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Selectivity of the enzymatic synthesis of ampicillin by *E. coli* PGA in the presence of high concentrations of substrates

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Abstract

Penicillin G acylase (PGA) catalyzes the synthesis/hydrolysis of acyl derivatives of phenylacetic acid through the formation of a covalent intermediate (the acyl–enzyme complex). When used for the kinetically controlled synthesis of β-lactam antibiotics, this enzyme promotes two undesired side reactions: the hydrolysis of the acyl side-chain precursor and of the antibiotic. Therefore, a high selectivity (synthesis/hydrolysis, S/H ratio) is very important for the process economics. Here, the enzymatic synthesis of ampicillin from D-phenylglycine methyl ester (PGME) and 6-aminopenicillic acid (6-APA), using PGA from *Escherichia coli* (EC 3.5.1.11) is studied. Kinetic assays provided S/H for high concentrations of substrates (up to 200 mM of 6-APA and 500 mM of PGME), using soluble PGA, at 25 °C, pH 6.5. S/H increased with 6-APA concentration, in accordance with the literature. However, when the concentration of 6-APA approached saturation, the rate of enzymatic hydrolysis tended towards zero (i.e., S/H tended to infinity). On the other hand, when the concentration of ester was augmented, S/H consistently decreased. This behavior, to the best of our knowledge still not reported, indicates that the acylation step may occur with 6-APA already positioned for the nucleophilic attack.
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Keywords: Ampicillin synthesis selectivity; Penicillin G acylase; 6-Aminopenicillanic acid; Synthesis/hydrolysis ratio

1. Introduction

The standard industrial practice to produce semi-synthetic β-lactam antibiotics employs a chemical route, with protection/de-protection of reactive groups, low temperatures (−30 °C or less), and organochloride solvents [1]. Hence, their enzymatic synthesis has received great attention as a possible "green chemistry" alternative.

In the kinetically controlled synthesis of ampicillin catalyzed by penicillin G acylase (PGA), either an ester or an amide reacts with the β-lactam nucleus, producing the antibiotic. In this reaction, PGA acts as a transferase. Nevertheless, this enzyme is also a hydrolase (see Fig. 1) – i.e., β-lactam nuclei and water molecules compete in the nucleophilic attack to the acyl–enzyme intermediate. Hence, the maximization of the selectivity towards the antibiotic is a key question for the economics of the process.

The mechanism of the three reactions in Fig. 1 (synthesis of the antibiotic, hydrolysis of the side-chain precursor and hydrolysis of the antibiotic) has been thoroughly studied in the literature. Yet, results for high concentrations of substrates are scarce, and one important question still remains open, i.e., whether the linkage of 6-APA to the enzyme may precede the acylation step in the synthetic path.

This work employs PGA of *Escherichia coli* (EC 3.5.1.11), an enzyme extensively used in the pharmaceutical industry, mainly in the hydrolysis of penicillin G to produce 6-APA. It promotes a nucleophilic attack to the carbonyl carbon of amide or ester bonds. In this aspect, it is similar to serine proteases, but instead of the catalytic triad, characteristic of these proteases, PGA has a single amino acid as a catalytic center: the Ser β1 residue, with the nucleophilicity of its Oγ enhanced by the α-amino group of this terminal

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Nomenclature

6-APA	6-aminopenicillanic acid
AB	d-phenylglycine methyl ester
AMP	ampicillin
AN	β-lactam antibiotic
AOH	D-phenylglycine
BH	methanol
C_i	denotes the concentration of component i
EA	acyl–enzyme complex
EAN	nucleus–acyl–enzyme complex
NH	6-aminepenicillanic acid
PG	D-phenylglycine
PGME	D-phenylglycine methyl ester

serine [2]. Hence, this enzyme may be classified as an N-terminal nucleophile hydrolase [3].

