Binding of Bacitracin to Cells and Protoplasts of Micrococcus lysodeikticus*

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SUMMARY

Bacitracin was tritiated by the Wilzbach technique and purified by carboxymethylcellulose chromatography. Binding studies between [3H]bacitracin and Micrococcus lysodeikticus cells and protoplasts indicated that the cells or protoplasts were saturated at 2 x 10^5 molecules per cell. This value correlates well with the number of C55-isoprenyl pyrophosphate molecules found in the membrane and adds further support to the hypothesis that the antibiotic activity of this peptide is due to complex formation with this functional lipid. The K_m for binding of bacitracin to cells or protoplasts was 3.7 x 10^{-8} molar which is consistent with the in vitro binding constant of 1 x 10^{-8} M^{-1} observed for the interaction of bacitracin A with the purified C55-isoprenyl pyrophosphate. In vitro binding studies between bacitracin A and purified bacterial phospholipids indicated that the antibiotic does not interact strongly with these lipids. It is concluded from these studies that binding of bacitracin to the bacterial cells can be accounted for quantitatively by virtue of a specific interaction with the membrane C55-isoprenyl pyrophosphate. Furthermore, the protoplast permeability changes induced by bacitracin, under certain conditions, may arise from interaction between bacitracin and the C55-isoprenyl pyrophosphate molecules in the membrane.

Bacitracin has been shown to inhibit cell wall biosynthesis in bacteria by forming a complex with the C55-isoprenyl pyrophosphate and a divalent cation (1–3). Binding between bacitracin and this functional phospholipid prevents the enzymatic dephosphorylation of the lipid, a step required in the biosynthesis of cell walls (4). This binding phenomena has been studied in some detail in vitro and it was concluded that the interaction exhibits considerable specificity for both the peptide and lipid structures as well as for the divalent cation. Thus, it has been proposed that the antibiotic activity of this peptide can be rationalized in terms of its interaction with a specific functional membrane lipid. However, protoplasts have been shown to be just as sensitive to bacitracin as the intact bacteria and the antibiotic also causes release of potassium ions from protoplasts (5, 6). These observations suggest that a component of the antibiotic activity of bacitracin peptides is damage to the cytoplasmic membrane resulting in permeability changes. These effects could be due either to interactions of bacitracin with a variety of membrane phospholipids or to a specific interaction of bacitracin with the C55-isoprenyl pyrophosphate which is an integral component of the cytoplasmic membrane.

In order to examine this question further, binding of bacitracin by intact cells and protoplasts has been quantitated to determine if the maximum number of bacitracin molecules bound per cell correlates with the number of C55-isoprenyl pyrophosphates present. In addition, the interaction of bacitracin with purified bacterial phospholipids has been examined in vitro by gel filtration studies. These studies suggest that bacitracin interacts specifically with the C55-isoprenyl pyrophosphate in the membrane without interacting strongly with other membrane phospholipids.

MATERIALS AND METHODS

Assay for Antibiotic Activity—Micrococcus lysodeikticus was grown in a medium containing 1% Difco Bactopeptone, 0.1% yeast extract, and 0.5% NaCl at pH 7.5. Ten milliliters of a 1% inoculum were incubated at 37°C in the presence of varying concentrations of the peptides. The turbidity of the solution was measured with a Klett photometric colorimeter after 16 hours. Antibiotic activity is expressed in terms of the minimum inhibitory concentration of the peptide.

Preparation of [3H]Bacitracin—A mixture of bacitracin A and B (80 mg) isolated from commercial bacitracin by carboxymethylcellulose chromatography (3, 7) was tritiated by the Wilzbach technique (New England Nuclear). This procedure resulted in considerable degradation of the peptide to inactive products. The crude peptide mixture was approximated one tenth as active as the starting material. The specific activity of this mixture was 1.25 mCi per mg. This sample was dissolved in 25 ml of distilled water and lyophilized 4 times to remove all exchangeable tritium and then applied to a carboxymethylcellulose column (0.9 x 100 cm) equilibrated with 0.06 M pyridine acetate buffer, pH 4.9. The column was eluted with the same buffer and the elution profile is shown in Fig. 1. All peaks were assayed for antibiotic activity using M. lysodeikticus as a test organism. Only Peaks I and II had significant anti-
and counted in a scintillation counter. At every concentration of \( ^{3}H \)bacitracin used, controls consisting of the cells, \( ^{3}H \)bacitracin and a large excess of unlabeled bacitracin (5 mg per ml) were run. Each sample was then corrected for weak non-specific binding by subtracting the counts bound in the presence of the large excess of unlabeled bacitracin.

