TWO-STEP INHIBITION OF *BACILLUS CEREUS* PENICILLINASE BY DICLOXACILLIN

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1. Introduction

Isoxazolyl penicillins, such as oxacillin, cloxacillin and dicloxacillin, besides themselves exerting an antibiotic action on some microorganisms [1], have also been proposed as synergistic agents which potentiate the action of other penicillins by inhibiting the enzymatic hydrolysis of the β-lactam bond [2,3]. This inhibition has generally been described as purely competitive [4–6], although some features, especially at long time intervals, indicate a more complex situation [7]. Gourevitch et al. [8] have described an irreversible inactivation of *Staphylococcus aureus* penicillinase by methicillin and/or by oxacillin, and have related the extent of inactivation to the show hydrolysis of the inactivating antibiotic; Sabath et al. [9] have however failed to confirm these results with a *Pseudomonas pyocyanea* enzyme, their data indicating only effects ascribed to purely competitive inhibition. In our hands [10], preliminary assays [8] had shown that the inhibitory effect exerted by dicloxacillin on several penicillinases, as evidenced by a conventional agar plate inhibition microbiological test, was time-dependent, and far more extensive than the effect evidenced by direct enzymatic tests. In the present work we have investigated whether prolonged contact of the enzyme with dicloxacillin modified the inhibitory pattern.

2. Materials and methods

The enzyme activity was estimated by a modification of the polarimetric method of Kersey et al. [11], by following, in a Cary 60 spectropolarimeter, the variations of optical rotation at 268 nm of an ampicillin solution after addition of the enzyme. Preliminary studies had shown that at this wavelength the optical rotatory dispersion spectrum of ampicillin had a positive shoulder, which disappeared upon enzymatic hydrolysis of the β-lactam bond. The differential specific rotation at this wavelength was 2.23×10^3 deg·cm^{-1}·M^{-1}; the rate of the reaction was expressed as millidegrees·cm^{-1}·min^{-1} at 25°C in a 0.07 M phosphate buffer at pH 7.0.

Ampicillin (lot no. CT2402) was a kind gift of Cyanamid Italia SpA, Catania, Italy; dicloxacillin (lot no. 402/1) from Lusofarmaco, Milan, Italy; 3 (2,6 dichlorophenyl), 5 methyl, 4 isoxazolcarboxylic acid (i.e. the side chain of dicloxacillin) from Italchemi, Milan, Italy. The enzyme was a partially purified penicillinase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6.) preparation (lot no. 9121147) from *Bacillus cereus* obtained from Baltimore Biological Laboratories, Baltimore, U.S.A; the stock solution contained 9 mg protein/ml, with a declared specific activity, estimated according to Kersey et al. [11], of 10^7 units/ml.

3. Results and discussion

Fig. 1 shows that, when the inhibitor (dicloxacillin) was added to the enzyme together with the substrate (ampicillin), a classical purely competitive inhibition was obtained, with a K_i around 0.33 mM, as compared with a K_m for ampicillin around 0.05 mM. When instead the enzyme was preincubated 45 min at 37°C and then overnight at room temperature (≅ 23°C)

