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BACITRACIN: AN INHIBITOR OF THE INSULIN DEGRADING ACTIVITY OF GLUTATHIONE-INSULIN TRANSHYDROGENASE

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SUMMARY

The antibiotic bacitracin, a known inhibitor of insulin degradation by both isolated cells and subcellular organelles, inhibited the ability of purified glutathione-insulin transhydrogenase to split insulin into its constituent A and B chains. This inhibition was demonstrated by measuring the formation of insulin degradative products that were both soluble in 5% trichloroacetic acid and chromatographed as the separate chains of insulin on Sephadex G-50. At concentrations of 90 and 300 μM, bacitracin inhibited 50 and 90%, respectively, of the degrading activity of the purified enzyme. Similarly, degradation by crude liver lysates was inhibited 50 and 90% by 70 and 250 μM bacitracin, respectively. Kinetic studies indicated that this inhibition was by a complex mechanism that decreased both the Vmax and affinity of the enzyme for insulin. These data raise the possibility that the inhibition of glutathione-insulin transhydrogenase by bacitracin could account for part or all of the effects of this antibiotic on inhibition of insulin degradation by target cells.

INTRODUCTION

Studies of the binding of ^{125}I-insulin to its receptor on either isolated cells or subcellular organelles are often complicated by concomitant degradation of the hormone (1-13). In fact, it appears as if all cells that are regulated by insulin also degrade the hormone (14). To facilitate in vitro studies of hormone action, agents have been sought to block this degradation.

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Of the compounds tested, the polypeptide antibiotic bacitracin has been found to be a highly effective agent (8,9,13,15). To understand the action of this antibiotic on the degradation process, it is necessary to know what specific enzyme or enzymes bacitracin inhibits.

At least four enzymes have been identified that can degrade insulin *in vitro* (16-19). One of these enzymes, glutathione-insulin transhydrogenase (or thiol: protein disulfide oxidoreductase), can be isolated in pure form from liver (20) and has been shown to promote the splitting of insulin into its two component chains (21). It has been hypothesized that this splitting is the first step in the degradation of insulin by liver cells (22). The present studies were designed, therefore, to test the effects of bacitracin on the degradative activity of this enzyme.

MATERIALS AND METHODS

The following were purchased: pork insulin (27.3 U.S.P. units per mg) from Elanco Products Co., Indianapolis, IN; Na (^{125}I) from New England Nuclear, Boston, MA; bacitracin (zinc free) from Sigma, St. Louis, MO, and Upjohn, Kalamazoo, MI; and Sephadex G-50 from Pharmacia, Piscataway, NJ.

Enzyme preparation. Glutathione-insulin transhydrogenase was purified from 35 male Sprague-Dawley rat livers by the procedure of Carmichael et al (20). Approximately 11 mg of purified enzyme were obtained from 580 g of liver. The enzyme preparation was greater than 90% pure as determined by densitometric scans of polyacrylamide gels of the enzyme. The enzyme co-electrophoresed with preparations of purified mouse liver glutathione-insulin transhydrogenase and reacted with antibodies to the mouse enzyme (23). The rat liver enzyme preparation degraded insulin with a specific activity of 9,500 units/mg protein where one unit is defined as the ability to degrade 1 µg insulin to trichloroacetic acid soluble fragments in the standard assay described below.

Rat liver lysates were prepared by the same initial steps used in purifying the enzyme. The livers were homogenized for 30 sec in a blender in a buffer containing 100 mM Na-PO_4 buffer, pH 7.5, 5 mM ethylenediamine tetraacetic acid and 1% Triton X-100, centrifuged for 20 min at 25,000 x g and passed through a nylon wool column. Prior to use, the mixture was dialyzed against 100 volumes of 100 mM Na-PO_4, pH 7.5, with 5 mM ethylenediamine tetraacetic acid.

Measurements of insulin degradation. Insulin degradation was routinely measured by the trichloroacetic acid precipitation method. Insulin was labeled with ^{125}I by a chloramine T method (24) and diluted with unlabeled carrier insulin to a specific activity of 3.5 Ci/mole. Aliquots containing 12.5 nmoles insulin (unless stated otherwise), enzyme or lysate (at the indicated concentrations), 0.05% bovine serum albumin, 100 mM Na-PO_4, pH 7.5, 150 mM NaCl, 5 mM ethylenediamine tetraacetic acid and bacitracin (where indicated) were incubated in a total volume of 0.65 ml at 37°C. The reaction

