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EFFECT OF ACETYLATION OF *BACILLUS SUBTILIS* α-AMYLASE ON THE KINETICS OF HEAT INACTIVATION

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SUMMARY

Bacillus subtilis α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) was acetylated with *p*-nitrophenyl acetate and the effect of acetylation on the thermostability of the enzyme was investigated. The thermostability of the α-amylase was increased by acetylation at temperatures higher than 70 °C and decreased at temperatures lower than 67 °C. The compensation effect was also observed for the heat inactivation of acetyl α-amylases and the temperature of compensation T_c was approximately 68 °C. This effect seems to be due to the conformational change of the enzyme caused by acetylation. The significance of the compensation effect and T_c in the study of the denaturation and stability of proteins was discussed.

INTRODUCTION

It is generally believed that thermostability of an enzyme is decided principally by its amino acid sequence and the specific conformation derived from the sequence. However, it seems to be very difficult to say what enzyme configuration makes it more heat stable. In order to provide a clue for elucidating the thermostability of an enzyme on a molecular basis, the physico-chemical properties of a heat-stable enzyme such as α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) from *Bacillus stearothermophilus* have been studied, and two opposing conclusions were presented. Manning *et al.*[1] reported that the enzyme in the native state exists as semi-randomly- or randomly-coiled, well-hydrated molecules, thus accounting for its resistance to heat inactivation. On the contrary, Ogasahara *et al.*[2] concluded that the α-amylase had a globular structure like many other α-amylases.

To clarify the cause of the thermostability of an enzyme, it is also very interesting to investigate the heat resistance of modified enzymes, which have the same primary structure with some altered amino acid side chains and whose conformation probably differs little from that of the native enzyme. Only a few studies[3–5] have been done concerning the effect of the chemical modification of a protein on its

thermostability. In this report, the amino groups of *Bacillus subtilis* α-amylase were acetylated and the heat inactivation kinetics of the enzyme were studied at various temperatures. The difference in thermostability between the native enzyme and its derivatives was explained in relation to their thermodynamic constants of activation.

MATERIALS AND METHODS

(1) Materials

Crystalline *B. subtilis* α-amylase was donated by Daiwa Kasei Co., Ltd, and was used without further treatment for the present study. The concentration of the enzyme was determined spectrophotometrically assuming $E^{1\%}_{1\,cm} = 25$ at 280 nm. The dioxane used was purified by distillation after refluxing with lithium aluminium hydride.

(2) Preparation of acetyl α-amylase

Acetyl α-amylase was prepared by adding *p*-nitrophenyl acetate in dioxane to the α-amylase solution in 0.2 M borate buffer (pH 8.5) containing 0.1 mM $CaCl_2$ at 40 °C. The final concentration of the enzyme and dioxane was 0.4% and 20% respectively. To obtain the samples with the required range of acetylation, the final concentration of *p*-nitrophenyl acetate was varied, as shown in Table I. After standing for 1 h, the dioxane concentration of the reaction mixture was made up to 70% by adding dioxane, then soluble starch dissolved in 70% dioxane was added to precipitate the acetyl enzymes as an enzyme–substrate complex. The precipitate thus obtained was washed thoroughly with 70% dioxane and dissolved in 0.2 M borate buffer solution (pH 8.5) containing 0.1 mM $CaCl_2$. Recovery of the protein through the above procedure was more than 80%.

Under the above conditions, dioxane had no effect on the enzyme and only amino groups of the enzyme seemed to be acetylated.

(3) Estimation of the number of amino groups acetylated

The number of free amino groups was determined using 2,4,6-trinitrobenzenesulfonic acid according to the procedure of Habeeb[6] with some modification, assuming the molecular weight of the enzyme to be $4.9 \cdot 10^4$ and the molar extinction coefficient of the trinitrophenylated amino group to be $\varepsilon = 1.2 \cdot 10^4$ at 340 nm. Then the number of amino groups acetylated per mole of the enzyme was estimated from the decrease of the number of free amino groups. *B. subtilis* α-amylase has 25 ε-amino groups of lysyl residues and an N-terminal α-amino group in each molecule[7]. According to our preliminary experiments, all amino groups in the enzyme molecule seemed to be able to react with 2,4,6-trinitrobenzenesulphonic acid or *p*-nitrophenyl acetate.

(4) Thermal inactivation

A native or acetyl α-amylase solution of about 0.1% protein concentration in 0.2 M borate buffer solution (pH 8.5) containing 0.1 mM $CaCl_2$, was diluted 5-fold with 0.2 M acetate buffer (pH 5.5) containing 0.1 mM calcium chloride, and the diluted solution was heated to the desired temperature. A portion was taken at precisely time intervals and chilled in ice water immediately.

