Calcium-binding parameter of *Bacillus amyloliquefaciens* α-amylase determined by inactivation kinetics

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The irreversible thermal inactivation and the thermodynamics of calcium ion binding of *Bacillus amyloliquefaciens* α-amylase in the absence of substrates were studied. The enzyme inactivation on heating was apparently followed by first-order kinetics. The enzyme was stabilized with an increased concentration of calcium ion and thus the inactivation was highly dependent on the state of calcium binding. The activation parameter for the inactivation suggests an unfolding of the enzyme protein upon heating. Values of both the activation enthalpy and entropy were increased with a higher calcium ion concentration. An inactivation kinetic model is based on the assumption of a two-stage unfolding transition in which the bivalent ion dissociation occurs in the first step followed by the secondary structural unfolding. This simple kinetic model provides both a qualitative and quantitative interpretation of calcium ion binding to the enzyme and its effect on the inactivation properties. The specific approximations of the kinetic model were strictly followed in the analysis to calculate the apparent inactivation rate at each calcium ion concentration in terms of the calcium-binding parameters. The enthalpy and entropy changes for the calcium ion binding were calculated to be -149 kJ/mol and -360 J · mol^{-1} · K^{-1} respectively and these values suggest a strong enthalpic affinity for the bivalent ion binding to the enzyme protein. The thermodynamical interpretation attempts to provide clear relations between the terms of an apparent inactivation rate and the calcium binding.

Key words: enthalpy, entropy, thermal inactivation, thermodynamic parameter, unfolding.

INTRODUCTION

α-Amylase (EC 3.2.1.1;1,4-α-D-glucan glucanohydrolase) hydrolyses starch by cleaving the internal α-1,4-glucosidic bonds [1–4]. This enzyme produced from the genus *Bacillus* has been studied for industrial applications involving sugar, brewing, alcohol, desizing and detergents [5,6]. The nucleotide sequence of the α-amylase gene from *Bacillus amyloliquefaciens* (BAA) has been determined previously [7,8]. Thus this bacterial α-amylase provides useful information about the relationship between the amino-acid sequence of a protein and the stability of its active conformation. Among the several enzymes used for thermostabilization, the calcium ion is known to be very effective owing to its structural stabilization [9,10]. Calcium has been shown to regulate the stability and reactivity of a wide variety of biological proteins. In particular, its binding to α-amylase is essential in activating and stabilizing the enzyme proteins [11,12]. Several reports have discussed thermodynamic analysis of calcium ion binding to EF-hand protein using calorimetry [13]. However, a firmly bound calcium ion could not be removed easily to estimate the binding constant. In the present study, we have developed a simple kinetic model that involves bivalent ion dissociation and secondary structural unfolding. We also estimated the binding constant by bivalent ion titration without any dialysis to remove the initially bound calcium ion.

α-Amylases are known to contain at least one calcium ion per molecule involved in the stabilization of the molecular structure [11]. The calcium-binding site of BAA has not been elucidated precisely, but the crystal structures of α-amylase from *Aspergillus oryzae* (TAA) [14] and α-amylase from porcine pancreas (PPA) [15] have revealed that the binding sites are similar in both the enzymes. Structural analysis of barley α-amylase has revealed that a single isoform of this amylase contains three calcium binding sites, including a site similar to that observed in other amylases [16]. In bacteria, fungi, plants and mammals, three conserved regions of the polypeptide chain contribute to the calcium binding [17]. In BAA, residues Gly^{97}–Ala^{109}, Ile^{217}–His^{235} and Ser^{314}–Ser^{334} are defined as the calcium-binding regions. Spectroscopic studies have suggested a conformational change of this protein upon removal of the bound calcium ion [12].

Irreversible inactivation of barley α-amylase is supposed to be caused by calcium ion depletion. It takes place upon heating as a balance between the rates at which the calcium ion binds to an apoenzyme of amylase and the apoenzyme undergoes denaturation [18]. In this sense, amylase inactivation may be caused by the two-step mechanism in which the reversible calcium ion depletion is the first step and the irreversible conformational change is the second step. In the present study, the effect of the calcium ion on the thermal inactivation of BAA in the absence of any substrates was studied. The binding constant was calculated by a simple kinetic model, which examines the effect of calcium ion on inactivation rates. To understand the kinetic behaviour when exposed to high temperature, a detailed analysis of the effect of temperature on the stability of BAA at various calcium ion concentrations was undertaken. Thermodynamic studies on the mechanism of the denaturation at various concentrations of calcium ion provide useful information about the stabilization of the enzyme structure bound to the bivalent ion.

