Mechanism of Action of Bacitracin: Complexation with Metal Ion and C_{55}-Isoprenyl Pyrophosphate

(molecular model/chelating agents/ternary complex/cell wall synthesis/peptidoglycan)

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ABSTRACT The inhibition by bacitracin of the enzymatic dephosphorylation of C_{55}-isoprenyl pyrophosphate is abolished by the addition of chelating agents. If, however, the chelating agent is added after a preincubation of bacitracin with a divalent cation and the lipid substrate, then its inhibition has little effect, indicating that bacitracin, metal ion, and C_{55}-isoprenyl pyrophosphate form a complex. Various divalent cations can participate in complex formation, but monovalent cations are ineffective. A direct demonstration of the formation of a complex between the C_{55}-isoprenyl pyrophosphate and bacitracin in the presence of metal ions was obtained. Molecular models that show one possible conformation for a complex between bacitracin and the C_{55}-isoprenyl pyrophosphate, in which the metal ion acts as a bridge between the two compounds, are presented.

Bacitracin is an antibiotic produced by strains of Bacillus licheniformis. It is a mixture of closely related compounds, the main component of which (bacitracin A) is a cyclic polypeptide with a peptide side chain (Fig. 1) (1-3). An unusual feature of the structure is the occurrence of a thiazoline ring formed between the L-cysteine and L-isoleucine residues at the N-terminal end of the acyclic peptide side-chain. The free amino group of L-isoleucine at the N-terminal end is adjacent to the thiazoline ring and is essential for antimicrobial activity. The transformation of bacitracin A to bacitracin F (in which the amino group is replaced by a carbonyl group) results in total loss of antimicrobial activity.

A metal ion may be essential for the antimicrobial activity of bacitracin (4-11). The antimicrobial activity is stimulated by various metal ions and inhibited by metal-chelating agents, such as EDTA. Spectroscopic evidence for the interaction of bacitracin with metal ions has been obtained. The chelation may involve both the nitrogen of the thiazoline ring and the adjacent free amino-group in isoleucine; this chelation could assist in stabilizing the thiazoline ring, thus hindering the deamination to bacitracin F (10). It has been further postulated that in the zinc-bacitracin A complex, the zinc also coordinates through the imidazole of the histidine residue and the peptide nitrogen of the histidine residue, thus forming a one-to-one complex with bacitracin (10).

Various biochemical lesions induced by bacitracin have been reported (9). Several effects, such as inhibition of induced enzyme synthesis and stimulation of exudation of K^{+} ions, could be ascribed to alterations in cell-membrane function. The antibiotic also inhibits incorporation of ^{14}C-amino acids into cell walls and induces the accumulation of uridine-nucleotide precursors of the wall under conditions in which incorporation of amino acids into protein is unaffected (12-15). However, since bacitracin affects protoplasts of bacteria, its action is not limited to effects on cell walls. The precise site of action in cell wall synthesis has been defined as inhibition of the dephosphorylation of C_{55}-isoprenyl pyrophosphate. This reaction is essential for regeneration of the lipid carrier required for the cyclic synthesis of peptidoglycan (16, 17). In the present paper, data are presented which suggest that bacitracin acts by forming a complex with C_{55}-isoprenyl pyrophosphate (a component of the cell membrane) and divalent cations. The metal ion may serve as a bridge between the antibiotic and the substrate.

MATERIALS AND METHODS

The C_{55}-isoprenyl pyrophosphatase used was that present in membranes of Streptococcus faecalis. The membranes were prepared and washed with EDTA and 2 M LiCl as described previously (through step 2 of the procedure) (Staudenbauer, W. L., and J. L., Strominger, J. Biol. Chem., submitted). The residual LiCl was removed by washing with water and then dialysis against water. Finally, the membranes were lyophilized. This freeze-dried preparation could be stored for at least a year. Before use, it was suspended in water to a protein concentration of 1.1 mg/ml.

The substrate, ^{32}P_{-}C_{55}-isoprenyl pyrophosphate, was prepared as described. † 1 liter of cells of Micrococcus luteus was grown at 37°C to half-maximum growth (8 hr after a 1% inoculum) in a medium containing 1% Difco Bacto-peptone, 0.1% Difco yeast extract, and 0.5% NaCl, adjusted to pH 7.5 with NaOH. Cells were harvested and transferred to medium that contained 10 mCi of ^{32}P_{-}inorganic phosphate and only 10% of the yeast extract used in the normal growth medium. 4 hr later, 160 mg of bacitracin was added and, after a further 3 hr, the cells were harvested by centrifugation. Lipids were extracted from the cells by acetone, chloroform/methanol, and pyridinium acetate-butanol. ‡ The pyridinium acetate-butanol fraction was washed four times with 0.5 volume of water. The lower aqueous phases were backwashed once with butanol, which was then combined with the organic phase. Pyridine (0.3 volume) was added and the mixture was taken to dryness on a rotary evaporator. Mild saponification ‡ hydrolyzed phosphatides to water-soluble products. The mixture was again taken to dryness, dissolved in chloroform/methanol 2:1 and washed five times with an equal volume of 50% methanol. The lower chloroform layer was evaporated to dryness (lipid A). The aqueous phase was backwashed.