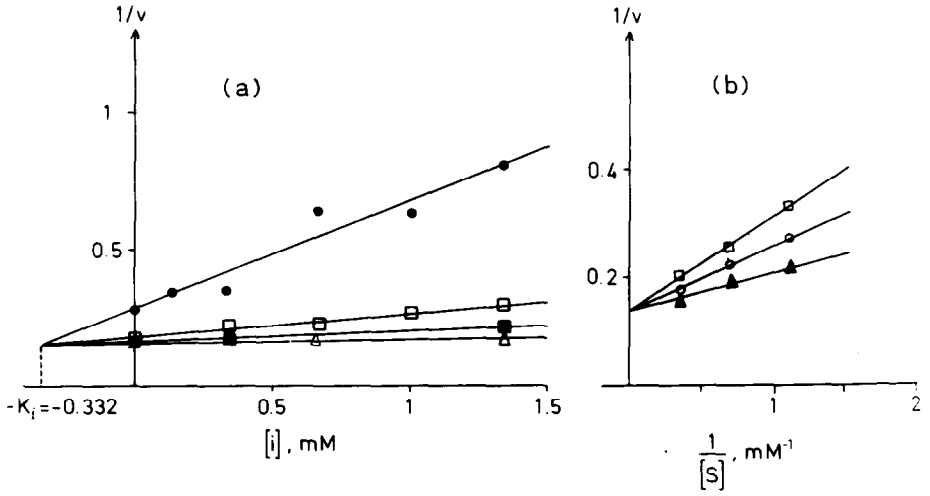

Fig. 1. $1/v$ vs [i] – fig. 1a – and $1/v$ vs $1/[S]$ – fig. 1b – plots of *B. cereus* penicillinase, upon mixing the enzyme simultaneously with substrate (ampicillin) and inhibitor (dicloxacillin). Substrate concentrations (fig. 1a): ●——●, 0.29 mM; ⊡——□, 0.94 mM; ■——■, 1.54 mM; Δ——Δ, 3.1 mM. Inhibitor concentrations (fig. 1b): ▲——▲, no inhibitor; ○——○, 0.66 mM; □——□, 1.34 mM. Enzyme final concentration: 6 μg protein/ml; optical pathlength: 1 cm. Rate of reaction (v) expressed as millidegrees/min.

in the absence of substrate and at various concentrations of inhibitor, a different pattern of inhibition was found (fig. 2). A set of parallel lines was obtained, both in the Lineweaver–Burk $1/v$ vs $1/[S]$ and in the Dixon $1/v$ vs [i] plots. It should also be noted that dicloxacillin exerts this inhibitory capacity at concentrations over two orders of magnitude lower than those reported in the experiments of fig. 1. Although a thorough study of the temperature dependence was not performed, it was found that exposure to 37°C was mandatory, while at 25°C the inhibition remained of the former type.

The pattern illustrated in fig. 2 is similar to that known as 'uncompetitive' inhibition. In our experiments, however, the enzyme had been preincubated with the inhibitor, and only afterwards had the activity been measured. In fact, the presence of μM concentrations of

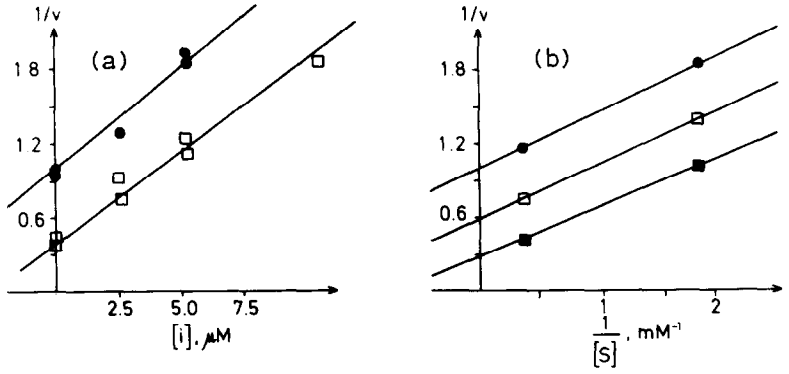

Fig. 2. $1/v$ vs [i] – fig. 2a – and $1/v$ vs $1/[S]$ – fig. 2b – plots of *B. cereus* penicillinase preincubated with dicloxacillin. Substrate concentrations (fig. 2a): ●——●, 0.575 mM; □——□, 2.9 mM. Inhibitor concentrations (fig. 2b): ■——■, no inhibitor; □——□, 2.53 μM; ●——●, 5 μM. The concentrations of dicloxacillin, both in fig. 2a and 2b, are the final ones in the assay mixture; during the preincubation with the enzyme they were three times higher. Other conditions as in fig. 1.

inhibitor during the assay can be neglected, since, as indicated in fig. 1 and as confirmed by other control experiments, only 200 times higher concentrations would have been effective. The mathematical treatment need therefore not consider the simultaneous presence of inhibitor and of substrate, but can take into account a simple enzyme + substrate reaction, where the enzyme has undergone different degrees of irreversible modification. In the classical Michaelis–Menten scheme

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P$$

the presence of inhibitor during the preincubation can be visualized as modifying the rate constants k_{+1}, k_{-1} and/or k_{+2}. The data of fig. 2b indicate that the maximal velocity, $V_{max} = k_{+2} \cdot [E_{tot}]$, is decreased, while

the slope, which is the ratio $\frac{K_m}{V_{max}} = \frac{1}{k_{+1}} + \frac{k_{-1}}{k_{-1} \cdot k_{+2}}$

remains constant.

In the simplest hypothesis, it can be assumed that the K_m/V_{max} ratio remains constant if both terms, $1/k_{+1}$ and $k_{-1}/k_{+1} \cdot k_{+2}$, are constant. Therefore: a) k_{+1} is not modified by the presence of inhibitor during the preincubation, and b) k_{-1} is modified to the same extent as k_{+2}. Alternatively, k_{-1} could also be invariant, but have such a low value, as compared to k_{+2}, that the term $k_{-1}/k_{+1} \cdot k_{+2}$ is negligible with respect to $1/k_{+1}$.

