Accumulation of glyceride-modified pre-penicillinase of *Bacillus licheniformis* in *Escherichia coli* treated with globomycin

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The membrane penicillinase of *Bacillus licheniformis* is a glyceride–cysteine lipoprotein whose NH_2 terminus is analogous to the major outer membrane lipoprotein of *Escherichia coli*. When *E. coli* cells producing *B. licheniformis* penicillinase were treated with the antibiotic, globomycin, a precursor of the penicillinase, pre-penicillinase, accumulated in the cell. It could be immunoprecipitated with anti-penicillinase antibodies; it contained palmitate; and one of its two cysteine residues was modified by glycerol. The action of globomycin, probably indirectly, also activates protease which acts differently on the pre-penicillinase than does the signal peptidase. The results strongly indicate that the pre-penicillinase is processed by the globomycin-sensitive signal peptidase in *E. coli*, and the modification of precursor by lipid precedes removal of the signal peptide as it does with the membrane lipoproteins of *E. coli*.

Pre-penicillinase Bacillus licheniformis Escherichia coli *Globomycin*
Lipid modification *Signal peptidase*

1. INTRODUCTION

The penicillinase (EC 3.5.2.6; *penP* product) of *Bacillus licheniformis* 749 can be detected in three forms: two excreted ones, exo-large and exo-small, found in the culture supernatant and a form bound to the cell membrane [1,2]. The membrane form of penicillinase has been found to be a lipoprotein [3–5] whose NH_2 terminus is similar to that of the major outer membrane lipoprotein of *Escherichia coli* [4,5] in which the NH_2-terminal cysteine residue is bonded to a glyceride through a thioether linkage. An antibiotic called globomycin [6] is known to prevent biosynthesis of the *E. coli* lipoprotein by inhibiting the specific signal peptidase; this results in the accumulation in the cell envelope of lipid-modified precursor containing the NH_2-terminal signal peptide [7–10]. The penicillinase of *B. licheniformis* is also synthesized as a precursor having an NH_2-terminal signal peptide [3,11,12] and is then processed to the membrane form [12], presumably by a specific signal peptidase. There is a remarkable homology of amino acid sequence between the *B. licheniformis* penicillinase and the major outer membrane lipoprotein of *E. coli* at the cleavage site of the respective precursors [4,13]; however, globomycin was not found to inhibit biosynthesis of penicillinase in *B. licheniformis* [5]. It rather suppressed the accumulation of the membrane penicillinase with a corresponding increase in the excreted form (exo-large) [5]. Here, we report that if *E. coli* cells, producing *B. licheniformis* 749 penicillinase, are treated with globomycin, a lipid-modified precursor of the penicillinase accumulates in the cell. Our findings indicate that the biosynthetic pathway followed by penicillinase is similar to that of the major outer membrane lipoprotein of *E. coli* as in [8,9].

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Abbreviation: SDS, sodium dodecyl sulfate

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and media*

Escherichia coli MRE600 (RNase I$^-$) [14] and JE5505 (*F$^-$ lpp-2 pps his proA argE thi gal lac xyl tsx*), a K-12 strain lacking the major outer membrane lipoprotein [15], was used. JE5505 was transformed with plasmid pRW83 which is a derivative of pBR325 devoid of its *bla* gene and containing an insert of *penP* gene from *Bacillus licheniformis* 749/C (P.S.F. Mezes, W. Wang, E.C.H. Yeh, J.O.L., in preparation). JE5505/pRW83 was grown at 37°C in either M-9 medium [16] supplemented with glucose, 2 g; histidine, proline, arginine, leucine, 50 mg each; and thiamine, 2 mg or L-broth [16]. Both contained 20 mg chloramphenicol/l.