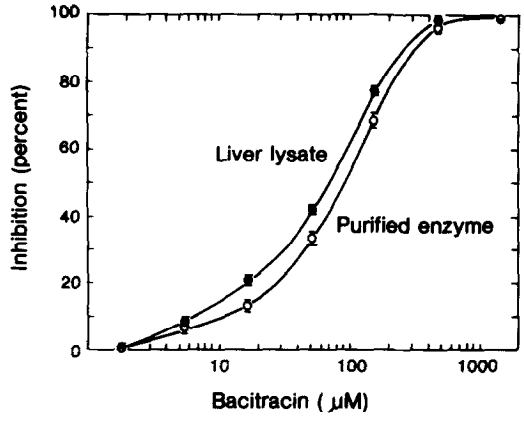

Figure 1. Inhibition of insulin degradation by bacitracin. The degradative activity of either 95 μg of rat liver lysate (●) or 0.9 μg of purified glutathione-insulin transhydrogenase (○) was measured in the presence of the indicated concentrations of bacitracin. In controls without bacitracin, 8.4% and 9.5% of the insulin was degraded by the liver lysate and purified enzyme, respectively. The results presented are means ± SEM of 3 separate experiments.

was started by addition of 50 μl of 14 mM reduced glutathione. After 5 min the reaction was terminated by the addition of 0.25 ml of 20% trichloroacetic acid. The precipitate formed after a 15 min incubation at 0°C was removed by centrifugation and aliquots of the supernatant were taken to measure the amount of acid soluble radioactivity generated. Control samples without enzyme were always included to determine the nonenzymatic increase in acid soluble radioactivity (less than 5% of the total) and this value was subtracted from the value obtained with enzyme to determine the specific increase. Degradation of insulin increased linearly with both time and enzyme concentration over the concentrations of enzyme and lysate employed in the experiments.

Gel filtration studies. The degradation reaction was performed exactly as described above except that at the end of the 5 min incubation the mixture was made 6 M in urea, 1 M in acetic acid and 1% in bovine serum albumin and then brought to 0°C. An aliquot of the mixture (400 μl) was then applied to a 1 x 55 cm column of Sephadex G-50 fine equilibrated with the urea-acetic acid buffer. One-half ml fractions were collected and their radioactivity measured.

RESULTS

Bacitracin, in a dose-dependent fashion, inhibited the ability of rat liver lysates to degrade insulin (Fig. 1). At concentrations of 70 and 250 μM, the degradation caused by the lysates was inhibited 50 and 90%, respectively. Since the protocol used to measure the degrading activity of the lysate preferentially measured the activity of glutathione-insulin trans-

hydrogenase, these results suggested that this enzyme was being inhibited by bacitracin.

To further investigate the possibility that the activity of glutathione-insulin transhydrogenase was affected by bacitracin, the enzyme was purified from rat liver by the procedure of Carmichael et al (20). The insulin degrading activity of this purified enzyme preparation was inhibited by bacitracin at concentrations similar to those that were effective against crude liver lysates; 90 and 300 μM bacitracin inhibited 50 and 90%, respectively, of the degrading activity of the purified enzyme (Fig. 1). In control experiments, bacitracin was added after the incubation of glutathione-insulin transhydrogenase with insulin but prior to the addition of trichloroacetic acid. This protocol resulted in no significant inhibition of the degradation process.

Since the degradation of insulin was monitored by the difference in solubility of insulin and its chains in trichloroacetic acid, it was possible that the inhibition observed was an artifact of this assay system. The reaction products were, therefore, also analyzed by chromatography on Sephadex G-50. After incubation with glutathione-insulin transhydrogenase, insulin eluted as two peaks, native insulin (peak I) and the chains of insulin (peak II) (Fig. 2). The presence of 470 μM bacitracin in the reaction mixture blocked the formation of 87% of the material eluting in peak II, a value close to the 90% inhibition found by the trichloroacetic acid precipitation procedure.

To investigate the mechanism of the inhibition of insulin degradation by bacitracin, kinetic studies were performed. Inspection of the results, presented in Fig. 3 as a double reciprocal plot, suggested a complex mechanism of inhibition. Both the Vmax and Km of the enzyme for insulin were effected by bacitracin. Although the change in Vmax was small, it appeared to be significant since additional analyses which fitted the data by a nonlinear least-squares method (25) also demonstrated a decrease in this parameter (Table I).

