Kinetic study of the irreversible thermal denaturation of *Bacillus licheniformis* α -amylase

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The irreversible thermal inactivation of *Bacillus licheniformis* α -amylase was studied. A two-step behaviour in the irreversible denaturation process was found. Our experimental results are consistent only with the two-step model and rule out the two-isoenzyme one. They suggest that the deactivation mechanism involves the existence of a temperature-dependent intermediate form. Therefore the enzyme could exist in a great number of active conformational states. We have shown that Ca^{2+} is necessary for the structural integrity of α -amylase. Indeed, dialysis against chelating agents leads to a reversible enzyme inactivation, though molecular sieving has no effect. Further, the key role of Ca^{2+} in the α -amylase thermostability is reported. The stabilizing effect of Ca^{2+} is reflected by the decrease of the denaturation constants of both the native and the intermediate forms. Below 75 °C, in the presence of 5 mM- CaCl_2 , α -amylase is completely thermostable. Neither other metal ions nor substrate have a positive effect on enzyme thermostability. The effect of temperature on the native enzyme and on one intermediate form was studied. Both forms exhibit the same optimum temperature. Identical activation parameters for the hydrolytic reaction catalysed by these two forms were found.

INTRODUCTION

α -Amylase (1,4- α -D-glucan glucanohydrolase, EC 3.2.1.1) hydrolyses starch by cleaving the internal α -1,4-glucosidic bonds. This enzyme is produced by a wide variety of organisms, but on account of its industrial applications the best-studied α -amylase is from the genus *Bacillus* [1–3]. Since starch is only soluble at temperatures close to 100 °C, one key in its industrial hydrolysis must be the use of highly thermostable enzymes. Therefore, in order to define rational strategies for enhancement of enzyme thermostability, the irreversible thermal denaturation process must be elucidated.

Enzyme thermal inactivation occurs in two steps according to the general model proposed by Lumry & Eyring [4]:

where N is the native catalytically active enzyme, U is the reversibly unfolded catalytically inactive enzyme and I is the irreversibly inactivated enzyme. In the first step there is a partial loss of activity due to the disruption of the non-covalent interactions maintaining the native conformation. This process is reversible, since the enzymic activity is completely recovered when the enzyme is cooled down [5]. Then, upon prolonged heating, another process occurs, leading to an irreversibly inactivated enzyme [6]. The molecular mechanisms of irreversible thermal denaturation of hen's egg-white lysozyme and bovine pancreatic ribonuclease A have been already elucidated [7,8]. Recently, similar studies have been performed on three bacterial α -amylases [9,10]. For *Bacillus licheniformis* α -amylase deamidation of aspara-

gine and glutamine residues emerges as the cause of inactivation [10].

If the kinetics of the reversible thermal denaturation have been extensively studied [5], little is known about the kinetics of the irreversible one. So, in order to understand the kinetic behaviour of this enzyme when exposed to high temperatures, a detailed analysis of the effect of temperature on the activity and stability of *B. licheniformis* α -amylase was undertaken. The effect of Ca^{2+} in the enhancement of enzyme thermostability was also studied.

MATERIALS AND METHODS

Enzyme

α -Amylase from *B. licheniformis* used in this study was purchased from Sigma Chemical Co. (type XII-A). The enzyme was homogeneous on polyacrylamide-gel electrophoresis in the absence and in the presence of SDS.

α -Amylase assay

Enzyme activity was determined colorimetrically with soluble potato starch as substrate. Reducing sugars were measured by the dinitrosalicylic acid method described by Bernfeld [11]. The reaction mixture contained 25 mM-Tris/acetate buffer, pH 8.2, 1 % (w/v) soluble starch and the enzyme solution. Incubations were carried out at different test temperatures (T_i) (20 to 80 °C). Specific activity (R) is expressed in terms of μmol of maltose equivalent produced/s per μmol of enzyme.

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Thermal denaturation kinetics

The time course of irreversible thermal inactivation of α -amylase was measured by incubating the enzyme in 10 mM-Tris/acetate buffer, pH 6.3, at various temperatures (T_0) (55 to 95 °C). The pH of the buffer was adjusted to the desired value at the temperature of thermoinactivation. Samples were removed at intervals, cooled in ice (15 min) and assayed for enzyme activity in the conditions previously described. Cooling in ice allowed the reversibly unfolded form U to fold into the native form N, so that only the irreversible denaturation was studied.

Calculation of rate constants and specific activities

In order to explain the biphasic deactivation patterns, two models can be proposed.