Abbreviations used: BAA, α-amylase from *Bacillus amyloliquefaciens*; PPA, α-amylase from porcine pancreas; TAA, α-amylase from *Aspergillus oryzae*.

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The binding properties of the calcium ion are presented as functions of the binding constants and interpreted in terms of changes in entropy and enthalpy.

MATERIALS AND METHODS

Enzyme source and substrates

A purified enzyme preparation of BAA was purchased from Seikagaku Kogyo (Tokyo, Japan). It was dissolved in 10 mM Tris/HCl buffer (pH 9.0) and applied on to a Toyopearl HW-55 (Tosoh, Tokyo, Japan) gel filtration column (2.6 cm × 80 cm), which had been equilibrated with the same buffer [19]. A fraction of α-amylase was dialysed against distilled water at 5 °C for 24 h. The molecular mass of the enzyme was estimated to be approx. 49000 Da by SDS/PAGE. Amylopectin from potato starch was purchased from Sigma and used as a substrate in the detection of enzyme activity.

Measurement of thermal inactivation of the enzyme

The reaction mixture consisting of 1.31×10^{-8} M BAA, 25 mM Tris/acetate buffer (pH 7.0) and 0.002–0.02 mM $CaCl_2$ was incubated at 45–65 °C for the appropriate time intervals. At various times of the incubation, the samples were removed, cooled in ice and assayed for residual hydrolytic activities. The residual activity was measured by the dinitrosalicylic acid method using 0.5% (w/v) amylopectin as a substrate in 25 mM Tris/acetate buffer (pH 7.0) at 30 °C [20]. The reducing ends of the product detected by this method were unaffected by the presence of calcium ion. Plots of logarithm of the residual activity against the incubation time were linear. They were used to estimate the apparent first-order rate constant k_{obs} for the inactivation process. The activation enthalpy $\Delta H^{\ddagger}$ and activation entropy $\Delta S^{\ddagger}$ for the inactivation were calculated using the k_{obs} values at the given temperatures according to the equation

$$k_{obs} = kT/h \exp(-\Delta H^{\ddagger}/RT + \Delta S^{\ddagger}/R) \quad (1)$$

where k, h, R and T are the Boltzmann constant, the Planck constant, the gas constant and the absolute temperature respectively.

Calculation of calcium-binding constants

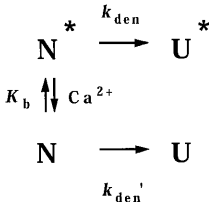
To explain the calcium-binding state for the inactivation process, the relationship between the calcium-bound native, calcium-depleted native and unfolded proteins is shown in Scheme 1. In this Scheme, N represents the folded native conformation in water, whereas U represents the unfolded state. The asterisks indicate apoenzymes that lack calcium ions. Based on the pre-equilibrium approximations of the kinetic model represented in Scheme 1, the following equation is derived to calculate the apparent inactivation rate in terms of calcium ion concentration:

$$k_{obs} = (k_{den} + K_b k'_{den}[Ca^{2+}])/(1 + K_b[Ca^{2+}]) \quad (2)$$

In this equation, k_{den} is the irreversible rate constant for the denaturation of the calcium-depleted enzyme, k'_{den} is that for the calcium-bound enzyme and K_b is the constant representing binding of the calcium ion to the enzyme protein. Since the inactivation rate of the calcium-depleted form is much greater than that of the calcium-bound form, we assumed that only the depleted form contributes to the inactivation, and the bound form contributions in eqn (2) were neglected. This assumption requires the approximation that the inactivation proceeds only as calcium is depleted from the protein. If this assumption is applied to the experimental data, the linear relationship between the values of reciprocal apparent first-order rate constant and calcium ion concentrations can be determined:

$$k_{obs}^{-1} = k_{den}^{-1} + K_b k_{den}^{-1}[Ca^{2+}] \quad (3)$$

This kinetic model requires two parameters, K_b and k_{den}. A linear fitting of the experimental data provided the calcium-binding parameter, by combining the obtained slope with the intercept.