2.2. *Globomycin treatment and labeling*

Globomycin [6] was a gift from Dr M. Arai (Sankyo Co., Tokyo). Typically, 1 ml cultures in supplemented M-9 medium were grown up to a cell density of A_{600} = 0.8, at which time a 1% (w/v) solution of globomycin in methanol was added to 250 μg/ml final conc. [^{35}S]Methionine (67 μCi . 71 nmol^{-1} . ml^{-1}) or [^{35}S]cysteine (110 μCi . 80 pmol^{-1} . ml^{-1}) (both from Amersham) was added 4 min after the addition of globomycin and labeling carried out for 10 min. For labeling with [9,10-^{3}H]palmitic acid (200 μCi . 10 nmol^{-1} . ml^{-1}) (from New England Nuclear), the palmitate was added just before the globomycin, and cells were labeled for 15 min. Cells were pelleted quickly by centrifugation in an Eppendorf microfuge for 2 min and boiled with 2% SDS for 5 min. The SDS-soluble fraction was used for immunoprecipitation with antipenicillinase antibodies as in [12]. Cytoplasm and membrane fractions were isolated as in [7].

2.3. *Electrophoresis*

Immunoprecipitated samples were analyzed in 15–22% acrylamide gradient urea–SDS slab gels as in [17]. Gels containing [^{3}H]palmitate-labeled samples were treated with En^{3}Hance (New England Nuclear) for fluorography. Protein bands labeled with [^{35}S]cysteine were cut from gel and prepared for high-voltage paper electrophoresis at pH 1.9 as in [13]. The dried paper was cut into 1 cm strips and counted for radioactivity.

2.4. *Cell-free protein synthesis*

The DNA-directed transcription–translation system in [18] was employed. S30 was made from *E. coli* MRE600. [^{35}S]Methionine was used for labeling. Reaction was stopped by addition of cold methionine and precipitation of protein with cold 10% trichloroacetic acid. The precipitate was washed with ether, dried, solubilized in 2% SDS and used for immunoprecipitation as above. For checking the action of Wickner's leader peptidase [19] the cell free reaction mixture was made 1% in Triton X-100 and the purified leader peptidase (gift of Dr W. Wickner) was added. As a control [^{35}S]methionine labeled procoat (gift of Dr W. Wickner) was treated similarly. Incubation was at 37°C for 1 h.

3. RESULTS

3.1. *Action of globomycin on penicillinase synthesis*

Globomycin is known to inhibit the signal peptidase that processes the precursors of *E. coli* membrane lipoproteins [7,20]. As the precursors accumulate in the envelope, cells become globular and lyse [6,7]. We took a mutant strain of *E. coli* K12, JE5505, which lacks the structural gene for the major outer membrane lipoprotein [15]. Thus the major target of globomycin and cause of cell lysis was absent. In fact, this strain is highly resistant to globomycin [7,21]. We transformed JE5505 with plasmid pRW83, a derivative of pBR325 whose *bla* gene had been rendered inactive and containing an insert of *penP* gene from *B. licheniformis* 749/C. When pRW83 is expressed in *E. coli* the membrane penicillinase can be detected in the cell envelope (P.S.F. Mezes, W. Wang, E.C.H. Yeh, J.O.L., in preparation). We pulse labeled the cells with [^{35}S]methionine or [^{35}S]cysteine, immunoprecipitated the SDS extract of the total cell with antipenicillinase antibodies and analyzed the precipitate on an acrylamide–urea–SDS gel (fig.1). The globomycin-treated cells (lanes 2,4) had a band above that for the membrane penicillinase. This band corresponded to the in vitro translation product using pRW83 DNA (lane 5). Since these bands were immunoprecipitated with anti-penicillinase antibodies, it is very likely that the upper band was the precursor of the membrane penicillinase; i.e., the pre-penicillinase.

