Inactivation of $\alpha$-Alanine Carboxypeptidase by Penicillins and Cephalosporins Is Not Lethal in Bacillus subtilis

(peptidoglycan/mucopeptide/cell wall/6-aminopenicillanic acid/cephalothin/cloxacin)

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ABSTRACT The $\alpha$-alanine carboxypeptidase of Bacillus subtilis is a particulate enzyme that is irreversibly inactivated by penicillins and cephalosporins. However, the lethal concentrations of these antibiotics are not the same as those that inhibit enzymatic activity in vitro. 6-Aminopenicillanic acid inactivates at least 95% of the enzyme at nonlethal concentrations. Conversely, cephalothin is lethal at concentrations that do not inactivate the enzyme. Experiments with intact, growing cells confirm the results obtained in vitro. Therefore, a killing site distinct from the carboxypeptidase must be postulated.

Crosslinking of two peptide chains coupled to the release of $\alpha$-alanine from the end of one of the chains is a terminal reaction in bacterial peptidoglycan synthesis. This reaction, catalyzed by a transpeptidase, is hypothesized to occur in two steps (1). First, the enzyme displaces $\alpha$-alanine from one peptide chain to form an acyl-enzyme intermediate. Then, the acyl group is transferred to an amino group in a second peptide chain, regenerating the enzyme and forming the peptide crossbridge. The transpeptidase was first demonstrated in cell-free preparations from Escherichia coli in which it is particulate and irreversibly inhibited by penicillin G and other $\beta$-lactam antibiotics (1).

A second enzymatic activity, that is catalyzed by a $\alpha$-alanine carboxypeptidase, is similar to that of the transpeptidase in that $\alpha$-alanine is released from the end of the peptide chain. However, crosslinking does not occur. This second activity could reflect assay of the transpeptidase under conditions in which water, rather than an amino group in the peptide acceptor, displaces the enzyme from the postulated acyl-enzyme intermediate. It could alternatively represent an independent activity whose function might be, for example, to limit the degree of crosslinking. In E. coli, this enzyme is soluble and reversibly and competitively inhibited by penicillins (1).

However, in Bacillus subtilis, this carboxypeptidase is particulate and inactivated by penicillins, suggesting that it could be an uncoupled transpeptidase (2).

The results presented in this paper show that 6-aminopenicillanic acid inactivates the B. subtilis carboxypeptidase without killing cells and, conversely, that cephalothin kills cells without inactivating this enzyme. They therefore argue against the hypothesis that the $\alpha$-alanine carboxypeptidase of B. subtilis is an uncoupled transpeptidase.

Abbreviation: 6-APA, 6-aminopenicillanic acid.
The activity of the particulate enzyme preparation was $3 \times 10^4$ cpm released/min/ml for 13–14 ml of enzyme prepared from cells from 1 liter of culture (1.5 g, wet weight) (specific activity, $7.5 \times 10^4$ dpm/min/mg of protein). The corresponding activity for the enzyme prepared from toulenuzied cells was $8.3 \times 10^4$ dpm/min/ml for 0.12 ml of enzyme prepared from cells from 5 ml of culture (specific activity, $7.6 \times 10^4$ dpm/min/mg) (75 dpm = about 1 pmol of alanine).

## RESULTS

### Irreversible inhibition of the D-alanine carboxypeptidase by penicillins and cephalosporins

Previous studies of the particulate D-alanine carboxypeptidase of *B. subtilis* have shown that the inhibition by penicillin is not reversed by penicillinase (2). If one postulates a mechanism involving reversible binding of penicillin, followed by acylation,

$$
\frac{K_I}{K_I} \frac{E + P}{E - P} \frac{h_n}{E + P},
$$

where $E = $ enzyme, $P = $ penicillin, $t = $ time, $E - P$ is a reversible complex between enzyme and antibiotic in which $K_I = (E)(P)/(E - P)$, $E_P$ is an irreversible complex formed from $E - P$ with rate constant $k_n$, then the rate of irreversible inactivation is described by the following equation (4), provided that $P \gg E$

$$
\ln \left( \frac{\text{total enzyme/active enzyme}}{\text{enzyme}} \right) = \frac{k_d \cdot P \cdot t}{K_I + P}.
$$

For the special case where $P \ll K_I$ (i.e., at low penicillin concentration), the expression simplifies to

$$
\ln \left( \frac{\text{total enzyme/active enzyme}}{\text{enzyme}} \right) = \frac{k_d \cdot P \cdot t}{K_I}.
$$

and, of course, where $P \gg K_I$ (i.e., at high penicillin concentrations)

$$
\ln \left( \frac{\text{total enzyme/active enzyme}}{\text{enzyme}} \right) = k_d t.
$$