The importance of this enzyme for industry has stimulated the research concerning its structure and catalytic action. There are many consensual aspects in the literature, mostly regarding the mechanism of enzyme acylation. However, one point remains uncertain with respect to the synthesis of semi-synthetic penicillins (see Fig. 2): several authors assume that 6-APA will only link to the enzyme after the formation of the acyl–enzyme complex [3–6]; the mechanism would be analogous to the one of serine-proteases (especially α-chymotrypsin), with competing nucleophiles (for instance, during the synthesis of peptides in aqueous medium). On the other hand, Gonçalves et al. [7,8], studying the synthesis of amoxicillin, assumed that the acyl–enzyme complex might be formed either after or before the binding of 6-APA to the enzyme, but only in the first case the antibiotic would be synthesized. The authors' reasoning was that the high concentration of water molecules (in aqueous medium) would strongly favor hydrolysis. Hence, for the synthesis to occur, 6-APA should be already in a favorable position for the nucleophilic attack.

According to the literature, the β-lactam nucleus must bind to the enzyme before its nucleophilic attack on the acyl-enzyme complex. Kasche et al. [9] put forth a test to this hypothesis, defining a ratio of apparent deacylation constants, $(k_H/k_S)_{app}$, given by $(k_H/k_S)_{app} = C_{NH}(v_{AOH}/v_{AN})$, that would be invariant with respect to the concentration of substrates, in the case of a direct attack of a free 6-APA molecule to the acyl–enzyme complex. On the other hand, if there is an acyl–enzyme–nucleus intermediary complex $(k_H/k_S)_{app}$ would vary with the concentration of 6-APA. Kasche et al. [9] and Gonçalves et al. [10], among others, plotting experimental data of $C_{NH}(v_{AOH}/v_{AN})$ against C_{NH}, showed a linear dependence of $(k_H/k_S)_{app}$ with respect to C_{NH}, and concluded that 6-APA must bind to the enzyme before deacylation. Yet, both papers reported results for only one fixed concentration of PGME.

A key question concerning the synthesis deacylation step is the mechanism of the nucleophilic attack of the acyl–enzyme intermediate by the β-lactam nucleus, in competition with water. For any appreciable synthesis to occur, one (or both) of two hypotheses should be valid: (a) the greater number of water molecules would be compensated by a significant difference in the deacylation rate constants (nucleus versus water) during this step; serine-proteases, when hydrolysis and transpeptidation (or synthesis) reactions occur simultaneously, may present this characteristic [11]. Yet, the results of Kasche et al. [9] and Gonçalves et al. [10], discarded this assumption; (b) a previous and/or facilitated attachment of the β-lactam nucleus to the enzyme would help the nucleus overcoming the competition by water. Still, the ques-

