REGULATION OF THE TERMINAL STEPS IN PEPTIDOGLYCAN BIOSYNTHESIS IN ETHER-TREATED CELLS OF *ESCHERICHIA COLI*

Edward E. ISHIGURO, David MIRELMAN+ and Robin E. HARKNESS
Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC V8W 2Y2 Canada and
+*Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel*

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1. Introduction

The terminal steps in cell wall peptidoglycan synthesis involve the crosslinking enzyme system which, in *Escherichia coli*, consists of three penicillin-sensitive enzyme activities [1]:

(i) Peptidoglycan transpeptidase (TPase) which catalyzes the transpeptidation reaction between the peptide moieties of peptidoglycan;
(ii) D-Alanine carboxypeptidase (CPase) which hydrolyzes the terminal D-alanine residue from the pentapeptide side chain of peptidoglycan;
(iii) Endopeptidase which hydrolyzes the cross-linkage between peptides.

The endopeptidase is apparently another manifestation of the CPase. Peptidoglycan synthesis in *E. coli* is under stringent control [2]. During amino acid deprivation, the synthesis of nucleotide precursors [3] and peptiodoglycan [4] was inhibited in a *relA*$^+$ strain but not in a *relA*$^-$ mutant [2].

Ether-treated bacteria (ETB) which are permeable to exogenous nucleotide precursors have been used for in vitro studies on the terminal steps in peptidoglycan synthesis [5]. ETB retain normal cellular controls involved in regulating peptidoglycan synthesis, in contrast to cell-free systems where such controls are lost as a result of the mechanical disruption of the cells [5]. Here we report on the regulation of the terminal steps in peptidoglycan synthesis during amino acid deprivation of *relA*$^+$ and *relA*$^-$ strains of *E. coli* using the ETB system. We show that:

(i) Amino acid deprivation resulted in a marked decrease in CPase activity of *relA*$^+$ bacteria but not of *relA*$^-$ bacteria;
(ii) The decreased CPase activity of amino acid-deprived *relA*$^+$ bacteria was associated with an increase in the crosslinkage of peptidoglycan synthesized in vitro by ETB;
(iii) Amino acid deprivation resulted in a decrease in the peptidoglycan biosynthetic activity of *relA*$^+$ ETB but not of *relA*$^-$ ETB.

2. Materials and methods

Escherichia coli K-12 strain LD5456 (*thi dapD lysA relA*) is an isogenic derivative of strain LD5 (*thi dapD lysA relA*$^+$) [2]. Bacteria were grown in 600 ml M9 minimal medium with required growth factors [2] at 37°C with shaking for 3 exponential doublings to a density of ~2 × 10^8 cells/ml. The cells were harvested, washed, and inoculated into 300 ml quantities of M9 medium, prewarmed to 37°C, with and without lysine [2]. The cultures were incubated in a 37°C shaker, and cells from 80 ml samples of the cultures were collected by centrifugation at 27 000 × *g* for 3 min at the indicated times. The cells were treated with ether as in [5,6]. Samples of ETB contained ~15 mg protein/ml as determined by the method in [7] with bovine serum albumin (Sigma Chemical Co.) as a standard.

The assay mixture for CPase and TPase contained ETB (0.5–1.0 mg protein), 50 mM Tris–HCl buffer (pH 8.3), 20 mM $MgCl_2$ 0.5 mM mercaptoethanol, and ^{14}C-labeled uridine diphosphate-*N*-acetylmuramyl-pentapeptide (UDP-MurNAc-pentapeptide, 141 cpm/pmol, 60 000 cpm) in 90 μl total vol. The ^{14}C-labeled UDP-MurNAc-pentapeptide (labeled in the two terminal D-alanine residues) was prepared as in [2]. The assays were performed with and without UDP-*N*-acetylglucosamine (UDP-GlcNAc, 0.53 mM) and in the presence and absence of ampicillin [5]. After

60 min incubation at 37°C, the assay mixtures were transferred to an ice bath and immediately applied to Whatman 3MM paper. The labeled components in the assay mixtures were separated by descending chromatography in isobutyric acid–1 N NH_4OH (5:3) for ~20 h. The D-[^{14}C]alanine spots were cut from the chromatogram and counted [2] in a Beckman LS 3145T scintillation counter.