When the total enzyme/active enzyme is plotted on a logarithmic scale as a function of antibiotic concentration $\times$ time for several $\beta$-lactam antibiotics, straight lines are obtained, in agreement with Eq. [3] (Fig. 1). The rates of inactivation

### Table 1. Sensitivity of cells and D-alanine carboxypeptidase to penicillins and cephalosporins

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Rate of inactivation*</th>
<th>IC_{50}†</th>
<th>P × t for 50% killing‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>0.074</td>
<td>9.4</td>
<td>0.003</td>
</tr>
<tr>
<td>Penicillin V</td>
<td>0.058</td>
<td>12</td>
<td>—</td>
</tr>
<tr>
<td>6-APA</td>
<td>0.0035</td>
<td>200</td>
<td>5.5</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>0.00026</td>
<td>2700</td>
<td>0.01</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>0.00014</td>
<td>5000</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Values were determined as described in Methods.

* ml/μg × min

† μg/ml × min, derived from Fig. 1. It should be noted, however, that straight lines yielding the same values were obtained if time was varied at a constant concentration of antibiotic.

‡ μg/ml, the concentration which inhibited growth by 50%.

§ μg/ml × min. A value of $t = 30$ min (the generation time) was assumed for the calculation.

(i.e., $k_d/K_I$, the slope of the line in Fig. 1) for various penicillins and cephalosporins are summarized in Table 1. Since straight lines were obtained for all of these antibiotics, the assumption that $K_I$ for all of these substances is much greater than the antibiotic concentration used (the assumption of Eq. [5]) must be correct. Since inactivation is a function of antibiotic concentration $\times$ time, it is relatively meaningless to refer to an antibiotic concentration that gives a certain degree of inhibition without reference to the time of exposure. The inactivation is fully irreversible over the periods of 30 min or less used in the assay. However, different penicillins differ in the rate at which enzyme is regenerated from $E$ by hydrolysis to $E$ and $P''$ (presumably a penicilloic acid). No appreciable activity is recovered during several days at 6°C with penicillin G and cephalethin. However, with cloxacillin, 50% hydrolysis occurs in 68 hr and with 6-APA, 50% hydrolysis occurs in 14 hr. The rate of hydrolysis of the 6-APA derivative of the enzyme is sufficiently rapid that it needs to be taken into account during enzyme assay.

### Sensitivity of *B. subtilis* to antibiotics

It is of some interest to compare the value of antibiotic concentration $\times$ time required for inactivation of the enzyme to the same value required for killing of the cells. The latter value was estimated as the concentration of antibiotic required for 50% reduction in viable colonies, as measured in the plating assay, times the doubling time of 30 min. It is compared in Table 1 to the value that gives 50% inhibition of the enzyme in 30 min. In the case of carboxypeptidase, the value of μg/ml × min required for inhibition of the enzyme is over 1000-times greater than the value required for killing of the cells. One is, therefore, forced to postulate that the site for killing is far more sensitive than is the carboxypeptidase
and the carboxypeptidase. If one assumes that the carboxypeptidase is synthesized as a fixed fraction of the bacterial cell mass, i.e., partial inactivation of the enzyme does not lead to an increased rate of its synthesis, then for log-phase cells

$$E_T(t) = E_T(0)e^{kt}$$  \[5\]

where $E_T(t)$ is total enzyme at time $t$, $E_T(0)$ is total enzyme at time 0, and $k_t$ is the instantaneous growth-rate constant of the culture. If the inhibition of the enzyme in intact cells, like that in vitro, is irreversible and obeys Eq. [3], then the rate of change of carboxypeptidase under such conditions is given by the equation:

$$\frac{d[E(t)]}{dt} = \frac{k_s}{K_i} \cdot P[E_T(t) - E(t)],$$  \[6\]

where $E(t)$ is the amount of inactivated enzyme at time $t$. For the initial condition (no inhibition at time 0) when one first adds antibiotic, the solution of the equation is

$$E_T(t) = E_T(0) \cdot \exp\left(k_t + \frac{1}{K_i} \cdot \frac{k_s}{(k_s/K_i)P} \cdot E_T(0) \cdot \exp\left(-k_t \cdot P \cdot t/K_i\right)\right),$$

where $E_T(t) = E_T(t) - E(t)$. The ratio of active enzyme, $E_T(t)$, to total enzyme, $E_T(t)$, in the presence of antibiotic is then:

