Effect of calcium ions on the irreversible denaturation of a recombinant *Bacillus halmapalus* α -amylase: a calorimetric investigation

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The effect of temperature and calcium ions on the denaturation of a recombinant α -amylase from *Bacillus halmapalus* α -amylase (BHA) has been studied using calorimetry. It was found that thermal inactivation of BHA is irreversible and that calcium ions have a significant effect on stability. Thus an apparent denaturation temperature (T_d) of 83 °C in the presence of excess calcium ions was observed, whereas T_d decreased to 48 °C when calcium was removed. The difference in thermal stability with and without calcium ions has been used to develop an isothermal titration calorimetric (ITC) procedure that allows simultaneous determination of kinetic parameters and enthalpy changes of the denaturation of calcium-depleted BHA. An activation energy E_A of 101 kJ/mol was found for the denaturation of calcium-depleted BHA. The results support a kinetic denaturation mechanism where the calcium-depleted amylase denatures irreversibly at

low temperature and if calcium ions are in excess, the amylase denatures irreversibly at high temperatures. The two denaturation reactions are coupled with the calcium-binding equilibrium between calcium-bound and -depleted amylase. A combination of the kinetic denaturation results and calcium-binding constants, determined by isothermal titration calorimetry, has been used to estimate kinetic stability, expressed in terms of the half-life of BHA as a function of temperature and free-calcium-ion concentration. Thus it is estimated that the apparent E_A can be increased to approx. 123 kJ/mol by increasing the free-calcium concentration.

Key words: activation energy, α -amylase, calcium binding, calorimetry, denaturation, thermodynamics.

INTRODUCTION

α -Amylases (1,4- α -D-glucan glucanohydrolase; EC 3.2.1.1) are enzymes that catalyse the hydrolysis of the internal α -1,4-glucosidic bond in starch. The enzymes have several important industrial applications, particularly in food and detergent industry [1–3]. Successful industrial use of amylases requires that they are sufficiently stable and active at application conditions, e.g. at high temperatures in starch-liquefaction processes. Besides the parameters, temperature and pH, the influence of bivalent metal ions is important in this context. Elucidation of the stabilization mechanisms involved may provide valuable tools to predict the stability under relevant conditions.

A characteristic feature of α -amylases is their requirement of calcium ions for activity and structural stability [4–9]. Vallee et al. [5] have shown previously that addition of excess EDTA to *Bacillus subtilis* amylases results in a 40 % decrease in the activity in 2 h at 25 °C. However, complete reactivation after 2 h was achieved on addition of excess calcium ions. A similar decrease in activity at 37 °C required only 40 min and it was not possible to obtain complete reactivation on addition of calcium ions. It has been suggested that irreversible inactivation of α -amylase follows a two-stage process [9–11], where the first process involves a reversible dissociation of bound calcium ions from the amylase. This is followed by a second process, where the amylase undergoes an irreversible denaturation.

All known α -amylases contain a structurally conserved calcium-binding site [12,13], but one or more additional calcium-binding sites have been identified depending on the origin of α -amylase. The X-ray structures of the α -amylases from the *Bacillus* species *licheniformis* and *subtilis* have revealed three calcium ions

[14,15]. The objective of the present study is to investigate the interrelationships between calcium binding and the irreversible inactivation of a recombinant *Bacillus halmapalus* α -amylase (BHA). In previous investigations on the effect of calcium ions on the inactivation of amylase [6,9–11], the preferred method has been hydrolytic activity measurements. In the present study, we have chosen a different approach, based on calorimetric methods. More specifically, we utilize the thermodynamic ‘fingerprint’, i.e. the characteristic time course of heat flow from a certain reaction to resolve the rate of this reaction in a multicomponent system. This requires the ‘fingerprint’ of each process be identified and that they are different significantly. This turned out to be the case for the ligand binding and protein denaturation studied here.

