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SUBSTRATE SPECIFICITY OF PENICILLIN AMIDASE FROM *E. COLI*

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Summary

1. The kinetic parameters of 12 substrates of penicillin amidase (penicillin amidohydrolase, EC 3.5.1.11) from *E. coli* have been determined. Most of the penicillin amidase amide substrates containing a phenylacetyl group in the acyl moiety have been shown to have similar catalytic constants of 50 s^{-1}. Substitution of the phenylacetyl group by 2-thienylacetyl group (cephalothin, cephaloridine) having a similar structure leads to a slight decrease in k_{cat}.

2. Nonspecific penicillin amidase substrates, which contain a free amino group in their acyl moiety, are characterized by a strong dependence of k_{cat}, on the structure of the leaving group with K_m being constant. To investigate the free amino group influence on the reaction kinetics, pH-dependences of k_{cat}/K_m of enzymatic hydrolysis of phenylacetic and D-(—)-α-aminophenylacetic acid *p*-nitroanilides have been studied. It has been shown that enzyme binds the deprotonated form of the substrate only.

3. Under thermodynamically favourable conditions for the synthesis of β-lactam antibiotics (at low pH), a concentration of the deprotonated substrate form is very low, and the reaction proceeds in the bimolecular regime. The value of the second-order rate constant for the substrate having a free amino group is small even at pH 7.5, and sharply decreases as does the pH. Hence, despite the favourable thermodynamic conditions for the production of all β-lactam antibiotics, low reaction rate is the basic hindrance for enzymatic synthesis of penicillins and cephalosporins having a free amino group in the acyl moiety.

Introduction

Penicillin amidase (penicillin amidohydrolase EC 3.5.1.11) from *E. coli* catalyzes the hydrolysis of various penicillins and cephalosporins, *N*-acylated

amino acids, amides and esters. The substrate specificity of this enzyme has been extensively studied [1—6]. It has been shown that penicillin amidase from this source is highly specific to phenylacetyl compounds [2,7]. The structure of the leaving group of substrates hardly affects the hydrolysis rate [7]. However, despite the progress in this field, there are significant differences in the kinetic parameter values that have been reported for the reactions catalyzed by penicillin amidase. For example, K_m values for benzylpenicillin determined by various authors lie in the range of $4.5 \cdot 10^{-3}$ M [8] and $7.4 \cdot 10^{-4}$ M [9] to $2 \cdot 10^{-5}$ M [10] and $4.6 \cdot 10^{-6}$ M [11], pH 7—8.5, i.e., differ by several orders of magnitude. Berezin et al. [11] suggested that high K_m values are due to strong competitive inhibition of the enzyme by the product of hydrolysis, phenylacetic acid ($K_i = 2.8 \cdot 10^{-5}$ M).

It should also be noted that in all previous papers only the values of the maximum rate (V) of hydrolysis of various substrates, with respect to benzylpenicillin, were measured. The absolute values of the kinetic constants and the effectiveness of catalysis of penicillin amidase compared with other enzymes, can hardly be inferred from the literature data since partially purified enzyme preparations of unknown concentration were frequently used.

After a rapid and effective method of determination of penicillin amidase concentration [12,13], by titration of the enzyme with a phenylmethylsulfonyl fluoride solution was developed in our laboratory, it became possible to measure the true values of the catalytic constants (k_{cat}) and the second-order rate constants (k_{cat}/K_m).

In the present work, data on the kinetic parameters of 12 penicillin amidase substrates are reported and the specificity of the interaction between this enzyme and substrates containing a free amino group in the acyl moiety is discussed.

Materials and Methods

Penicillin amidase from *E. coli* was isolated and purified as described previously [11]. Commercial preparations of cephaloridine (Ceporin), cephalexin (Ceporex) and cephalothin (Keflin) manufactured by Eli Lilly and Co. and Pliva were used. Ethyl phenylacetate was from Koch-Light and phenylmethylsulfonyl fluoride from Sigma. 7-Phenylacetamidodeacetoxycephalosporanic acid (7-PADCA) and penicillin amidase preparations were a kind gift from the All-Union Scientific Research Institute of Antibiotics.