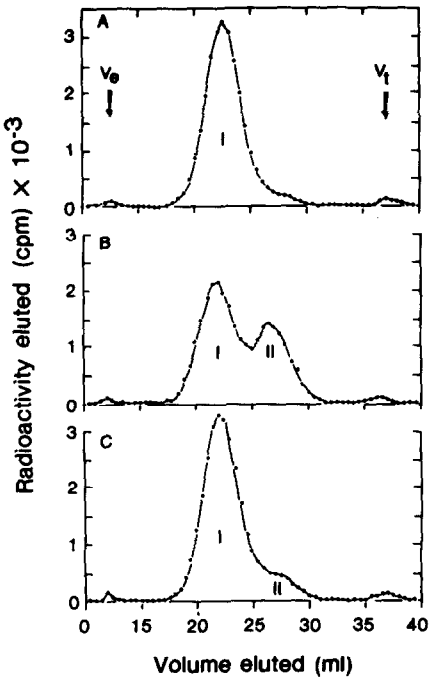

Figure 2. Elution profile on Sephadex G-50 of ^{125}I-insulin, ^{125}I-insulin incubated with purified glutathione-insulin transhydrogenase and ^{125}I-insulin incubated with enzyme in the presence of bacitracin. Each sample contained 22,000 cpm.
A. Insulin control.
B. Insulin incubated with 3 μg of purified glutathione-insulin transhydrogenase in the standard reaction mixture.
C. Insulin incubated with 3 μg of purified enzyme in the reaction mixture plus 470 μM bacitracin.

DISCUSSION

Bacitracin, a peptide antibiotic obtained from Bacillus licheniformis, has several biological activities. This cyclic polypeptide inhibits bacterial peptidoglycan synthesis (26), mammalian transglutaminase activity (27), and various proteolytic enzymes including subtilisin, papain and leucine aminopeptidase (28). Because of this latter inhibitory activity, bacitracin has been used to block degradation in numerous studies on peptide hormone binding, action and uptake (8,9,13,15,29,30). In the case of insulin, bacitracin was found to inhibit degradation of the hormone by rat adipocytes (9), cultured human lymphocytes (13), breast cancer cells (8) and rat hepatocytes (29). The concentration of bacitracin required to inhibit 50% of the degradation in

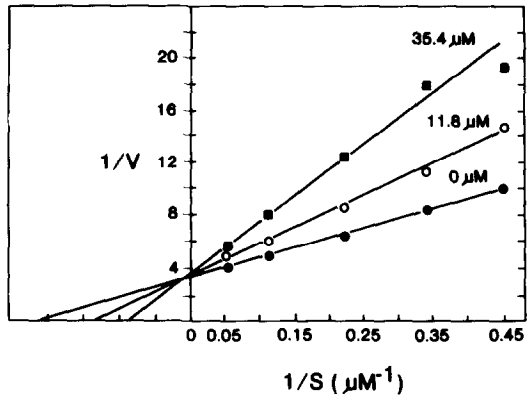

Figure 3. Double reciprocal plot of inhibition by bacitracin of insulin degradation. Varying concentrations of insulin were incubated with 250 ng of purified glutathione-insulin transhydrogenase in the presence of zero (●), 11.8 (○) or 35.4 (■) µM bacitracin. The velocity (V) is expressed in picomoles of insulin degraded per minute per microgram of enzyme. Each value is the mean of 3 separate experiments.

these studies varied from 30 µM for rat adipocytes (9) to 300 µM for cultured human breast cancer cells (8).

The present studies demonstrate that bacitracin is an effective inhibitor of the insulin degrading activity of glutathione-insulin transhydrogenase. It has been proposed that this enzyme promotes in vivo the first step in insulin degradation, the splitting of the hormone into its constituent chains (22). At a concentration of 90 µM, bacitracin was found to inhibit 50% of the activity of purified preparations of this enzyme. This value was within the range observed for inhibition of degradation by various cell types and only slightly higher than the 70 µM required to inhibit a comparable amount of insulin degradation by rat liver lysates. This small difference could result from a slight alteration of the structure of the enzyme during the purification procedure.

The kinetic studies suggested that the inhibition observed resulted from both a decrease in the Vmax and affinity of the enzyme for insulin. This complex mechanism of inhibition is not surprising considering the complexity of the reaction of insulin with the enzyme (21). The thiazoline

TABLE I

Kinetic Constants for Insulin at Various Bacitracin Concentrations

Bacitracin Concentration (μM)	Apparent V_{max} ± SEM (pmol min^{-1} μg^{-1})	Apparent Km ± SEM (μM)
0	283 ± 13	3.98 ± 0.42
11.8	254 ± 13	5.54 ± 0.56
35.4	184 ± 13	5.99 ± 0.83

ring of the bacitracin molecule (31) could bind to either the insulin or glutathione reactive site on the enzyme or the antibiotic could be interacting with an allosteric site on the enzyme (21).

In conclusion, these results demonstrate that bacitracin inhibits the splitting of insulin into its constituent chains by glutathione-insulin transhydrogenase. It is, therefore, possible that inhibition of this enzyme by bacitracin accounts for part or all of the effect of this antibiotic on inhibition of insulin degradation by isolated cells or subcellular organelles.

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