To determine peptidoglycan synthesis, the above assay mixture (with 0.53 mM UDP-GlcNAc) was prepared in duplicate and incubated at 37°C for 60 min. One of the assays was terminated by adding 1 ml of 4% sodium dodecyl sulfate (SDS) and boiling for 30 min. To the other assay, 2 ml cold 5% trichloroacetic acid (TCA) was added, and the mixture was kept on ice for 30 min. The SDS-insoluble and trichloroacetic acid-insoluble fractions were collected on 0.45 μm Millipore filters, and radioactivity was determined as in [5].

For the determination of peptidoglycan crosslinkage, ETB were incubated at 37°C for 60 min in the above assay mixture with 0.56 mM UDP-MurNAc-pentapeptide and UDP-*N*-acetyl-D-[6-^{3}H(*N*)]glucosamine (New England Nuclear, 7.5 cpm/pmol, 150 000 cpm) as substrates. The labeled peptidoglycan was isolated and digested with lysozyme as in [5]. The amounts of crosslinked (dimer) and uncrosslinked (monomer) fragments in the lysozyme digest were quantified after paper chromatography [8].

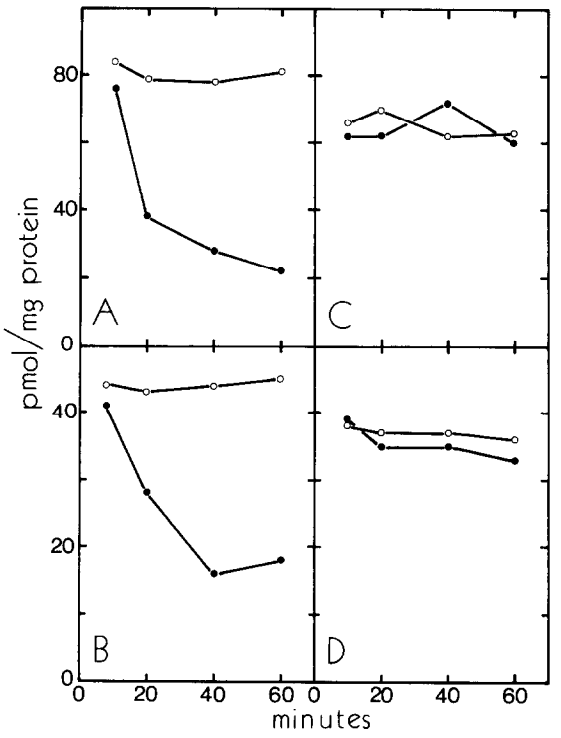

Fig.1. Effect of amino acid deprivation on specific activities of CPase (A,C) and TPase (B,D) in strains LD5 *relA*$^+$ (A,B) and LD5456 *relA*$^-$ (C,D). Enzyme activities were determined in ETB prepared at designated times from cultures grown with (○) and without (●) L-lysine. Activities are expressed as pmol D-[^{14}C]alanine released/mg protein from ^{14}C-labeled UDP-MurNAc-pentapeptide.

3. Results and discussion

Fig.1 compares the specific activities of CPase and TPase in ETB prepared from cultures of strains LD5 (*relA*$^+$) and LD5456 (*relA*$^-$) grown in the presence and absence of lysine. For both strains, the activities of CPase and TPase remained most constant in cells grown with lysine. In lysine-deprived ETB, a marked decrease in CPase activity (70%) occurred in strain LD5 (*relA*$^+$) but not in strain LD5456 (*relA*$^-$). Similarly, TPase activity decreased ~60% during lysine deprivation in strain LD5 (*relA*$^+$) but <10% in strain LD5456 (*relA*$^-$). In strain LD5 (*relA*$^+$), most of the decrease in the activities of CPase and TPase occurred during the first 40 min lysine deprivation.

Fig.2 compares the peptidoglycan biosynthetic activities of ETB prepared from cultures grown with and without lysine. For both LD5 (*relA*$^+$) and LD5456 (*relA*$^-$), the amounts of trichloroacetic acid-insoluble (total macromolecular peptidoglycan) and SDS–insoluble peptidoglycan (total crosslinked to pre-existing peptidoglycan) synthesized by ETB from cultures grown with lysine remained almost constant throughout the experimental period (A,D). As in other *E. coli* [5], the SDS-insoluble peptidoglycan accounted for ~40–50% of the total peptidoglycan synthesized in both strains (C,F). In strain LD5 (*relA*$^+$), the synthesis of trichloroacetic acid-insoluble and SDS-insoluble peptidoglycan decreased by ~75% and 50%, respectively, during lysine deprivation (B). The proportion of the peptidoglycan synthesized by *relA*$^+$ ETB which was SDS-insoluble increased to >70% during lysine deprivation (C). In contrast, the synthesis of both TCA-insoluble and SDS-insoluble peptidoglycan remained about the same in lysine-deprived ETB of the *relA*$^-$ strain (E,F). The degree of crosslinkage was determined in lysozyme digests of

