Vol. 80, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1978 Pages 573-579

INDUCTION OF PENICILLINASE BY BACITRACIN

Yoav Citri

School of Medicine, Faculty of Health Sciences
Ben-Gurion University of the Negev
Beersheva, Israel

Received December 28,1977

Bacitracin preparations are shown to induce penicillinase (β-lactamase I) formation in strain 569 of Bacillus cereus. At high bacitracin concentrations (2-4 mg/ml) the level of induced enzyme obtained reaches a maximum which is comparable to that induced by optimal concentrations (1-2 mcg/ml) of β-lactam antibiotics. Penicillinase formation induced by short exposure to bacitracin, continues at a normal rate after all free bacitracin has been removed. The inducing activity of bacitracin is highly, but not completely, resistant to β-lactamase and can be entirely eliminated by prolonged treatment with penicillinase of B. cereus. The site of induction by bacitracin is, however, different from that mediating induction by β-lactam antibiotics. The inducing component has been isolated by thin layer chromatography; it seems to be closely related to, but not identical with, bacitracin A,B or F.

Bacitracin (1) is a complex of polypeptide antibiotics produced by Bacillus licheniformis and active mainly against gram-positive bacteria (2,3). It may serve a physiological function, such as regulating sporulation (4) or facilitating cation transport (5) in the producing cell. It is known to inhibit cell wall formation in susceptible bacteria (6) by blocking peptidoglycan synthesis at the phospholipid acceptor site. In this report I describe the hitherto unrecognized activity of bacitracin as an inducer of penicillinase. Significantly, penicillinase can abolish the activity of the inducing fraction of bacitracin. These observations are discussed in the context of specificity of penicillinase and its induction.

Materials and Methods

Penicillinase (penicillin aminohydrolase E.C. 3.5.2.6.) was prepared and purified as previously described (7). Benzylpenicillin was purchased

0006-291X/78/0803-0573$01.00/0

from Rafa Laboratories, Israel. Bacitracin (Teva, Israel), lot # 1676 was used throughout this work; the main experiments were subsequently repeated with other lots from that source and with preparations from other suppliers (N.B. Co., Ohio; B.B.L., Maryland). Freshly prepared solutions in phosphate buffer (0.03 M, pH 6.0) were used. Other chemicals were all CP grade commercial preparations.

Induction was studied in log phase cultures of Bacillus cereus, strain 569, grown in 50 ml side-arm flasks, containing 10 ml of CH/C medium (8). Inducers were added at cell density of 70-80 Klett units and incubation continued in a reciprocal shaker-bath at 35° and 120 strokes/min. 0.2 ml samples taken at 10-30 min. intervals were assayed directly by the timed iodometric (7,9) or the colorimetric method (9). In competition experiments, the inducers were added to precooled cultures. After 60-300 sec at 4° the cells were washed twice with cold CH/C medium, and incubated as before. In earlier experiments, penicillinase treatment (10 min at 37°) was interposed between washes to ensure elimination of free penicillin, but that turned out to be superfluous.

Analytical thin layer chromatography runs of bacitracin were as described (10) (solvent-butanol:acetic acid:water, 4:1:2). For preparative chromatographic separation the same solvent system was used with 2.0 mm plates. Individual UV absorbing bands were scraped off and extracted with methanol: water (1:1 by volume). Fresh aqueous solutions of the freeze-dried extracts were tested for induction. For inactivation of the inducer, bacitracin was incubated (at 30° for 6 hours) with penicillinase in 5 ml of 0.03 M phosphate buffer, pH 6. The concentrations of bacitracin (40-100 mg/ml) and of penicillinase (20 u/ml) were such that at the dilutions used for induction the added enzyme activity was negligible. In all cases, controls received identically incubated preparations minus bacitracin.

Results and Discussion

Induction of penicillinase by bacitracin (2 mg/ml) is shown in Fig. 1.

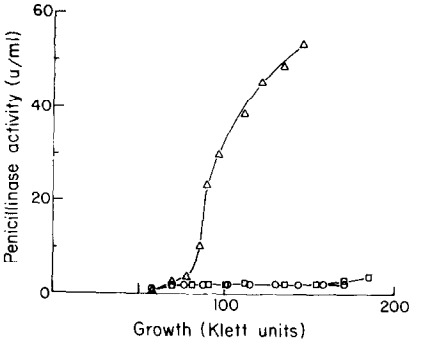

Fig. 1 Induction by bacitracin is abolished by penicillinase. The details of induction and pretreatment with penicillinase are described in Materials and Methods. (Δ) Bacitracin (2 mg/ml); (◘) Bacitracin (2 mg/ml) pretreated with penicillinase; (0) No additions.