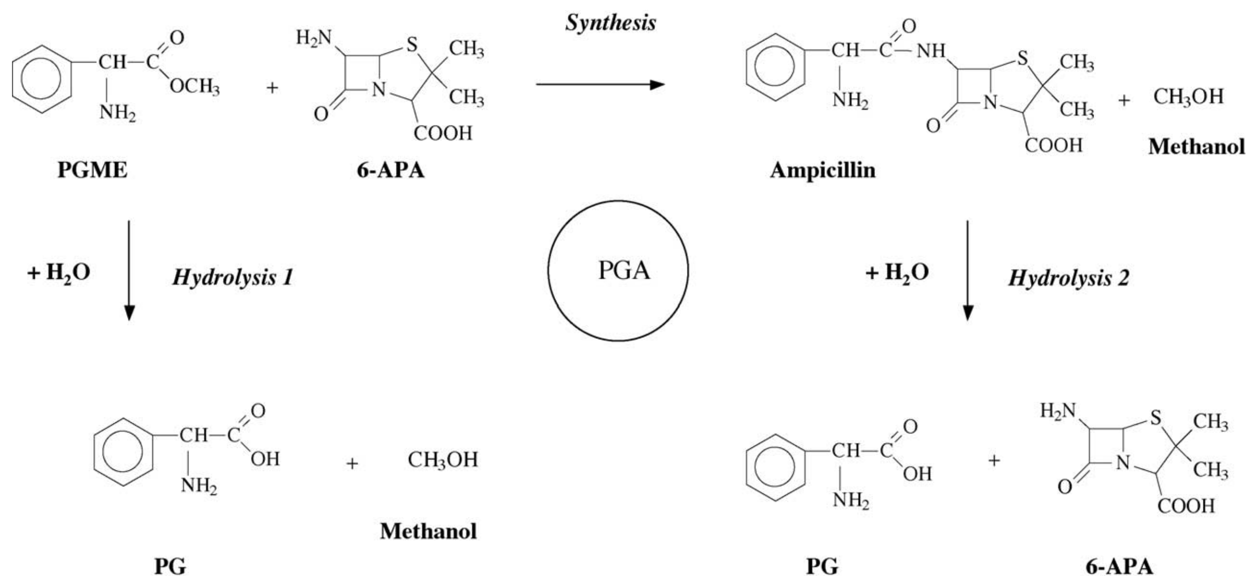

Fig. 1. Overall reactions in the synthesis of ampicillin, where PGME = D-phenylglycine methyl ester, 6-APA = 6-aminopenicillanic acid and PG = D-phenylglycine.

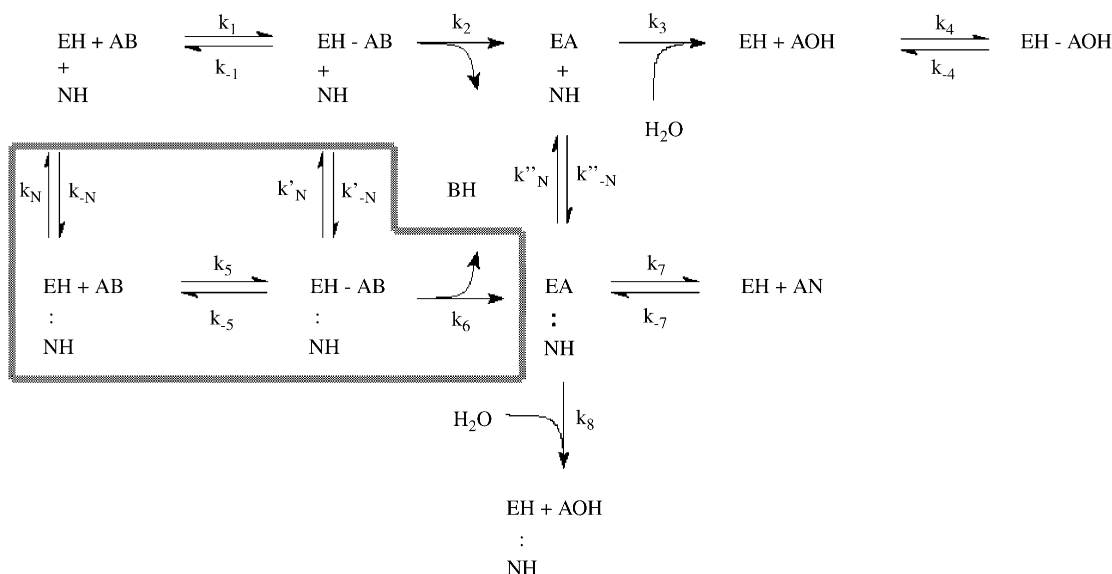

Fig. 2. Mechanistic scheme for the enzymatic synthesis of β-lactam antibiotics. The highlighted part of the figure indicates the steps that are not consensual in the literature. For ampicillin synthesis, departing from the side-chain methyl ester, one has: EH = free enzyme; AB = PGME; NH = 6-APA; BH = methanol; AOH = PG; AN = ampicillin; EA = acyl–enzyme complex; EA· · ·NH = acyl–enzyme–nucleus complex. Non-covalent bonds are depicted by dots.

tion whether the nucleus–enzyme complex is formed before or after the acyl–enzyme intermediate is not a consensus.

