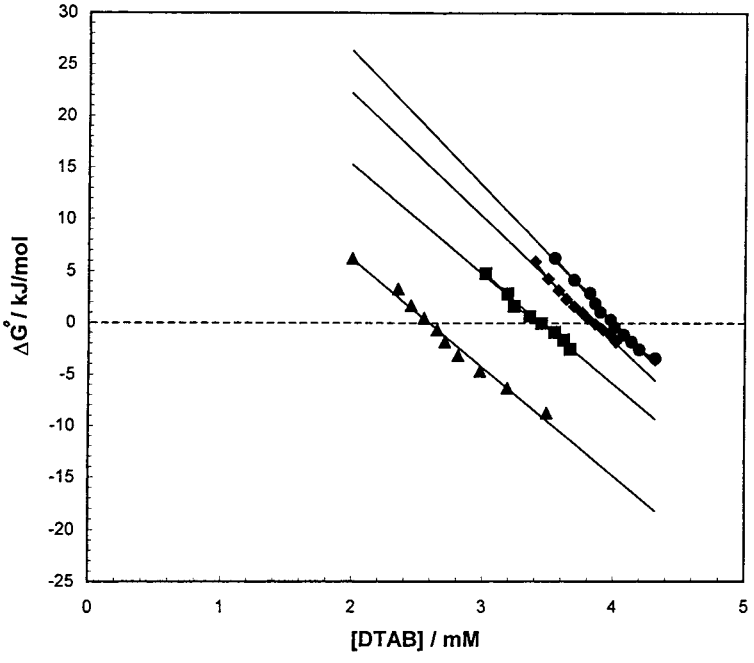

Fig. 4. The free energy of unfolding (calculated from Eq. (11) by assuming a two-state mechanism) vs. DTAB concentration, at different fixed concentrations of calcium ion: 0 mM (▲); 2 mM (■); 4 mM (◆); and 6 mM (●) in the base of data shown in Fig. 3b.

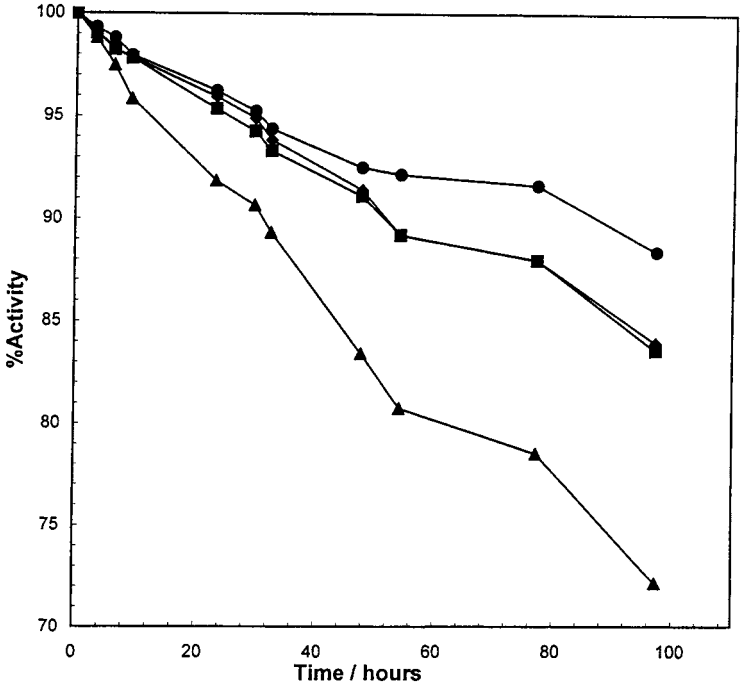

Fig. 5. Spontaneous inactivation of BAA at different fixed concentrations of calcium ion: 0 mM (▲),;2 mM (■); 4 mM (◆); and 6 mM (●).

assume that linear dependence continues to zero concentration and to use a least-squares analysis to fit the data to the equation [26]

$$\Delta G^0 = \Delta G^0(H_2O) - m[\mathrm{DTAB}] \quad (12)$$

where m is a measure of the dependence of ΔG^0 on DTAB concentration. The values of ΔG^0 (H_2O) are 27.2, 36.5, 46.1 and 52.7±0.2 kJ/mol in the absence, and in the presence of 2, 4 and 6 mM calcium, respectively. Therefore, the existence of calcium led to more stability of the enzyme.

The relative activities of the BAA were determined in the presence of different concentrations of divalent calcium cation. These results are shown in Fig. 5. As it can be noticed, excess binding of calcium ion to BAA led to a decrease in spontaneous inactivation of the enzyme.

It is concluded that the binding of divalent calcium cation on the surface of BAA globular macromolecule causes an increase in enzyme thermal stability due to less flexibility (increasing of T_m) and also an increase in enzyme stability against DTAB as a surfactant denaturant.

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