Phenylacetic acid *p*-nitroanilide, *p*-nitrophenyl phenylacetate and phenylacetylglycine were synthesized by us according to the standard techniques. *p*-Nitroanilide of D-(—)-α-aminophenylacetic acid was synthesized according to the method of Tuppy et al. [14]. Benzylpenicilloic acid was obtained by enzymatic hydrolysis of benzylpenicillin with β-lactamase.

The kinetics of enzymatic hydrolysis were studied under standard assay conditions with excess substrate over the enzyme ($[S]_0 >> [E]_0$). The catalytic parameters (k_{cat}; K_m) were presented as Lineweaver-Burk plots. Analysis of the experimental data and calculation of the mean-square errors were carried out by a PDP 8/E computer.

The kinetics of enzymatic hydrolysis of benzylpenicillin, 7-PADCA, cephalo-

thin, cephaloridine, benzylpenicilloic acid, phenylacetic acid, *p*-nitroanilide and ethyl and *p*-nitrophenyl phenylacetates were studied by alkaline titration (0.01 KOH), using a Radiometer pH-stat (TTT-1c, Denmark). All the experiments were carried out at 25 ± 0.2°C in 0.1 M KCl solution in a buffer-free medium.

The kinetics of hydrolysis of *p*-nitrophenyl phenylacetate and of *p*-nitroanilides of phenylacetic and D-(—)-α-aminophenylacetic acid were studied on a fast 'GEMSAEC' analyzer at a 400 nm wavelength.

In this instrument, the spectrophotometric cells (15 working cells and one reference cell) were filled with the solutions automatically. The rotor, which has 16 cells divided into chambers (A, B and C) is filled by four syringes (vol. 0.02—1 ml). The buffer solution and the substrate (475 μl) were fed to chamber C, and the enzyme solution of a certain concentration to chamber B. The kinetic curve was recorded after movement of the rotor to the measuring block, stirring and temperature stabilization. It is possible to determine 20 values of the absorbance with a constant time interval (5—10 s) between them. Plots of absorbance vs. time obtained in each cell were treated in a PDP8/E computer according to a program. Not only the absorbance values and those of the initial reaction rate were typed out but also the resulting (k_{cat} and K_m) values with respective errors.

When we determined the pH dependences of k_{cat}/K_m, a solution of the substrate in a universal buffer mixture (0.04 M solution of phosphoric, boric and acetic acids and 0.2 M NaOH) was used. To study the kinetics of ampicillin and cephalexin hydrolysis the indicator reaction with *p*-dimethylaminobenzaldehyde was used [15,16].

To calculate the pK_a and pK_b values, a special program was devised for a PDP8/E computer. The resulting values are optimal, that is they give the best fit with the experimental data.

Results and Discussion

Determination of the kinetic parameters of various penicillin amidase substrates

The kinetic parameters of the penicillin amidase substrates studied are presented in Table I.

The native penicillin amidase substrate, benzylpenicillin, has the highest bimolecular constant value, k_{cat}/K_m, of $1 \cdot 10^7\ M^{-1} \cdot s^{-1}$ compared to all the compounds investigated. Most of the amide penicillin amidase substrates, containing phenylacetic group in the acyl moiety have similar values of the catalytic constants of $50\ s^{-1}$. The values of both esteric substrates are also identical and equal to $170\ s^{-1}$. Comparison of k_{cat} for benzylpenicillin, benzylpenicilloic acid and phenylacetic acid *p*-nitroanilide shows that it is not affected by the system of β-lactam and thiazolidine rings. Substitution of the phenylacetic group by 2-thienylacetic group (cephalothin, cephaloridine), having a similar structure and electron properties, leads to a slight decrease in k_{cat}. On the other hand, substitution of the leaving group in the benzylpenicillin results in a much weaker binding and in a decrease of the second-order rate constant.

Consequently, the opinion expressed more than once in the literature, that the specificity of the penicillin amidase substrate depends entirely on the substrate acyl moiety, is true of the saturating concentration of the substrates

TABLE I

SUBSTRATE SPECIFICITY OF PENICILLIN AMIDASE

(pH 7.5, 25°C)

