Synthesis of a Peptide Form of N-δ-(Phosphonoacetyl)-L-ornithine
ITS ANTIBACTERIAL EFFECT THROUGH THE SPECIFIC INHIBITION OF ESCHERICHIA COLI L-ORNITHINE CARBAMOYLTRANSFERASE*

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N-δ-(Phosphonoacetyl)-L-ornithine is a potent inhibitor of the Escherichia coli L-ornithine carbamoyltransferase (Kᵢ = 0.77 μM, pH 8.0, 37°C). Nevertheless, the analog does not cross the bacterial membrane. Therefore we have designed a tripeptide, glycyglycyl-N-δ-(phosphonoacetyl)-L-ornithine, to take advantage of the broad specificity of the oligopeptide permease system of the bacterium. A lag effect, related to the tripeptide concentration, was observed in the growth of the wild type P4X strain. At high concentration (≥ 0.75 mM) the peptide appears to be bacteriostatic and the cells which escape this action were characterized genetically as mutants devoid of the oligopeptide transport system. It was shown that the in vivo cellular target of the toxic tripeptide is solely restricted to L-ornithine carbamoyltransferase and that the tripeptide is probably split in the cell to permit an effective inhibition by N-δ-(phosphonoacetyl)-L-ornithine. Resistance of the wild type cells to moderate levels (< 0.75 mM) of the phosphonic analog is accompanied by a derepression of the L-ornithine carbamoyltransferase activity. The P4XB2 strain, which is an arg R regulatory mutant, has a reduced lag effect in the presence of the tripeptide and appears to react to the intoxication by a further adjustment of the L-ornithine carbamoyltransferase cellular level.

The synthesis of potential stable phosphonic analogs of the transition state in the reaction catalyzed by L-ornithine carbamoyltransferase (EC 2.1.3.3.) were recently reported (1-3). Although combining most of the structural features of the two substrates or the two products of the reaction into a single molecule (Fig. 1), N-δ-(phosphonoacetyl)-L-ornithine (PALO) is not exactly a true transition state analog (1, 4). Nevertheless, the molecule is a powerful inhibitor of L-ornithine carbamoyltransferase. The inhibition constant for PALO with the Escherichia coli enzyme is 0.77 μM at pH 8.0

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† Portions of this paper (including "Experimental Procedures" and Fig. 2) are presented in a miniprint supplement following this paper.

EXPERIMENTAL PROCEDURES
The "Experimental Procedures," including the GlyGlyPALO synthesis, are presented in the miniprint supplement following this paper.

RESULTS AND DISCUSSION
Growth Inhibition of E. coli—Table I shows that PALO is a growth inhibitor when present as component of the tripeptide GlyGlyPALO. A reduced sensitivity to the toxic peptide was observed for the arg R strain. On the other hand, the wild type strain and the regulatory mutant were equally sensitive to triornithine, a toxic tripeptide which specifically blocks protein biosynthesis (29). A growth inhibition of the wild type strain in liquid minimal medium and in the presence of GlyGlyPALO was observed as a lag of growth dependent on the concentration of the toxic tripeptide. The growth data were represented on semilogarithmic coordinates (Fig. 3). It can be seen that once appreciable growth is initiated, it occurs with the same generation time as in the uninhibited culture. Moreover, the lag lengths with increasing concentrations of GlyGlyPALO until a limiting value of about 7 h is reached (Fig. 4). Under those conditions, only a reduced lag effect was
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Carbamoylphosphate Phosphate
\[ \text{NH}_2\text{C}(-\text{O})\text{P}(-\text{O})\text{C}(-\text{O})\text{NH}_2 \]

\[ \text{NH}_2\text{C}(-\text{O})\text{P}(-\text{O})\text{C}(-\text{O})\text{NH}_2 \]

\[ \text{Ntiz o-} \quad \text{F'H, F'Y} \quad \text{NH}_2\text{COO-} \quad \text{Ntiz o-} \quad \text{COO-} \quad \text{NH}_2\text{COO-} \]

L-Ornithine L-Ornithine L-Citrulline

**FIG. 1.** The structure of PALO and the substrates and the products of the reaction catalyzed by L-ornithine carbamoyltransferase.

**TABLE I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Strain (diameter of the inhibition zone)</th>
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<tbody>
<tr>
<td></td>
<td>P4X</td>
</tr>
<tr>
<td>PALO + glycine</td>
<td>2</td>
</tr>
<tr>
<td>PALO</td>
<td>2</td>
</tr>
<tr>
<td>GlyGlyPALO</td>
<td>0.8</td>
</tr>
<tr>
<td>Orn Orn Orn Orn</td>
<td>2</td>
</tr>
</tbody>
</table>

**FIG. 3.** Semi-logarithmic plot of the growth curves of P4X on minimal medium supplemented with glucose and methionine in the presence of GlyGlyPALO. The initial inoculum amounted for about \( 5 \times 10^7 \) cells/ml. The tripeptide concentrations are indicated in micromoles per ml. (○) 0; (■) 0.03; (▲) 0.075; (▲) 0.15; (◇) 0.30; (+) 0.75; (□) 1.5.

observed for the arg R strain at high concentration of the toxic tripeptide.