At lower concentrations (down to 200 mcg/ml) the rate of penicillinase formation was roughly proportional to the concentration of bacitracin added. At higher concentrations, inhibition of growth made it progressively difficult to study induction. This difficulty was overcome following the observation that induced enzyme continues to be formed at the initial rate after all free bacitracin has been removed (see Table I). And, since removal of bacitracin relieves the inhibition of growth, it was possible to establish that maximal induction is obtained with 2-4 mg/ml of bacitracin. The optimal response to a brief exposure (90 sec. at 4°) to bacitracin implies that the inducer is bound firmly, perhaps irreversibly, to the site of induction. Thus, maximal induction will be observed when all induction sites are saturated.

Induction as a single irreversible act is very unusual. It was first described in the classical work of Pollock on the induction of penicillinase in B. cereus (11). The inducer in that work was benzylpenicillin and the observation was subsequently confirmed with other β-lactam antibiotics (12,13). There is no real evidence that any other inducers and inducible systems interact in a similar manner.

The analogy between induction by bacitracin and a β-lactam antibiotic

Table I Induction by bacitracin and penicillin is additive

Logarithmically growing cells of B. cereus 569 were exposed (at 4°) to bacitracin (10 mg/ml) or benzylpenicillin (5 mcg/ml). After 5 min the cells were washed and similarly exposed to the second inducer. The wash was repeated and the cells tested for penicillinase formation in the absence of free inducer. The differential rate of penicillinase formation is given by $\frac{\Delta E_I - \Delta E_B}{\Delta OD}$, where ΔE_I and ΔE_B represent increase in penicillinase activity (U/ml) of induced (E_I) and uninduced (E_B) cultures, and ΔOD (Klett units) represents growth of the induced culture during that time interval. For other details, see Materials and Methods.

First inducer	Second inducer	Differential rate of penicillinase formation
Bacitracin	None	0.73
Bacitracin	Bacitracin	0.72
Benzylpenicillin	None	1.17
Benzylpenicillin	Benzylpenicillin	1.07
Bacitracin	Benzylpenicillin	1.50

is further extended by the following observation. The inducing activity of bacitracin, although highly resistant to penicillinase, can be completely eliminated by a suitably extensive incubation with that enzyme (Fig. 1). The antibiotic activity of bacitracin, as assayed against B. cereus strain 569, B. cereus strain T and against a strain of Sarcina lutea, was not affected by a similar preincubation with penicillinase.

The above observations suggested that induction by bacitracin preparations is due to a minor component which cannot be equated with the antibiotically active components of bacitracin. Indeed, the inducing properties of bacitracin observed so far could be fully explained by the presence of a minute amount of a β-lactam derivative in the preparations. In the following experiments the relation between the inducing factor, the family of bacitracins and that of β-lactams is examined.

Table I shows an experiment designed to answer the question whether

bacitracin induces at the same site as the known inducers of penicillinase. It has been shown (12,13) that, as expected, all β-lactam antibiotics bind to the same induction site in B. cereus. Thus it is possible to saturate all sites with one inducer so that after its removal, an additional exposure of the cells to the same inducer (or any other β-lactam), will have no effect on the rate of penicillinase formation. Furthermore, since β-lactam derivatives differ in their inducing efficiency, a poor inducer will competitively suppress induction by a more efficient derivative. The suppressing effect will, of course, be maximal when the less efficient inducer is added first, and at saturating concentrations. In such experiments the induction sites were saturated with either benzylpenicillin (5 mcg/ml) or bacitracin (10 mg/ml). The results, illustrated in Table I, demonstrate that, at saturation, bacitracin is the less efficient inducer. Nevertheless, saturation with bacitracin did not prevent further induction by the subsequently added benzylpenicillin. Moreover, the effect of the two inducers is nearly additive and thus mediated, largely if not entirely by distinct binding sites.