Model 1 implies the existence of two isoenzymes. If there are differences in both specific activities and thermal stabilities, the isoenzymes would give rise to biphasic deactivation processes:

Model 2 suggests the formation of an intermediate (X) on the pathway between the native (N) and the denatured (D) enzyme forms:

The changes in the specific activity (R) of the mixture with respect to time can be described by the following equations:

$$R = R_1 \cdot e^{-k_1 t} + R_2 \cdot e^{-k_2 t} \quad (1)$$

$$R = \left(R_1 - \frac{R_2 \cdot k_1}{k_1 - k_2} \right) e^{-k_1 t} + \left(R_2 \cdot \frac{k_1}{k_1 - k_2} \right) e^{-k_2 t} \quad (2)$$

These two models require four parameters: R_1 and R_2 are the specific activities of the two native isoenzymes (N_1 and N_2) in the case of model 1 or of the native (N) and the intermediate (X) forms in the case of model 2. k_1 and k_2 are the corresponding deactivation rate constants. For both models, the four parameters are obtained by a non-linear fitting of the data to eqns. (1) and (2) with a Victor V286C computer. An estimation of the initial parameters was made in accordance with Tanford [5]. For each model, the criterion used for the best fit is the minimum value of the sum of the residue squares.

RESULTS AND DISCUSSION

Thermal denaturation kinetics

Preliminary experiments were carried out in order to select a suitable initial enzyme concentration. The results obtained after an incubation for 15 min at 80 °C show that the maintenance of enzyme activity is strongly dependent on the initial protein concentration (Fig. 1). Above 2 μ M the inactivation process is accompanied by heavy aggregation of the enzyme. As previously described [6], working with diluted enzyme solutions lowered the rate of non-covalent intermolecular reactions. In all further experiments an enzyme concentration of 0.17 μ M was used.

A first set of runs was performed by deactivating the

Fig. 1. Effect of the initial concentration of *B. licheniformis* α -amylase on its thermostability

α -Amylase solutions (0.1–7.5 μ M) in 10 mM-Tris/acetate buffer, pH 6.3, were incubated at 80 °C. After an incubation of 15 min, specific activity was assayed at 30 °C and pH 8.2. The activity of the non-heated α -amylase was considered to be 100 %.

enzyme at various temperatures (from 55 to 85 °C) and testing the specific activity at one temperature (T_t 30 °C). The results obtained for the deactivation at 80 °C are shown in Fig. 2. The plot of $\ln R$ versus incubation time is distinctly biphasic (Fig. 2 inset), and the denaturation process cannot be described in terms of a single

Fig. 2. Kinetics of the thermal denaturation of *B. licheniformis* α -amylase

α -Amylase (0.17 μ M) in 10 mM-Tris/acetate buffer, pH 6.3, was incubated at 80 °C for various times. Specific activity was assayed at 30 °C and pH 8.2. Inset: semi-logarithmic plot; the curves are theoretical and obtained with the numerical values given in Table 1 (two-step model).

Table 1. Calculated values of the kinetic parameters for both deactivation models for *B. licheniformis* α -amylase

k_1 , k_2 , R_1 and R_2 were obtained by non-linear fitting of the experimental results to eqns. (1) and (2). All correlation coefficients were higher than 0.999.

Temperature (°C)	Rate constant (s ⁻¹)		Specific activity ($\mu\text{mol/s per } \mu\text{mol}$)			
			Two-step model		Two-isoenzyme model	
	$10^3 \times k_1$	$10^4 \times k_2$	R_1	R_2	R_1	R_2
55	1.36 ± 0.33	0.17 ± 0.09	372.4 ± 6.7	266.8 ± 11.0	102.3 ± 12.5	270.1 ± 12.1
60	1.73 ± 0.88	1.34 ± 0.20	368.0 ± 5.3	291.5 ± 23.9	51.9 ± 15.8	316.1 ± 15.5
65	1.77 ± 0.67	0.97 ± 0.08	368.9 ± 6.0	293.9 ± 14.6	58.0 ± 11.1	310.8 ± 10.6
70	4.35 ± 0.55	1.85 ± 0.40	368.8 ± 3.6	212.5 ± 10.7	146.8 ± 11.2	221.9 ± 12.0
75	3.65 ± 0.23	3.94 ± 1.0	361.5 ± 10.2	242.9 ± 49.7	89.2 ± 35.4	272.3 ± 44.7
80	4.62 ± 0.83	12.3 ± 3.0	368.2 ± 5.2	109.7 ± 34.7	218.6 ± 49.6	149.5 ± 50.8
85	7.60 ± 1.85	18.2 ± 6.2	369.0 ± 6.4	115.7 ± 43.9	216.9 ± 61.3	152.0 ± 62.5