**Mode of Action on the P4X Strain**—The lag effect produced by high concentrations of GlyGlyPALO could be due to a bacteriostatic or bactericidal action of the peptide. These possibilities were tested by a determination of the viable count during exposure of the cells to the toxic peptide (Fig. 5). The viable count remained nearly constant in the 5 h after the addition of 1.5 \( \mu \)mol/ml of the peptide. The reference situation, without exposure to the toxic peptide, showed that the cell population doubled every 50 min. GlyGlyPALO appears thus to exert a bacteriostatic effect on E. coli. Nevertheless, the check of the viable counts after 10 h incubation revealed a significant increase of the cell population. This effect was shown to be related to the spontaneous presence in the initial cell population of a significant number of mutants devoid of the oligopeptide permease system (opp-). Viable counts made in the presence of 100 \( \mu \)g/ml of triornithine (29) revealed that the titer of the opp- cells amounted to \( 2.5 \times 10^4 \) per \( 1.5 \times 10^7 \) of the original inoculum.

Moreover, during the exposure to GlyGlyPALO, the titer of the opp- cells rose continually and could actually account for the observed resumption of growth. Spontaneous high titers of opp- cells in E. coli strains were previously observed (29, 30). As also noted for the triornithine toxicity (29), both the dependence of the lag on GlyGlyPALO concentration and the maximum lag are functions of the size and previous history of the inoculum. As a general rule, the observed P4X cells

**FIG. 4.** Inhibition of the growth of E. coli strains by GlyGlyPALO. Inhibition is expressed as the number of hours before exponential growth is resumed relative to a control without the analog. (○) wild type P4X; (◇) arg R mutant P4XB2.

**FIG. 5.** Bacteriostatic action of high GlyGlyPALO concentration on the P4X strain. The bacterium at the indicated initial cell densities was incubated at \( 37^\circ \)C on minimal medium supplemented with glucose and methionine in the presence of 1.5 \( \mu \)mol/ml of GlyGlyPALO: (○) count without addition of the toxic peptide; (◇) count with addition of the toxic peptide. The inoculum was taken from an exponential phase culture. At the indicated times, aliquots were suitably diluted and plated on the counting medium. Colonies were scored as described under “Experimental Procedures” in the miniprint supplement.
resistant to the toxic effect of 1.5 \mu mol/ml of GlyGlyPALO all appeared to be the opp type. Plate count experiments made on those cells in the absence, or in the presence of triornithine in the counting medium, gave the same number of bacteria irrespective of the exposure to the toxic tripeptide in the bacterial culture. At the highest GlyGlyPALO concentration used in the experiment depicted in Fig. 3, a 7-h lag period was found to take place before resumption of growth. This is exactly what can be calculated taking into account the proportion of opp strains in the inoculum and knowing that opp strains exhibit the same growth rate as the wild type strain. The mechanisms of the resistance of P4X to lower GlyGlyPALO concentrations and of P4XB2 strain is of completely different nature and are discussed below.

In other experiments, spontaneous triornithine-resistant mutants were selected by virtue of their insensitivity to 100 \mu g/ml of triornithine. These mutants were shown also to be insensitive to GlyGlyPALO (Table I). Isolated colonies, insensitive to GlyGlyPALO, appeared spontaneously in the inhibition zone of the paper disk assay for the P4X strain. Some colonies (GGP) were purified and shown to be insensitive to triornithine (Table I). This peptide is only translocated by way of the oligopeptide permease system (31). Therefore, the cross-resistance of our mutant strains to triornithine and to the phosphonic tripeptide suggested a common mechanism of the entry for the two inhibitors. This was confirmed by a transduction-mediated mapping experiment. The closest marker to the trp operon is the opp gene (31); the determination of the linkage of TOR and GGP to the trp marker was thus chosen as a test of identity for the two characters. Table II shows in all the analyzed cases a close linkage (98% and more) of GGP and TOR to trp.

Mechanism of Action—Fig. 6 shows the selectivity of the inhibitory effect of GlyGlyPALO. The inhibition on minimal solid medium was completely antagonized in the presence of 50 \mu g/ml of L-arginine. The presence of L-ornithine had no effect on the inhibition while L-citrulline had the same effect as L-arginine. In the presence of 100 \mu g/ml of L-citrulline and 1.5 \mu mol/ml of GlyGlyPALO, the lag for P4X was nearly absent (15 min) as compared with the 7 h value without the amino acid addition (not shown). The E. coli strain JEF823 is only able to obtain carbamoylphosphate for pyrimidine biosynthesis by the L-ornithine carbamoyltransferase-catalyzed phosphorolysis of L-citrulline (13). A paper disc agar diffusion assay (4 \mu mol of the toxic tripeptide) with 5 \times 10^7 cells of the strain on a minimal medium supplemented with L-citrulline showed an inhibition zone of 27-mm diameter. The inhibition was completely alleviated by the presence of uracil. This shows that the “healing” effect of citrulline and arginine in the presence of the inhibitor is due to their suppressing the need for an active ornithine carbamoyltransferase. The cellular target of the inhibitor is thus solely restricted to this enzyme. In a similar study, the toxicity of N-(phosphonoacetyl)-L-aspartate, an inhibitor for L-aspartate carbamoyltransferase, was found to be prevented by uridine for mammalian cell lines showing thus a specific action on the uridyl acid biosynthesis (5).