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twice with chloroform. The combined chloroform layers were taken to dryness (lipid B). Lipid A and lipid B were separately suspended by sonication in acetone and passed through a 1-ml column of basic silica-gel G (Woelm), which had been washed with acetone. Elution was done batchwise. Acetone (3 ml) eluted nonradioactive, nonpolar lipids; chloroform-methanol 1:1 (3 ml) and methanol (3 ml) eluted radioactive lipid-P-P. The chloroform–methanol and methanol fractions were combined and examined by thin-layer chromatography on silica gel G, with isobutyl ketone–acetic acid–water 8:5:1 as the developing solvent. Radioautography of the chromatogram showed that both lipid A (5.0 × 10⁶ cpm) and lipid B (2.1 × 10⁶ cpm) were predominantly C₄₀-isoprenyl-P–P (Rf = 0.33) although lipid A contained traces (about 5%) of material that chromatographed with [³²P]C₄₀-isoprenyl-P (Rf = 0.41). An important observation was that when highly radioactive lipid-P-P was filtered through glass wool or chromatographed on a column of silica gel, the lipid-P-P was irreversibly bound to the glass unless it had previously been silanized with a 5% solution of dimethyldichlorosilane in toluene.

Bacitracin was generously given by the Upjohn Co., Kalamazoo, Mich. The general methods and materials used have been described (17, 18).

RESULTS

Effect of Metal-Chelating Agents on Bacitracin Inhibition of Dephosphorylation of Lipid Pyrophosphate. Bacitracin at low concentrations inhibits the dephosphorylation of C₄₀-isoprenoid alcohol pyrophosphate by a specific pyrophosphatase (17). This dephosphorylation does not require metal ions, as evidenced by the fact that concentrations of EDTA and other metal chelating agents up to 0.05 M have no effect on its activity. However, the addition of EDTA or other chelating agents to a system that had been inhibited to the extent of more than 95% by the addition of bacitracin resulted in restoration of activity to the amount found in the absence of bacitracin (Fig. 2). This effect of EDTA is not due to some chemical alteration of the bacitracin molecule produced by EDTA, since after prior incubation of bacitracin with EDTA, the addition of magnesium ions in slight excess of the amount of EDTA added resulted in restoration of the inhibition. The effect was not specific for Mg⁺⁺, but could also be obtained with Cu⁺⁺.

Effect of Prior Incubation of Bacitracin with Various Components of the Reaction Mixture in the Presence of Metal Ions. The sequence of addition of components of the reaction mixture was important in obtaining reproducible results. All of the above experiments had, therefore, been done by addition of the lipid substrate, metal, chelating agent, bacitracin, and enzyme in the sequence indicated. However, if bacitracin, metal ions, and substrate were incubated together before the addition of EDTA, EDTA reversed the inhibition due to bacitracin by only about 30%. On the other hand, prior incubation of enzyme with bacitracin in the presence of a metal ion resulted in normal enzymatic activity when EDTA was added before substrate. These data suggest that a complex is formed between bacitracin, metal ion, and substrate that can be reversed to only a small extent by the addition of EDTA. Similar results were obtained with hydroxyethylendiamine-triacetate and ethylene-bis-(oxethylenenitritelo)tetracetate.

Effects of Various Metals on Formation of the Inactive Complex. When bacitracin was incubated with substrate alone, even in the absence of added metal, considerable inhibition of

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**Fig. 1.** Proposed structure of bacitracin A. →→ signifies a C–N bond. The structure of the l-cysteine and l-isoleucine residues at the end of the acyclic side chain are detailed in order to illustrate the thiazoline ring.