exponential decay. As previously described for potato acid phosphatase [12] and guinea-pig liver transglutaminase [13], two models are possible: the two-step model and the two-isoenzyme model. In order to discriminate between the two hypotheses, the deactivation constants (k_1 and k_2) and the specific activities (R_1 and R_2) were determined for each temperature and for both models by fitting the experimental data to eqns. (1) and (2). The results are presented in Table 1. The values of k_1 and k_2 given by the fitting are identical for both models. Since for both models all the correlation coefficients are higher than 0.999, only the analysis of the variations of R_1 and R_2 with respect to temperature allowed us to choose between the two hypotheses.

In the case of model 1 (two-isoenzyme model), since R_1 and R_2 are the specific activities of the two native isoenzymes (N_1 and N_2), it is obvious that R_1 and R_2 must not vary with the deactivation temperature (T_d). As this is not the case, this model must be ruled out. Only the two-step model can explain our experimental data. Indeed, in this case R_1 , which is the specific activity of the native enzyme (N), is constant whatever the T_d . On the other hand, the variations of R_2 with respect to T_d suggest the existence of more than one intermediate form. The structure of this form would depend on the deactivation temperature according to model 2':

Increasing this temperature would lead to less-active intermediate forms that are deactivated with their own rate constants so that k_2 cannot follow the Arrhenius law. As it has been shown that irreversible thermal denaturation of *B. licheniformis* α -amylase at pH 6.5 is caused by deamidation of asparagine and/or glutamine residues [10], one could imagine that our intermediate forms correspond to deamidated species with different degrees of deamidation.

We may note that above 85 °C the plots of $\ln R$ versus incubation time were characterized by only one straight line (result not shown). No intermediate form could be detected: denaturation occurred very quickly and the specific activity of X became too low to be measured. Similar results have already been reported for different glutamine synthetases [14].

Effect of temperature on the activity of the native and the intermediate forms

The effect of temperature on the activity of one intermediate form was studied and compared with the temperature-activity profile of the native form. Fig. 3(a) shows the results obtained when the enzyme was deactivated at 65 °C and the specific activity assayed at various temperatures in the range from 30 to 80 °C. The plots of $\ln R$ versus incubation time are parallel since the residual activity exhibits the same temperature-dependence at all levels. Fitting of these data to eqn. (2) allowed us to calculate the four parameters k_1 , k_2 , R_1 and R_2 at each test temperature. The variations of R_1 and R_2 with T_i are shown in Fig. 3(b). The temperature profile of the intermediate form (X_{65}) and the native form are similar and the optimum temperature is near 80 °C. The Arrhenius plot representing the variations of the specific activities R_1 and R_2 with respect to the reciprocal of the temperature (between 30 and 80 °C) was drawn (result not shown). The activation energies of the hydrolysis reaction catalysed by the intermediate form X_{65} and by the native form are quite similar [26.8 ± 1.8 and 30.0 ± 1.5 kJ·mol⁻¹ respectively; Table 2, part (a)]. The specific activities of the native and the X_{65} forms at 80 °C are not very different [1549 and 1096 $\mu\text{mol/s per } \mu\text{mol}$ respectively; Fig. 3(b)] so that there is not a great difference between the corresponding $\Delta G^\ddagger$ (65.2 ± 3.1 and 66.3 ± 3.6 kJ·mol⁻¹ respectively).

Since $E_a^\ddagger$ and $\Delta H^\ddagger$ are equal, the same quantity of energy is necessary for the conversion of the native enzyme-substrate complex and the intermediate form-substrate complex into the 'activated complex'. Moreover, the transition state is a more ordered structure than the ground state ($\Delta S^\ddagger$ is strongly negative).