As for the factor by which k_{+2} and possibly k_{-1} are modified, it may be assumed that they are decreased proportionally to the extent of binding of inhibitor to the enzyme, i.e. k_{+2} is changed into

$$k_{+2,i} = \frac{k_{+2}}{1 + [i]/K_i} \text{ and } k_{-1} \text{ into } k_{-1,i} = \frac{k_{-1}}{1 + [i]/K_i}.$$

In this case the new rate of reaction, v_i, is such that

$$\frac{1}{v_i} = \frac{1}{k_{+2} \cdot [E_{tot}]} \cdot \frac{K_i + [i]}{K_i} + \frac{1}{[S]} \cdot \frac{k_{-1} + k_{+2}}{k_{+1} \cdot k_{+2} \cdot [E_{tot}]}.$$

The slope in the $1/v$ vs $1/(S)$ plot, i.e. $\frac{k_{-1} + k_{+2}}{k_{+1} \cdot k_{+2} \cdot [E_{tot}]}$

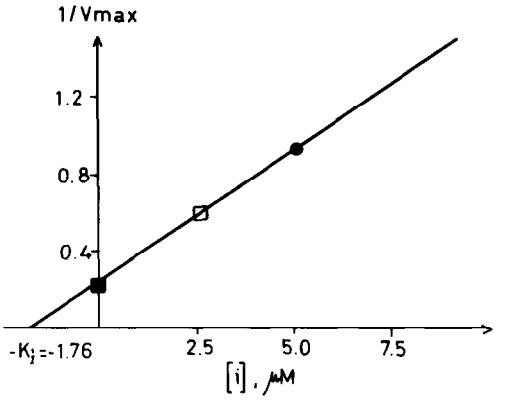

Fig. 3. $1/V_{max}$ vs [i] plot of the data of fig. 2b. Symbols and conditions as in fig. 2b.

is independent of [i] – i.e. the lines are parallel, and the slope in the $1/v$ vs [i] plot, $1/K_i \cdot k_{+2} \cdot [E_{tot}]$, is independent of [S] – and also yields parallel lines. The intercepts on the Y axis are instead dependent upon [i] and [S], respectively. Extrapolation of the V_{max} values in a Dixon plot yielded (fig. 3) a K_i value of 1.76 μM, a figure 200 times lower than the K_i obtained without preincubation.

The preceding assumptions would indicate that, upon preincubation at 37°C of the enzyme with the inhibitor, the latter combines irreversibly with the protein, modifying the rates of dissociation of the enzyme–substrate complex. If we further assume that $k_{-1} < k_{+2}$, the results, as mentioned before, would also be accounted for by a specific effect on k_{+2} alone, the invariance of k_{-1} being damped off by the low value of this rate constant. In this case, dicloxacillin would be visualized as reacting irreversibly with the catalytic site of the enzyme, without affecting at all the substrate-binding site. In both cases, preincubation with dicloxacillin leads to a decreased ability of the ES complex to dissociate, either in both directions (E + S and E + P) or only in the step leading to formation of product.

Experiments with 3(2,6 dichlorophenyl), 5 methyl, 4 isoxazolcarboxylic acid (i.e. with the isolated side chain of dicloxacillin) did not reveal any inhibition even when preincubation was performed with a 1 mM concentration. This may indicate that penetration of dicloxacillin into the active site of the enzyme is mandatory for its further irreversible effect on the protein.

It is possible that, as suggested for methicillin by Gourevitch et al. [8], the inactivating agent is not dicloxacillin itself, but a hydrolysis product, by some kind of suicide mechanism: there is however, so far, no evidence that dicloxacillin is hydrolyzed at all.

The results reported in the present work indicate that dicloxacillin can inhibit *B. cereus* penicillinase by two different mechanisms, either competing with the substrate for the binding site of the enzyme, or combining with the protein, possibly through the highly reactive dichlorophenyl moiety, and making the enzyme unable to carry on its hydrolytic function. The latter type of inhibition, requiring much smaller concentrations of the inhibitor, appears to be far more important for the therapeutic point of view, the K_i being proximal to the serum levels of dicloxacillin [12]. It may therefore bear some relevance to the question of whether dicloxacillin (and possibly other semisynthetic penicillins), being inhibitory to penicillinase(s), really exerts in vivo a good antibacterial synergism [2,3]. Moreover, since as it had also been noted by Gourevitch et al. [8], the absence of substrate seems to be mandatory for the irreversible inhibition to occur, these data would suggest a schedule of non-simultaneous administration of antibiotic associations.

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