$$\frac{E_T(t)/E_T(0)}{1 + \alpha + 1 + \alpha} = \exp\left(-1 + \alpha k_t \cdot t\right),$$  \[7\]

where $\alpha = (k_s/K_i)/(P \cdot k_s)$.† The solid line in Fig. 2 is the graph obtained from the equation at various concentrations of 6-APA (P), the value of $k_s/K_i$ for the inhibition of the enzyme by 6-APA in vitro (Table 1), a value of $k_s = 0.0224 \text{ min}^{-1}$ (for a generation time of 30 min.), and a value $t = 30 \text{ min}$. The experimental points shown in Fig. 2 were obtained by exposing growing cells of AP-AI to different concentrations of 6-APA for 30 min, then harvesting and measuring residual enzyme activity (See Methods). The close agreement of the experimental values determined in intact cells with the theoretical curve (obtained using constants measured in vitro) indicates that neither permeability nor artifacts induced during enzyme preparation introduce any large difficulties in these experiments. Moreover, 95% inactivation of the carboxypeptidase was obtained during exposure to 100 $\mu$g/ml of 6-APA for 30 min.‡ In liquid culture, at this concentration of 6-APA.
6-APA, the cells grow at a rate not more than 10% lower than the control. Thus, it is clear that virtually all of the carboxypeptidase was inhibited by 6-APA under conditions in which this antibiotic had negligible effect on growth.

Failure of cephalothin to inhibit carboxypeptidase in intact, growing cells under conditions that result in cell death
The reciprocal result to that obtained with 6-APA was obtained with cephalothin; namely, this antibiotic inhibited growth in intact cells without inhibiting carboxypeptidase, as predicted from the data of Table 1. However, the experiments are technically more difficult to perform because the assumption that cells continue to synthesize carboxypeptidase at a rate independent of antibiotic concentration once the antibiotic has reached lethal concentrations is not true. Moreover, cephalothin induces formation of protoplasts, which burst, so that it is difficult to recover cells quantitatively for measurement of total enzyme activity. Stabilization by 0.7 mM sucrose and 1 mM MgCl₂ aids considerably, but some loss of cells (and consequently, of enzymatic activity) due to lysis still occurs. Nevertheless, it has been possible to recover 70–85% of carboxypeptidase activity under conditions in which only 15% of the cells survive. One could argue that more than 85% of the carboxypeptidase activity of the control is necessary for cell survival, but the experiments with 6-APA indicate that the 95% inhibition of carboxypeptidase is not lethal. These two experiments together, therefore, must lead to the conclusion that the n-alanine carboxypeptidase of *B. subtilis* is not the site of killing by these antibiotics. These results are in agreement with earlier findings that *B. subtilis* possesses multiple penicillin-binding components, some of which are more resistant to cephalothin than others (ref. 5 and H. Suginaka and J. L. Strominger, in preparation).

§ Cells were grown to mid-log phase at 37°C in medium supplemented with 0.7 M sucrose and 1 mM MgCl₂. Cephalothin was added to a final concentration of 0.4 mg/ml and incubation was continued for 40–50 min. The cells were then harvested and carboxypeptidase was assayed as described in Methods. The total activities recovered from 5 ml of cephalothin-treated cultures in two experiments were 6850 and 6870 dpm of ¹⁴C-alanine released per min (compared to control values of 9560 and 7930 dpm per min, respectively). The values for cephalothin-treated cultures were not corrected for losses due to cell lysis.

**DISCUSSION**

It has been suggested that the particulate n-alanine carboxypeptidase of *B. subtilis* (2) and the soluble n-alanine carboxypeptidase of *Streptomyces* (6, 7) could be uncoupled transpeptidases. That is, if the postulated acyl-enzyme intermediate formed in the transpeptidation reaction can be attacked by water, then hydrolysis would occur and the over-all reaction would be that of a n-alanine carboxypeptidase rather than a transpeptidase. The present experiments with *B. subtilis* indicate that the killing site is distinct from the carboxypeptidase. Therefore, if the killing site is the transpeptidase, the transpeptidase in *B. subtilis* cannot be the carboxypeptidase. Various physiological data suggest that the inhibition of transpeptidase is closely related to killing (1). It seems likely, however, that only genetic information showing whether or not the carboxypeptidase and transpeptidase can mutate independently will finally resolve the question of their identity or nonidentity. It remains an unlikely possibility that the particulate carboxypeptidase and the transpeptidase are products of the same gene, but that some modification in state or structure of some of the protein after synthesis (e.g., by limited proteolysis) alters both its catalytic activity and its sensitivity to penicillin. This question is currently under study.

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