While a direct kinship of the inducing component with the β-lactam family is ruled out by the evidence in Table I, a close relationship with the bacitracin family is indicated by the observations which follow. The TLC analysis of the bacitracin preparation (see Fig. 2) revealed that the inducer is not identical with the major components of the preparation, namely bacitracins A, B or F. It appears, however, to be closely related to the known bacitracins on the basis of the ninhydrin reaction, UV absorption and solubility (unpublished). Most significantly, the Rf of the inducing component is not appreciably changed following inactivation by penicillinase. This is clearly in contrast with the effect of penicillinase on β-lactam antibiotics, where the opening of the imide bond causes a major change in the Rf. No such change is expected if the inducer is a bacitracin, and the overall structure of this large and complex compound is not markedly modified by penicillinase. Indeed, it has been proposed in the past that penicillinase is a non-specific

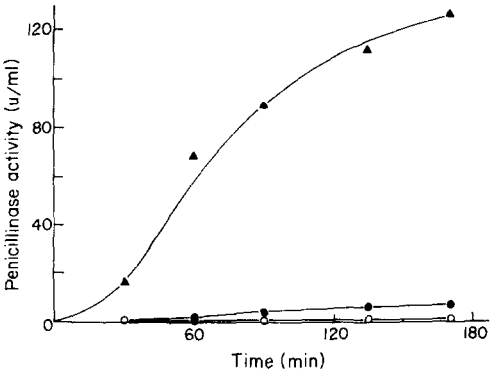

Fig. 2 Induction of penicillinase by a chromatographically homogeneous fraction of bacitracin. The separation by preparative TLC yielded 5 UV-absorbing, ninhydrin positive bands, which were extracted and tested for induction (see Materials and Methods). Bands 2, 3 and 4 from bottom, which include bacitracin B and F were combined (O). The bottom band (●) represents bacitracin A. The top band (▲) had an Rf of 0.68.

peptidase (14, 15). The bulk of evidence does not support that proposition (16, 17, 18), but the specificity of penicillinase may need to be re-examined. While not necessarily "non-specific", penicillinase may be more than a β-lactamase. Its range of specificity appears to be wider, but nevertheless quite restricted. It may be significant that the "non-specific peptide-inducers" of penicillinase all contain D-amino acid residues (14,15 and present work). The reason for that is not known, but a structural analogy between such peptide segments and penicillin can be drawn in some cases, and indeed has been proposed (19) as the basis for the antibacterial action of β-lactam antibiotics.

Acknowledgments: This work was supported by a grant from the Gulton Foundation. It was carried out in the laboratory of Prof. N. Citri at the Hebrew University-Hadassah Medical School, Jerusalem. I deeply thank my host and his collaborators for their hospitality and advice.

References.

1) Johnson F.A., Anker H. and Meleney F.L., Science 102,376 (1945).
2) Craig L.C., Weisiger J.R., Hausmann W. and Harfenist E.F., J. Biol. Chem. 199,259 (1952).
3) Newton G.G.F. and Abraham E.P., Biochem. J. 53,597 (1953).
4) Bernlohr R.W. and Novelli G.D., Arch. Biochem. Biophys. 103,94 (1963).

5) Haavick H.I. and Froyshov O., Nature 254,79 (1975).
6) Siewert G. and Strominger J.L., Proc. Nat. Acad. Sci. (USA) 57,767 (1967).
7) Citri N., Garber N. and Sela M., J. Biol. Chem. 235,3454 (1960).
8) Kogut M., Pollock M.R. and Tridgell E.J., Biochem. J. 62,391 (1956).
9) Citri N., in "Methods in Medical Research" Eiesen H.W. ed., vol. 10,221 (1964).
10) Simlot M.M., Pfaender P. and Specht D., FEBS lett. 35,231 (1973).
11) Pollock M.R., Brit. J. Exper. Pathol. 31,739 (1950).
12) Pollock M.R., Biochem. J. 66,419 (1957).
13) Citri N. and Pollock M.R., in "Advances in Enzymology" 28,237 (1966).
14) Saz A.K. and Lowery D.L., Biochem. Biophys. Res. Commun., 15,525 (1964).
15) Saz A.K. and Lowery D.L., Biochem. Biophys. Res. Commun., 19,102 (1965).
16) Pollock M.R., Brit. Med. J. 4,71 (1967).
17) Pollock M.R., Ann. N.Y. Acad. of Sci., 151,502 (1968).
18) Citri N., in "The Enzymes" vol. 4,23 (1971).
19) Tipper D.J. and Strominger J.L., Proc. Natl. Acad. Sci. (USA), 54,1133 (1965).