**Fig. 2.** Restoration of activity of bacitracin-inhibited \( ^{32} \)C₄₀-isoprenyl pyrophosphatase by chelating agents. Incubation mixtures contained 10,000 cpm of \( ^{32} \)P-C₄₀-isoprenyl pyrophosphate (dried at the bottom of the tube); 10 μl of a mixture containing 0.4 M Tris-HCl (pH 8.2), 0.1% 2-mercaptoethanol, and 1.2% deoxycholate; 5 μl of 6 mM bacitracin; 10 μl of membrane preparation from S. faecalis (1.1 mg of protein per ml); and 10 μl of chelating agent, to give the final concentration indicated. After incubation for 30 min at 37°C, the reactions were stopped by heating in a boiling water bath for 2 min. Assays were performed by spotting the incubation mixtures at the origin of a paper chromatogram, then chromatographing overnight in isobutyric acid–1 N ammonium hydroxide 5:3. The compounds were located by radioautography; the Rf values were lipid–³²P, 1.0, lipid–³²P–³⁵S, 0.95, and inorganic phosphate–³²P, 0.3. The areas of radioactivity were cut out and counted in a liquid scintillation spectrometer. EDTA, ethylenediaminetetraacetate; HEDTA, hydroxyethylendiaminetetraacetate; EBONTA, ethylene-bis-(oxethylenenitritelo)tetracetate. Two other chelating agents, \( \alpha, \alpha' \)-dipyridyl and (2-hydroxyethylamino)-diacetate had no effect. Solid lines are the experimental tubes. The dotted line is an incubation from which bacitracin was omitted, but to which chelating agent was added; all of the chelating agents gave identical control lines.
activity on subsequent addition of enzyme was observed (Table 1). Treatment of enzyme, substrate, and bacitracin separately with EDTA failed to alter the result of this experiment. It is presumed that this effect of bacitracin in the absence of added metal is due to the presence of small amounts of metal ions that could not be removed under the conditions used. As bacitracin is known to have a very high affinity for metals (5, 7), it would presumably also scavenge traces of metal ions in the buffers, glassware, etc.

Addition of several divalent cations, Mg++, Ca++, Cu++, and Zn++, further enhanced the inhibition. In each case, the inhibition was relieved if EDTA was added before addition of bacitracin, but not if it was added after bacitracin. Several other metals, Mn++, Fe++, Co++, and Cd++, also enhanced the inhibition by bacitracin, although under our conditions these metals, especially Cd++, also caused some inhibition of the enzyme reaction by themselves. The monovalent cations, Li+, Na+, and K+, failed to enhance the inhibition by bacitracin. More striking, however, is the fact that they relieved the inhibition observed in the absence of added metal ion. This effect was presumably due to displacement from bacitracin of trace amounts of divalent cations by an excess of these monovalent cations.

**The Effects of Bacitracin on Enzyme Systems Involving Other Substrates.** A model to be presented below suggests that the divalent cation forms a bridge between the pyrophosphate residue in the C55-isoprenyl pyrophosphate and bacitracin. If this is so, then other compounds containing pyrophosphate residues might also be expected to complex with bacitracin. The effects of bacitracin on enzymatic reactions involving inorganic pyrophosphate, ATP, and ADP were therefore examined. Adenylate kinase, ATPase, pyruvate phosphokinase, and inorganic pyrophosphatase were not inhibited by bacitracin at concentrations up to 10 mg/ml. All of these enzymes require Mg++ or another divalent cation for activity and were tested in its presence. In addition, it was already known from the point of inhibition of bacitracin in the cycle of cell wall synthesis that bacitracin does not affect any of the other reactions in the cycle that involve either C55-isoprenyl phosphate or C55-isoprenyl pyrophosphoryl-sugar compounds, e.g., the initial reaction in which UDP-MurNAc-pentapeptide reacts with C55-isoprenyl phosphate to form lipid-P-P-MurNAc-pentapeptide, or any of the subsequent reactions in which

![FIG. 3](https://example.com/figure3.png)

**Fig. 3.** Complexation of bacitracin with [32P]C55-isoprenyl pyrophosphate. (A) The radioactive lipid substrate (5.8 × 10^6 cpm) in 6.5 ml of 0.1 M Tris buffer (pH 8.2), containing 0.35% deoxycholate, 0.02 mM MgCl2, and 0.5 mM 2-mercaptoethanol was applied to a column of Sephadex G-25 (1.4 ml in a 1-ml graduated pipet). Fractions of 40 μl were collected in a micro-pipet every 1.5 min: the entire fraction was counted. The first 1 ml of the elution fluid was discarded. The elution of radioactivity was constant after collection of an additional 40 fractions. Bacitracin, 7.2 mg, dissolved in 40 μl of the radioactive elution solution was added to the top of the column, and elution was then continued. (B) The same experiment was performed except that MgCl2 was omitted from the radioactive buffer-substrate mixture, and EDTA, at a final concentration of 40 mM was added. (C) The experiment was done as described in A, except that [32P]C55-isoprenyl phosphate (19) (4.5 × 10^6 cpm) was substituted for the [32P]C55-isoprenyl pyrophosphate.