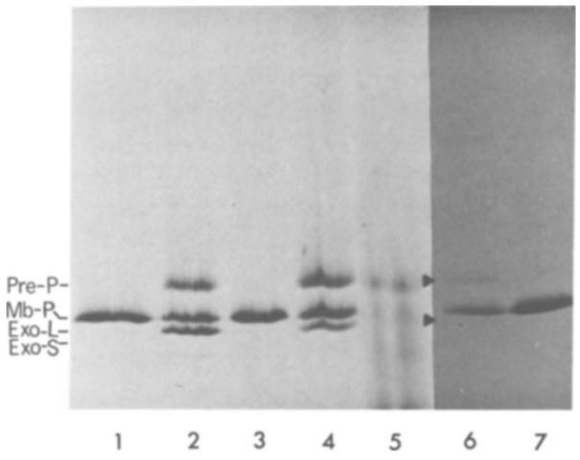

Fig.1. Effect of globomycin (250 μg/ml) on penicillinase synthesis. *E. coli* JE5505/pRW83 cells were labeled with [^{35}S]methionine (lanes 1,2) or [^{35}S]cysteine (lanes 3,4) or [^{3}H]palmitic acid (lanes 6,7) in presence (lanes 2,4,6) or absence of globomycin (lanes 1,3,7). After extraction with 2% SDS and immunoprecipitation with anti-penicillinase antibodies the samples were analyzed in a 15–22% acrylamide gradient urea–SDS gel. Lane 5 contains anti-penicillinase immunoprecipitate of protein synthesized in the coupled transcription–translation system using pRW83 plasmid DNA. Lanes 1 and 3 contained 5000 cpm; for lanes 2 and 4, 10000 cpm was used to facilitate demonstration of pre-penicillinase; lanes 5,7, 2000 cpm; lane 6, 3000 cpm. The gel piece containing lanes 6 and 7 was treated for fluorography and exposed to X-ray film for 8 days. The rest was exposed for 5 days: Pre-P, pre-penicillinase; Mb-P, membrane penicillinase; Exo-L, exo-large; Exo-S, exo-small; (⟶) in lane 6 show the [^{3}H]palmitate-labeled bands of pre-penicillinase and the degradation product.

When materials similar to those in lanes 1 and 2 of fig.1 were precipitated with acetone, resuspended in 40 mM Tris–HCl (pH 8.0)/10 mM CaAc buffer and treated with trypsin (40 μg/ml) all bands were cut down to a size identical to exo-small (not shown). Exo-small, which includes the main globular portion of pre-penicillinase and the COOH terminus, is resistant to proteolysis [22,23]. This also shows that the bulk of the material in lane 2 (fig.1) is pre-penicillinase and membrane penicillinase.

3.2. *Lipid modification of the precursor*

To check whether the pre-penicillinase band contained a glyceride unit as in the mature membrane penicillinase, we labeled the cells with [^{3}H]palmitic acid. The immunoprecipitated bands are shown in fig.1 (lanes 6,7). The pre-penicillinase band was lightly labeled with [^{3}H]palmitate in the globomycin-treated cells (lane 6) and is discussed later. To further check whether the cysteine residue was modified by lipid in the pre-penicillinase, the [^{35}S]cysteine labeled pre-penicillinase band from lane 4 was cut out from the gel, oxidized with performic acid and hydrolyzed for the detection of glyceryl cysteine sulfone by high-voltage paper electrophoresis (fig.2). The bands of membrane penicillinase, from lanes 3 and 4 of fig.1 (fig.2A and 2C, respectively) were taken as a control. It can be seen that the ^{35}S-label was found mostly in the position of glyceryl cysteine sulfone as expected of the membrane penicillinase (fig.2A). The