**Binding Assay for Interaction of \( ^{3}H \)Bacitracin with M. lysodeikticus Proteolysis**—One liter of \( M. \) lysodeikticus was grown to 260 Klett units, washed twice with 10 mM Tris-HCl, pH 8.0. The pellet was suspended in 300 ml of 20 mM Tris-HCl, pH 8.0 containing 20% sucrose. Lysozyme and sodium EDTA were added to bring the final concentrations to 0.5 mg per ml and 10 mM, respectively. The suspension was stirred gently for 1 hour at room temperature. The proteolysis were then centrifuged at 10,000 \( \times g \) for 30 min and then washed three times with 0.1 M Tris at pH 7.5 containing 20 mM MgCl\(_2\) and 20% sucrose. The final pellet was suspended in 200 ml of the above buffer giving a final concentration of 2 \( \times 10^8 \) protoplasts per ml based upon the number of starting cells. Tubes containing 20 \% of the proteolysis suspension, \( ^{3}H \)bacitracin and 400 \( \mu \)l of a 0.1 M Tris buffer at pH 7.5 containing 1 mM MgCl\(_2\) and 20% sucrose were incubated at 25°C for 30 min. The assay mixture was then centrifuged for 45 min at 30,000 \( \times g \) and the pellet was washed once with 400 \( \mu \)l of the Tris buffer and counted in 10 ml of Aquasol. Controls containing a large excess of unlabeled bacitracin were run as with the whole cell binding studies.

**Preparation of \( ^{3}P \)-Labeled Bacterial Phospholipids**—One liter of *E. coli* or *Bacillus subtilis* was grown to late log phase in the presence of 15 mCl of \( H_3^{2}P\)O\(_4\). The bacteria were harvested and then washed four times with distilled water. The cells were mixed with 20 ml of methanol and heated for 15 min at 50°C followed by addition of 40 ml of chloroform and stirring for 20 min at room temperature. The organic phase was separated from the cells by filtration through Whatman No. 1 filter paper. The chloroform-methanol extract was then back extracted three times with 30 ml of Folch's upper phase (chloroform-methanol-water, 3:48:47). The organic solvents used in this extraction procedure contained 50 \( \mu \)g per ml of 2,6 di-tert-butyl cresol as an antioxidant. The chloroform-methanol extract was then concentrated and run on silica gel plates (200 \( \mu \) thick) in several solvent systems to fractionate the phospholipids. The solvent systems used were as follows: System I consisted of neutral plates (without binder) developed with chloroform-methanol-acetic acid-water, 80:13:8:0.3 (v/v); System II employed basic plates and was developed with pyridine-petroleum ether, 3:1 (v/v) (8); and System III used Brinkmann F-254 silica gel plates developed with chloroform-methanol-acetic acid, 65:25:8 (v/v). After running the total lipid extract on the silica gel plate, the phospholipid fractions were located by autoradiography. The individual fractions were eluted from the silica gel; the extracts were reduced to dryness and stored at 4°C under nitrogen.

**Assay for Binding Between \( ^{3}P \)-Labeled Phospholipids and Peptide Antibiotics**—Binding between bacitracin A or polymyxin B and phospholipids was examined using the general technique described by Hummel and Dreyer (9). The \( ^{3}P \)-labeled lipid was sonicated into a 0.1 M Tris buffer at pH 7.5 containing 1 mM MgCl\(_2\). Neutrally dispersed lipid was removed by filtration through Whatman No. 1 filter paper. The buffer containing the \( ^{3}P \)-labeled phospholipid micelles was then passed through a Sephadex G-25 column (5 \( \times \) 50 mm) and 0.270 ml fractions were collected and counted in 10 ml of Aquasol. After a base line of radioactivity was obtained, a weighed amount of the peptide was added to the column in 60 ml of the radioactive buffer. The phospholipid micelles were excluded from the gel. If the interaction between the peptide and antibiotic was sufficiently strong,
phospholipid was extracted by the peptide from the micelle and included in the gel. Under these circumstances the elution profile consists of a trough of radioactivity followed by a peak (see Fig. 5 below). The ratio of peptide bound lipid to unbound lipid can be approximated knowing the average counts per min in the peak, trough and base line. Knowing the ratio of bound lipid to unbound lipid and the amount of peptide present in the binding assay, it is possible to derive approximate binding constants for the following equilibrium:

\[
(lipid)_n + peptide \rightleftharpoons (peptide\cdot lipid)_n + (lipid)_{n-1}
\]