Scheme 1 The proposed kinetic unfolding mechanism of BAA induced by the dissociation of calcium ion

N represents the folded native conformation in water and U the unfolded state. The asterisks indicate the apoenzyme which lacks calcium ion.

RESULTS AND DISCUSSION

To examine the effect of calcium on the thermal inactivation of BAA, we first studied the kinetics of inactivation of amylase activity at various calcium ion concentrations (Figure 1). Little spontaneous inactivation of BAA in the absence of calcium was observed at room temperature, but a significant irreversible thermal inactivation was observed above 45 °C. The optimum temperature of BAA is approx. 60 °C in the presence of calcium and starch, and the inactivation kinetics of BAA have been studied at temperatures above 60 °C [21,22]. However, in the absence of any added bivalent ion and substrates, the thermal inactivation of BAA occurred even below 60 °C. The irreversible

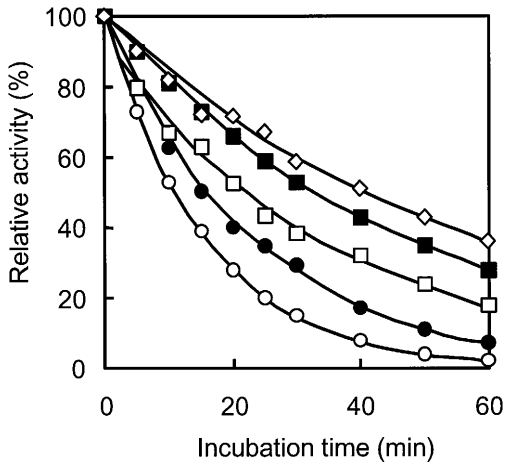


Figure 1 Kinetics of the thermal inactivation of BAA in the presence of calcium ion at 50 °C and pH 7.0

Calcium ion concentrations were: ○, 0.002 mM; ●, 0.005 mM; □, 0.01 mM; ■, 0.015 mM; ◇, 0.02 mM. Protein concentration was 1.31×10^{-8} M in 25 mM Tris/HCl buffer. The remaining activity was the initial hydrolytic velocity against soluble amylopectin as a substrate. The reducing sugar was measured by dinitrosalicylic acid method.

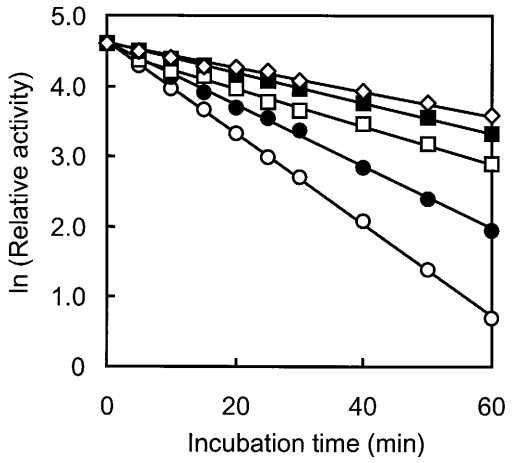


Figure 2 Logarithmic plot of the remaining activity after the incubation against incubation time

Calcium ion concentrations were: ○, 0.002 mM; ●, 0.005 mM; □, 0.01 mM; ■, 0.015 mM; ◇, 0.02 mM. Protein concentration, buffer and temperature are the same as in the legend to Figure 1.

thermal inactivation of substrate-free BAA and the conformational changes in the secondary structure were reported to occur even below 60 °C [12]. The inactivation rate is significantly reduced by the addition of calcium ion, and hence the bivalent ion is effective in stabilizing the enzyme. In all our measurements in the present study, a single linear relationship for the logarithmic plot was obtained (Figure 2), suggesting that the inactivation kinetics were fitted in single exponential curves. In a previous study [23], the thermal inactivation profile of *B. licheniformis* α-amylase demonstrated that the logarithmic plot was distinctly biphasic, and it was impossible to describe the denaturation process in terms of a single exponential decay. However, in the case of BAA, our observation fitted well with the single exponential curve, and did not justify consideration of a more complex inactivation scheme.

