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Chemical mechanism of penicillin V acylase from *Streptomyces lavendulae*: pH-dependence of kinetic parameters

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Abstract

The variation of kinetic parameters of penicillin V acylase from *Streptomyces lavendulae* with pH was used to gain information about the chemical mechanism of the hydrolysis of penicillin V catalyzed by this enzyme. The pH-dependence of V_{max} showed that a group with a pK value of 6.45 (pK_1) must be unprotonated for activity. The pH-dependence of V_{max}/K_m showed that a group with a pK value of 7.1 (pK_1) must be unprotonated and a group with a pK of 10.83 (pK_2) must be protonated for activity. The lower pK value corresponded to a group in the enzyme involved in catalysis and whose protonation state also affects binding. The higher pK value was only involved in binding. Results from chemical modification studies showed the importance of serine residues in the catalytic mechanism of the enzyme and pointed to the identity of the groups responsible for pK_1 and pK_2 as the α-amino nitrogen of the N-terminal residue and a lysine residue, respectively.

Keywords: *Streptomyces lavendulae*; Penicillin V acylase; Kinetic parameters; Essential aminoacids

1. Introduction

Penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) catalyzes the hydrolysis of penicillins into 6-aminopenicillanic acid (6-APA) and an organic acid that depends on the type of penicillin [1]. Two types of enzymes, penicillin V acylase (PVA) and penicillin G acylase (PGA), with different substrate specificities, account for the enzymatic industrial production of 6-APA, the precursor of semisynthetic penicillins [2].

Penicillin V acylase is widely distributed among microorganisms, molds, yeast, bacteria and actinomycetes being intracellularly produced in most cases. However, the enzyme from *Streptomyces lavendulae* is extracellular and able to hydrolyze phenoxymethyl penicillin (PV) and aliphatic penicillins (heptyl, 2-pentenyl or pentyl penicillins) [3,4]. This hydrolytic capability revealed this enzyme as very interesting for industrial production of 6-APA from the bulk raw material penicillin V that always contains significative amounts (2–3%) of these aliphatic penicillins.

Recently, we have shown the enhancement of the production of penicillin V acylase from *S. lavendulae* [4] and some of its properties have been described [5].

The enzyme catalyses the hydrolysis of penicillins and synthetic analogs as 2-nitro-5-(phenoxyacetamido) benzoic acid (NIPOAB) through an ordered Uni Bi

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; DIFP, diisopropylfluorophospate; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNM, tetranitromethane; DEP, diethylpyrocarbonate; NBS, *N*-bromosuccinimide; DMSO, dimethylsulfoxide

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mechanism (Torres-Guzmán et al., in press), providing evidence in favor of an acyl-enzyme catalytic intermediate as in the case of penicillin G acylase from *Escherichia coli* and *Kluyvera citrophyla* [6,7].

A class of amidohydrolases, the so-called N-terminal nucleophile hydrolases (Ntn-hydrolases) have been recognized recently [8,9]. Two such enzymes are penicillin acylase and the prokaryotic proteasome catalytic subunit. In these two enzymes, the side chain that provides the nucleophile is the N-terminal residue whose α-amino nitrogen acts as a base. This N-terminal nucleophile has been identified as a serine in penicillin G acylase from *E. coli* [6,10] and from *K. citrophyla* [11], being a cysteine in penicillin V acylase from *Bacillus sphaericus* [12].

The present paper reports evidence about the chemical mechanism of penicillin V acylase from *S. lavendulae* by examining the pH-dependence of kinetic constants of the enzyme and the variation with pH of K_i for phenoxyacetic acid as competitive inhibitor. The results are supported using the chemical modification approach with amino acid specific reagents in order to selectively modify amino acids involved in the catalytic mechanism of the enzyme.

2. Materials and methods

2.1. Chemicals

Penicillin V potassium salt, phenoxyacetic acid, fluorescamine, Caps, tricine, PMSF, DEP, were from Sigma (USA). *N*-acetyl imidazole, iodoacetic acid, TNBS, DIFP, cyanate, TNM and sodium *p*-hydroxy mercurybenzoate were from Fluka (Germany). All other reagents and products were from Merk (Germany).

2.2. Enzyme purification

Penicillin V acylase from *S. lavendulae* spp. lavendulae ATCC 13664 was purified from culture supernatants as previously described [5].

