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pH Stability of Penicillin Acylase from *Escherichia coli*

D. T. Guranda, T. S. Volovik, and V. K. Švedas*

Faculty of Bioengineering and Bioinformatics and Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (7-095) 939-2355; E-mail: vytas@belozersky.msu.ru

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Abstract—The inactivation kinetics of penicillin acylase from *Escherichia coli* have been investigated over a wide pH range at 25 and 50°C. The enzyme was very stable in neutral solutions and quickly lost its catalytic activity in acidic and alkaline solutions. In all cases, the inactivation proceeded according to first order reaction kinetics. Analysis of the pH dependence of enzyme stability provides evidence that stable penicillin acylase conformation is maintained by salt bridges. Destruction of the salt bridges due to protonation/deprotonation of the amino acid residues forming these ion pairs causes inactivation by formation of the unstable "acidic" EH_4^{3+}, EH_3^{2+}, EH_2^+ and "alkaline" E^- enzyme forms. At temperatures above 35°C penicillin acylase apparently undergoes a conformational change that is accompanied by destruction of one of these salt bridges and change in the catalytic properties.

Key words: penicillin acylase, pH stability, thermostability

Penicillin acylase (PA) from *Escherichia coli* (EC 3.5.1.11) is widely used in the pharmaceutical industry for production of 6-aminopenicillanic and 7-aminodesacetoxycephalosporanic acids—the key intermediates for the synthesis of new penicillins and cephalosporins [1]. The enzyme displays wide substrate specificity and high stereoselectivity, which provides good opportunities of its use in fine organic synthesis for resolution of α-, β-, and γ-amino acids and aminophosphonous and aminophosphinic acids [2-5]. Elevated catalytic activity of PA from *E. coli* in acidic medium reported recently [6] opens new perspectives for modification of β-lactam antibiotics, as well as for enantioselective acylation and resolution of racemic aminonitriles [7]. A related enzyme from *Alcaligenes*, advantageously distinguished from *E. coli* PA by unique catalytic properties in alkaline medium [8], made possible the effective biocatalytic resolution of racemic non-functionalized amines [9]. Taking into account the biocatalytic potential of PA in media of different acidity, pH stability studies for the enzymes of this family are of practical importance.

Hitherto, there has been no clear perception of the enzyme inactivation mechanism and the relationship between its stability and catalytic activity. This is a part of the reason why the task of preparing PA preparations stable and active over a broad pH range has not been solved. In the early 80s PA preparations stable in an acidic medium were prepared by incorporating the enzyme in polyelectrolyte complexes and purposefully changing its microenvironment during immobilization [10, 11]. However, there was no clear application of these preparations, and this research was not further developed at that time. In recent years, interest in thermostable PA preparations prepared by multipoint attachment to a matrix has increased [11, 12]; however, these preparations apparently possess low catalytic activity [13, 14]. Recently reported inactivation of *E. coli* PA at high substrate concentrations [15] is one more aspect challenging the design of stabilized enzyme preparations.

Kinetic studies, which would elucidate factors governing stability and catalytic activity of PA over a wide range of experimental conditions, are of particular interest.

MATERIALS AND METHODS

Chemicals. *p*-Nitro-*m*-carboxyanilide of phenylacetic acid (NIPAB), sodium azide, *m*-carboxy-*p*-nitroaniline, and phenylmethylsulfonyl fluoride were from Sigma (USA). Other chemicals and components of buffer solutions of "pure for analysis" grade were from Reakhim (Russia). Penicillin acylase from *E. coli* was obtained from the Russian Research Institute of Antibiotics (Moscow). The purification of PA was carried

Abbreviations: PA) penicillin acylase; k_{in}) first order inactivation constant; $t_{1/2}$) half-life period; μ) ionic strength; NIPAB) *p*-nitro-*m*-carboxyanilide of phenylacetic acid.

* To whom correspondence should be addressed.

out as described earlier [16]. The concentration of PA active sites was determined by titration with phenylmethylsulfonyl fluoride [17].