In the present study, we utilize a novel calorimetric methodology to determine both thermodynamic and kinetic parameters associated with irreversible denaturation of calcium-depleted α -amylase. The methodology utilizes both scanning and titration calorimetry, and is based on the difference in thermostability of the α -amylase with and without bound calcium ions. Hence, if an experiment is performed at a temperature where the protein is mainly in the folded state when metal ions are bound, removal of these ions will destabilize the protein. The calorimetric response will therefore include heat changes associated with the metal ion removal process and the subsequent denaturation process. This allows one to determine both the rate and enthalpy of denaturation at a specific temperature in a single experimental trial.

In the present study, we have used a combination of results from denaturation kinetics, calcium binding and thermal stability to elucidate the denaturation mechanism of a recombinant α -amylase and to show how calcium ions are involved.

Abbreviations used: BAA, *Bacillus amyloliquefaciens* α -amylase; BHA, *Bacillus halmapalus* α -amylase; BLA, *Bacillus licheniformis* α -amylase; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry.

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MATERIALS AND METHODS

Enzyme and chemicals

Recombinant BHA was expressed in *Bacillus licheniformis* α -amylase (BLA) and purified to > 95 %, determined by SDS/PAGE (Novozymes A/S, Bagsvaerd, Denmark). The protein was dialysed extensively, at 5 °C, against one of the following buffers: 20 mM Hepes, 40 μ M CaSO₄ (pH 8.0) or 20 mM Hepes, 60 μ M EGTA (pH 8.0). The following chemicals were used: Hepes (> 99.5 %; Sigma), EDTA (> 99 %; Merck, Darmstadt, Germany), EGTA (> 97 %; Sigma) and calcium sulphate (> 99 %; Fluka, Buchs, Switzerland).

Thermostability

Differential scanning calorimetry (DSC) experiments were performed with a MicroCal VP-DSC (Northampton, MA, U.S.A.) [16]. All enzyme solutions were dialysed against the desired buffer, and the dialysate was used as reference. Before scanning, all solutions were degassed by stirring under vacuum. To determine if the denaturation of BHA was reversible, a second scan was performed after cooling the samples. Furthermore, scans were performed at scan rates from 10 to 90 °C · h⁻¹ to determine if the BHA denaturation process is scan-rate-dependent. In all experiments, a BHA concentration of 20 μ M was used and buffer scans were subtracted from BHA scans. The DSC data were analysed using the Origin software from MicroCal supplied with the calorimeter. Molar excess heat capacities (C_p) were obtained by normalizing with the BHA concentration and the volume of the calorimeter cell. Apparent denaturation temperatures (T_d) were determined as the temperature corresponding to maximum C_p . The denaturation enthalpy was determined by integration after subtraction of a cubic baseline. However, it was not possible to determine the denaturation enthalpy in the presence of calcium ions due to exothermic aggregation effects.

Chelator-induced denaturation

Consider a protein P with a denaturation temperature of X °C in the presence of a ligand L. When L is removed, the denaturation temperature decreases to Y °C (i.e. $Y < X$). L can be removed by addition in excess of a molecule B, with high affinity to L [eqn (1)]. If L is removed from the protein at a temperature between X and Y , the protein will begin to denature [eqn (2)]. The following reactions take place after the addition of B:

where D is the denatured state of the protein and k is a first-order rate constant. Both processes (1) and (2) will generate or absorb heat. Therefore the processes can be monitored as the heat flow ($\Delta q / \Delta t$) from a solution of PL after the injection of B. Initially, the heat flow will be dominated by the heat released after the displacement of B with P [eqn (1)]. Subsequently, the protein will begin to unfold [eqn (2)] and hence generate an endothermic heat flow.

The output of isothermal titration calorimetry (ITC) is

$$\frac{\Delta q}{\Delta t}(t) \quad (3)$$

and the heat flow generated during a first-order irreversible denaturation process is given by

$$\frac{\Delta q}{\Delta t} = k Q x(t) \quad (4)$$

where Q is the total heat adsorbed during the denaturation process and $x(t)$ is the time-dependent fraction of folded protein in the solution:

$$x(t) = \frac{[P(t)]}{[P]_0} \quad (5)$$

Here $[P]_0$ is the initial concentration of the folded protein.