Substrate	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Benzylpenicillin	48	$4.6 \cdot 10^{-6}$	$1.0 \cdot 10^{7}$
7-PADCA	50	$1.0 \cdot 10^{-5}$	$5.0 \cdot 10^{6}$
Ethyl phenylacetate	170	$4.5 \cdot 10^{-5}$	$3.8 \cdot 10^{6}$
p-Nitrophenyl phenylacetate	170	$3.1 \cdot 10^{-5}$	$5.5 \cdot 10^{6}$
Phenylacetate *p*-nitroanilide	55	$9.7 \cdot 10^{-5}$	$5.7 \cdot 10^{5}$
Phenylacetylglycine	47	$8.0 \cdot 10^{-5}$	$5.9 \cdot 10^{5}$
Benzylpenicilloic acid	40	$2.0 \cdot 10^{-3}$	$2.0 \cdot 10^{4}$
Cephalothin	25	$4.2 \cdot 10^{-5}$	$0.6 \cdot 10^{6}$
Cephaloridine	33	$1.0 \cdot 10^{-4}$	$3.3 \cdot 10^{5}$
Ampicillin	11	$5.2 \cdot 10^{-3}$	$2.2 \cdot 10^{3}$
Cephalexin	54	$2.1 \cdot 10^{-3}$	$2.6 \cdot 10^{4}$
D-(-)-α-aminophenylacetic acid *p*-nitroanilide	0.54	$3.2 \cdot 10^{-3}$	157

only. To judge by the second-order rate constants, the leaving group strongly influences the specificity.

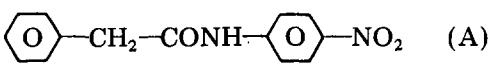
Penicillins and cephalosporins, containing a free amino group in their acyl moiety (ampicillin, cephalexin etc.), represent another large and important group of β-lactam antibiotics. These compounds are nonspecific penicillin amidase substrates. It is noteworthy that in this case, k_{cat} values of the enzymatic hydrolysis are determined not only by the structure of the acyl moiety but by the structure of its leaving group. Thus, on substitution of cephalexin by D-(—)-α-aminophenylacetic acid *p*-nitroanilide, the k_{cat} value decreases by two orders, and the K_m value remains the same.

Another interesting feature of the substrates containing a free amino group in the acyl moiety is pH-dependence of the second-order rate constant of hydrolysis.

pH-dependence of the second-order rate constant of hydrolysis

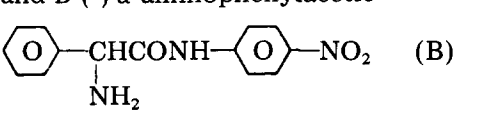
It is known that study of pH dependences of the kinetic constants of enzymic reactions can yield essential information about the mechanism of enzyme-substrate interaction. In this work, we tried to elucidate the distinguishing features of the interaction of penicillin amidase, with compounds containing an amino group in the acyl moiety, compared to the interaction of the enzyme with its specific substrates.

We studied pH-dependences of the second-order rate constants of enzymatic hydrolysis of the two model reactions. *p*-Nitroanilides of the phenylacetic



(A)

and D-(-)-α-aminophenylacetic



(B)

acids were chosen as models.

In the first case, the pH-dependence of k_{cat}/K_m is a standard bell-shaped curve, which is typical of other penicillin amidase substrates. It can be described by 2 pK values equal to 5.3 and 9.6, and k_2/K_s of $57 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ (Fig. 1), which markedly differ from those obtained for benzylpenicillin [17]. These pK values correspond to the ionogenic groups of the native enzyme which are responsible for transitions between three equilibrium forms of the enzyme, i.e., 'acid', 'neutral' and 'base' of which only the neutral form is active with respect to the substrate.

In case B, the pH-dependence of k_{cat}/K_m differs greatly from that in case A. For the description of such a dependence, it is necessary to take into account the dissociation of the substrate amino groups. Indeed, in these compounds, the pK of the amino group is close to 7 (for ampicillin, pK 7.2; for B it is 7.1). This means that over the wide pH range used two forms of the substrate exist, i.e., protonated and deprotonated. Let us assume that only one of these forms can be bound by an enzyme. If only the deprotonated form is bound, the pH-dependence of k_{cat}/K_m is described by Eqn. 1. In the case of the protonated form, it is described by Eqn. 2.

$$\frac{k_{cat}}{K_m} = \frac{k_2/K_s}{\left(1 + \frac{H^+}{K_a} + \frac{K_b}{H^+}\right)\left(1 + \frac{H^+}{K_c}\right)} \quad (1)$$

$$\frac{k_{cat}}{K_m} = \frac{k_2/K_s}{\left(1 + \frac{H}{K_a} + \frac{K_b}{H^+}\right)\left(1 + \frac{K_c}{H^+}\right)} \quad (2)$$

where K_a and K_b are the ionization constants of free enzyme ionogenic groups and K_c is the dissociation constant of the substrate amino group. It is possible