Direct X-ray crystallography of the enzyme-(β-lactam) binding could give an insight on this point. However, the solubility of β-lactam nuclei and of PGA is similar, turning unfeasible their co-crystallization, as well as the soaking of protein crystals [12]. Hence, though a number of papers show the crystal structure of the enzyme complexed with side-chains ligands [2,12–15], no data are available for an enzyme-(β-lactam) complex.

The influence of saturating PGA with 6-APA, previously to the synthesis of amoxicillin, was tested by Gonçalves [16]. No statistically meaningful difference was detected between S/H ratios at pH 7.0, but at pH 6.0, the selectivity increased when PGA was previously saturated with 6-APA. This is another indication that the binding of the β-lactam nucleus to the enzyme is important in the overall synthesis mechanism. At lower pHs, when less neutral Ser β1 amines are available, the importance of the binding of the nuclei to PGA would become noticeable. Hence, a complete kinetic mechanism of this system of reactions, taking into account all possible interactions, would be quite complex [9,10,17,18].

Several mechanistic models were proposed for the enzymatic synthesis of β-lactam antibiotics. In the absence of antibiotic, i.e., considering only initial velocities, models derived from Fig. 2, and ignoring the highlighted part of mechanism will have S/H given by [3,7,9]:

$$\left(\frac{\mathrm{S}}{\mathrm{H}}\right)_{C_{\mathrm{AN}}=0} = \left(\frac{v_{\mathrm{AN}}}{v_{\mathrm{AOH}}}\right)_{C_{\mathrm{AN}}=0} = \frac{P_1 C_{\mathrm{NH}}}{P_2 + P_3 C_{\mathrm{NH}}} \tag{1}$$

where P_1, P_2 and P_3 are lumped kinetic constants. Therefore, for all those models, S/H would be invariant with respect to PGME.

Gonçalves et al. [7,8] hypothesized that the acyl–enzyme complex might be formed either after or before the adsorption of 6-APA. For the rate of synthesis of antibiotic, v_{S}, the authors used a semi-empirical model with a “partition term” ($T_{\max}X$):

$$v_{\mathrm{S}} = \frac{k_{\mathrm{cat1}} C_{\mathrm{AB}} C_{\mathrm{E0}}}{K_{\mathrm{m1}}\left(1 + \frac{C_{\mathrm{AN}}}{k_{\mathrm{AN}}} + \frac{C_{\mathrm{AOH}}}{k_{\mathrm{AOH}}}\right) + C_{\mathrm{AB}}} T_{\max} X \tag{2}$$

with $0 \le T_{\max} \le 1$ and $X = C_{\mathrm{NH}}/(K_{\mathrm{EN}} + C_{\mathrm{NH}})$.

$T_{\max}$ quantifies the amount of (6-APA)–acyl–enzyme complex (EANH) that enters the synthetic route. Consequently, $1 - T_{\max}$ is the ratio of EANH suffering a nucleophilic attack by water. All acyl–enzyme, EA, which does not have NH already in position, would be hydrolyzed. Following this semi-empirical approach, S/H would also have a functional form similar to Eq. (1).

To ensure the economical operation of the enzymatic reactor, repeated runs in fed-batch mode are necessary, starting with high concentration of substrates (or even crystallized substrates) and promoting the crystallization of products. Ferreira et al. [19] used experimental design to map selectivity, productivity and 6-APA yield in the region of low substrate concentrations (up to 50 mM). In this work, S/H values for high concentrations of substrates are reported.

2. Materials and methods

2.1. Materials

D-Phenylglycine methyl ester, PGME (Aldrich), 6-APA (Winlab), ampicillin (Winlab), D-phenylglycine, PG (Aldrich). All other chemicals were of laboratory grade

from different commercial suppliers. Penicillin G acylase (PGA) from *E. coli* was donated by Antibioticos S.A., Spain.

2.2. Methods

2.2.1. Enzymatic activity

Enzyme activity was assessed via colorimetric analysis of *p*-nitro-aniline benzoic acid, released by the hydrolysis of 33 mg/l of 6-nitro-3-(phenylacetamide benzoic acid) (NIPAB), in 50 mM phosphate buffer, pH 7.5 at 25 °C. 1 IU corresponds to the amount of enzyme that hydrolyses 1 μmol of penicillin G (5%, m/v) per minute at pH 8.0 and 38 °C.

