Regulation of Bacitracin Synthetase by Divalent Metal Ions in *Bacillus licheniformis*

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The activity *in vitro* of the bacitracin synthetase of *Bacillus licheniformis* ATCC 10716 is influenced by divalent metal ions (Mg^{2+} , Mn^{2+} , Fe^{2+} or Co^{2+}) and by bacitracin. It is possible that complexes between bacitracin and metal ions exert feedback control on the synthetase. An overall control mechanism for bacitracin synthetase may consist of substrate-metal ion complexes and product-metal ion complexes.

INTRODUCTION

The peptide antibiotic bacitracin is produced by some strains of *Bacillus subtilis* and *Bacillus licheniformis* (Hickey, 1964). Bacitracin is synthesized by an enzyme complex, bacitracin synthetase, which is composed of three multifunctional enzymes (Frøyshov & Laland, 1974; Frøyshov, 1975). The antibiotic may promote divalent metal ion transport during growth of the producer strain (Haavik & Frøyshov, 1975; Haavik, 1976).

At present much interest is focused on possible control mechanisms for antibiotic formation. Synthesis of several antibiotics may be controlled by catabolite regulation and phosphate regulation (Demain, 1972; Martín, 1977). However, in the case of bacitracin production by *B. licheniformis* ATCC 10716, both the glucose effect (catabolite regulation) and the phosphate effect may be due to a more general response of the cells to environmental factors (Haavik, 1974*a*, *b*).

Several antibiotics may exert feedback effects on their own synthesis (Demain, 1976) and trace metals may also have some regulatory functions (Weinberg, 1978). We have therefore examined the effect of bacitracin and divalent metal ions on bacitracin formation by the bacitracin synthetase of *B. licheniformis* ATCC 10716.

METHODS

Organism. The bacitracin-producing strain *Bacillus licheniformis* ATCC 10716 was kept as a spore suspension at 4 °C.

Media and growth conditions. The chemically defined medium contained (g l⁻¹ in distilled water): L-glutamic acid, 20.0; L-alanine, 0.2; citric acid, 1.0; NaH₂PO₄·2H₂O, 2.0; KCl, 0.5; Na₂SO₄, 0.5; MgCl₂·6H₂O, 0.2; CaCl₂·2H₂O, 0.01; FeSO₄·7H₂O, 0.01; MnSO₄·H₂O, 0.01. The pH was adjusted to 6.0 with NaOH before autoclaving. Spores (about 2 × 10⁷) were suspended in 200 ml medium in a 2 l Erlenmeyer flask. After incubation at 37 °C at 360 rev. min⁻¹ on a New Brunswick rotatory shaker (model G-53), the culture was used to inoculate 8 l medium in a New Brunswick fermenter (Microferm). The cells were grown at 37 °C with stirring (500 rev. min⁻¹) and aeration (8 l air min⁻¹). They were harvested early in the exponential phase of growth (*A*₆₅₀ 1.2, 1 cm path length, Spectronic 20 spectrophotometer), washed with 0.04 M-potassium phosphate buffer pH 7.2, containing 2 mM-MgSO₄ (buffer A) and then stored at -20 °C.

Fig. 1

Fig. 2

Fig. 1. Effect of divalent metal ions on bacitracin formation by bacitracin synthetase *in vitro*. Mn^{2+} was omitted from the normal reaction mixture described in Methods and Mn^{2+} ($\circ$), Mg^{2+} ($\bullet$), Fe^{2+} ($\square$) or Co^{2+} ($\blacksquare$) was added at the concentration indicated. The total radioactivity in each incubation mixture was 92×10^3 c.p.m. The results have been corrected by subtracting the radioactivity measured for control tubes without metal ions (250 to 300 c.p.m.).

Fig. 2. Effect of divalent metal ions on Mn^{2+} -stimulated (3 mM) bacitracin synthetase. Mn^{2+} ($\circ$), Mg^{2+} ($\bullet$), Fe^{2+} ($\square$) or Co^{2+} ($\blacksquare$) was added at the concentration indicated. The total radioactivity in each incubation mixture was 98×10^3 c.p.m. The results have been corrected by subtracting the radioactivity measured for control tubes without metal ions.