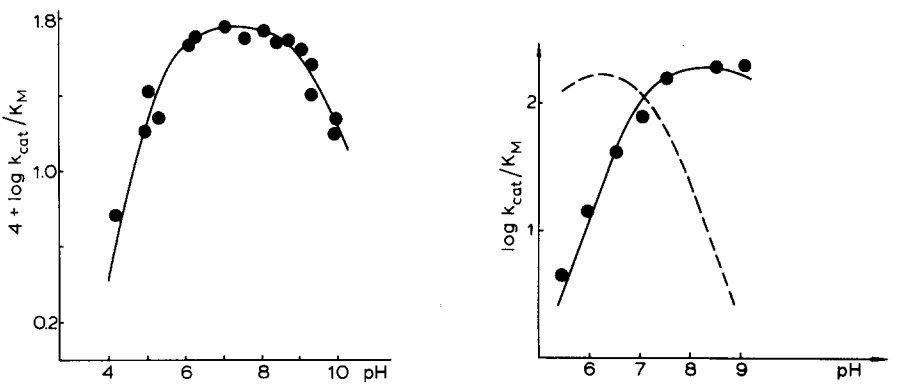


Fig. 1. pH-dependence of the second-order rate constant of phenylacetic acid *p*-nitroanilide hydrolysis. Solid line is a theoretical curve for pK_a 5.3, pK_b 9.6 and k_{cat}/K_m $5.7 \cdot 10^5$ $M^{-1} \cdot s^{-1}$. The values of k_{cat}/K_m are presented in $M^{-1} \cdot s^{-1}$. Conditions: 25°C, 0.1 M KCl. $[S]_0 = 0.22 \cdot 10^{-4}$ M, $[E]_0 = 0.3 \cdot 10^{-8}$ M.

Fig. 2. pH-dependence of the second-order rate constant of D-(—)-α-aminophenylacetic acid *p*-nitroanilide hydrolysis. Theoretical curves are calculated for pK_a 5.3, pK_b 9.6; pK_c 7.1 and k_{cat}/K_m 220 $M^{-1} \cdot s^{-1}$ in the case of the deprotonated form binding (solid line) and protonated from (broken line) only. The values of k_{cat}/K_m are presented in $M^{-1} \cdot s^{-1}$. Conditions: 25°C, 0.1 M KCl. $[S]_0 = 0.2$—5 mM. $[E]_0 = 5 \cdot 10^{-8}$ M.

to plot the theoretical dependences of k_{cat}/K_m vs. pH for both cases, on the basis of pK_c 7.1, k_2/K_s determined from the experimental data of Fig. 1, and of pK_a 5.3 and pK_b 9.6, assuming that they do not depend on the nature of the substrate (Fig. 2). The experimental data in this figure unambiguously point to the binding of the deprotonated form only. This conclusion is proved by extremely high hydrophobicity of the penicillin amidase sorption site previously obtained in this laboratory [18].

Some comments on enzymatic synthesis of ampicillin

It is seen from the literature that the substrate specificity of hydrolysis of penicillin amidase is identical to that of synthesis [19]. The data obtained allow some assumptions to be made about enzymatic synthesis of ampicillin and other β-lactam antibiotics containing a free amino group in their acyl moiety.

Until now, numerous attempts to synthesize ampicillin directly from the free acid with the help of penicillin amidase from *E. coli* have failed.

Now we can explain this, the pK value of D-(—)-α-aminophenylacetic acid is 9.1. The maximum velocity of the enzymatic reaction does not depend on the form of the substrate bound with the enzyme. But since K_m depends on the concentration of the deprotonated form, at low pH values we always have the bimolecular reaction. This means that at pH 6.0 the solution will contain less than 0.1% of the deprotonated substrate form, i.e., the form which makes the reaction possible. Hence, a considerable increase in the effective binding constant and a decrease in k_{cat}/K_m.

The synthesis is impossible at rather high pH values (pH > 7.0) for thermodynamic reasons, when the concentration of the deprotonated form of the acid is high [16]. Under thermodynamically optimal conditions, the synthesis has an extremely low rate.

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