2.2.2. Analysis

Concentrations of PGME, PG, 6-APA and ampicillin were determined using HPLC: Waters Nova-Pak C18 60 Å, 4 μm, 3.9 mm × 150 mm, 1 ml/min of mobile phase containing 35% acetonitrile, 2‰ SDS, H_3PO_4 10 mM, $K_2H_2PO_4$ 5 mM, at 25 °C and λ = 215 nm.

2.2.3. Ampicillin synthesis

Batch assays at 25 °C and pH 6.5 (using a Titrino pHStat with NaOH 1 M) in a 20 ml jacketed, stirred batch reactor. 0.1–1.0 ml of the enzyme solution (the enzyme was in soluble form), containing 630 IU/ml, was used in the assays. At these conditions, the reactants were soluble for all the range of concentrations used here. The assays took 15 min, a time span for which the assumption of initial rates was valid (the concentration versus time curves where straight lines). Samples were collected each 3 min. Another reactor was run in parallel, in blank assays with 2 ml of the initial solution of reactants (without enzyme), measuring the rate of unspecific hydrolysis of ester (PGME). This is a parallel reaction, not related to the enzymatic mechanism, which rate has been subtracted from the "total" rate of hydrolysis obtained from enzymatic assays.

3. Results and discussion

3.1. Unspecific hydrolysis of PGME

The non-enzymatic hydrolysis of PGME, although small, should be considered for an accurate assessment of S/H ratios. Fig. 3 shows the results of blank assays (without enzyme, 25 °C and pH 6.5) for various concentrations of 6-APA. Since no correlation between unspecific hydrolysis and 6-APA concentration was observed (within the range of experimental error), a single empirical model was fitted for all data.

The "enzymatic hydrolysis" of PGME is calculated hereon as the "total" (measured) rate of hydrolysis obtained from enzymatic assays minus the unspecific rate, predict by the correlation shown in Fig. 3.

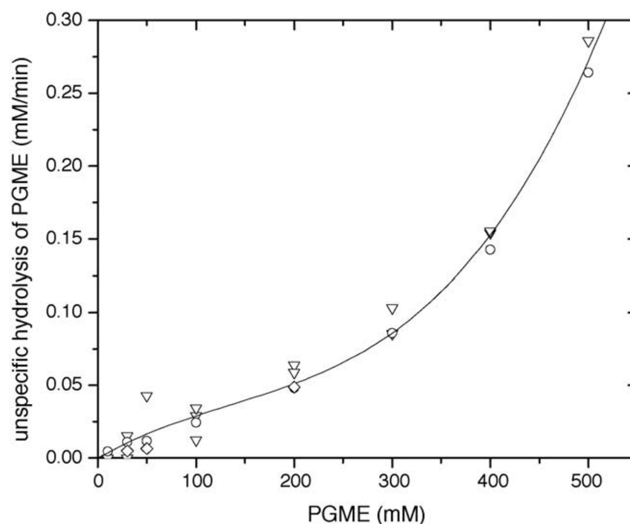

Fig. 3. Rate of unspecific hydrolysis of PGME, 25 °C, pH 6.5. 6-APA concentrations: 25 mM (○), 100 mM (▽) and 200 mM (◊); (—) $v_{\text{unspecific}}$ (mM/min) = $3.9 \times 10^{-4} C_{AB} - 1.33 \times 10^{-6}\ C_{AB}^2 + 3.3 \times 10^{-9} C_{AB}^3$. $r^2 = 0.98815$.