(5) Assay for amylase activity

The activity of α-amylase was determined at 40 °C in 0.2 M acetate buffer solution (pH 5.5) containing 0.1 mM $CaCl_2$ by the decreasing rate of the absorbance of the iodine–starch complex at 660 nm.

RESULTS

Acetyl α-amylase having different numbers of acetylated amino groups was prepared as described in Materials and Methods using varying *p*-nitrophenyl acetate concentrations as shown in Table I. Table I also shows that acetylation of up to 9 acetylated amino groups per enzyme molecule had only a small effect on the enzyme activity.

TABLE I

PREPARATION AND ACTIVITY OF ACETYL α-AMYLASE

The α-amylase concentration was about 0.08 mM in the acetylation reaction. The number of amino groups acetylated was determined using 2,4,6-trinitrobenzenesulphonic acid. Further details are given in the text.

Sample	*Concn of p-nitrophenyl acetate (mM)*	*No. of $-NH_2$ acetylated*	*Relative enzyme activity (%)*
A- 0	0	0	100
A- 1.5	0.16	1.5	99
A- 6.5	1.6	6.5	100
A- 9	2.4	9	92
A-11.5	4.0	11.5	79

The effect of acetylation on the thermostability of the enzyme was investigated at various temperatures ranging from 60 to 75 °C. Fig. 1 shows the time course of heat inactivation of these acetylated α-amylases. The heat inactivation curves of A-0, which had been treated as other acetyl enzymes without *p*-nitrophenyl acetate, were essentially the same as that of the original enzyme at every temperature tested. An increased thermostability of the acetylated enzyme at temperatures higher than 70 °C and a decreased thermostability at temperatures lower than 67 °C were observed. This tendency was more remarkable when the acetylation number was increased. The acetylated amylase samples used were mixtures of enzymes having different numbers and sites of acetylation, so the semi-logarithmic plots of their heat inactivation were somewhat concurved. The apparent heat inactivation rate constant k' was estimated from the results shown in Fig. 1 assuming first order kinetics. The nominal values of k' are summarized in Table II.

The activation parameters for the activated complex(es) involved in the heat inactivation reaction were determined from the Arrhenius plots as shown in Fig. 2 applying the Eyring[8] absolute rate equation:

$$k' = \kappa \frac{kT}{h} e^{-\Delta H^*/RT} e^{\Delta S^*/R}$$

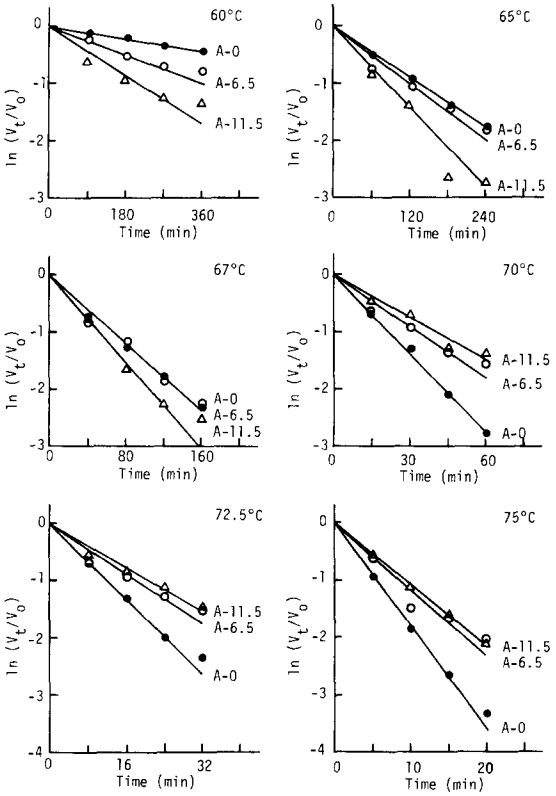

Fig. 1. Time course of heat inactivation of acetyl α-amylase. The solution of acetyl α-amylase was heated at 60 °C, 65 °C, 67 °C, 70 °C, 72.5 °C and 75 °C. The other experimental conditions are described in the text under Methods. The ordinate is scaled as the natural logarithm of the remaining activity fraction, V_t being the remaining enzyme activity at time t, and V_0 being the enzyme activity at time zero. The samples described in Table I are identified by number in the figure. ●, A-0; ○, A-6.5; △, A-11.5.

TABLE II

THE APPARENT HEAT INACTIVATION RATE CONSTANT k' OF ACETYL α-AMYLASE AT VARIOUS TEMPERATURES

The values of k' were obtained from the data shown in Fig. 1. The numbers in the first column refer to the acetyl α-amylases described in Table I.