Activity of PA was determined spectrophotometrically by following accumulation of the colored product during hydrolysis of the specific chromogenic substrate NIPAB [16]. The initial rate of enzymatic hydrolysis was determined at 400 nm using a Shimadzu UV-1601 spectrophotometer at 25°C in 0.01 M phosphate buffer, pH 7.5, 0.1 M KCl. The initial substrate concentration was 1 mM.

Kinetic parameters (k_{cat} and K_m) of the enzymatic hydrolysis of the chromogenic substrate were determined analyzing the dependence of the initial rates on the substrate concentration according to the Michaelis–Menten equation ($[S]_0 >> [E]_0$) at 25°C (unless indicated otherwise) and constant ionic strength 0.12 M in a corresponding buffer.

PA stability was investigated in the pH interval 2-12, at 25 and 50°C, ionic strength 0.12 M. The typical experiment was as follows: 2 ml of PA solution (0.2-1 μM) was incubated at appropriate pH and temperature in a corresponding buffer solution. The residual PA activity was assayed at different incubation times. Inactivation rate constants (k_{in}) of native PA were calculated by processing experimental data in coordinates $\{\ln(A/A_0); t\}$ in accord with equation $A_t = A_0 \cdot \exp(-k_{in}^{eff} \cdot t)$, where A_0 and A_t are the initial ($t = 0$) and current enzyme activity during inactivation, and t is incubation time. Incubation time usually covered several half-life periods, and, depending on the pH of a solution, varied from the few minutes to a few days.

Buffer solutions. The experimental studies of catalytic activity and stability of PA were carried out in an appropriate buffer containing 0.02% sodium azide at constant ionic strength 0.12 M (the ionic strength value was adjusted using KCl). Buffering conditions were as follows: in the acidic media (pH 3-6), 0.01 M acetate; in the range pH 6.0-8.5, 0.01 M phosphate; in the range pH 8.5-11, 0.04 M phosphate; at pH 11.5, 0.03 M phosphate; at pH 2 and 12, buffer free solutions with pH monitoring. In all cases, the pH value was constant during incubation. The phosphate, acetate, and chloride anions did not affect the enzyme stability and catalytic activity at used concentrations, this being demonstrated by independent experiments.

RESULTS

Temperature profile of catalytic activity. The temperature profile of PA catalytic activity was investigated at pH 7.5 in the interval 5-70°C. The rate of the enzymatic hydrolysis of the colorimetric substrate increased with increasing temperature, and the enzyme displayed the maximum activity at 60°C (Fig. 1a). The Arrhenius plot for hydrolysis of NIPAB (Fig. 1b) was characterized by a broken line, indicating that a conformational transition in the active center above 35°C apparently takes place, in agreement with earlier observations [18]. The empiric