3.2. Enzymatic synthesis assays

Initial rates kinetic experiments were carried out here to verify the trend of the selectivity S/H when the concentrations of substrates were changed. Figs. 4 and 5 show the rate of production of ampicillin, v_{AN}, and D-phenylglycine, v_{AOH}, as functions of 6-APA and PGME concentrations. The inhibitory effect of 6-APA is evident from these figures: both rates decreased significantly when C_{NH} increased. However, since v_{AOH} decreased faster than v_{AN}, the selectivity increased with C_{NH} (see Fig. 6), as expected [1,9,10].

Fig. 5 clearly shows that the enzymatic rate of hydrolysis decreases to zero for concentrations of 6-APA above 100 mM.

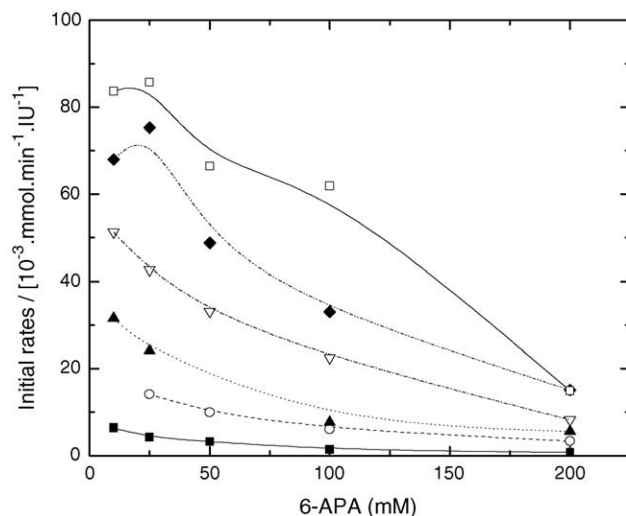

Fig. 4. Initial rates of formation of ampicillin (divided by the enzymatic load, E_0). Concentrations of PGME: 10 mM (■), 30 mM (○), 50 mM (▲), 100 mM (▽), 200 mM (◆) and 400 mM (□) (25 °C and pH 6.5).

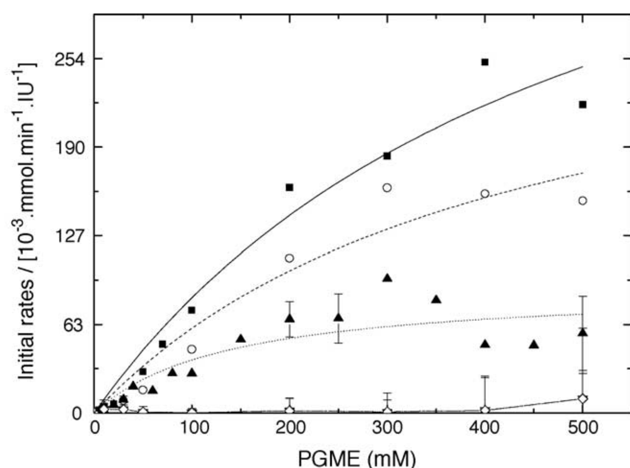

Fig. 5. Enzymatic initial rates of formation of PG (divided by E_0; unspecific hydrolysis rates were subtracted). Concentrations of 6-APA: 10 mM (■), 25 mM (◯), 50 mM (▲), 100 mM (▽) and 200 mM (◊) (25 °C and pH 6.5). Error bars: S.D., estimated from triplicates.

This is not the behavior reported in the literature. Kasche et al. [9], showed experimental data for which the rate of hydrolysis did not tend to zero when the concentration of nucleus was enhanced (at least for concentrations of 6-APA up to 100 mM, but using only one concentration of PGME, 10 mM). Based on this evidence, the authors concluded that the acyl–enzyme–nucleus complex could be hydrolyzed by water, as well (elementary step 8 in Fig. 2). However, the unspecific hydrolysis was not taken into account, and this conclusion should be reconsidered.