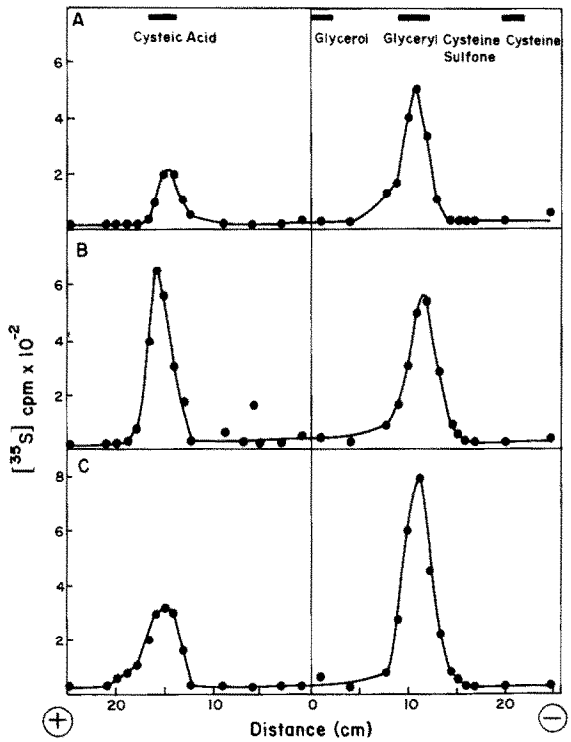

Fig.2. High-voltage paper electrophoresis of the oxidation products of [^{35}S]cysteine-labeled membrane penicillinase and pre-penicillinase. [^{35}S]Cysteine-labeled bands of membrane penicillinase from fig.1, lane 3 (A) and lane 4 (C) and of pre-penicillinase from fig.1, lane 4 (B) were cut out, and the protein was oxidized with performic acid and hydrolyzed with acid. Electrophoresis was at 3500 V for 1 h at pH 1.9.

pre-penicillinase gave two peaks of identical intensity: one corresponding to glyceryl cysteine sulfone and the other to cysteic acid (fig.2B). There is one more cysteine residue (Cys 21) in pre-penicillinase than in the membrane penicillinase; this is present in the signal peptide [3]. Since the sizes of the two peaks in fig.2B are almost equal, it is likely that one of the two cysteine residues in the whole molecule was modified by glycerol/glyceride and the other was not. We assume from this that the pre-penicillinase accumulating in the presence of globomycin was modified by lipid at the cysteine residue (Cys 27) that is retained in the membrane penicillinase [12].

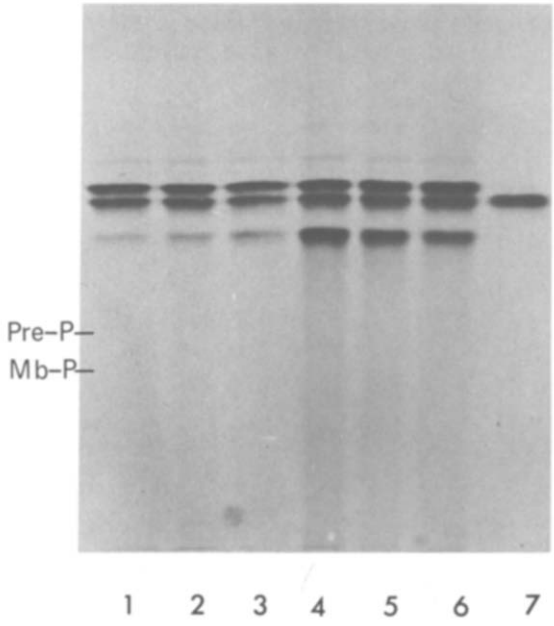

Fig.3. Pulse/chase labeling of pre-penicillinase. To *E. coli* JE5505/pRW83 cells were added globomycin (250 μg/ml) and [^{35}S]methionine either immediately (lane 2), after 10 min (lane 3), or after 20 min (lane 4); labeling was for 10 min. To a portion of the cells used for lane 2, unlabeled methionine was added after the 10 min labeling, and incubation (chase) was continued for 10 min (lane 5), 20 min (lane 6), or 30 min (lane 7). SDS extracts of cells were immunoprecipitated, analyzed by gradient gel electrophoresis and autoradiographed. Lane 1 represents a control culture (without globomycin treatment) labeled for 10 min. This sample contained 10000 cpm; each of the others contained 20000 cpm. The gel was exposed to X-ray film for 4 days: Mb-P, membrane penicillinase; Pre-P, pre-penicillinase.

3.3. *Degradation of pre-penicillinase*

A band migrating faster than the membrane penicillinase is evident in pre-penicillinase-containing lanes of fig.1; i.e., in cells treated with globomycin. This band migrated slightly slower than the larger of the two exo-penicillinases (exo-large). This gel system gives a good separation of all the forms of penicillinase, including the membrane penicillinase and exo-large. The exo-penicillinases have no cysteine residues, while this band could be labeled with [^{35}S]cysteine and [^{3}H]palmitate (fig.1, lanes 4,6). From these properties and its migration in the gels we consider it a degraded form of pre-penicillinase (or membrane penicillinase), shortened from the COOH terminus and possibly from the NH_2 terminus as well.