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<tr>
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Experiments were performed essentially as described in the legend to Fig. 2.

The control was obtained by mixing substrate and metal ion to a final concentration of 1 mM, then adding EDTA before bacitracin and enzyme. In the experimental tube, substrate and metal ion were mixed and then bacitracin was added before EDTA and enzyme. The concentration of EDTA was 1.4 mM.

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6 EDTA was removed from enzyme and bacitracin by filtration on a Biogel P-2 column, and from the lipid substrate by partition between CHCl3 and methanol-water followed by chromatography of the organic phase on silica-gel G.
acetylglucosamine is added to the lipid intermediate (18). The effects of bacitracin on C\textsubscript{55}-isoprenyl phosphate phosphatase (Willoughby, E. and J. L. Strominger J. Biol. Chem., submitted) and on the ATP-dependent phosphokinase that catalyzes the phosphorylation of free C\textsubscript{55}-isoprenyl alcohol (19) were also examined; neither enzyme was inhibited by up to 1 mg/ml of bacitracin. On the other hand, marked inhibition of the biosynthesis of squalene and sterols from mevalonic acid, involving short-chain prenyl pyrophosphates as intermediates, has been observed and will be reported subsequently.

**Complex Formation between Bacitracin and C\textsubscript{55}-isoprenyl Pyrophosphate.** When a sample of [\textsuperscript{32P}]C\textsubscript{55}-isoprenyl pyrophosphate was filtered through a column of Sephadex G-25, in a buffer containing deoxycholate, until it was eluted at a constant rate, the addition of bacitracin resulted in a trough of radioactivity followed by a peak, indicative of the forma-
tion of a complex (Fig. 3A) (20). This result is the opposite of the effect observed in the earlier use of this method, in which a peak was followed by a trough (20), and must indicate that the complex is included in the gel to a greater extent than is the uncomplexed C15-isoprenyl pyrophosphate. Presumably, the free C15-isoprenyl pyrophosphate is included in a relatively large detergent micelle, which cannot penetrate the gel. In the presence of EDTA, complexation was reduced (Fig. 3B). No complex formation was observed when C15-isoprenyl phosphate was used (Fig. 3C).

Molecular Models of Bacitracin and of C15-isoprenyl Pyrophosphate. Acid hydrolysis of bacitracin A yields small amounts of two peptides containing the sequence phenylalanine-[

...through four points of attachment so that it forms a bridge between the bacitracin and the pyrophosphate. Attachment is made through oxygens of adjacent phosphates in the substrate and through peptide nitrogens of the α-asparagine residue due attached to the cyclic ring, and of the amino group of L-lysine in the ring at the point where the acyclic peptide side chain is attached to this nitrogen. This coordination is shown in Fig. 4B, with the hydrophobic C15-isoprenoid chain in an extended conformation. The requirement of isoprenyl pyrophosphates for binding, however, indicate that the isoprenoid chain must also interact with the bacitracin. Subsequent studies (manuscript in preparation) have shown that neither inorganic pyrophosphate nor isopentenyl pyrophosphate is complexed in the presence of metal to bacitracin, but C15-farnesyl pyrophosphate forms a strong complex. It should be emphasized that no data are available regarding the conformation of bacitracin. Other conformations are possible in which the isoprenyl pyrophosphate can be complexed through the metal ion to bacitracin, including one in which the acyclic peptide side chain is folded on the opposite side of the peptide ring to interact with the phenylalanine residue and another in which the histidine residue (just above the α-asparagine residue as photographed) is brought forward to provide coordination sites for the metal ion. The latter conformation would account for the known interaction of the histidine residue in bacitracin with the metal ion (10, 11), but the possibility also needs to be considered that the metal–histidine coordination site would be replaced by metal–pyrophosphate coordination sites in the complex. The present model, presented as a basis for further study, accounts for the failure of bacitracin–metal to complex with the C15-isoprenyl pyrophosphoryl–sugar derivative (since there is no space for the sugar derivatives in the groove into which pyrophosphate fits) or with C15-isoprenyl phosphate (which cannot complex tightly with the metal ion).

Finally, the general effect of bacitracin on various membrane functions (9) could be explained by the distortion of membrane structure that might be expected from the complexation of C15-isoprenyl pyrophosphate with bacitracin, either due to the removal of the lipid from the membrane or due to the penetration of the membrane by bacitracin. Similarly, the toxicity of bacitracin for animal cells could be due to the complexation of bacitracin with short-chain prenyl pyrophosphates found in animal tissues.

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