Effect of Ca²⁺ on α -amylase thermostability

α -Amylases are known to contain at least one Ca²⁺ ion per molecule (intrinsic Ca²⁺), which is involved in stabilization of the molecular structure [15]. Our results confirm the key role of Ca²⁺ in the enzyme structure. Indeed, although extensive dialysis of the native α -amylase at 4 °C against continuous supplies of 25 mM-Tris/acetate buffer, pH 8.2, containing 5 mM-EDTA led to a total inactivation of the enzyme, molecular sieving

Fig. 3. Temperature-dependence of the activity of the intermediate form of *B. licheniformis* α -amylase

(a) α -Amylase was incubated at 65 °C. For each incubation time, specific activity was assayed at various temperatures: Δ , 30 °C; ∇ , 40 °C; $\square$, 50 °C; $\blacksquare$, 60 °C; $\circ$, 70 °C; $\bullet$, 80 °C. (b) Activity profiles of the native form ($\bullet$) and the intermediate form ($\circ$). Specific activities of the native form and the intermediate form (R_1 and R_2 respectively) were obtained from (a) by fitting the experimental data to eqn. (2).

on Sephadex G-25 had no effect (results not shown). Since the dissociation constant for the Ca^{2+} -binding site is very low ($2.5 \times 10^{-8} \text{ M}$) [16], intrinsically bound Ca^{2+} could not be removed easily. However, inactivation was reversible. Addition of Ca^{2+} to dialysed enzyme led to a complete re-activation of the enzyme.

It is well known that addition of Ca^{2+} is required for an enhanced thermostability of α -amylases [17,18]. Therefore, in order to obtain information concerning the role of this cation in the enzyme stabilization, a study of the kinetics of irreversible thermal denaturation of α -amylase in the presence of Ca^{2+} at different temperatures was carried out.

The dependence on Ca^{2+} concentration of α -amylase thermostability at 90 °C was studied. In order to remove the extrinsic Ca^{2+} that may be present in the commercial enzyme preparation, α -amylase was desalted on Sephadex G-25. When this enzyme (with its intrinsic Ca^{2+}) was incubated for 15 min at 90 °C in the presence of 5 mM- CaCl_2 , 50% activity remained, whereas in the absence of added Ca^{2+} no activity could be detected. However, increased concentrations of Ca^{2+} above 5 mM have no further stabilizing effect (results not shown).

α -Amylase was deactivated at incubation temperatures from 75 to 95 °C in the presence of 5 mM- CaCl_2 . The specific activity was assayed at 30 °C. The results obtained are shown in Fig. 4. The ability of added Ca^{2+} to protect *B. licheniformis* α -amylase is striking, since at 75 °C no inactivation occurred. The stabilizing effect of Ca^{2+} being optimal for 5 mM, all the enzyme molecules should be present as enzyme- Ca^{2+} complexes in accordance with model 3:

with R'_1 and R'_2 being the specific activities of NCa and XCa respectively. The specific activity of the mixture varies with time in accordance with eqn. (2). Kinetic parameters are listed in Table 3.

At 80 and 85 °C, in the presence of Ca^{2+} , the native enzyme was inactivated with a slower deactivation constant as compared with that obtained in the absence of added Ca^{2+} ($0.75 \times 10^{-3} \pm 0.28 \times 10^{-3}$ and $4.62 \times 10^{-3} \pm 0.83 \times 10^{-3} \text{ s}^{-1}$ at 80 °C, and $1.22 \times 10^{-3} \pm 0.19 \times 10^{-3}$ and $7.60 \times 10^{-3} \pm 1.85 \times 10^{-3} \text{ s}^{-1}$ at 85 °C). On the other hand, the intermediate form X_{80} and to a smaller extent X_{85} are thermostable, since their deactivation constants are low ($3.0 \times 10^{-6} \pm 0.2 \times 10^{-6}$ and $70.0 \times 10^{-6} \pm 20 \times 10^{-6} \text{ s}^{-1}$). The stabilizing effect of Ca^{2+} seems to be stronger on the intermediate form than on the native one (Tables 1 and 3). We can note that the

Table 2. Thermodynamic activation parameters for *B. licheniformis* α -amylase

Activation energies were calculated from the slopes of Arrhenius plots. $\Delta G^\ddagger$, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values are given at 80 °C.

Reaction	$E_a^\ddagger$ (kJ·mol ⁻¹)	$\Delta G^\ddagger$ (kJ·mol ⁻¹)	$\Delta H^\ddagger$ (kJ·mol ⁻¹)	$\Delta S^\ddagger$ (J·mol ⁻¹ ·K ⁻¹)
(a) Catalysis:				
Native form	30.0 ± 1.5	65.2 ± 3.1	27.1 ± 1.5	-108.6 ± 4.4
Intermediate form	26.8 ± 1.8	66.3 ± 3.6	23.4 ± 1.8	-119.0 ± 5.0
(b) Thermal denaturation:				
Without additive	53.1 ± 9.5	103.2 ± 0.5	50.4 ± 9.5	-151.0 ± 25.2
With CaCl_2^*	155.4 ± 17.2	108.1 ± 1.0	150.5 ± 17.0	$+124.0 \pm 45.1$

* Time course of inactivation was monitored at various temperatures in the presence of 5 mM- CaCl_2 .