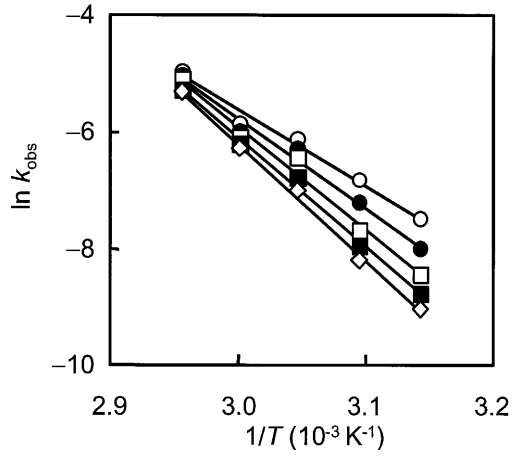


Figure 3 Arrhenius plots for the thermal inactivation of BAA in the presence of calcium ion at pH 7.0

Calcium ion concentrations were: ○, 0.002 mM; ●, 0.005 mM; □, 0.01 mM; ■, 0.015 mM; ◇, 0.02 mM in 25 mM Tris/acetate buffer (pH 7.0) at 45–65 °C. Protein concentration is the same as in the legend to Figure 1.

Table 1 Rate constants and activation parameters for the thermal inactivation of BAA at 50 °C

Measurements were carried out in 25 mM Tris/acetate buffer, pH 7.0, in the presence of calcium ions.

$[Ca^{2+}] \times 10^5$ (M)	$k_{obs} \times 10^4$ (s^{-1})	$\Delta G^{\ddagger}$ (kJ/mol)	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ ($J \cdot mol^{-1} \cdot K^{-1}$)
0.2	10.8 ± 0.1	97.7 ± 0.1	105 ± 8	22.1 ± 1.2
0.5	7.32 ± 0.12	98.7 ± 0.1	126 ± 10	83.8 ± 1.4
1.0	4.58 ± 0.14	100 ± 0	146 ± 11	145 ± 2
1.5	3.53 ± 0.01	101 ± 0	154 ± 7	166 ± 1
2.0	2.75 ± 0.08	101 ± 0	165 ± 6	199 ± 1

Thermodynamic and activation parameters provide a detailed mechanism for many kinds of chemical and biological reactions. It is evident that the inactivation rate is retarded by the calcium ion. In order to understand the process involving the binding of the calcium ion to the enzyme protein, we examined the effect of temperature on the inactivation process. The Arrhenius plot shows a linear relationship in temperature range 45–65 °C (Figure 3). According to the transition state theory, the rates of inactivation observed in the temperature range yield the activation enthalpy and entropy. The increased slope and the Y intercept suggest an increased activation energy and pre-exponential factor due to the addition of the bivalent ion. Table 1 lists the observed inactivation rate, obtained activation enthalpy, entropy and Gibbs free energy. The Gibbs free energies at various calcium ion concentrations were determined to be approx. 100 kJ/mol. The positive apparent activation entropy increased when the calcium ion concentration increased and was compensated by a higher activation enthalpy. There are several possible mechanisms of inactivation, including chemical modification and protein unfolding. The parameters for the inactivation yield insights into the nature of the inactivation process. The activation enthalpy $\Delta H^{\ddagger}$, approx. 100 kJ/mol, is in agreement with previously measured activation energies for the unfolding of soluble proteins [24,25]. Activation Gibbs free energies $\Delta G^{\ddagger}$, which are measures of the spontaneity of the inactivation processes, are lower than the $\Delta H^{\ddagger}$ values. This is due to the positive entropic contribution during the inactivation process. The activation entropy represents the difference in the extent of local disordering between the transition state and the ground state for the inactivation pathway. Thus the positive $\Delta S^{\ddagger}$ is in agreement with increasing local disorder in the transition state when compared with the ground state [26,27]. The increasing $\Delta S^{\ddagger}$ value as a function of calcium concentration is considered to be due to the degree of disorder in the structure needed to reach the transition state for the unfolding because the structural packing in the ground state was firmly stabilized by the bivalent ion.