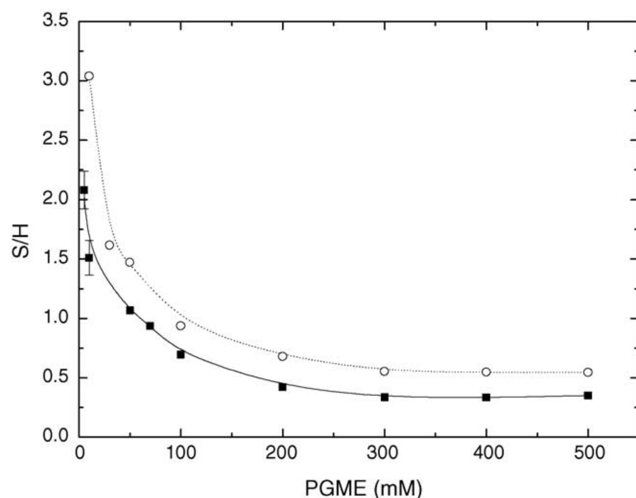

Fig. 6. S/H ratio. 6-APA: 10 mM (■) and 25 mM (◯). Enzymatic synthesis of ampicillin at 25 °C and pH 6.5. Error bars: S.D., estimated from triplicates. Concentrations of 6-APA above 50 mM have hydrolysis rates tending to zero, i.e., S/H → ∞ and are not shown in the figure.

Fig. 6 brings about an important discussion. If 6-APA would only link to the enzyme after the formation of the acyl–enzyme complex, as it is assumed by several authors [3–6], the S/H ratio would be invariant with respect to the concentration of PGME (see Eq. (1)). However, our results show that S/H declines with PGME, in disagreement with Youshko et al. [3]. These authors, however, apparently did not take into account the unspecific hydrolysis of PGME, either. It should be noticed that for concentrations of 6-APA above 50 mM, the enzymatic release of D-phenylglycine becomes negligible (see Fig. 5). In other words, S/H tends to infinity in this region and the scattering of the S/H data increases.

None of the mechanisms reported by the literature provide an equation where S/H, in the absence of products, is dependent on the concentration of PGME, as our experimental results have disclosed.

Our results show that high concentrations of 6-APA and low concentrations of PGME would improve the selectivity of the enzymatic synthesis of ampicillin. However, high concentrations of 6-APA also inhibit the rate of antibiotic synthesis. Therefore, the optimal concentrations of substrates will come from a trade-off between selectivity and productivity.

A more complex model could be derived from Fig. 2, when the highlighted part of the mechanism is accounted for. From that scheme, S/H in the absence of products becomes:

$$\left(\frac{\mathrm{S}}{\mathrm{H}}\right)_{C_{\mathrm{AN}}=0} = \frac{C_{\mathrm{NH}}(P_1 + P_2C_{\mathrm{AB}} + P_3C_{\mathrm{NH}} + P_4C_{\mathrm{AB}}C_{\mathrm{NH}} + P_5C_{\mathrm{NH}}^2)}{P_6 + P_7C_{\mathrm{AB}} + P_8C_{\mathrm{NH}} + P_9C_{\mathrm{NH}}^2 + P_{10}C_{\mathrm{NH}}^3 + P_{11}C_{\mathrm{AB}}C_{\mathrm{NH}} + P_{12}C_{\mathrm{NH}}^2C_{\mathrm{AB}}} \tag{3}$$

where P_i ($i = 1, 2, \ldots, 12$) are lumped kinetic constants.

To derive Eq. (3), the Briggs–Haldane steady state approach was employed. Therefore, no intermediate step is assumed to be in equilibrium. The dependence of S/H on PGME concentration, C_{AB}, only arises when elementary steps 5 and 6 in Fig. 2 are present (that is, k_5, k_{-5} and k_6 are not null). Other simplifications of the overall mechanism in Fig. 2, such as assuming a non-competitive inhibition by 6-APA of the formation of acyl–enzyme complex ($k_6 = 0$), or competitive inhibition by 6-APA of the linkage of PGME to the enzyme ($k_5 = k_{-5} = 0$), all provide S/H-ratios independent of C_{AB}. Thus, the complete scheme in Fig. 2 has to be considered in order to represent our data.