**RESULTS**

**Binding of [3H]Bacitracin to Whole Cells of M. lysodeikticus**

The binding curve for the interaction of [3H]bacitracin with M. lysodeikticus is hyperbolic with no evidence of cooperativity between binding sites (Fig. 3). When the data were plotted in a Hill plot a slope \( n = 1 \) was obtained (10). The \( K_m \) for binding occurred at a concentration of \( 3.7 \times 10^{-4} \) \( \mu \) bacitracin which compares favorably with the binding constant of \( 1 \times 10^{-6} \) determined in vitro for the interaction of bacitracin A with the C\(_{15}\)-isoprenyl pyrophosphate from M. lysodeikticus (3). These two numbers are also reasonable when compared with the minimum inhibitory concentration of the peptide (\( 1 \times 10^{-7} \) M).

The bacteria are completely saturated with bacitracin at approximately \( 2 \times 10^6 \) molecules per cell. This value was checked by plotting the data of Fig. 3 in a double reciprocal plot (Fig. 4). The linearity of the double reciprocal plot is also indicative of noninteracting binding sites (11). The amount of C\(_{15}\)-isoprenyl pyrophosphate per cell can be estimated at approximately \( 5 \times 10^5 \) molecules per cell (12). Thus, the saturation binding of bacitracin to M. lysodeikticus could be accounted for in quantitative terms by an interaction with the C\(_{15}\)-isoprenyl pyrophosphate in the membrane.

![Fig. 3. Binding of [3H]bacitracin by M. lysodeikticus cells and protoplasts.](image)

**Binding of [3H]Bacitracin to M. lysodeikticus Protoplasts**

The binding of bacitracin to protoplasts paralleled the binding seen with whole cells (Fig. 3). Thus, the protoplasts are saturated at approximately \( 2 \times 10^5 \) molecules per cell with half-saturation occurring at approximately \( 3.7 \times 10^{-4} \) molar. These data suggest two important aspects for the binding of bacitracin to bacterial cells. First, bacitracin does not bind to cell walls. Secondly, the presence of the cell wall does not reduce binding of bacitracin to cells indicating the peptide can diffuse freely through the cell wall to the cytoplasmic membrane. When the protoplasts were osmotically lysed, the membranes still retained their capacity to bind bacitracin.

**Effect of EDTA on Binding of [3H]Bacitracin to M. lysodeikticus**

In vitro binding studies between bacitracin A and the C\(_{15}\)-isoprenyl pyrophosphate have demonstrated that complexation requires a divalent cation (2, 3). Furthermore, the antibiotic activity of bacitracin is lost at low levels of EDTA (13). Because of these observations, binding of [3H]bacitracin by whole cells of M. lysodeikticus was examined in the presence of 0.05 mM EDTA (Fig. 3). The presence of EDTA in the binding assay dramatically decreased binding. In the presence of EDTA, saturation binding was not observed over the concentration range used. Furthermore, the effective \( K_m \) for binding in the presence of EDTA was at least several orders of magnitude lower. Thus, these results suggest that binding of bacitracin to whole cells requires a divalent cation and is consistent with the metal ion requirement for in vitro binding between the peptide and the C\(_{15}\)-isoprenyl pyrophosphate as well as the metal ion requirement for antibiotic activity.