According to first-order rate kinetics,

$$[P(t)] = [P]_0 e^{-kt} \quad (6)$$

Q is related to the enthalpy of denaturation (ΔH_{den}) by the following equation:

$$Q = \Delta H_{\text{den}} [P]_0 V_{\text{cell}} \quad (7)$$

where V_{cell} is the volume of the calorimeter cell.

The following equation for the heat flow can be obtained by substitution of eqns (5–7) into eqn (4):

$$\frac{\Delta q}{\Delta t} = k \Delta H_{\text{den}} V_{\text{cell}} [P]_0 e^{-kt} \quad (8)$$

ΔH_{den} and k can then be determined by non-linear least-squares regression of the ITC data using eqn (8). ΔH_{den} can also be determined, independent of any model, by integrating the thermogram from t_0 , and thus provides an easy way to verify the value obtained from regression analysis. Other authors have derived equations similar to eqn (8) and discussed the applicability of isothermal heat conduction microcalorimetry to studies of slow reactions such as drug degradation [17].

Before a regression analysis is performed, it is necessary to account for the part of the thermogram corresponding to the initial removal of ligand [eqn (1)] and define zero time (t_0) for the denaturation process. For the current systems, reaction rates are sufficiently different for t_0 to be determined by the linear extrapolations illustrated in the inset of Figure 2. The shift from the binding process [eqn (1)] to the denaturation process [eqn (2)] is associated with a rapid change in heat flow. Owing to the response time of the calorimeter, it is therefore necessary to ignore the initial heat flow of the denaturation process. The time required to reach a consistent heat flow is defined as the lag time (t_{lag}) and is disregarded in the regression analysis (see inset of Figure 2).

All isothermal denaturation experiments were conducted on an MCS-ITC equipment (MicroCal) [18]. Before titration, all solutions were degassed by stirring under vacuum. In a typical experiment, the sample cell was loaded with a solution of 20 μ M BHA with 40 μ M CaSO₄. One aliquot of 50 μ l EDTA solution was added (giving a 10:1 molar ratio of EDTA/BHA in the calorimeter cell) and the heat flow was recorded. The dilution heat was determined by making a second injection of the same volume after the protein had been denatured completely. Analysis of the thermograms was done using the Origin 7.0 software package (OriginLab Corporation, Northampton, MA, U.S.A.). All the reported uncertainties represent the standard deviations of regression analysis for three experiments.

Figure 1 DSC thermograms of 20 μ M BHA denaturation in 20 mM Hepes (pH 8.0) in the absence and presence of calcium ions

Calorimetric trace obtained in (A) 8 times excess EDTA and (B) 2 times excess CaSO_4 . The exothermic signal following denaturation in the presence of calcium ions reflects precipitation in the calorimeter cell. The T_d values are listed above the curves.

Calcium binding

Calcium-binding parameters of BHA were determined using ITC. The reference cell was filled with water. In a typical experiment, the sample cell was loaded with a solution containing a mixture of 60 μ M EGTA and 30 μ M BHA. The cell solution was titrated with 40 aliquots of 5 μ l of 1.25 mM CaSO_4 solution. All solutions were degassed by stirring under vacuum before the experiments. The resulting binding isotherms showed a biphasic behaviour reflecting the binding of Ca^{2+} to EGTA and when the chelator had been saturated to BHA. Binding isotherms were analysed using a multicomponent-binding model as described previously [19]. A non-linear least-squares minimization routine was used to obtain the maximum likelihood values of binding constants K , enthalpies ΔH and stoichiometry n . Changes in the standard free energy ΔG° and entropy ΔS° were determined as $\Delta G^\circ = -RT \cdot \ln \cdot K$ and $T\Delta S^\circ = \Delta H - \Delta G^\circ$ (under the assumption that $\Delta H = \Delta H^\circ$). All the reported uncertainties represent three standard deviations of the regression analysis.

