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The Influence of pH on the Thermal Stability of Penicillin Acylase from *Alcaligenes faecalis*

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Abstract—Penicillin G acylase (PA, EC 3.5.1.11) from *Alcaligenes faecalis* (AfPA) is one of the most thermostable bacterial penicillin acylases. However, systematic data about the thermal stability of AfPA are not found in the literature. A systematic study of the influence of pH on the thermal stability of AfPA was done in the pH range 7.5–9.5. It was found that in all pH ranges studied the enzyme inactivation follows first-order kinetics. The dependence of the inactivation rate constant on pH has an *S*-shape with an inflection point at pH 8.3–8.5. The temperature dependences of the inactivation rate constant at four pH values were obtained and activation parameters $\Delta H^{\#}$ and $\Delta S^{\#}$ were calculated for each pH value. The decrease of both values, $\Delta H^{\#}$ and $\Delta S^{\#}$ with pH growth shows that a minimum of one iogenic group is essential for the enzyme's thermal stability.

Key words: penicillin acylase, *Alcaligenes faecalis*, thermal stability, pH, inactivation.

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Pennicillin acylase (PA) refers to a superfamily of enzymes with an N-terminal nucleophile (Ntn-hydrolase), which are activated by autocatalytic cleavage of the original inactive polypeptide precursor [1]. PA is widely used in the production of antibiotics of penicillin and cephalosporin series, in fine organic synthesis, and in obtaining optically pure compounds [2–4] at the expense of broad substrate specificity and high stereoselectivity.

PA from the bacteria *Alcaligenes faecalis* (AfPA) is one of the most thermostable penicillin acylases [5], but the published data on the thermal stability of this enzyme is restricted to the time values of half inactivation for two or three temperatures. In our laboratory the AfPA gene from strain *Alcaligenes faecalis* VKM-B1518 was cloned and expressed in *E. coli* [6]; the results of preliminary experiments at pH 8.0 indicate that inactivation of the enzyme proceeds via a monomolecular mechanism. We have also shown that the theory of the activated complex can be used for calculation of the activation parameters of inactivation $\Delta H^{\#}$ and $\Delta S^{\#}$. The effective application of the enzyme in practice requires more detailed information about the stability of the enzyme depending on parameters such as the temperature and pH of the solution. This work is devoted to a systematic investigation of the influence of pH on the thermal stability of AfPA in the temperature range 49–54°C.

MATERIALS AND METHODS

Cultivation. Growing *E. coli* cells with recombinant AfPA was performed in 1-liter shaking flasks containing two or four baffle plates on a Multitrone shaker (Infofs, Switzerland). The working volume of the YE^{++} medium (30 g/l of yeast extract, 5 g/l of sodium chloride, 5 g/l of glycerol, and 2 mM calcium chloride, at pH 7.5) was 100 ml. Inoculum grew overnight (37°C, 180 rpm). The medium contained 35 μg/ml of chloramphenicol. We used isopropyl-β-thiogalactoside (IPTG, the final concentration was 0.1 mM) as an inducer of protein biosynthesis, which was added after the suspension of cells reached an absorption of 0.6–0.8 at 600 nm (A_{600}). The cells were then cultured for 55–60 h at 15–17°C. The cells were precipitated by centrifugation (7500 rpm, 20 min, 4°C) in a Beckman J-21 centrifuge (Germany).

Separation and purification of the enzyme. The procedure for separation and purification of recombinant AfPA included the destruction of bacterial cells by osmotic shock, fractionation with ammonium sulfate (35–65% of saturation) and desalting on a column with Sephadex G 25 (Amersham Biosciences, Sweden). We used 0.1 M Tris–HCl as a demineralizing buffer, which was then brought to the appropriate pH in the range from 7.5 to 9.5 units. Purity control was performed using analytical electrophoresis in a 12% polyacrylamide gel in the presence of sodium dodecyl sulphate in an electrophoresis apparatus (BioRad).