Sample	k'					
	60 °C	*65 °C*	*67 °C*	*70 °C*	*72.5 °C*	*75 °C*
A- 0	$2.2 \cdot 10^{-5}$	$1.28 \cdot 10^{-4}$	$2.5 \cdot 10^{-4}$	$7.6 \cdot 10^{-4}$	$1.44 \cdot 10^{-3}$	$2.9 \cdot 10^{-3}$
A- 1.5	$2.5 \cdot 10^{-5}$	$1.26 \cdot 10^{-4}$	$2.5 \cdot 10^{-4}$	$6.9 \cdot 10^{-4}$	$1.26 \cdot 10^{-3}$	$2.9 \cdot 10^{-3}$
A- 6.5	$4.4 \cdot 10^{-5}$	$1.37 \cdot 10^{-4}$	$2.5 \cdot 10^{-4}$	$5.3 \cdot 10^{-4}$	$0.92 \cdot 10^{-3}$	$1.88 \cdot 10^{-3}$
A- 9	$5.2 \cdot 10^{-5}$	$1.62 \cdot 10^{-4}$	$2.6 \cdot 10^{-4}$	$4.6 \cdot 10^{-4}$	$0.80 \cdot 10^{-3}$	$1.84 \cdot 10^{-3}$
A-11.5	$7.8 \cdot 10^{-5}$	$1.94 \cdot 10^{-4}$	$3.2 \cdot 10^{-4}$	$4.2 \cdot 10^{-4}$	$0.81 \cdot 10^{-3}$	$1.80 \cdot 10^{-3}$

TABLE III

ACTIVATION PARAMETERS FOR HEAT INACTIVATION OF ACETYL α-AMYLASES

The activation parameters were calculated from the results shown in Fig. 2. ΔG^* was determined for 70 °C. The numbers in the first column refer to the acetyl α-amylases described in Table I.

Sample	ΔH^* *(kcal/mole)*	ΔS^* *(cal/mole deg.)*	ΔG^* *(kcal/mole)*
A- 0	83	168	25
A- 1.5	78	153	25
A- 6.5	62	107	25
A- 9	55	86	25
A-11.5	46	60	25

where κ is the transmission coefficient (assumed to be unity), k is Boltzmann's constant, h is Planck's constant, R is the gas constant, T is the absolute temperature, and ΔH^* and ΔS^* are, respectively, the enthalpy and entropy of activation of the process. ΔG^*, the free energy of activation, was obtained from the relationship $\Delta G^* = \Delta H^* - T\Delta S^*$. The calculated data are given in Table III. These results show that the values of ΔH^* and ΔS^* decreased as the number of acetylation of the enzyme increased, and the values of ΔG^* remained constant for all five samples studied, approximating 25 kcal per mole at 70 °C.

Of particular interest is that the Arrhenius plots for these acetyl α-amylases met at about 68 °C as shown in Fig. 2. According to Cremer[9] this temperature is termed to be the temperature of compensation (T_c) or isokinetic temperature, so Fig. 2 demonstrates the existence of the so-called compensation effect[9]. An alternative representation of the compensation effect is the linear relationship between ΔH^* and ΔS^*[10] as shown in Fig. 3, the slope $\Delta H^*/\Delta S^*$ being T_c. The value of T_c obtained from Fig. 3 was also approx. 70 °C.

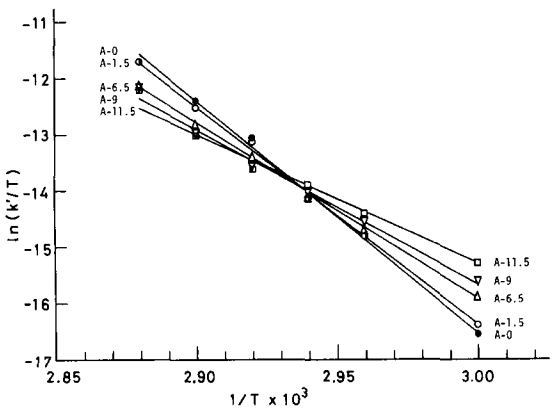

Fig. 2. Arrhenius plots for heat inactivation of acetyl α-amylase. The samples described in Table I are identified by number in the figure. ●, A-0; ○, A-1.5; △, A-6.5; ▽, A-9; □, A-11.5.