RESULTS AND DISCUSSION

Denaturation of BHA and calcium-depleted BHA was determined by DSC as illustrated in Figure 1. In the presence of 2-fold excess calcium ions (CaSO_4/BHA in the ratio 2 : 1), T_d was found to be 83 °C. It was difficult to determine the precise value due to irreversible aggregation after denaturation, and the reported value should therefore be regarded as a minimum value. T_d decreases to 48 °C when EDTA is in excess (EDTA/BHA in the ratio 8 : 1), and the denaturation was also found to be irreversible. The T_d values are scan-rate-dependent due to the irreversibility of the processes. Therefore all scans were recorded at low scan rate ($10^\circ\text{C} \cdot \text{h}^{-1}$) to obtain T_d values approaching isothermal conditions. Increasing the scan rate to $90^\circ\text{C} \cdot \text{h}^{-1}$ resulted in approx. 10°C higher T_d values for both processes. The DSC results obtained, support a kinetic inactivation mechanism similar to the one proposed recently for *Bacillus amyloliquefaciens* α -amylase (BAA) [11].

In Scheme 1, P and D represent the native and denatured conformations of the amylase respectively. The asterisk denotes calcium-depleted amylase. K_{Ca} is the calcium-binding constant,

Scheme 1 Proposed mechanism for inactivation of BHA with and without calcium ions

Figure 2 Denaturation of BHA on calcium depletion followed by ITC in 20 mM Hepes (pH 8.0) at 60 °C

The thermogram was obtained by injection of 50 μ l of 5.2 mM EDTA into a 20 μ M BHA and 40 μ M CaSO_4 solution. The initial exothermic part of the thermogram corresponds to displacement of calcium ions from BHA to EDTA. This is followed by a large endothermic heat flow owing to denaturation of calcium-depleted BHA. The inset shows the initial part of the denaturation process. t_0 defines the time at which the denaturation process initiates and it can be determined graphically by drawing a straight line through the data points obtained before the denaturation process and a second line through the points obtained during the beginning of the denaturation process as indicated. t_{lag} defines the time required to reach a consistent heat flow after the initiation of the denaturation process.

k_{den} and k_{den}^* represent the rate constants for denaturation of calcium-bound and calcium-depleted amylase respectively. It is not known if D and D^* are different. However, it is irrelevant to the analysis of the results of the present study.

The difference in T_d with and without calcium ions has been used to study the kinetics of denaturation on calcium depletion. Injection of EDTA, the calcium chelator, into a solution of BHA/ CaCl_2 at 60 °C results in characteristic thermograms that allow a clear separation of the initial binding reaction (binding and displacement of calcium ions to EDTA, see eqn 1) and the subsequent denaturation of the protein (Figure 2). First, the binding reaction generates an exothermic heat flow that is observed as a sharp peak. It has been observed previously that the displacement reaction is in fact exothermic [19]. Secondly, a positive heat flow is generated due to the endothermic denaturation of BHA. Thermograms obtained at temperatures from 50 to 70 °C

Figure 3 Denaturation thermograms at 40–70 °C

At each temperature, three thermograms are presented, but these are so similar as to appear as a single line in each panel. At 40 °C, no significant denaturation appears to take place since only an exothermic heat flow is observed. The thermograms were obtained as described in Figure 2.

share the same characteristics (see Figure 3). However, when the temperature is increased, the heat flow at the beginning of the denaturation is increased and the heat flow approaches the baseline faster. If a similar experiment is performed at 40 °C only the initial binding reaction is observed. This is in accordance with the observation (Figure 1) that the temperature is well below the T_d of calcium-depleted BHA. Spectroscopic studies on BAA have indicated that the protein undergoes a conformational change after the removal of bound calcium ions [8]. Unfortunately, the experimental temperature of the work was not given by the authors.