Fig. 4. Kinetics of the thermal denaturation of *B. licheniformis* α -amylase in the presence of Ca^{2+}

α -Amylase ($0.17 \mu\text{M}$) was incubated at various temperatures (75 to 95°C) in the presence of 5 mM-CaCl_2 for various times. Δ , 75°C ; $\blacksquare$, 80°C ; $\square$, 85°C ; $\circ$, 90°C ; $\bullet$, 95°C . The curves are theoretical and were obtained from the numerical values given in Table 3.

specific activities of any one of the intermediate forms obtained at one temperature in the presence and in the absence of added Ca^{2+} are significantly different (for example, $326.4 \pm 14.1 \text{ s}^{-1}$ for X_{80} in the presence of 5 mM-CaCl_2 and $109.7 \pm 34.7 \text{ s}^{-1}$ in its absence). All these results (Tables 1 and 3) may mean that α -amylase at high temperatures could exist under a great but finite number of active and stable (the activity of enzyme stored in ice after heating was constant) conformational states. Each of them depends on the thermal treatment of the native enzyme and on the presence or the absence of added Ca^{2+} .

In the presence or in the absence of added Ca^{2+} , only the first deactivation constant followed the Arrhenius law (Fig. 5). The activation parameters of the thermal denaturation are listed in Table 2, part (b). The stabilizing effect of Ca^{2+} is reflected by the increased values of the activation energy, which is 3 times higher in the presence of 5 mM-CaCl_2 . A similar stabilizing effect has been described for guinea-pig liver transglutaminase [13]. The

Fig. 5. Arrhenius plots for the thermal denaturation of *B. licheniformis* α -amylase

The first deactivation constants in the presence ($\bullet$) and in the absence ($\circ$) of Ca^{2+} were obtained in both cases from Tables 1 and 3. Activation energies were determined by linear-regression analysis.

$\Delta G^\ddagger$ value of about $100 \text{ kJ} \cdot \text{mol}^{-1}$ is close to the values usually obtained for irreversible denaturation of various proteins [19]. The strongly negative value of $\Delta S^\ddagger$ in the absence of Ca^{2+} suggests that the transition state reached from N to X must have a highly folded structure whereas this is not the case in the presence of Ca^{2+} .

The thermostabilization seems to be Ca^{2+} -specific since neither other metal ions (Fe^{2+} , Mg^{2+} , Zn^{2+} , Ba^{2+} and Mn^{2+}) nor the substrate (1% starch) provided any thermal protection (results not shown).

The three-dimensional structure of pig pancreatic α -amylase has recently been elucidated [20]. It was shown that Ca^{2+} stabilized the active-site cleft by inducing an ionic bridge between the two domains A and B. Although no similar study has been carried out on *B. licheniformis* α -amylase, one may consider that Ca^{2+} plays a similar role. Indeed, this cation is necessary for the structural integrity of all α -amylases, and the conserved sequence regions are involved in the architecture of the Ca^{2+} -binding site and of the active site. Our results clearly indicate that Ca^{2+} ions play an important role in the enzyme's thermostability. Probably by binding to the protein, it stabilizes the three-dimensional structure and prevents the enzyme from unfolding.

Table 3. Calculated values of the kinetic parameters for deactivation of *B. licheniformis* α -amylase in the presence of CaCl_2

k_1' , k_2' , R_1' and R_2' were obtained by non-linear fitting of the experimental results to eqn. (2). All correlation coefficients were higher than 0.999.

Temperature ($^\circ\text{C}$)	$10^3 \times k_1' (\text{s}^{-1})$	$10^3 \times k_2' (\text{s}^{-1})$	$R_1' (\text{s}^{-1})$	$R_2' (\text{s}^{-1})$
80	0.75 ± 0.28	0.0030 ± 0.0002	368.0 ± 1.0	326.4 ± 14.1
85	1.22 ± 0.19	0.07 ± 0.02	367.2 ± 1.8	255.5 ± 12.1
90	2.52 ± 0.52	0.12 ± 0.01	366.1 ± 6.5	148.5 ± 28.0
95	6.50 ± 1.66	1.17 ± 0.57	377.4 ± 8.0	125.0 ± 47.2

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Received 8 February 1989/18 May 1989; accepted 30 May 1989