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 × 10⁶ 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 Kₘ for binding of bacitracin to cells or protoplasts was 3.7 × 10⁻¹⁰ molar which is consistent with the in vitro binding constant of 1 × 10⁻¹ⁱ 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.

MATERIALS AND METHODS

Array 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 dissolved in 0.9% NaCl and applied to a carboxymethylcellulose column (0.9 × 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. lysodeikiticus as a test organism. Only Peaks I and II had significant anti-
and counted in a scintillation counter. At every concentration of \([\text{aH}]\text{bacitracin}\) used, controls consisting of the cells, \([\text{3H}]\text{bacitracin}\) and a large excess of unlabeled bacitracin were used for protein determination.

Binding Assay for Interaction of \([\text{aH}]\text{Bacitracin}\) with \(M. \text{lysodeikticus}\) Protoplasts—One liter of \(M. \text{lysodeikticus}\) 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 protoplasts 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 \(\times\) \(10^9\) protoplasts per ml based upon the number of starting cells. Tubes containing 20 \(\mu\)l of the protoplast suspension, \([\text{aH}]\text{bacitracin}\) 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° 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 \(\text{3P-Labeled Bacterial Phospholipids}\)—One liter of \(E. \text{coli}\) or \(B. \text{subtilis}\) was grown to late log phase in the presence of 15 mCi of \(\text{H}_3\text{P}_{2}O_4\). 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° followed by addition of 40 ml of chloroform and stirring for 20 min at room temperature. The organic phase was separated from the cell suspension 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° under nitrogen.

Assay for Binding Between \(\text{3P-Labeled Phospholipids and Peptide Antibiotics}\)—Binding between bacitracin A or polymyxin B and phospholipids was examined using the general technique described by Hummel and Dreyer (9). The \(\text{3P-labeled lipid}\) was sonicated into a 0.1 M Tris buffer at pH 7.5 containing 1 mM MgCl\(_2\). Nondispersed lipid was removed by filtration through Whatman No. 1 filter paper. The buffer containing the \(\text{3P-labeled phospholipid micelles}\) 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 established 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:

\[
\text{lipid}_a + \text{peptide} \rightleftharpoons (\text{peptide}.\text{lipid}_a) + (\text{lipid})_a
\]

\[ \text{micelle} \quad \text{micelle} \]

**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 \( 3 \times 10^{-5} \) molar 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.

**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,
The data in this report favor the latter in-specific interactions between the membrane phospholipids and the membrane until the cell was saturated. Instead, there 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 nonspecific 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 $K_u$ for binding of bacitracin to cells is $3 \times 10^{-6}$ M and the in vitro association constant for binding between bacitracin and the C55-isoprenyl pyrophosphate is $1 \times 10^5$ 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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