**Lack of Binding between Bacitracin A and Membrane Phospholipids**

Interaction between bacitracin A and E. coli phosphatidylethanolamine, B. subtilis phosphatidylglycerol, and cardiolipin was examined in vitro by gel filtration. No strong interaction between bacitracin A and these phospholipids was detected. For example, as shown in Fig. 5A, polymyxin B interacted strongly with B. subtilis phosphatidylglycerol (as well as with phosphatidylethanolamine and cardiolipin), whereas no binding was seen with bacitracin A (Fig. 5B). These binding curves require further explanation in order to understand what is actually being measured. The phospholipid micelles are excluded from the gel. When polymyxin was added to the column,
it interacted with the lipid micelle in such a way that some fraction of the total phospholipid was included in the gel. Thus, the interaction between polymyxin and phosphatidylglycerol must be quite strong, strong enough so that the peptide is capable of disrupting the phospholipid micelle. Bacitracin does not exhibit this behavior with any of the phospholipids examined. These results do not indicate that bacitracin does not interact with the phospholipid micelle since binding of the peptide to the detergent micelle is not detected in this assay. They do indicate, however, that bacitracin A does not interact with the phospholipids strongly enough to extract lipid from the micelle. The ability to perturb lipid micelles is a property consistent with the mechanism of action attributed to polymyxin which involves permeability changes induced by strong interactions with membrane phospholipids (14, 15).

**DISCUSSION**

There is little doubt that bacitracin inhibits cell wall biosynthesis in vivo and in vitro by complexation with the C55-isoprenyl pyrophosphate. Inhibition of cell wall biosynthesis would certainly be sufficient to terminate further bacterial growth. The question under consideration is whether the protoplast permeability changes induced by bacitracin are due to non-specific interactions between the membrane phospholipids and the peptide or to a specific interaction with the C55-isoprenyl pyrophosphate. The data in this report favor the latter interpretation.

If the bacitracin did interact nonspecifically with membrane phospholipids, it would be expected that the peptide would coat the membrane until the cell was saturated. Instead, there appear to be a limited number of “receptors” in the membrane for bacitracin. Estimates of approximately $5 \times 10^4$ C55-isoprenyl pyrophosphates per cell correlate well with the value of $2 \times 10^4$ molecules of bacitracin bound at saturation. Furthermore, the $K_m$ for binding of bacitracin to cells is $3 \times 10^{-4}$ M and the in vitro association constant for binding between bacitracin and the C55-isoprenyl pyrophosphate is $1 \times 10^6$ M$^{-1}$. Both binding phenomena depended very strongly upon the presence of divalent cations.

Nothing is known about the orientation of the C55-isoprenyl pyrophosphate in the membrane but in light of the function of the lipid it seems highly likely that the pyrophosphate group of the lipid would be situated on the outside surface of the membrane since transfer of the sugar-peptide unit from the C55-isoprenyl pyrophosphate to the growing peptidoglycan must occur externally to the cytoplasmic membrane. Thus, the pyrophosphate function would be readily accessible for complex formation with bacitracin and a divalent cation. In vitro binding studies have suggested that the peptide-lipid complex also involves secondary hydrophobic interactions between nonpolar residues of bacitracin and the lipid alkyl side chain. It is anticipated that in vivo, bacitracin not only complexes the pyrophosphate group of the lipid but also penetrates to some extent into the bilayer interacting hydrophobically with the alkyl side chain of C55-isoprenyl pyrophosphate. Such penetration of the peptide into the membrane could clearly disrupt the membrane structure sufficiently to produce permeability changes.

The effects of bacitracin on protoplasts have to be interpreted with some caution. Reynolds has pointed out that protoplasts are initially fragile and require a lag period after their formation before they become stabilized and adjusted to the new osmotic support which has been substituted for the peptidoglycan (16). For example, if bacitracin was added to the suspension of protoplasts after they had become fully active metabolically, the peptide had little effect on the turbidity of the suspension.

It would be anticipated from the specificity exhibited for the interaction of bacitracin A with the C55-isoprenyl pyrophosphate in vitro that the peptide would not interact strongly and nonspecifically with membrane lipids. The binding data presented in this study support that view. Membrane perturbing antibiotics such as polymyxin B and the new antibiotic, EM 49, disrupt phospholipid micelle structure (17). Bacitracin does not.

If indeed bacitracin induces permeability changes in the plasma membrane and inhibits biosynthesis of peptidoglycan by virtue of its interaction with the C55-isoprenyl pyrophosphate in the membrane, then both effects may contribute to killing of the cells.

**REFERENCES**


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