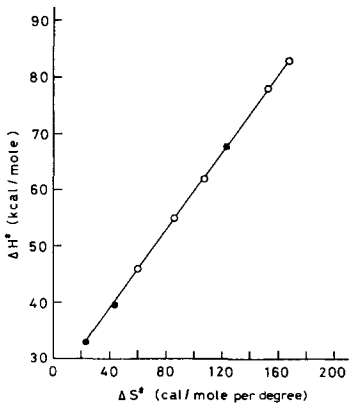

Fig. 3. Plot of ΔH^* *versus* ΔS^* for inactivation of native and acetyl α-amylases. The values of ΔH^* and ΔS^* were obtained from Table III (○) and Yamanaka *et al.*[11] (●).

DISCUSSION

The thermostability of *B. subtilis* α-amylase was considerably affected by acetylation of its amino groups but the effect may be evaluated in quite the opposite directions, a decrease or increase in the stability, depending upon the temperature at which the thermostability was studied (Fig. 1). These effects of acetylation on the thermostability of the enzyme were found to be due to a decrease in the values of ΔH^* and ΔS^* for heat inactivation as shown in Table III. The decreases in ΔH^* and ΔS^* indicate that by acetylation the conformation of the enzyme molecule was somewhat altered in the direction of a partially unfolded activated state. The conformational change caused by acetylation may or may not be detectable by the measurement of the physicochemical properties of the modified enzymes as have been shown in several reports[3,12–15]. However, kinetic studies as described in this report permit the detection of conformational changes which might not be detectable by a physicochemical determination.

Figs 2 and 3 demonstrate the compensation (or isokinetic) effect for the heat inactivation of the acetyl α-amylases. The existence of the compensation effect is evidence favoring the idea that there is a constant mechanism or an identical nature of the transition state for the related series of reactions in which, for example, the solvent or the structure of the reactants changes[10]. Therefore, it seems to be reasonable that the native and acetyl α-amylases have the same transition state for heat inactivation. Yamanaka *et al.*[11] investigated the denaturation of *B. subtilis* α-amylase under various conditions, and some thermodynamic data of the denaturation were calculated. Some of these data obtained under three denaturation conditions (0.05 M $CaCl_2$, pH 8.0; 0.05 M $CaCl_2$, pH 4.5; 6 M urea, 0.05 M $CaCl_2$, pH 8.0) are plotted in Fig. 3 (filled circles). It is significant that the denaturation of the α-amylase under various conditions, including pH change, with or without 6 M urea, and varying number of acetyl groups, exhibits the isokinetic relationship as shown in Fig. 3. This suggests the presence of a common mechanism of denaturation for the α-amylase under these conditions.

The linear relationship between ΔH^* and ΔS^* for the denaturation of a protein or an enzyme was generally observed from the data given by several authors[11,16,17].

TABLE IV

T_c FOR THE DENATURATION OF PROTEINS

Substance	*pH*	*Conditions of denaturation*			T_c (°C)
		$(NH_4)_2SO_4$ (*M*)	*Urea* (*M*)	*Alcohol* (*vol. %*)	
Hemoglobin (ref. 17)	4.08–8.0	0–1.52	0	0	77
Hemoglobin (ref. 17)	6.0 –7.0	0	0	0–30	–9
Egg albumin (ref. 17)	1.02–9.8	0 or 1	0 or 10	0	79
Invertase (ref. 17)	3.0 –5.7	0	0	0	55
Pancreatic ribonuclease (ref. 16)	7.3	0	0–8.0	0	40*
B. subtilis α-amylase (ref. 11)	4.5 –8.5	0	0–6	0	70**

* Determined using the data for denaturation and renaturation.
** The same as the value for acetyl α-amylase obtained from this experiment.

From these data, the temperature of compensation, T_c, was calculated; these results are given in Table IV. In the case of pancreatic ribonuclease, a single isokinetic line was obtained using the activation parameters for denaturation and renaturation at pH 7.3 in urea solutions of various concentrations; this suggests that the same activation state is formed in both reactions. On the other hand, deviation from the isokinetic line was observed in the cases of urea denaturation of pancreatic ribonuclease at pH 5.6 and alcohol denaturation of hemoglobin. The deviation from the isokinetic line indicates a different mechainism for the reaction, so it is of interest to classify denaturants by evaluating ΔH^* and ΔS^* for denaturation.

Barnes *et al.*[18] proposed the significance of T_c in biological systems. They suggested that the determination of T_c might be of value in the prediction and classification of reactions. The values of T_c for the denaturation of proteins seem to be in the range between −10 °C and 100 °C as shown in Table IV. It is clear from the existence of T_c in the range of experimental conditions that certain precautions should be taken in the interpretation of the effect of environmental conditions or modification of a protein on its stability, because no effect will be seen at T_c and opposite effects will be observed at both sides of T_c as was shown in the present experiment.

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