Preparation of cell-free extracts. Frozen cells were thawed then lysed with the aid of lysozyme, and a 43 to 49% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction was prepared and solubilized in 4 ml buffer A according to Frøyskov & Laland (1974).

Cell-free synthesis of bacitracin. The reaction mixture normally contained (in a final volume of 100 μl) 20 mM-ATP, 10 mM- MnCl_2 (the pH of these two solutions was adjusted to 7.4 with KOH), 50 mM-potassium phosphate buffer pH 7.4, 0.1 mM-dithiothreitol, 0.2 mM L-forms of all the amino acids in bacitracin except L-isoleucine, 0.1 mM-L-[U- ^{14}C]isoleucine [10 Ci mol^{-1} (370 GBq mol^{-1}), New England Nuclear] and 10 μl enzyme solution. Modifications of this reaction mixture are described in the text. The mixture was incubated for 30 min at 37 °C and bacitracin was isolated and identified as described previously (Frøyskov & Laland, 1974).

RESULTS

Effect of divalent metal ions on formation of bacitracin by bacitracin synthetase

Bacitracin production in the cell-free enzyme preparation from *B. licheniformis* ATCC 10716 was dependent on the presence of Mn^{2+} , Mg^{2+} , Fe^{2+} or Co^{2+} (Fig. 1). These metal ions could not be substituted by Ca^{2+} , Cd^{2+} , Ni^{2+} , Cu^{2+} or Zn^{2+} . The efficiency of bacitracin synthetase increased with increasing concentrations of Mn^{2+} , Mg^{2+} or Fe^{2+} up to at least 10 mM, but with Co^{2+} above 5 mM, bacitracin synthetase activity decreased.

Addition of Mg^{2+} to the Mn^{2+} -stimulated bacitracin synthetase increased the activity of the enzyme preparation as much as the addition of the same concentration of Mn^{2+} (Fig. 2). This was not the case with Fe^{2+} or Co^{2+} . By adding Fe^{2+} to the Mn^{2+} -stimulated bacitracin synthetase, bacitracin production was somewhat inhibited, and Co^{2+} caused a dramatic inhibition of the Mn^{2+} -stimulated bacitracin synthetase (Fig. 2). Similar results were obtained with the Mg^{2+} -stimulated bacitracin synthetase (results not shown).

Table 1. *Effect of divalent metal ions on the activation of histidine as amino-acyl adenylate by bacitracin synthetase*

The activation of histidine was measured by the [32 P]PP $_i$ -ATP exchange reaction (see Frøyskov & Laland, 1974).

Metal ion added (50 mM)	[32 P] ATP formation (c.p.m.)
Mg $^{2+}$	1800
Fe $^{2+}$	1250
Co $^{2+}$	750
Mn $^{2+}$	650
Zn $^{2+}$	350
Cd $^{2+}$	160
Ca $^{2+}$	0
Ni $^{2+}$	0
Cu $^{2+}$	0

Fig. 3. Effect of bacitracin on divalent metal ion-stimulated bacitracin production by bacitracin synthetase. The metal ions (each at 5 mM) were Mn $^{2+}$ (○), Mg $^{2+}$ (●), Fe $^{2+}$ (□) or Co $^{2+}$ (■). The total radioactivity in each incubation mixture was 90×10^3 c.p.m. The results have been corrected by subtracting the radioactivity measured for control tubes without metal ions.

Effect of divalent metal ions on the activation of amino acids by bacitracin synthetase

The constituent amino acids of bacitracin are activated as amino-acyl adenylates by bacitracin synthetase. Mg $^{2+}$ was required for this reaction to occur (Frøyskov & Laland, 1974). Table 1 shows the effect of several divalent metal ions on this reaction between L-histidine, ATP and bacitracin synthetase. Mg $^{2+}$, Fe $^{2+}$, Co $^{2+}$ or Mn $^{2+}$ were the most effective, Zn $^{2+}$ or Cd $^{2+}$ were less effective and no activation occurred with Ca $^{2+}$, Ni $^{2+}$ or Cu $^{2+}$. Optimum activation was obtained at 30 to 50 mM metal ion. Similar results were obtained with the other constituent amino acids of bacitracin (results not shown).