When cultures were treated with globomycin for 30 or 40 min and then pulse labeled, there was no pre-penicillinase and diminished amounts of membrane penicillinase (not shown). To further understand this phenomenon we performed two experiments. In one we added [^{35}S]methionine at 0, 10 and 20 min after addition of globomycin and allowed labeling for 10 min. The cells were spun down, boiled with 2% SDS and the penicillinase immunoprecipitated and analyzed by electrophoresis (fig.3, lanes 2–4): there is little difference in the relative intensity of the bands. This indicates that the pre-penicillinase is synthesized throughout the 30 min incubation with globomycin. In a parallel experiment [^{35}S]methionine was added with globomycin and, after 10 min labeling, cold methionine was added and samples taken every 10 min for 40 min (fig.3, lanes 5–7). Comparison of lanes 2 and 5 shows that during the 10 min chase, there is a decrease in the relative amount of pre-penicillinase. Further chase brought very little change. As equal amounts of radioactivity were put in all lanes (lanes 2–7) quantitation is difficult, but it is clear that the pre-penicillinase was not very stable in the cell.

4. DISCUSSION

It is now known that in the biosynthesis of the membrane lipoprotein of *E. coli* the precursors are first modified by lipid at a cysteine residue which becomes the NH_2 terminus of the mature protein [7,8]. The globomycin antibiotic, inhibits the signal peptidase for the lipoprotein [8–10]. Here,

we find that when *E. coli* cells are producing *B. licheniformis* penicillinase, globomycin inhibits the processing of pre-penicillinase to the membrane penicillinase, which is a lipoprotein similar to the major outer membrane lipoprotein [3–5]. But unlike the studies with the *E. coli* membrane lipoprotein, we did not get a total inhibition (fig.1), possibly because of the relative impermeability of JE5505 to globomycin [21]. Another type of signal peptidase, known as leader peptidase [19], is present in *E. coli* cells. Globomycin does not inhibit the leader peptidase, which does not act on the *E. coli* lipoprotein [24]. We had found that the leader peptidase could not process the primary translation product of *penP*, while it did process the procoat protein (from bacteriophage M13) under similar conditions (not shown). So it is possible that some other type of protease was activated as the precursors accumulated because of the action of globomycin. Our results suggest that the protease(s) acts differently from signal peptidase. Instances of such proteolysis of unusual proteins (e.g., the MalE-LacZ hybrid protein [25]) in *E. coli* are known.

The pre-penicillinase accumulating due to globomycin was modified by glyceride at the cysteine residue (fig.1, lane 6; fig.2B); this is similar to what had been found with *E. coli* membrane lipoproteins [7,20]. The [^{3}H]palmitate labeling of pre-penicillinase was disproportionately fainter when compared with [^{35}S]methionine- or [^{35}S]cysteine-labeled bands (fig.1, lanes 2,4). In cells treated with globomycin, pre-penicillinase can be found in the cytoplasm as well as the membrane fraction (not shown). This unusual localization could be the reason for low palmitate labeling. How pre-penicillinase is modified by glycerol (fig.2B) remains unexplained. However, the results indicate that the biosynthesis of penicillinase is similar to that of the membrane lipoprotein of *E. coli* as described in [8,9]; i.e., the primary translation product of *penP* is first modified by lipid and then the signal peptide is removed by the signal peptidase which is sensitive to globomycin.

Presumably, membrane penicillinase is biosynthesized in *B. licheniformis* with intermediates similar to those in *E. coli*, although there is evidence that membrane penicillinase is not an obligate intermediate [5]. In *B. licheniformis* globomycin was found to reduce synthesis of membrane penicillinase with a corresponding increase in production of the secreted form, exo-large [5]. Possibly in the presence of globomycin, the enzyme(s) that ordinarily cleaves membrane penicillinase now cleaves the pre-penicillinase (modified or unmodified) to give exo-large directly, as speculated in [5,26]. This process would bypass the membrane penicillinase while allowing total penicillinase production to remain constant.

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