2.3. Kinetic assays

The activity of penicillin V acylase was determined at 40°C in incubation mixtures containing 0.8–1 μg/ml of enzyme (showing over 60 IU/mg) and increasing concentrations of penicillin V (1–100 mM) in 100 mM potassium phosphate buffer pH 8 for 20 min. The reaction was stopped by addition of 0.5 M sodium acetate buffer pH 4.5. After centrifugation, the release of 6-APA was monitored by adding Fluram (0.1% fluorescamine in acetone) to the supernatant. After 40 min of reaction at room temperature, absorbance was recorded at 378 nm in a Beckman DU-70 spectrophotometer [13]. The product formation proceeded linearly with time under all the conditions used in kinetic experiments.

The pH-dependence of kinetic parameters was determined in 25 mM phosphate/pyrophosphate (neutral acid) or Caps/imidazole/trizine (cationic) buffers in the temperature range 30–42°C. The enzyme was stable in the temperature and pH range assayed. The ionic strength at each pH was adjusted to 400 mM by addition of NaCl in amounts calculated using a Visual Basic program developed in our laboratory, which allows analysis systems with up to four tetraprotic species. Solvent perturbation studies with 20% DMSO were performed in both neutral acid and cationic buffer systems. The organic solvent was added after determination of the pH in the assay mixture. DMSO (20%) causes no loss of enzyme activity and the enzyme was stable during the reaction time. The pH-dependence of K_i was examined for phenoxyacetic acid as competitive inhibitor of penicillin V acylase. Assays were run at variable concentrations of penicillin V (1–100 mM) with phenoxyacetic acid varied from 0 to 200 mM at 40°C for 20 min. Results in figures and tables are the mean value of triplicate experiments, and the error intervals were lesser than 10% for all data points in the figures. Penicillin V and 6-APA were stable under the experimental conditions (pH, temperature and time of reaction). Moreover, as stated above, product formation proceed linearly, at least for 35 min, at 40–42°C, pH 6, 10 and 11 both in the absence and in the presence of 20% DMSO.

2.4. Statistical analysis of data

Values of kinetic constants were determined by fitting initial velocity data to the Woolf–Augustinson–Hoffstee equation [14] by a weighted least-squares method developed in our laboratory. Substrate saturation kinetics were fitted to Eq. (1). Inhibition constants

(K_i) at each pH were calculated by fitting the corresponding kinetic data in the presence of a competitive inhibitor to Eq. (2):

$$V = \frac{V_{max} S}{K_m + S} \quad (1)$$

$$V = \frac{V_{max} S}{K_m(1 + I/K_i) + S} \quad (2)$$

Data for pH profiles showing a decrease in $\log V_{max}$ with a slope of 1 as the pH is lowered were fitted to Eq. (3):

$$Y = \frac{C}{1 + ([H^+]/K_1)} \quad (3)$$

Data for pH profiles showing a decrease in $\log(V_{max}/K_m)$ with a slope of 1 as the pH is lowered and a slope of -1 as it is raised were fitted to Eq. (4):

$$Y = \frac{C}{1 + (([H^+]/K_1) + (K_2/[H^+]))} \quad (4)$$

pK_i for phenoxyacetic acid as competitive inhibitor was calculated by fitting the data from the corresponding pH profiles to Eq. (4). The enthalpies of ionization of enzyme residues were determined by fitting the pK values obtained from pH profiles at different temperatures to Eq. (5):

$$pK = \frac{\Delta H_{ion}}{2.3RT} \quad (5)$$

In Eqs. (1) and (2), V and V_{max} are initial and maximum velocity, respectively, K_m the Michaelis constant, S the substrate concentration, I the inhibitor concentration and K_i is the inhibition constant. In Eqs. (3) and (4), K_1 and K_2 are the dissociation constants of groups that must be deprotonated and protonated, respectively, for activity, Y is V_{max} or V_{max}/K_m and C is the value of Y attained at the optimum state of protonation. In Eq. (5) ΔH_{ion} is the enthalpy change of dissociation, R the gas constant and T is the absolute temperature.

2.5. Chemical modification studies

2.5.1. Serine modification

Penicillin V acylase (1.4 μg/ml) in 20 μl of 2 mM potassium phosphate buffer pH 7, was incubated at room temperature for 30 min with 5 μl of 15 μM to 100 mM PMSF in DMSO or DIFP ethanolic solution. The concentration of organic solvents in the enzymatic mixture did not exceed 20% (v/v) and was found to have no noticeable effect on stability or activity of the enzyme. The reaction was stopped by adding 45 μl 200 mM potassium phosphate buffer pH 8 and ice cooling. The enzyme activity was measured in 10 μl aliquots after addition of 30 mM substrate under standard assay conditions.