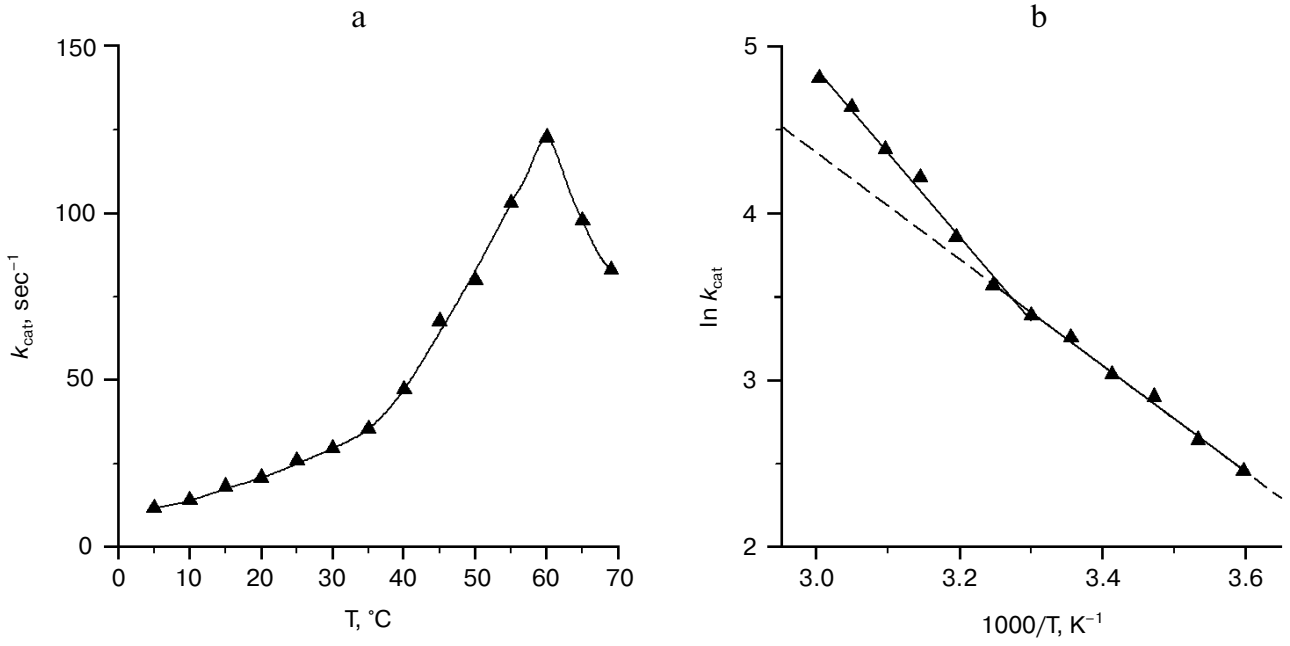


Fig. 1. Temperature profile of PA catalytic activity (a) and Arrhenius plot (b) for enzymatic hydrolysis of NIPAB. Conditions: 0.1 M KH_2PO_4, 0.1 M KCl, pH 7.5.

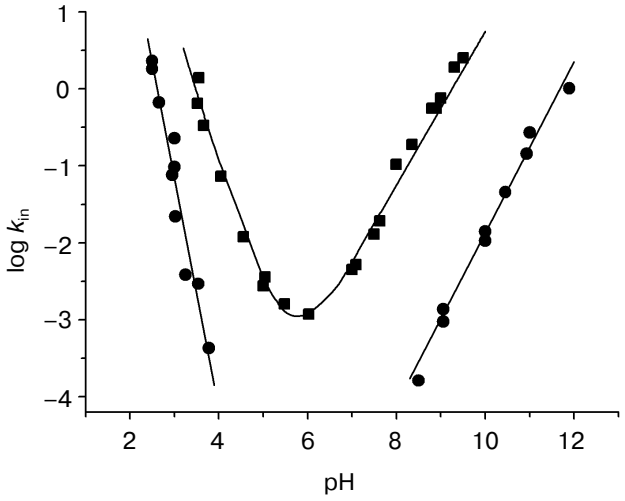


Fig. 2. pH dependence of the effective first order inactivation rate constant of PA at 25 and 50°C, ionic strength 0.12 M. The curve describing experimental data at 50°C is calculated according to the equation $k_{in} = (k_{2a}\cdot[H^+]^2/K_{2a}\cdot K_{1a} + k_{1a}\cdot[H^+]/K_{1a} + k_{1b}\cdot K_{1b}/[H^+])/(1 + [H^+]/K_{1a} + K_{1b}/[H^+] + [H^+]^2/K_{2a}\cdot K_{1a})$, where $k_{2a} = k_{1b} = 200$ min^{-1}, $k_{1a} = 0.10$ min^{-1}, p$K_{1a} = 3.5$, p$K_{2a} = 1.3$, p$K_{1b} = 11.6$ (see the Scheme). At 25°C the experimental data are approximated by linear dependencies according to the equations: log$k_{in} = -3.0\cdot$pH + 7.85, log$k_{in} = 1.11\cdot$pH − 13 in acidic and alkaline media, respectively.