To test the validity of eqn (3), we measured the apparent inactivation rate as a function of calcium ion concentration. Figure 4 shows the plot of reciprocal apparent inactivation rate constants k_{obs} versus calcium ion concentrations according to eqn (3). The linear relationships of the plots in the Figure correspond to Scheme 1 and to the approximation presented in the Materials and methods section. Many α-amylases have been known to contain one firmly bound calcium ion per enzyme molecule. The calcium ion stabilizes the secondary and tertiary structures of the enzyme upon heating. Therefore it is presumed that the simple inactivation process represented in Scheme 1 is

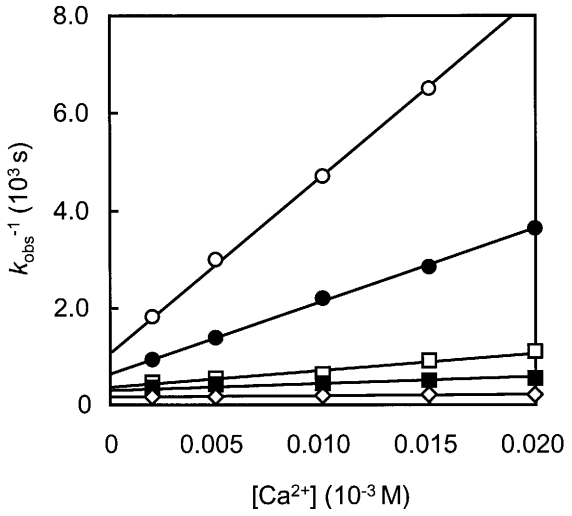


Figure 4 Plots of reciprocal apparent rate constants for the thermal inactivation of BAA against calcium ion concentrations

Solid lines are calculated by a least-squares fit to eqn (3). BAA in 25 mM Tris/acetate buffer (pH 7.0) was incubated at various temperatures in the presence of calcium ion; ○, at 45 °C; ●, at 50 °C; □, at 55 °C; ■, at 60 °C; ◇, at 65 °C.

appropriate in explaining the one calcium ion bound to the protein. However, in barley α-amylase and PPA, several structural investigations seemed to indicate the presence of more than one calcium-ion-binding site [16,28]. Moreover, in TAA, a detailed description of the stereochemistry by X-ray diffraction analysis indicated a secondary calcium binding site [29]. In BAA, there seemed to be uncertainty regarding the exact number of calcium ions in the protein, in spite of the similarity in its primary and secondary structures to PPA and TAA. Previous studies [29] have shown that the secondary binding site displays the inhibitory effect of calcium on the catalytic activity observed at higher concentrations. We tested the change in the catalytic activity of BAA on soluble amylose (DP18) as a function of calcium ion concentration (results not shown). The calcium concentration required for the decrease in catalytic rate was at least one order of magnitude higher than that for the decrease in the inactivation rate. Thus the influence of secondary binding on the inactivation process is considered to be negligible in this study. In this sense, the validity of the thermal inactivation process represented in the scheme was confirmed.

In Figure 4, the straight lines with correlation coefficients greater than 0.96 are completely consistent with the assumption of the model and the approximation expressed by eqn (3). All the plots were linear, with high correlation coefficients for the given range of $CaCl_2$ concentration and temperature. In this case, the actual calcium binding is considered to include the folding of enzyme protein, because the calcium-depleted apoenzyme is known to be inactive and easily unfolded. Thus the obtained value includes the process of calcium binding itself and structural folding of the protein. Bush et al. [18] explained the irreversible inactivation of barley α-amylase by a simple two-step mechanism: calcium ion depletion followed by an irreversible conformational change. This mechanism indicated that the calcium depletion accompanied by a conformational change led to the irreversible inactivation.