To analyze protection by substrate (penicillin V) or by a competitive inhibitor (phenoxyacetic acid) against enzyme inactivation by the chemical reagent, 100 mM penicillin V or phenoxyacetic acid in the corresponding buffer, was present during the incubation of the enzyme with the reagent. The inactivation conditions were as described above.

2.5.2. Histidine modification

Enzyme modification with DEP and protection of enzyme against inactivation were carried out as described for serine modification. Taking into account that DEP decomposes spontaneously, stock solutions were titrated with histidine according to the method described by Miles [15].

2.5.3. Lysine modification

Penicillin V acylase was modified with TNBS (in methanolic solution) in the same conditions as described above. Protection with penicillin V or phenoxyacetic acid was carried out in the same conditions as described above.

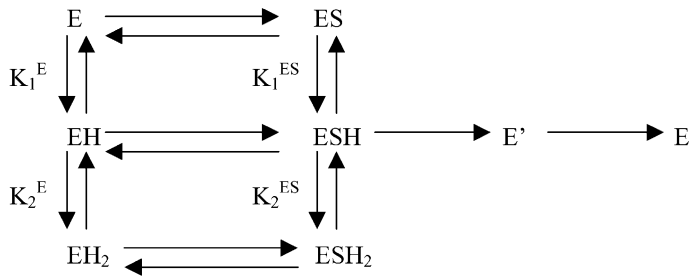
2.5.4. α-Amino modification

Penicillin V acylase was modified with potassium cyanate in the same conditions as described above, but in this case the incubations with the chemical reagent were carried out overnight. Protection experiments were run as described above.

3. Results

The experiments reported here demonstrate that penicillin V acylase lost its activity at low and high pH according to the mechanism given in Scheme 1.

If K_1^E and K_2^E are far apart the equation that describes the variation of the corresponding kinetic parameter with pH is Eq. (4). When the activity is lost



Scheme 1.

at low pH, the pH-dependence of the corresponding kinetic parameter is described by Eq. (3) [16].

The pH-dependence of kinetic parameters, V_{max} and V_{max}/K_m was determined over a pH range from 6 to 11. No irreversible inactivation was observed within this pH range at the assayed times of incubation. Fig. 1 shows the pH variation of V_{max} and V_{max}/K_m. The maximum velocity decreased at low pH, with a limiting slope close to 1, indicating that protonation of a single group causes loss of activity. The data were fitted to Eq. (3) (Fig. 1a) and indicated that a group with an apparent pK value of 6.45 ± 0.12 (pK_1) must be unprotonated for activity. The V_{max}/K_m profile (Fig. 1b) decreased both at low and high pH, with limiting