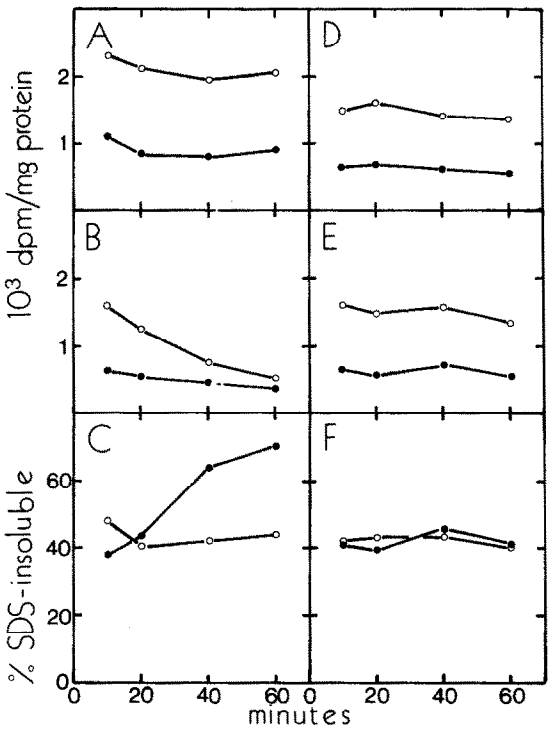

Fig.2. Effect of lysine deprivation on peptidoglycan biosynthesis in strains LD5 *relA*$^+$ (A,B,C) and LD5456 *relA*$^-$ (D,E,F). ETB were prepared at designated times from cultures grown with (A,D) and without (B,E) L-lysine. The trichloroacetic acid-insoluble (○) and SDS-insoluble (●) peptidoglycan synthesized in vitro by these ETB were quantified. (C,F) percentages of the peptidoglycan which were crosslinked to pre-existing peptidoglycan as a function of time (lysine-deprived ETB (●); control ETB (○).

labeled peptidoglycan synthesized in vitro. The ETB used in these experiments were prepared from cultures grown in the presence and absence of lysine for 60 min. As noted in [9], the dimer:monomer ratios of the peptidoglycan synthesized by lysine-deprived and unstarved ETB of strain LD5 (*relA*$^+$) were 1.42 and 0.82, respectively. In contrast, the dimer:monomer ratios for lysine-deprived and unstarved ETB for strain LD5456 (*relA*$^-$) were essentially identical (0.90 and 0.97, respectively). Thus, the markedly lower level of CPase activity in lysine-deprived *relA*$^+$ ETB was associated with an enhancement of transpeptidation (despite the decreased TPase activity in these ETB). Collectively, these results support the proposal [1,5,9–12] that CPase may be involved in regulating peptidoglycan transpeptidation, possibly by controlling the amount of substrate available for TPase.

As discussed in [5], the ETB system offers one distinct advantage over the cell-free systems in that the regulatory mechanism for peptidoglycan synthesis appears to remain functional. Thus, the decrease in peptidoglycan biosynthetic activity in lysine-deprived *relA*$^+$ ETB could be a reflection of stringent control, and this could involve, at least partly, the inhibition of TPase. The observation that peptidoglycan biosynthetic activity decreased <10% in lysine-deprived *relA*$^-$ ETB is consistent with this view. We have not yet determined the levels of guanosine 5′-diphosphate 3′-diphosphate (ppGpp) in ETB. However, if ether treatment results in the release of low M_r compounds from bacteria [6], this would probably include ppGpp, the proposed mediator of stringent control [13]. If so, the results of this study suggest that stringent control is probably not due to the inhibition of a key peptidoglycan biosynthetic enzyme resulting from a direct interaction of the enzyme with ppGpp [2]. Since phospholipid synthesis is also under stringent control [14], an alternative possibility is that a change in membrane composition and structure occurs during amino acid deprivation in *relA*$^+$ bacteria but not in *relA*$^-$ bacteria. This change could affect the activity of certain particulate enzymes involved in peptidoglycan synthesis. For example, we have shown that the decrease in CPase activity during amino acid deprivation of strain LD5 (*relA*$^+$) was due to the conversion of CPase to a cryptic form which could be activated by sonic disruption of the ETB [9]. Cryptic ity of CPase could be the result of a change in membrane structure.

Acknowledgements

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