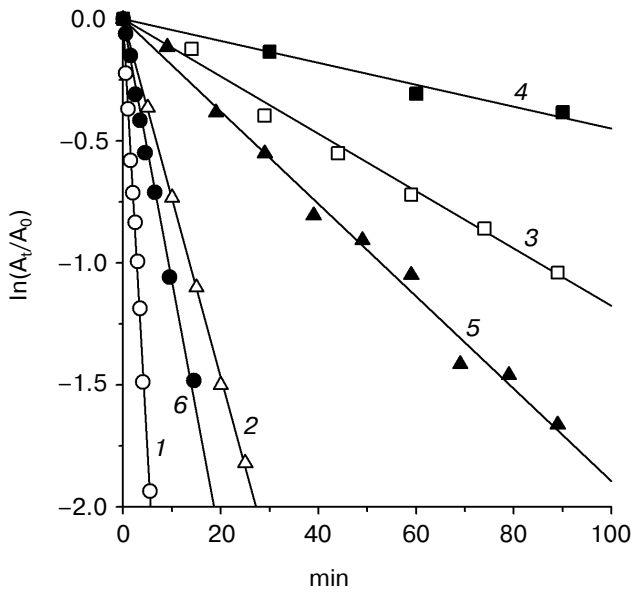
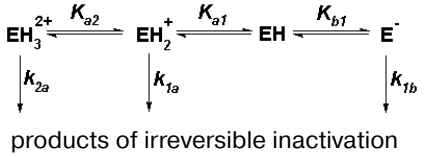


Fig. 3. Kinetics of irreversible PA inactivation at different pH values. Activity measurements: 25°C, 0.01 M phosphate buffer, pH 7.5, 0.1 M KCl. Inactivation conditions: 50°C, 0.1 M KCl, 0.02% NaN_3, $[E]_0 = 0.2$-2 μM, 0.01 M acetate (pH 3.65 (*1*), pH 4.05 (*2*), pH 4.55 (*3*)), and 0.01 M phosphate (pH 7.0 (*4*), pH 7.6 (*5*), pH 8.0 (*6*)).

activation energy for NIPAB hydrolysis by the two conformers was 26 and 40 kJ/mol, respectively.

pH stability dependence. The rate constants of the irreversible PA inactivation were determined over a broad pH interval, at 25 and 50°C, and constant ionic strength (Fig. 2). The irreversible enzyme inactivation followed the kinetics of first order reactions (Fig. 3). The enzyme stability strongly depended on the pH as well as on the temperature. The logarithm of the PA inactivation constant is characterized by a reciprocal bell-shaped pH dependence. The left and right branches of this experimental dependence are approximated by straight lines with a slope −3/+1 at 25°C and −2/+1 at 50°C, respectively. Such dependencies indicate existence of at least 4 ionic forms of PA (acidic EH_4^{3+}, EH_3^{2+}, EH_2^+, and alkaline E^-), as well as a neutral one (EH), which differ from each other in their inactivation rate constant. The pH profile of PA thermostability was described according to the minimal kinetic Scheme below.



"Minimal" kinetic scheme of the irreversible PA thermal inactivation taking into account the following ionic forms of the enzyme in equilibrium: acidic EH_3^{2+} and EH_2^+, neutral EH, and alkaline E^-

Basing on estimated k_{in} values it could be assumed that the observed inactivation is completely due to the inactivation of the acidic and alkaline forms of PA, even in the pH stability optimum when the overall concentration of the acidic and the alkaline forms is very low compared to the concentration of the neutral one.

pH profile of activity. PA was very stable in the pH optimum of the catalytic activity (Table 1), whereas at the extreme pH values the enzyme displayed low activity and stability. However, the low activity in the acidic as well as in the alkaline media was not due to the enzyme inactivation. At the pH equal to the pK_a of PA ionogenic group, controlling enzymatic activity in alkaline medium, the half-life period $t_{1/2}$ exceeded the reaction time by a factor of more than a thousand.