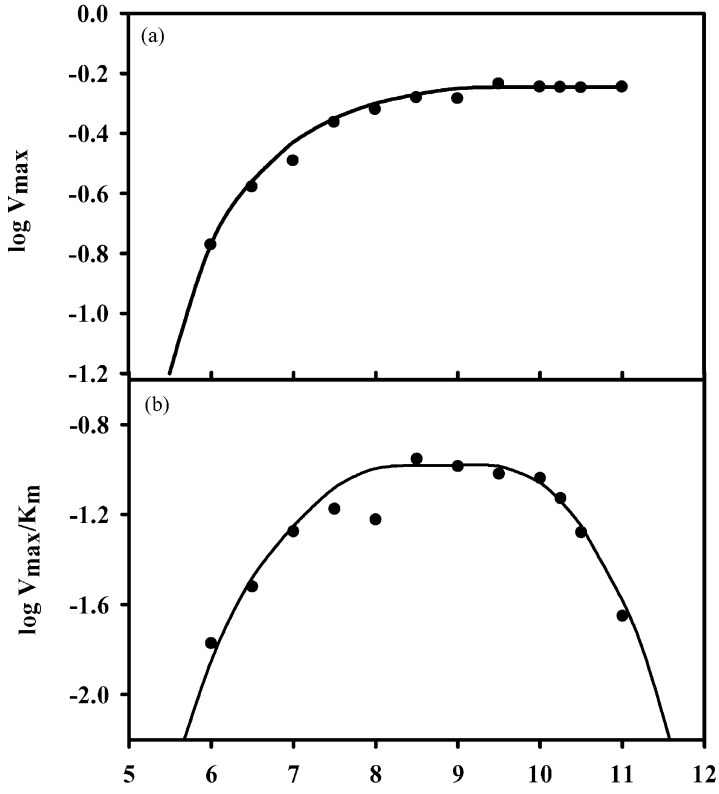


Fig. 1. pH-dependence of log V_{max} (a) and log(V_{max}/K_m) (b). Individual data points represent fits of initial velocities at different concentrations (1–100 mM) of penicillin V to Eq. (1). The curve through the data is an iterated fit to Eq. (3) for log V_{max} and to Eq. (4) for log(V_{max}/K_m).

slopes close to 1 and −1, respectively, and the data were fitted to Eq. (4). The data indicated an apparent pK of 7.10 ± 0.20 (pK_1) on the acidic side and an apparent pK of 10.83 ± 0.40 (pK_2) on the basic side. As substrate has no ionizable groups at these pK values it follows that the catalytic activity of penicillin acylase depends on ionization of essential residues on the enzyme. To gain information about the identity of the groups responsible for these pK values, the temperature-dependence of V_{max} and V_{max}/K_m profiles was examined. Experiments were carried out at 30, 35, 37, 40 and 42°C (Fig. 2). No irreversible thermal inactivation was observed at these temperatures at the pH range used. Data were fitted to Eq. (5) in order to obtain the corresponding ionization enthalpies (ΔH_{ion}):

from V_{max} profiles :

$$\Delta H_{ion} = 6.27 \pm 0.73 \text{ kcal/mol for p}K_1$$

from V_{max}/K_m profiles :

$$\Delta H_{ion} = 9.70 \pm 0.70 \text{ kcal/mol for p}K_1 \text{ and}$$

$$\Delta H_{ion} = 9.88 \pm 0.8 \text{ kcal/mol for p}K_2$$

Further evidence on the nature of the catalytic groups was obtained by examining the effect of organic

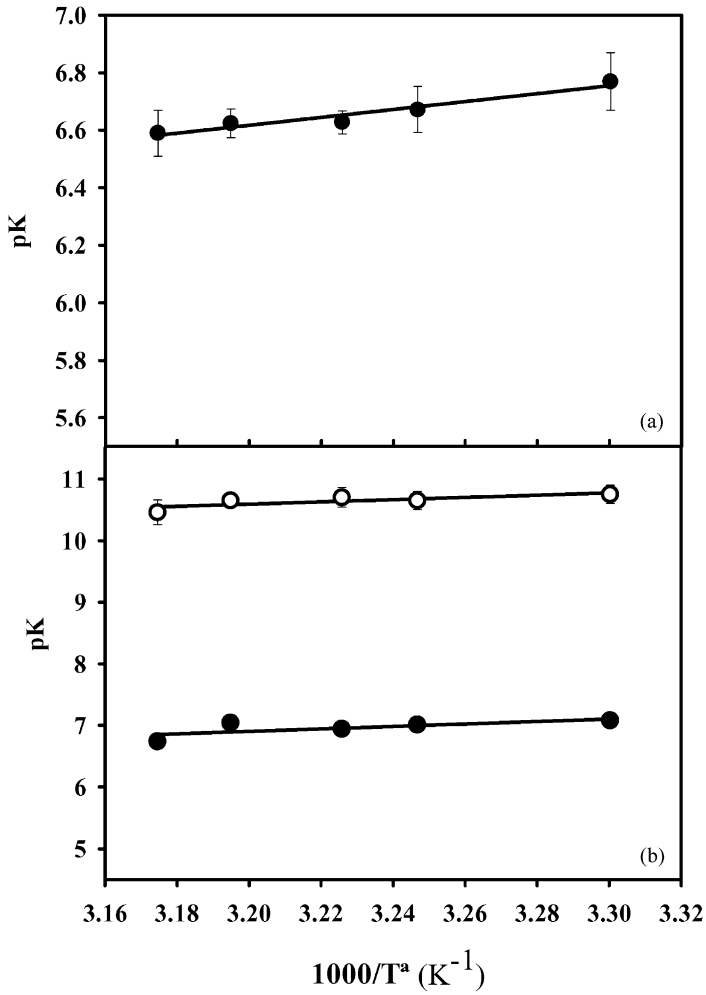


Fig. 2. Temperature-dependence of pK values from V_{max} (a) and V_{max}/K_m (b). Experiments similar to those in Fig. 1 were carried out at several temperatures (30–42°C) and the calculated values of pK_1 (●) and pK_2 (○) were plotted.