As a first step in the thermogram analysis, t_0 and t_{lag} have been determined as described in the Materials and methods section (see inset in Figure 2). t_0 remained close to 25 s at all temperatures, whereas t_{lag} varied from 280 s at 50 °C to 40 s at the highest temperatures. Secondly, non-linear least-squares regression, using eqn (8), was used to obtain the maximum likelihood values for the parameters k and ΔH_{den} . The k and ΔH_{den} values obtained at 50–70 °C are listed in Table 1. The correlation coefficient for the regression analysis is higher than 0.998 in all experiments and the ability of eqn (8) to account for the results is illustrated in Figure 4. Furthermore, the method proved to be highly reproducible and the mean standard deviation is 3 %. The dilution heat was found to be approx. 5 % of the denaturation enthalpy. Plotting $\ln(\Delta q/\Delta t)$ versus t provides a simple test to determine if the observed process is of first-order [17], since a linear correlation is expected only

Table 1 Rate constants and unfolding enthalpies for thermal inactivation of calcium-depleted BHA in 20 mM Hepes buffer (pH 8.0)

Temperature (°C)	ΔH_{den} (kJ/mol)*	ΔH_{den} (kJ/mol)†	$k \times 10^4$ (s ⁻¹)†	$t_{1/2}$ (min)
50	1066 ± 55	1139 ± 43	9.23 ± 0.59	12.5
55	1476 ± 31	1474 ± 26	15.0 ± 0.2	7.7
60	1683 ± 63	1692 ± 20	27.4 ± 0.3	4.2
65	1761 ± 43	1747 ± 50	47.2 ± 1.6	2.4
70	1797 ± 76	1768 ± 67	80.6 ± 1.7	1.4

* Obtained by integration.

† Obtained by regression analysis using eqn (8).

Figure 4 Analysis of the denaturation thermogram at 60 °C

The data points correspond to the heat flow (see Figure 2). Non-linear least-squares regression based on eqn (8) was used to determine the denaturation rate and enthalpy.

for first-order processes. These types of plots (results not shown) were indeed linear. This is in agreement with recent studies on BAA, which indicated that inactivation is a one-step irreversible process [11]. However, it should be noted that a previous study on BLA concluded that the inactivation proceeds as a two-step process [6].

The ΔH_{den} values obtained by regression analysis are in good agreement with the values determined by integration (see Figure 5). ΔH_{den} increases with temperature from approx. 1100 kJ/mol at 50 °C to almost 1800 kJ/mol at 70 °C. The correlation is not linear and the enthalpy seems to become practically temperature-independent above 65 °C. The enthalpy changes measured by ITC are further supported by the value (1310 kJ/mol at 48 °C) determined by DSC.

An Arrhenius plot was used to calculate the activation energy E_A of denaturation of calcium-depleted BHA (see Figure 6) and found to be 101 kJ/mol. This value is comparable with a previously reported E_A value of 113 kJ/mol for the inactivation of BLA in the presence of excess EDTA [10]. However, Violet and Meunier [6] found an E_A value of 53.1 kJ/mol for the inactivation of BLA without the added calcium ions. The temperature-dependent rate constant can, according to transition-state theory, be used to calculate $\Delta H^\ddagger$ and $\Delta S^\ddagger$ of a thermoinactivation process [20]

$$k = \frac{k_B T}{h} \exp\left(\frac{-\Delta H^\ddagger}{RT}\right) \exp\left(\frac{\Delta S^\ddagger}{R}\right) \quad (9)$$

where k_B is the Boltzmann constant and h the Planck constant. $\Delta H^\ddagger$ and $\Delta S^\ddagger$ were determined using the above equation to be

Figure 5 Temperature dependence of denaturation enthalpies

Correlation of denaturation enthalpies (Table 1) obtained by integration (○) and by fitting using eqn (8) (■).