The estimated binding parameters K_b according to eqn (3) at the specific temperatures are listed in Table 2. The thermo-

Table 2 Parameters from least-squares fits and K_b values according to eqn (3)

Temperature (°C)	Slope $\times 10^{-6}$ ($s \cdot M^{-1}$)	Intercept $\times 10^{-2}$ (s)	Correlation coefficient	$K_b \times 10^{-3}$ (M^{-1})
45	368 ± 6	10.7 ± 0.8	0.991	345
50	150 ± 3	6.33 ± 0.41	0.998	236
55	36.2 ± 4.0	3.43 ± 0.58	0.965	106
60	9.94 ± 0.89	3.40 ± 0.13	0.976	29.2
65	2.23 ± 0.11	1.48 ± 0.10	0.988	15.1

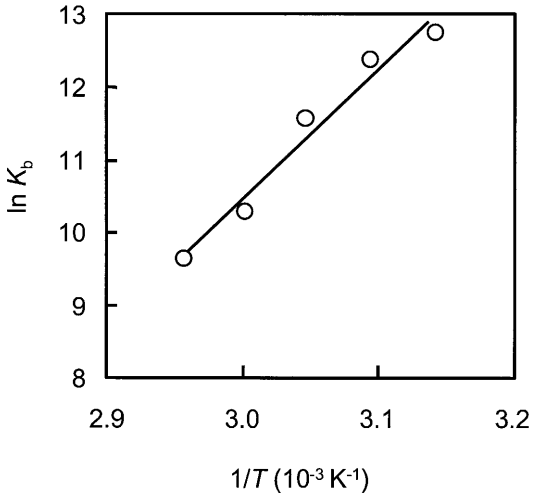


Figure 5 Temperature dependence of calcium ion-binding constants (K_b) of BAA

The binding constants were calculated from the parameters for thermal inactivation kinetics according to eqn (3) at each temperature.

dynamic parameters were calculated with K_b values at the required temperatures (45–65 °C) given by eqn (3) (Figure 5), and the standard enthalpy and entropy changes $\Delta H°$ and $\Delta S°$ were estimated to be -149 ± 16 kJ/mol and -360 ± 2 J · mol^{-1} · K^{-1} respectively using the following equation:

$$K_b = \exp(-\Delta H°/RT + \Delta S°/R) \quad (4)$$

The large negative value of $\Delta H°$ (−149 kJ/mol) suggests strong enthalpic affinities of the bivalent ion to the enzyme protein for thermostability. The thermodynamic changes in the binding of the calcium ion to globular proteins, such as equine lysozyme, α-lactalbumin, human and hen lysozyme, T4 lysozyme and ribonuclease A, have been estimated by some workers and they demonstrated that the bindings are primarily due to enthalpic contributions [30–33]. Our estimated parameters for the calcium binding may consist both of contributions of calcium binding itself and of protein foldings. Kuroki et al. [13] estimated the calcium-binding contribution of some proteins by removing the contribution of conformational changes. The enthalpic ($\Delta H°$) and entropic ($T\Delta S°$) contributions of the calcium binding itself, to $\Delta G°$ at 30 °C, were estimated to be 8 kJ/mol (2 kcal/mol) and 42 kJ/mol (10 kcal/mol) respectively. Both parameters for the binding contribution showed positive values. Hence, the negative

change in both enthalpy and entropy, seen in this study, is characteristic of values seen in the thermodynamic change on the conformational change. Previous studies [13,34] have shown that enthalpy–entropy compensation is often observed in some proteins due to the linkage system involving either a conformational change or change of state. Kuroki et al. demonstrated that the free energy changes due to the binding of the calcium ion to some EF-hand proteins were nearly equal to 0 after subtracting the values for calcium binding itself (8 kJ/mol from $\Delta H°$ and 42 kJ/mol from $T\Delta S°$) [13]. Our estimated values after subtracting these values at 30 °C were −157 kJ/mol for $\Delta H°$ (−149−8 kJ/mol) and −151 kJ/mol for $T\Delta S°$ (−109−42 kJ/mol). Therefore, the enthalpy–entropy compensation was obtained within experimental error and was found to match the results of a previous report [13]. Agarwal and Henkin estimated the calcium-binding constants of human salivary α-amylase and PPA and the binding constants of these amylases were approx. 10^7 M^{-1} at 22 °C [35]. These values agree with our estimated value for BAA, which is extrapolated to 22 °C ($K_b = 3.4 \times 10^7$ M^{-1}). A particular strongly bound calcium ion identified in amylase induces the packing of residues and varies the degree of protein distortion. The calcium-binding site of BAA is considered to be the same as those of PPA and TAA, because these amylases have conserved polypeptide chains in the binding sites.