Table 1
Effect of 20% (v/v) DMSO on p*K* values in either neutral or cationic acid buffers[a]

		Neutral buffer		Cationic buffer	
		−DMSO	+DMSO	−DMSO	+DMSO
$\log V_{max}$	pK_1	6.45 ± 0.12	5.97 ± 0.13	6.93 ± 0.15	6.64 ± 0.2
	pK_2	–	–	–	–
$\log(V_{max}/K_m)$	pK_1	7.09 ± 0.19	6.47 ± 0.16	6.69 ± 0.15	6.34 ± 0.10
	pK_2	10.83 ± 0.3	10.87 ± 0.21	10.90 ± 0.1	11.01 ± 0.16

[a] p*K* values and their standard deviations were obtained by statistical analysis of the parameter data in accordance with Eq. (3) or (4).

solvents on the p*K* values in both neutral and cationic acid buffers (Table 1). Addition of 20% DMSO caused a decrease in the pK_1 value observed in neutral acid buffer, whereas this p*K* did not change significantly in cationic acid buffer, suggesting that the group responsible for this p*K* is the cationic acid type. However, pK_2 remained unchanged after the addition of 20% DMSO, in V_{max}/K_m profiles, either in neutral and cationic buffers.

The pK_i profile for a competitive inhibitor shows the effect of ionizations on binding only and has the virtue of giving correct p*K* values [17]. V_{max} and V_{max}/K_m profiles show the effect of ionizations on catalysis as well as in binding and thus a comparison of the two types of profiles is very useful to discriminate between groups responsible for catalysis from those involved in binding. The pH-dependence of K_i was studied with phenoxyacetic acid as a competitive inhibitor of the reaction with penicillin V (Fig. 3). Data were fitted to Eq. (4); the pK_i values were 6.81 ± 0.40 for the acid side and 10.39 ± 0.5 for the basic side, similar values to those observed for pK_1 and pK_2, respectively, in the V_{max}/K_m profile, indicating that the ionization of the same groups are seen in both profiles.

To ascertain the identity of the groups responsible for these p*K*'s, chemical modification studies were carried out by using chemical-directed reagents.

Penicillin V acylase was inhibited in the presence of PMSF. At concentrations above 10 mM PMSF, the enzyme lost all its activity. Protection of the enzyme

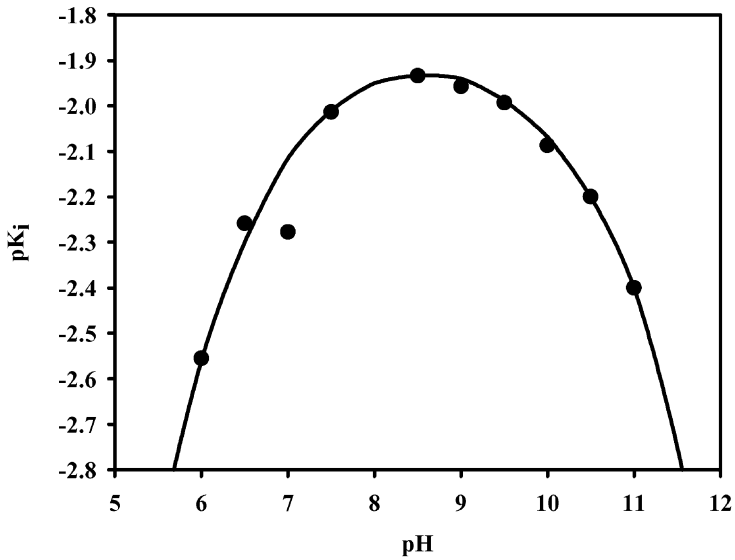


Fig. 3. Variation of pK_i with pH for phenoxyacetic acid. Experiments were carried out as in Fig. 1 with phenoxyacetic acid from 0 to 200 mM. K_i values were calculated by fitting the data to Eq. (2) and pK_i values were plotted vs. pH and fitted to Eq. (4).