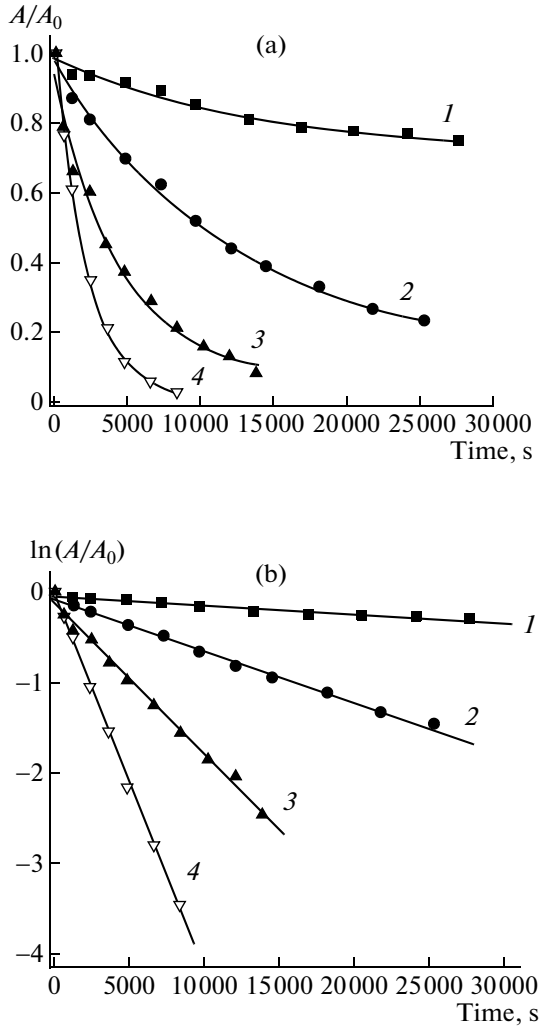

Fig. 1. Inactivation of penicillin acylaze of bacteria *A. faecalis* at 49°C in 0.1 M Tris–HCl buffer at different pH values (*1*, 7.5; *2*, 8.0; *3*, 8.5; and *4*, 9.5), the dependence of residual activity and time: (a) in the coordinates $A/A_0 - t$; (b) in semilogarithmic coordinates.

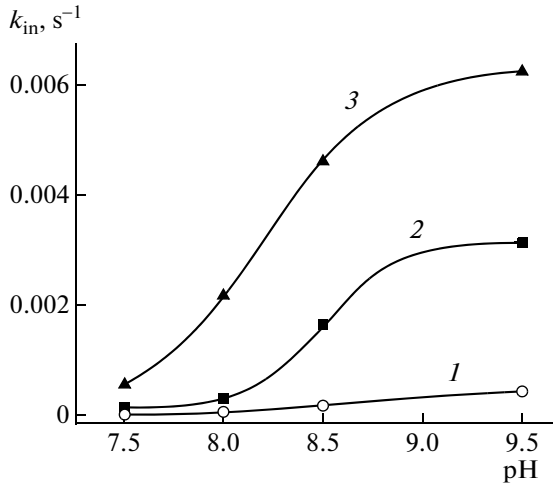

Fig. 2. The dependence of the rate constant of inactivation on pH (0.1 M Tris–HCl) at different temperatures, °C: *1*, 49; *2*, 52; *3*, 54.

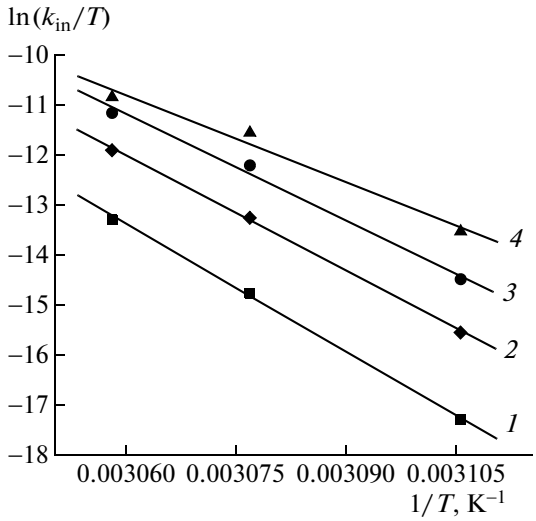

Fig. 3. The dependence of the rate constant of inactivation on the temperature in the coordinates $\ln(k_{in}/T)-1/T$, K^{-1} at various pH values: *1*, 75; *2*, 8.0; *3*, 8.5, *4*, 9.5.

The purity of the enzyme preparations was not less than 90%.

Determination of enzyme activity. AfPA activity was determined spectrophotometrically by the accumulation of chromophore during the process of the hydrolysis of a 0.24 mM solution of *p*-nitro-*m*-carboxyanilide of phenacetic acid (NIPAB) at 400 nm on a UV-1601 spectrophotometer (Shimadzu, Japan). The reaction was conducted at 30°C in 0.01 M phosphate buffer (pH 8.0 and 0.1 M KCl).

Study of thermal stability. To study thermal stability, a series of experiments to determine the dependence of the residual enzyme activity on time in an appropriate buffer was carried out. To conduct the experiment a series of twenty plastic tubes with a volume of 500 μl with 50 μl of the enzyme were prepared. The tubes were placed in a water bath, heated to the desired temperature (temperature control accuracy ± 0.1°C), and at certain time intervals two tubes were sampled. The interval between sampling was determined such that the total time of the experiment was three periods of half inactivation. The tubes were placed for cooling in ice water for 1 min, then they were centrifuged for 2 min at 14000 rpm in an Eppendorf 5415D centrifuge to remove sediment. The samples of 20 μl were then taken from the test tube and the residual enzymatic activity was measured as described above.