Figure 6 Arrhenius plot of the denaturation rate constants of calcium-depleted BHA in 20 mM Hepes (pH 8.0)

The rate constants are obtained from Table 1.

100 kJ/mol and $6.9 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ respectively. Previously, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values of 105 kJ/mol and $22.1 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ respectively have been reported for the irreversible thermal inactivation of BAA in $2 \mu\text{M}$ CaCl_2 at pH 7.0 [11].

The calcium-binding parameters of BHA have been determined by isothermal-calorimetric titration of BHA/EGTA mixtures with CaSO_4 in the temperature range $24\text{--}40^\circ\text{C}$. The latter temperature was chosen since the DSC and kinetic results indicate that no significant denaturation takes place below 40°C . Table 2 summarizes the binding parameters obtained. The binding affinity is of the order of 10^5 M^{-1} in the temperature range studied and decreases approximately linearly with temperature. An apparent stoichiometry of 1.4 is observed. Crystal structure of the structurally related BLA has revealed three calcium-binding sites [14]. Under the assumption that BHA also contains three binding sites, it is not unexpected to observe a stoichiometry higher than 1. The observed stoichiometry provides information about the ratio between the calcium affinity of BHA and EGTA and suggests that BHA has one binding site with lower affinity when compared with EGTA, one site with an affinity comparable with EGTA and probably one site with an affinity higher than EGTA [19]. EGTA binds calcium with an affinity of $10^7\text{--}10^8 \text{ M}^{-1}$ under the current experimental conditions (see Table 2). Hence, the observed stoichiometry indicates that BHA has one calcium site with an affinity of approx. 10^5 M^{-1} and one site with an affinity of approx. $10^7\text{--}10^8 \text{ M}^{-1}$.

Both the binding enthalpy ($\Delta H = -11.1 \text{ kJ/mol}$ at 27°C) and the changes in standard entropy ($T\Delta S^\circ = 22.4 \text{ kJ/mol}$ at 27°C) contributed favourably to the binding. $T\Delta S^\circ$ accounts for approx. two-thirds of the change in standard free energy ($\Delta G^\circ = -33.5 \text{ kJ/mol}$). The large positive ΔS value suggests that a dehydration process is involved in the binding of calcium to BHA. One such contribution may arise from the release of six co-ordinated water molecules around the calcium ion [21] on binding to BHA. Furthermore, release of water molecules from the calcium-binding site may contribute to the increase in entropy on calcium binding. Another possible source of entropy increase is dehydration of non-polar surfaces on calcium binding either at the binding site or as a result of coupled conformational changes in the protein. Changes in C_p values can be used to estimate the significance of hydrophobic effects and it has been reported that dehydration of non-polar surfaces results in negative change in C_p , which scales with the dehydrated surface area [22,23]. Apparently, ΔH increases slightly with temperature corresponding to a small positive change in heat capacity of approx. $30 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. Therefore it seems unlikely that dehydration of non-polar surfaces contributes significantly in the calcium-binding process.

Tanaka and Hoshino [11] have estimated the thermodynamic calcium-binding parameters of BAA by inactivation kinetics at $45\text{--}65^\circ\text{C}$. The estimated van't Hoff ΔH° and ΔS° values were

Table 2 Calcium-binding parameters of BHA and EGTA in 20 mM Hepes buffer (pH 8.0)