inactivation with substrate or analogs is regarded as evidence of modification of residues at or near the active site. Penicillin V (100 mM) and phenoxyacetic acid (100 mM) protected the enzyme against inactivation, being penicillin V better protective agent than phenoxyacetic acid (70 and 40% of residual activity, respectively). This is consistent with the tight binding of penicillin V to the enzyme (K_m for penicillin V is 3 mM; K_i for phenoxyacetic acid is 40 mM at pH 8). These results point to serine residues as important for enzyme activity. Protection from PMSF inactivation in the presence of substrate or a competitive inhibitor indicates that some of the serine residues undergoing modification are located at or near the active site. However, penicillin V acylase retains most of its activity (until 60%) when incubated with 50 mM DIFP, another sited-directed reagent for serine residues. Similar results were found for penicillin G acylase from *E. coli* [18] and from *K. citrophyla* [19].

On the other hand, penicillin acylase has been recently recognized as an Ntn-hydrolase [8]. In this class of enzymes, the side chain that provides the nucleophile to attack the carbonyl carbon of the substrate is a N-terminal residue whose α-amino nitrogen atom acts as a base. Thus, nucleophile and base exist in the same amino acid. This amino acid seems to be the N-terminal serine from β-chain in penicillin G acylase [10].

Since penicillin V acylase from *S. lavendulae* shown to have essential serines, we have modified the enzyme with cyanate in order to test the possible involvement of the α-amino in catalysis, for analogy with penicillin G acylase. At pH 7 or below, the α-amino groups of peptides and proteins react with cyanate about 100 times faster than ε-amino groups and can be modified selectively [20]. Cyanate (10 mM) abolished penicillin V acylase activity. Penicillin V (100 mM) and phenoxyacetic acid (100 mM) protected the enzyme against inactivation with cyanate (65 and 35% of residual activity, respectively), suggesting that the α-amino takes part in the mechanism of hydrolysis of penicillin V by penicillin V acylase from *S. lavendulae*.

Other essential residues involved in penicillin V acylase activity are lysines, since the enzyme was inactivated in the presence of TNBS (at 10 mM TNBS remained 20% of residual activity) and was protected against inactivation in the presence of 100 mM penicillin V or phenoxyacetic acid (72 and 40% of residual activity, respectively).

DEP, a specific reagent for histidine residues under determined experimental conditions, inhibited penicillin V acylase activity (50% remaining activity at 10 mM DEP and 10% remaining activity at 25 mM DEP). However, both penicillin V and phenoxyacetic acid failed to protect the enzyme, suggesting the modified histidine residues are not involved in the catalytic events at the active site but to maintain the catalytically active conformation of enzyme.

Finally, penicillin V acylase was treated with other specific reagents to test the possible involvement of other amino acid residues in the active site of the enzyme. Phenylglyoxal, a modifier of arginine residues, *N*-acetyl imidazole and TNM as modifiers of tyrosine residues, iodoacetate or *p*-hydroxy mercurybenzoate as modifiers of cysteine residues and NBS, specific for tryptophan residues, failed to inhibit penicillin V acylase activity at any inhibitor concentration, pH, temperature and time tested.

4. Discussion

A very useful approach to chemical mechanism of enzymes is to analyze the pH-dependence of steady-state kinetic parameters to gain information on the nature of ionizable groups that are involved in the catalytic mechanism.

The plot of $\log V_{max}$ showed a change in slope in the acidic side, however, the plot of $\log(V_{max}/K_m)$ showed changes in slope at both low and high pH. The one at low pH corresponds to a pK value of 6.45 in the V_{max} profile, and 7.10 in V_{max}/K_m profile. Therefore, a group on the enzyme or in the substrate must be unprotonated for activity. As the substrate has no ionizable group with this pK value, it follows that a group on the enzyme is essential for activity. The results of solvent perturbation studies (Table 1) and the ΔH_{ion} value (6.27–9.70 kcal/mol) indicated that this group is a cationic acid. The difference in ΔH_{ion} values from V_{max} and V_{max}/K_m profiles can be ascribed to the different pK_1 value from both profiles (6.45–7.10). Although the determination of enthalpies of ionization is a helpful strategy when pK values are the same, it is not a completely reliable way to distinguish groups whose pK values have been altered by the environ-

ment, because this displacement may also change the ionization enthalpy [21]. The pK_1 from the V_{max} profile is displaced outward by about 0.65 pH units from the value seen in the corresponding V_{max}/K_m profile, which presumably represents the true pK value of the enzyme [17]. This displacement suggest that binding of the substrate favors the release of the corresponding proton. The data are thus consistent with the presence of a cationic group on the enzyme that is responsible for binding, for catalysis or for both.