Fitting all kinetic parameters of such a complex rate equation is an almost intractable problem, and is out of our scope here. An illustrative fit of this complete model (based on the mechanism in Fig. 2) to our $(k_{\mathrm{H}}/k_{\mathrm{S}})_{\mathrm{app}}$ data is shown in Fig. 7. The parameters were highly correlated, as expected, and are not shown here, but the important point is that only the “complete” mechanism could qualitatively explain our observations (see Fig. 7).

Again, the hypothesis that all antibiotics come from acyl–enzyme complexes formed before the linking of the β-lactam nucleus cannot explain the results in Figs. 6 and 7. Indeed, without the highlighted steps in Fig. 2, $(k_{\mathrm{H}}/k_{\mathrm{S}})_{\mathrm{app}}$ would be invariant, for constant 6-APA (see Eq. (1)). The semi-empirical approach of Gonçalves et al. [7,8] is not ad-

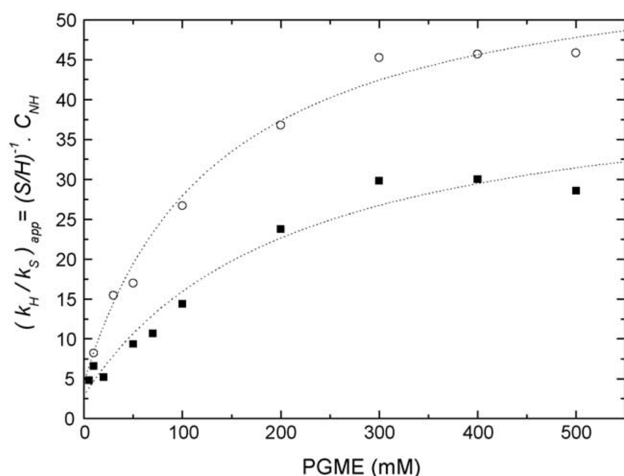

Fig. 7. Apparent ratio of deacylation constants. Concentrations of 6-APA: 10 mM (■) and 25 mM (◯) (pH 6.5 and 25 °C). Illustrative fit of Eq. (3).

equate, either, at least in the region of high concentrations of substrates. Furthermore, in the present case, the simplified models presented up to now in the literature would not be adequate if one intends to span all the range of substrate concentrations.

4. Conclusions

Initial rates of the enzymatic synthesis of ampicillin from D-phenylglycine methyl ester (PGME) and 6-aminopenicillanic acid (6-APA) were measured, at pH 6.5 and 25 °C. Our results showed that high concentrations of ester reduce the selectivity (S/H) towards the antibiotic, when the concentration of 6-APA is maintained constant. The dependence of S/H on ester concentrations that was observed here is in disagreement with former results from the literature. A kinetic model that would fit this behavior should be more complex than the simple rate equations that were proposed up to now. A hybrid, neural-network model such could be a reasonable solution for this problem [18,19].

Our results indicate that the assumption that all acyl–enzyme is formed before the adsorption of 6-APA is not adequate. The hypothesis that the positioning of 6-APA on its site does not change the acylation rate is not satisfactory, either. These two simplified models, however, may fit the data for a narrower range of ester concentrations (lower than 200 mM).

Increasing concentrations of 6-APA leaded to smaller acylation rates, but affected more intensively the hydrolysis of the ester than the synthesis of ampicillin. Above 100 mM of 6-APA, the enzymatic hydrolysis of PGME became negligible, and the selectivity tended towards infinity. This behavior, which has important consequences for the operation of the industrial reactor, can be explained if the positioning of the β-lactam nucleus on the active site (probably Phe-α146, Arg-α145 and Phe-β71) occurs before the acylation step. Linked 6-APA molecules would hinder – but not completely prevent – the correct positioning of the acyl derivative (PGME, in our case) for the nucleophilic attack promoted by the Oγ of Ser β1. Hence, high concentrations of 6-APA, approaching the enzyme saturation, would increase the probability of a nucleophilic attack to the acyl–enzyme coming from the nucleus (already correctly positioned), rather than from a molecule of water.

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