The rate constants of thermal inactivation k_{in} were determined from the slope of the linear graph of the natural logarithm of the relative residual activity versus

Table 1. The rate constants of penicillin acylaze thermal inactivation at different values of pH and temperature

Buffer solution	Tris–HCl pH 7.5	Tris–HCl pH 8.0	Tris–HCl pH 8.5	Tris–HCl pH 9.5
		49°C		
k_{in}, s^{-1}	$(9.90 \pm 0.80) \times 10^{-6}$	$(5.75 \pm 0.15) \times 10^{-5}$	$(1.67 \pm 0.04) \times 10^{-4}$	$(4.16 \pm 0.08) \times 10^{-4}$
		52°C		
k_{in}, s^{-1}	$(1.28 \pm 0.01) \times 10^{-4}$	$(5.69 \pm 0.16) \times 10^{-4}$	$(1.61 \pm 0.03) \times 10^{-3}$	$(3.11 \pm 0.03) \times 10^{-3}$
		54°C		
k_{in}, s^{-1}	$(5.53 \pm 0.09) \times 10^{-4}$	$(2.16 \pm 0.02) \times 10^{-3}$	$(4.61 \pm 0.16) \times 10^{-3}$	$(6.24 \pm 0.14) \times 10^{-3}$

time ($\ln(A/A_0) - t$) by linear regression using the program OriginPro 7.0.

RESULTS AND DISCUSSION

The kinetics of inactivation of recombinant AfPA was studied at three temperatures 49, 52, and 54°C, within a pH range from 7.5 to 9.5 units. The studies of the dependence of AfPA activity on pH in the range of 7.5–9.5 showed that in this range the enzyme activity is constant. Figures 1a and 1b shows the dependence of the residual enzyme activity on time for 49°C and four pH values in simple and semi-logarithmic coordinates. It turned out that at all the studied pH values these dependences in the semilogarithmic coordinates $\ln(A/A_0)$–t are direct, i.e., the enzyme is inactivated in accordance with the kinetics of a first-order reaction. According to the slope rate constant of inactivation of the first order were determined. Similar curves were obtained for two other temperatures. The numerical values of rate constants of inactivation are given In Table 1. Figure 2 shows the dependence of these constants on pH at different temperatures. As can be seen from the graph, the rate constant of inactivation increases with increasing pH (i.e., it decreases the stability of the enzyme), and on the curves for 52 and 54°C a bend is clearly visible in the pH range of 8.3–8.5. The presence of such an inflection means that with increasing pH the enzyme changes into another form. This transition is likely to be due to deprotonation of a group of the enzyme, resulting in a decrease in peniñillin acylase stability. The closest pK values to the observed value inflection points are found for the sulfhydryl group of cysteine residue (8.5–9.0) and the amino group of lysine residues (9.0–9.5). Since there are no free AfPA sulfhydryl groups (the protein has only two cysteine residues, which are linked by disulfide bond) [5], the most likely candidate is the amino group of the lysine residues.

The truly monomolecular nature of the process of inactivation over the entire range of temperatures allows one to apply the theory of an activated complex for its analysis. According to the theory of an activated complex (TAC), the observed rate constant thermal inactivation–temperature equation is as follows:

$$k = \frac{k_B \mathbf{T}}{h} \exp\left(-\frac{\Delta G^{\neq}}{RT}\right) = \frac{k_B \mathbf{T}}{h} e^{\frac{\Delta S^{\neq}}{R_e} - \frac{\Delta H^{\neq}}{RT}}.$$

This equation can be represented in linear form:

$$\ln\left(\frac{k}{T}\right) = \ln\left(\frac{k_B}{T}\right) + \frac{\Delta S^{\neq}}{R} - \frac{\Delta H^{\neq}}{RT} = \text{const} - \frac{\Delta H^{\neq}}{R}\frac{1}{T},$$

where

$$\text{const} = \ln\left(\frac{k_B}{h}\right) + \frac{\Delta S^{\neq}}{R}.$$

In this case, an experimental plot in coordinates $\ln(k_{in}/T) - 1/T$, K^{-1} will be a straight line with a slope equal to $\Delta H^{\#}/R$ (Fig. 3). The $\Delta S^{\#}$ value can be obtained by approximation of the line in Fig. 3 to the zero ordinate. Figure 3 clearly shows that for all the investigated pHs, the rate constant of the inactivation dependence is described by the TAC equation.

The values of activation parameters of thermal inactivation of the AfPA are presented in Table 2. The high values of the enthalpy and entropy of activation indicate that the inactivation of the enzyme at elevated temperatures is associated with the denaturation of protein molecules. Table 2 also shows that with increasing pH a decrease occurs for the entropy and the enthalpy of activation. This may be due to the loss of the charge of one of the amino groups of lysine res-

Table 2. The values of activation parameters of the process of thermal inactivation of AfPA at different pH values (0.1 M Tris–HCl)

pH	$\Delta H^{\neq}$, kJ/mol	$\Delta S^{\neq}$, J/mol
7.5	705 ± 26	1848 ± 83
8.0	635 ± 21	1644 ± 63
8.5	585 ± 52	1499 ± 166
9.5	480 ± 75	1182 ± 233

idues, which may interact with the carboxyl group residue of aspartic or glutamic acid.

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