DISCUSSION

Hydrophobic interactions and electrostatic contact of oppositely charged groups are the most important factors of protein folding and maintaining its structure [19, 20]. Their role is reflected in the protein amino acid composition (Table 2), and, in particular, the higher content of charged amino acids should be mentioned in thermophilic proteins compared to mesophilic ones. The

Table 1. pH profile of PA catalytic activity and stability (25°C, ionic strength 0.12 M)

pH 8.0			pH = pK_b*			pH 10		
k_{cat}, sec^{-1}	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$	$t_{1/2}$, days	k_{cat}, sec^{-1}	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$	$t_{1/2}$, min	k_{cat}, sec^{-1}	k_{cat}/K_m, $M^{-1}\cdot sec^{-1}$	$t_{1/2}$, h
27	$1.4 \cdot 10^6$	~6.0**	26	$6.5 \cdot 10^5$	550	16	$1.6 \cdot 10^5$	0.90

* pK_a and pK_b values are equal to 6.1 and 9.1 [27].
** Expected values of half-life according to the linear dependence presented in Fig. 2.

Table 2. Relative amino acid composition of PA in comparison with proteins of mesophiles and thermophiles

Enzyme	Amino acid residues*, %		
	hydrophobic	polar	charged
E. coli PA	42	36	22
Thermophiles**	43	27	30
Mesophiles**	45	31	24

* Amino acid residues are relatively grouped as hydrophobic (L, M, I, V, W, P, A, F), polar (G, S, T, N, Q, Y, C), and charged (D, E, K, R, H).
** Data from [19].

resulting free energy of overall interactions, including (i) interactions of amino acid residues with the environment and (ii) entropy factor, could be considered as a free energy of stabilization of globular proteins in solutions estimated as 50 kJ/mol [19].

Some stabilization effect of salting out reagents against heat denaturation of PA [21-23] reveals an important role of hydrophobic interactions, and the impact of the ionization state of corresponding groups discloses the role of ion pairs for maintaining the active enzyme conformation. Basing on these data it can be suggested that PA inactivation proceeds in the following way: native PA conformation at 25°C is maintained by three salt bridges between ionogenic groups of the enzyme, and at neutral pH the enzyme is extremely stable. Destruction of the salt bridges due to the protonation or deprotonation of the amino acid residues forming these ion pairs causes inactivation by formation of the unstable enzyme forms. A conformational transition in protein structure apparently takes place with increasing temperature, and as a result one of the three ion pairs is destroyed. This explains the difference in the pH stability profile at 25 and 50°C. The proposed mechanism of maintaining enzyme tertiary structure is in agreement with investigations on pH- and temperature-controlled conformational transitions in the PA active center [18] and with conceptions concerning the conformational changes in the enzyme active center based on X-ray data [24-26].

Adequate fitting of our experimental data can be obtained assuming existence of ion pairs formed by acidic groups with pK_a 1.3, 1.3, 3.5, and alkaline group with pK_b higher than 11.6 (presumably carboxylates of Asp/Glu and guanidine of an Arg residue). Asymmetric dependence in Fig. 2 indicates that the gegenion of two carboxylate anions has an aprotonic nature. Calcium ion could be such a positively charged center, which coordinates side chains of glutamate and three aspartates on the basis of X-ray analysis [25], and of which the functional role is unknown.

This investigation disclosed the role of ion pairs for maintaining the stable conformation of PA active center and characterized the pH stability profile of the enzyme quantitatively. The question about the role of Ca^{2+} and, probably, other metal ions, as well the role of individual amino acid residues of the protein for its stability and catalytic properties requires more detailed consideration and is the scope of further investigations.

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REFERENCES

1. Bruggink, A., Roos, E. C., and de Vroom, E. (1998) *Org. Process Res. Dev.*, **2**, 128-133.
2. Solodenko, V. A., Belik, M. Y., Galushko, S. V., Kukhar, V. P., Kozlova, E. V., Mironenko, D. A., and Švedas, V. K. (1993) *Tetrahedron: Asymmetry*, **4**, 1965-1968.
3. Švedas, V. K., Savchenko, M. V., Beltser, A. I., and Guranda, D. F. (1996) *Ann. N.-Y. Acad. Sci.*, **799**, 659-669.
4. Baldaro, E., D'arrigo, P., Pedrocchi-Fantoni, G., Rossell, C. M., Servi, S., Tagliani, A., and Terreni, M. (1993) *Tetrahedron: Asymmetry*, **4**, 1031-1034.
5. Soloshonok, V. A., Soloshonok, I. V., Kukhar, V. P., and Švedas, V. K. (1998) *J. Org. Chem.*, **63**, 1878-1884.