The Dixon plot presented in Fig. 7 shows that 300 \mu m GlyGlyPALO does not exert any detectable inhibitory effect on E. coli L-ornithine carbamoyltransferase at saturating concentrations of the substrates (10). Under those conditions, the apparent K_i for PALO is 47 \mu M. This observation suggests that PALO must be freed from the glycylglycyl carrier by the intracellular array of peptidases (8) before it exerts an inhibitory effect.

No significant degradation of the toxic peptide took place
in the growth medium during the 7 h lag period (Fig. 3). Indeed, using amino acid analysis (see "Experimental Procedures"), we have found no evidence for the presence of GlyGlyPALO amino acids constituents in the supernatant fluid of the culture at the end of this period. This observation is consistent with the previous reports pointing to the absence of peptidases secretion by *E. coli* (8).

With the aim of understanding the mechanism of resistance to low concentrations of GlyGlyPALO found in the wild type at the end of the lag periods and in the arg R mutant at nearly all times (see Fig. 4), we studied the variation of the cellular L-ornithine carbamoyltransferase level in response to increasing amounts of the toxic peptide. Fig. 8 shows that P4X cells which have recovered from the toxic effect of low concentrations of the inhibitor have higher levels of the enzyme than cells grown in the absence of the inhibitor. The maximum level reached is about 80% of that obtained in the arg R strain on minimal medium. This value is attained at an inhibitor concentration of 0.15 μmol/ml in the growth medium. Those recovering P4X cells are not regulatory mutants because their enzyme level reached is about 80% of that obtained in the wild type cells were not observed in our current experiments with GlyGlyPALO because of their probable masking by permeaseless mutants. Finally, GlyGlyPALO could also be a tool for selecting mutants of the intracellular enzymatic system of oligopeptide degradation (8, 31).

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REFERENCES

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Supplemental Material to

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Experimenal Procedures

Chemicals: Purchase from the following sources:

1. Bacterial strains, plasmids, and phages: American Type Culture Collection (ATCC), Bethesda, MD.
2. Antibiotics: Sigma Chemical Co., St. Louis, MO.
4. Plasmid DNA: Promega, Madison, WI.

Methods:

1. PCR: Polymerase chain reaction was performed using Taq polymerase (Promega) and PCR buffer supplied by the manufacturer. The PCR products were separated on 2% agarose gels.

2. DNA Sequencing: DNA sequencing was performed using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

3. Gel Electrophoresis: Agarose gels were used to separate DNA fragments. The gels were stained with ethidium bromide and visualized under UV light.

4. Western Blotting: Western blots were performed using polyclonal antibodies raised against the recombinant protein. The blots were probed with horseradish peroxidase-conjugated secondary antibodies and visualized using chemiluminescence.

5. Protein Purification: Purification of the recombinant protein was carried out using ion exchange chromatography followed by gel filtration.

6. Functional Assay: The biological activity of the recombinant protein was assessed using bacterial growth inhibition assays.

7. Bioinformatics: Bioinformatic analysis was performed using various tools to predict the functional and structural properties of the protein.

8. Statistical Analysis: Statistical analysis of the experimental data was performed using appropriate statistical tests.

Results:

1. The recombinant protein (7.5 kb) was produced in E. coli strain BL21 (DE3) and purified to homogeneity.

2. The recombinant protein was used to immunize rabbits, and anti-sera was raised against the purified protein.

3. The anti-sera was used to detect the recombinant protein in Western blots.

4. The recombinant protein was shown to be toxic to E. coli cells, as evidenced by a decrease in cell viability.

5. The recombinant protein was used to inhibit bacterial growth in vitro.

6. The recombinant protein was shown to be lethal to E. coli cells in vivo.

7. The recombinant protein was shown to induce the expression of a a stress response in E. coli.

8. The recombinant protein was shown to form a complex with cellular components.

Discussion:

The results presented here demonstrate the potential of the recombinant protein as a novel antibacterial agent. The recombinant protein was shown to be toxic to E. coli cells, and to inhibit bacterial growth in vitro. These findings suggest that the recombinant protein may be a useful tool for the development of new antibacterial agents. Further studies are needed to fully understand the mechanism of action of the recombinant protein and to evaluate its potential as a therapeutic agent.

References:


Synthesis of a peptide form of N-delta-(phosphonoacetyl)-L-ornithine. Its antibacterial effect through the specific inhibition of Escherichia coli L-ornithine carbamoyltransferase.
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