Temperature ($^\circ\text{C}$)	BHA–Ca binding parameters				EGTA–Ca binding parameters			
	ΔH (kJ/mol)	K_{Ca} (M^{-1})	ΔG° (kJ/mol)	$-T\Delta S^\circ$ (kJ/mol)	ΔS° ($\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$)	Apparent stoichiometry	ΔH (kJ/mol)	K (M^{-1})
19*	-11.5 ± 0.1	$(7.6 \pm 0.6) \times 10^5$	-32.9 ± 0.2	-21.4 ± 0.3	73.3 ± 1.0	1.4 ± 0.1		
24	-11.3 ± 0.3	$(7.3 \pm 0.2) \times 10^5$	-33.4 ± 0.1	-22.1 ± 0.4	74.4 ± 1.3	1.3 ± 0.1	-29.9 ± 0.6	$(1.2 \pm 0.1) \times 10^8$
27	-11.1 ± 0.2	$(6.8 \pm 0.8) \times 10^5$	-33.5 ± 0.3	-22.4 ± 0.5	74.6 ± 1.7	1.4 ± 0.1	-30.0 ± 0.4	$(8.4 \pm 2.0) \times 10^7$
33	-11.0 ± 0.1	$(5.7 \pm 0.4) \times 10^5$	-33.7 ± 0.2	-22.7 ± 0.3	74.1 ± 1.0	1.4 ± 0.1	-30.9 ± 0.3	$(7.0 \pm 0.3) \times 10^7$
40	-10.8 ± 0.3	$(5.1 \pm 0.3) \times 10^5$	-34.2 ± 0.1	-23.4 ± 0.4	74.7 ± 1.3	1.3 ± 0.1	-31.8 ± 0.6	$(5.3 \pm 2.0) \times 10^7$

* Values at 19°C from [19].

Figure 7 van't Hoff plot of the obtained BHA-calcium-binding constants

The binding constants are obtained from Table 2.

−149 kJ/mol and −360 J · mol^{−1} · K^{−1} respectively. However, the authors note that their values may include contributions from both calcium binding and structural changes in the protein. In contrast, our reported ΔH values are not expected to contain significant contributions from structural changes in the protein, since both the DSC and kinetic results indicate no significant structural changes in both calcium-bound and calcium-depleted amylase below 40 °C where all binding experiments were performed. When the obtained K_{Ca} values in Table 2 are introduced into a van't Hoff plot, ΔH° and ΔS° values of -16 ± 2 kJ/mol and 60 ± 5 J · mol^{−1} · K^{−1} respectively are obtained (see Figure 7). Thus the van't Hoff values are in good agreement with the calorimetric values listed in Table 2. Furthermore, our binding enthalpy values compare well with a reported ΔH value of −16 kJ/mol (at 27 °C) for BAA calcium binding, which has been determined using microcalorimetry [24].

A combination of kinetic denaturation results and calcium-binding constants can be used to predict the combined effect of free calcium and temperature on the apparent inactivation rate constant k_{app} for BHA. According to Scheme 1, k_{app} can be determined using the following equation, which is based on pre-equilibrium approximations [11]:

$$k_{app} = \frac{k_{den}^* + K_{Ca}k_{den}[Ca^{2+}]}{K_{Ca}[Ca^{2+}] + 1} \quad (10)$$

Calculation of k_{app} thus requires knowledge of both inactivation rate constants of calcium-bound and calcium-depleted BHA and also the calcium-binding constant. At low temperature, the contribution from denaturation of calcium-bound BHA is insignificant, since inactivation proceeds almost exclusively as denaturation of calcium-depleted BHA. Based on the DSC thermograms (Figure 1), it is expected that the denaturation of calcium-bound BHA is negligible below 65 °C. Hence, eqn (10) reduces to

$$k_{app} = \frac{k_{den}^*}{K_{Ca}[Ca^{2+}] + 1} \quad (11)$$

where only knowledge of the inactivation constant of calcium-depleted BHA and the calcium-binding constant is required. In the absence of free calcium ions ($[Ca^{2+}] = 0$), eqn (11) further reduces to $k_{app} = k_{den}^*$.

Table 3 Kinetic unfolding and calcium-binding parameters of BHA at 20–60 °C in 20 mM Hepes buffer (pH 8.0)

Temperature (°C)	$k_{den}^* \times 10^5$ (s ^{−1})	$K_{Ca} \times 10^{-5}$ (M ^{−1})	$t_{1/2}$ (min)
20	2.0†	7.6‡	590
30	7.7†	6.3‡	150
40	28†	5.0‡	42
50	92§	3.8	13
60	290§	2.5	4.0

† Extrapolated value from Arrhenius plot (Figure 6).