6. Chilov, G. G., and Švedas, V. K. (2002) *Can. J. Chem.*, **80**, 699-707.
7. Chilov, G. G., Moody, H. M., Boesten, W. H. J., and Švedas, V. K. (2003) *Tetrahedron: Asymmetry*, **14**, 2613-2617.
8. Švedas, V., Guranda, D., van Langen, L., van Rantwijk, F., and Sheldon, R. (1997) *FEBS Lett.*, **417**, 414-418.
9. Guranda, D. T., van Langen, L. M., van Rantwijk, F., Sheldon, R. A., and Švedas, V. K. (2001) *Tetrahedron: Asymmetry*, **12**, 1645-1650.
10. Margolin, A. L., Izumrudov, V. A., Švedas, V. K., Zenin, A. B., Kabanov, V. A., and Berezin, I. V. (1981) *Biochim. Biophys. Acta*, **660**, 359-365.
11. Yamskov, I. A., Budanov, M. V., and Davankov, V. A. (1981) *Biokhimiya*, **46**, 1603-1608.
12. Guisan, J. M., Alvaro, G., Fernandez-Lafuente, R., Rossel, C. M., Garcia, J. L., and Tagliani, A. (1993) *Biotechnol. Bioeng.*, **42**, 455-464.
13. Kazan, D., Ertan, H., and Erarslan, A. (1997) *Appl. Microbiol. Biotechnol.*, **48**, 191-197.
14. Ospina, S. S., Lopez-Munguia, A., Gonzalez, R. L., and Quintero, R. (1992) *Chem. Tech. Biotechnol.*, **53**, 205-214.
15. Scherbakova, T. A., Korennykh, A. V., van Langen, L. M., Sheldon, R. A., and Švedas, V. K. (2004) *J. Mol. Cat. B. Enzym.*, in press, MOLCAB1137.
16. Yousko, M. I., Shamolina, T. A., Guranda, D. F., Synev, A. V., and Švedas, V. K. (1998) *Biochemistry (Moscow)*, **63**, 1104-1109.
17. Švedas, V. K., Margolin, A. L., Sherstyuk, S. F., Klyosov, A. A., and Berezin, I. V. (1977) *Bioorg. Khim.*, **3**, 546-553.
18. Berezin, I. V., Klibanov, A. M., Klyosov, A. A., Martinek, K., and Švedas, V. K. (1975) *FEBS Lett.*, **49**, 325-328.
19. Jaenicke, R. (2000) *J. Biotechnol.*, **79**, 193-203.
20. Petersen, S. B., Jonson, P. H., Fojan, P., Petersen, E. I., Petersen, M. T. N., Hansen, S., Ishak, R. J., and Hough, E. (1998) *J. Biotechnol.*, **66**, 11-26.
21. Andersson, E., and Hahn-Hagerdal, B. (1987) *Biochim. Biophys. Acta*, **912**, 317-324.
22. Azevedo, A. M., Fonseca, L. P., and Prazeres, D. M. F. (1999) *J. Chem. Technol. Biotechnol.*, **74**, 1110-1116.
23. Kheirolomoom, A., Ardjamand, M., Vossoughi, M., and Kazemeini, M. (1998) *Biochem. Eng. J.*, **2**, 81-88.
24. Done, S. H., Brannigan, J. A., Moody, P. C. E., and Hubbard, R. E. (1998) *J. Mol. Biol.*, **284**, 463-475.
25. McVey, C. E., Walsh, M. A., Dodson, G. G., Wilson, K. S., and Brannigan, J. A. (2001) *J. Mol. Biol.*, **313**, 139-150.
26. Alkema, W. B. L., Hensgens, C. M. H., Kroezinga, E. H., de Vries, E., Floris, R., van der Laan, J.-M., Dijkstra, B. W., and Janssen, D. B. (2000) *Prot. Eng.*, **13**, 857-863.
27. Morillas, M., Goble, M. L., and Virden, R. (1999) *Biochem. J.*, **338**, 235-239.