The following conclusions can be drawn from this study. First, the results are well described by the model represented in Scheme 1. Secondly, using a reasonable approximation of the rate equation, the binding constant of the calcium ion to BAA can be estimated. Thirdly, the values of the binding constant and thermodynamic parameters for the binding are close to the calcium-binding parameters determined by other methods. From the results of the thermal inactivation kinetics of BAA as a function of calcium ion concentration, we determined the binding parameters of the calcium ion to BAA. Our estimated parameters may consist of the contributions of both ion binding and structural folding of the protein. The contribution of ion binding to the thermodynamic change has been determined previously, and the positive entropic change is considered to be due to the release of some water molecules from the calcium ion and proteins on the bindings. Thus the large negative values of enthalpy and entropy change are considered to be due to the folding in the tertiary structural level on the bindings. The simple kinetic model that involves the bivalent ion dissociation and secondary-structural unfolding provided the binding constant by using bivalent ion titration without any dialysis to remove the initially bound calcium ion. The thermodynamical interpretation attempts to present a clear relationship between the terms of apparent inactivation rates and calcium binding.

REFERENCES

1 Robyt, J. F. and Whelan, W. J. (1968) The α-amylases. In Starch and its Derivatives (Radley, J. A., ed.), 4th edn., pp. 430–476, Chapman & Hall, London
2 Takagi, T., Toda, H. and Isemura, T. (1971) Bacterial and mold amylases. In The Enzymes (Boyer, P. D., ed.), pp. 235–271, Academic Press, New York
3 Fogarty, W. M. and Kelly, C. T. (1980) Amylases, amyloglucosidases and related glucanases. In Microbial Enzymes and Bioconversions (Rose, A. H., ed.), pp. 115–170, Academic Press, London
4 Kindle, K. L. (1983) Characteristics and production of thermostable α-amylase. Appl. Biochem. Biotech. **8**, 153–170
5 Kulp, K. (1975) Carbohydrases. In Enzymes in Food Processing (Reed, G., ed.), 2nd edn., pp. 53–122, Academic Press, New York
6 Reichelt, J. R. (1983) Industrial applications. In Industrial Enzymology (Godfrey, T. and Reichelt, J. R., eds.), pp. 375–409, Nature Press, New York
7 Takkinen, K., Pettersson, R. F., Kalkkinen, N., Palva, I., Soderlund, H. and Kaariainen, L. (1983) Amino acid sequence of α-amylase from *Bacillus amyloliquefaciens* deduced from the nucleotide sequence of the cloned gene. J. Biol. Chem. **258**, 1007–1013
8 Yuuki, T., Nomura, T., Tezuka, H., Tsuboi, A., Yamagata, H., Tsukagoshi, N. and Udaka, S. (1985) Complete nucleotide sequence of a gene coding for heat- and pH-stable α-amylase of *Bacillus licheniformis*. J. Biochem (Tokyo) **98**, 1147–1156
9 Levine, B. A. and Williams, R. J. P. (1982) Calcium binding to proteins and other large biological anion centers. In Calcium and Cell Function (Cheung, W.-Y., ed.), pp. 1–38, Academic Press, New York
10 Klee, C. B. and Vanaman, T. C. (1982) Calmodulin. Adv. Protein Chem. **35**, 213–321
11 Vallee, B. L., Stein, E. A., Sumerwell, W. N. and Fischer, E. H. (1959) Metal content of α-amylases of various origins. J. Biol. Chem. **234**, 2901–2905
12 Oh, H. S., Kim, K. H., Suh, S. W. and Choi, M.-U. (1991) Spectroscopic and electrophoretic studies on structural stability of α-amylase from *Bacillus amyloliquefaciens*. Korean Biochem. J. **24**, 158–167
13 Kuroki, R., Nitta, K. and Yutani, K. (1992) Thermodynamic changes in the binding of Ca^{2+} to a mutant human lysozyme. J. Biol. Chem. **267**, 24297–24301