Effect of bacitracin on bacitracin formation by bacitracin synthetase

Addition of bacitracin to bacitracin synthetase inhibited the enzyme activity whether it was stimulated by Mn $^{2+}$, Mg $^{2+}$, Fe $^{2+}$ or Co $^{2+}$ (Fig. 3). The inhibitory effect of bacitracin was dependent on the concentration of Mg $^{2+}$ or Mn $^{2+}$ (Table 2); it increased with increasing concentrations of metal ions to a constant level. Bacitracin was significantly more inhibitory to bacitracin production in the presence of Mn $^{2+}$ than in the presence of Mg $^{2+}$ (Table 2).

Table 2. *Effect of bacitracin on bacitracin production by bacitracin synthetase at different metal ion concentrations*

Bacitracin production was measured by [^{14}C]isoleucine incorporation (Frøyshov & Laland, 1974). The results are expressed as a percentage of the bacitracin produced in the absence of added bacitracin.

Metal ion concn (mM)	Bacitracin production (%) in the presence of bacitracin (300 i.u. ml ⁻¹) and metal ion:	
	Mg ²⁺	Mn ²⁺
1	87.9	75.5
2	75.4	65.0
4	61.3	51.7
6	57.1	38.6
8	63.5	41.5
10	62.2	38.0

DISCUSSION

Bacitracin is synthesized from 10 different amino acids by the thiotemplate mechanism (Frøyshov & Laland, 1974). At least 53 catalytic functions on bacitracin synthetase are involved in the formation of bacitracin (Frøyshov *et al.*, 1978). Very little is known about the control mechanisms acting on this multifunctional enzyme complex.

The concentration of metal ions had a strong influence on the activity of the bacitracin synthetase (Fig. 1). Furthermore, different metal ions resulted in different levels of activity. Figure 2 shows that Mn²⁺ and Mg²⁺ may be able to substitute for each other. It is therefore likely that these two ions influence the same catalytic functions on the synthetase. This was not the case with Fe²⁺ or Co²⁺; the latter was particularly strongly inhibitory to bacitracin formation in the presence of Mn²⁺ or Mg²⁺. Thus Co²⁺ antagonized the stimulatory effect of Mn²⁺ or Mg²⁺ and concentrations of Co²⁺ which were stimulatory to bacitracin formation by the synthetase when added alone became inhibitory when added together with Mn²⁺ or Mg²⁺. This could reflect an important control function of Co²⁺.

Whether the metal ions influence all the catalytic functions of the bacitracin synthetase or just a few is not known. It is reported that Mg²⁺ is necessary for the activation of amino acids as amino acyl adenylates (Frøyshov & Laland, 1974). In this reaction Mg²⁺ could be substituted by Mn²⁺, Fe²⁺, Co²⁺ or Zn²⁺. Thus it seems as if metal ions may play a vital role in the use of chemical energy by the synthetase.

Addition of bacitracin to the bacitracin synthetase inhibited the enzyme activity, probably by a feedback inhibition mechanism. As metal ion-activated enzymes may be controlled by end-products with chelating properties (Wyatt, 1964) and as bacitracin is a chelator of divalent metal ions (Garbutt *et al.*, 1961; Wasylshen & Graham, 1975), bacitracin could influence the activity of the bacitracin synthetase by binding the very metal ions necessary for its own synthesis. Therefore one might expect that the addition of excess metal ions would then restore the activity of the synthetase. However, this was not the case as the same amount of bacitracin showed a stronger inhibitory effect on the bacitracin synthetase in the presence of high concentrations of Mn²⁺ and Mg²⁺ than in the presence of low concentrations.

Since the inhibitory effect of bacitracin increased with increasing concentrations of Mn²⁺ and Mg²⁺, it is possible that a complex between bacitracin and metal ion exerts feedback inhibition on the enzyme activity. This is supported by the observation that bacitracin is less inhibitory in the presence of Mg²⁺ than in the presence of Mn²⁺.

Several metal ions stimulated bacitracin synthetase when added alone. One would expect that molecules with chelating properties could interfere with this. This may explain why several free amino acids seem to have a marked effect on the amount of bacitracin produced *in vivo* (Haavik, 1979). Thus an overall control mechanism of the multifunctional

bacitracin synthetase could consist of substrate-metal ion complexes and product-metal ion complexes.

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