‡ Interpolated value from van't Hoff plot (Figure 7).

§ Interpolated value from Arrhenius plot (Figure 6).

|| Extrapolated value from van't Hoff plot (Figure 7).

Figure 8 Correlation between free-calcium concentration and estimated apparent half-life of BHA in 20 mM Hepes (pH 8.0) versus temperature

Temperatures: 20 (■), 30 (○), 40 (▲), 50 (□) and 60 °C (●).

Using the Arrhenius equation and the E_A value of calcium-depleted BHA, we have estimated k_{den}^* values in the temperatures range 20–60 °C. Extrapolation of the van't Hoff plot (Figure 7) has been used to estimate K_{Ca} values in the same temperature range.

k_{app} values of the calcium-depleted BHA were estimated using eqn (11) and the corresponding half-life ($t_{1/2}$) calculated. The estimated values are listed in Table 3. Similarly, apparent $t_{1/2}$ values at free-calcium concentration up to 100 μM were estimated. Figure 8 illustrates the increase in $t_{1/2}$ on increasing the free-calcium concentration in the temperature range 20–60 °C. At 20 °C, the free-calcium concentration has a major effect on the half-life of BHA. Thus increasing the free-calcium concentration from 0 to 100 μM increases the apparent half-life from approx. 10 to 750 h. At 60 °C, a similar increase in the free-calcium concentration increases $t_{1/2}$ from 4 min to 2 h. To obtain an apparent half-life of 10 h at 60 °C, a free-calcium concentration > 500 μM is required. The estimated k_{app} values have also provided a basis for the construction of Arrhenius plots at different concentrations of free-calcium ions. Hence, it has been possible to estimate apparent E_A values as a function of free-calcium concentration (see Figure 9). At low calcium concentrations even small changes have a notable effect on E_A .

Figure 9 Estimated apparent E_A of BHA denaturation as a function of free-calcium concentration in 20 mM Hepes (pH 8.0)

Only temperatures below 65 °C are included, since only denaturation of calcium-depleted BHA is expected to take place.

For example, increasing the free-calcium concentration from 0 to 2 μ M increases E_A from 101 to 112 kJ/mol. Only minor increases in E_A are observed at higher calcium concentrations, and the E_A appear to approach asymptotically a value of 123 kJ/mol at high calcium concentrations (see Figure 9). The results suggest that it is possible to increase the apparent E_A by almost 22 kJ/mol by increasing the free-calcium concentration. Violet and Meunier [6] have reported previously an E_A value of 155.4 kJ/mol for BLA in the presence of 5 mM CaCl_2 .

In summary, it was found that calcium ions have significant effect on the thermal stability of BHA. Thus removal of calcium ions from a BHA solution leads to an apparent decrease in stability of approx. 35 °C. This calcium-dependent difference in thermal stability has been used to investigate the irreversible denaturation of BHA on calcium depletion. The results support the kinetic denaturation mechanism proposed by Tanaka and Hoshino [11]. In this model, the calcium-depleted enzyme unfolds irreversibly at low temperature. When calcium ions are in excess, the enzyme unfolds irreversibly at high temperatures. Therefore the two denaturation reactions are coupled with the calcium-binding equilibrium. This equilibrium has been quantified in the present study and used, together with the kinetic results, to estimate the half-life of BHA as a function of temperature and free-calcium-ion concentration.

The calorimetric approach developed in the present study appears to be a robust method in the investigation of the effect of temperature and calcium ions on the stability of amylase. Hence, relatively few experimental trials are needed to obtain detailed kinetic and thermodynamic information. It seems probable that the procedures used in the present study could be applicable to other protein systems where calcium ions or other bivalent metal ions have significant effect on the thermal stability. As an example, it has been reported that removal of calcium ions from bovine α -lactalbumin decreases the thermostability by approx. 40 °C [25].

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