14 Matsuura, Y., Kusunoki, M., Harada, W. and Kakudo, M. (1984) Structure and possible catalytic residues of Taka-amylase A. J. Biochem (Tokyo) **95**, 699–702
15 Buisson, G., Duee, E., Haser, R. and Payan, F. (1987) Three dimensional structure of porcine pancreatic α-amylase at 2.9 Å resolution. EMBO J. **6**, 3909–3916
16 Kadziola, A., Abe, J., Svensson, B. and Haser, R. (1994) Crystal and molecular structure of barley α-amylase. J. Mol. Biol. **239**, 104–121
17 MacGregor, E. A. (1988) α-Amylase structure and activity. J. Protein Chem. **7**, 399–415
18 Bush, D. S., Sticher, L., van Huystee, R., Wagner, D. and Jones, R. L. (1989) The calcium requirement for stability and enzymatic activity of two isoforms of barley aleurone α-amylase. J. Biol. Chem. **264**, 19392–19398
19 Suzuki, A., Yamane, T., Ito, Y., Nishio, T., Fujiwara, H. and Ashida, T. (1990) Crystallization and preliminary crystallographic study of bacterial α-amylases. J. Biochem (Tokyo) **108**, 379–381
20 Bernfeld, P. (1955) Amylases, α and β. Methods Enzymol. **1**, 149–158
21 Tomazic, S. J. and Klibanov, A. M. (1988) Mechanisms of irreversible thermal inactivation of *Bacillus* α-amylases. J. Biol. Chem. **263**, 3086–3091
22 Suzuki, Y., Ito, N., Yuuki, T., Yamagata, H. and Udaka, S. (1989) Amino acid residues stabilizing a *Bacillus* α-amylase against irreversible thermoinactivation. J. Biol. Chem. **264**, 18933–18938
23 Violet, M. and Meunier, J.-C. (1989) Kinetic study of the irreversible thermal denaturation of *Bacillus licheniformis* α-amylase. Biochem. J. **263**, 665–670
24 Segel, I. H. (1975) Thermodynamics of enzyme inactivation. In Enzyme Kinetics, p. 941, John Wiley and Sons, New York
25 Keleti, T. (1985) Stability, heat stability and heat sensitivity of proteins. Acta Biochim. Biophys. Acad. Sci. Hung. **20**, 183–186
26 Chen, X. and Matthews, C. R. (1994) Thermodynamic properties of the transition state for the rate-limiting step in the folding of the α-subunit of tryptophan synthase. Biochemistry **33**, 6356–6362
27 Stein, R. A. and Staros, J. V. (1996) Thermal inactivation of the protein tyrosine kinase of the epidermal growth factor receptor. Biochemistry **35**, 2878–2884
28 Payan, F., Haser, R., Pierrot, M., Frey, M., Astier, J. P., Abadie, B., Duee, E. and Buisson, G. (1980) The three-dimensional structure of α-amylase from porcine pancreas at 5 Å resolution. Acta Crystallogr. B **36**, 416–421
29 Boel, E., Brady, L., Brzozowski, A. M., Derewenda, Z., Dodson, G. G., Jensen, V. J., Petersen, S. B., Swift, H., Thim, L. and Woldike, H. F. (1990) Calcium binding in α-amylases: an X-ray diffraction study at 2.1-Å resolution of two enzymes from *Aspergillus*. Biochemistry **29**, 6244–6249
30 Kuroki, R., Kawakita, S., Nakamura, H. and Yutani, K. (1992) Entropic stabilization of a mutant human lysozyme induced by calcium binding. Proc. Natl. Acad. Sci. U.S.A. **89**, 6803–6807
31 Lumry, R. and Rajender, S. (1970) Enthalpy-entropy compensation phenomena in water solutions of proteins and small molecules. Biopolymers **9**, 1125–1227
32 Elwell, M. L. and Schellman, J. A. (1977) Stability of phage T4 lysozymes. Biochim. Biophys. Acta **494**, 367–383
33 Privalov, P. L. and Khechinashvili, N. N. (1974) Thermodynamic approach to the problem of stabilization of globular protein structure. J. Mol. Biol. **86**, 665–684
34 Lumry, R. and Gregory, R. B. (1989) Dynamical factors in protein–protein association. J. Mol. Liq. **42**, 113–144
35 Agarwal, R. P. and Henkin, R. I. (1987) Metal binding characteristics of human salivary and porcine pancreatic amylase. J. Biol. Chem. **262**, 2568–2575

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