The binding of substrate is prevented when a group with a pK_i value of 6.80 becomes protonated (Fig. 3). This pK value is similar to the lower pK value of 7.10 from V_{max}/K_m profile, suggesting the involvement of the cationic acid residue in the binding of the substrate.

If the cationic acid group was also involved with catalysis, its ionization should be observed in the V_{max} profile. The V_{max} profile (Fig. 1) shows that maximum enzyme activity is achieved when a group with a pK of 6.45 becomes unprotonated. Therefore, the ionization of the same group is being observed in the V_{max}, pK_i and V_{max}/K_m profiles at the lower pH. Since both V_{max} and V_{max}/K_m profiles decreased below pK_1, penicillin V binds to the enzyme both in the protonated and unprotonated form, although prefers to bind the last one [17] (Scheme 1).

With respect to the identity of the group responsible of the low pK, results of pH studies could point to histidine residues. Nevertheless, results from chemical modification studies point to the α-amino group of the enzyme. For analogy to penicillin G acylase, could be the α-amino group of the catalytic N-terminal serine residue [10]. Analysis of the N-terminal region of penicillin V acylase from *S. lavendulae* have shown, that a serine group is the N-terminal amino acid (data not shown). Although the value of pK_1 is somewhat less than expected for an α-amino group, a decreased value is plausible in a protein environment that is less polar than that for the aqueous solvent. Nevertheless, an histidine residue as responsible of the pK_1 that would be important on the maintenance of the active conformation of the enzyme cannot be discarded although, if this was true, a higher ΔH_{ion} had to be observed since conformational changes in the protein have large ΔH_{ion} values that are often 15–25 kcal/mol [16].

With respect to the identity of pK_2, plot of $\log(V_{max}/K_m)$ showed a change in slope at high pH with a pK value of 10.83, that is a group of the enzyme, since the substrate has no ionizable groups with this pK value. However, there was no sign of a pK value in V_{max} profile at high pH, indicating that the group on the enzyme with a pK of 10.83 is involved only in binding. Actually, the binding of the substrate is prevented when a group with a pK of 10.39 becomes deprotonated (Fig. 3). The corresponding ΔH_{ion} of this group points to a lysine residue [16], however, this conclusion could not be confirmed from the effect of the organic solvents on pK_2. Results of chemical modification studies point also to the presence of a lysine residue at or near the active site since TNBS inhibited the enzyme activity being protected against inactivation in the presence of substrate or a competitive inhibitor. Svedas et al. [22] reported the presence of a positively charged group in the subsite ρ 3 of penicillin G acylase from *E. coli*, that would be responsible of a rather effective electrostatic interaction with the negative charged group of carboxyle-side of the substrate (the 6-APA portion of the substrate). They argued that the chiral discrimination of the substrate in penicillin acylase active site is based on the balance of the electrostatic interactions of the substrate with the positive charge in subsite ρ 3 and sterically controlled hydrophobic interactions at subsite ρ 2.

Recently pH-kinetic studies for penicillin G acylase have shown that the activity of this enzyme from *K. citrophyla* [23], *Alcaligenes faecalis* [24] and *E. coli* [25] depends on two pK values (6.1–6.7 for pK_1 and 9.0–10.6 for pK_2). These pK values are similar to that for penicillin V acylase from *S. lavendulae*; however, evidence was not given about the nature of the amino acid residues responsible for these pK's. Only in the case of penicillin G acylase from *E. coli*, Morillas et al. [25] pointed to the possibility that the low pK would correspond to the α-amino group of the enzyme.

In conclusion, results present here indicated that in the catalytic mechanism of penicillin V acylase from *S. lavendulae* are involved two ionizable groups. The N-terminal α-amino group as a plausible candidate for a catalytic base acting in both acylation and deacylation steps [10,25], that could enhance the nucleophilic character of the nucleophilic residue responsible of the attack to the carbonyl group of the substrate. Results of chemical modification studies point to a serine residue as that nucleophile. The second ionizable group could be a lysine involved in the binding of the substrate to the active site of the enzyme.

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