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DUAL ENZYME ACTIVITIES OF CELL WALL PEPTIDOGLYCAN SYNTHESIS, PEPTIDOGLYCAN TRANSGLYCOSYLASE AND PENICILLIN-SENSITIVE TRANSPEPTIDASE, IN PURIFIED PREPARATIONS OF *ESCHERICHIA COLI* PENICILLIN-BINDING PROTEIN 1A

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SUMMARY: Two different cell wall peptidoglycan synthetase systems are carried by penicillin-binding proteins 1A and 1B purified from *Escherichia coli*. Both systems consist of two enzyme activities carrying out successive reactions of peptidoglycan synthesis from the lipid-linked precursor, N-acetylglucosaminyl-N-acetylmuramyl(-pentapeptide)-diphosphate-undecaprenol, namely, those of peptidoglycan transglycosylase and β-lactam antibiotic-sensitive transpeptidase. The activities of the two enzyme systems differ in optimal conditions and sensitivities to β-lactam antibiotics. The properties of purified PBP-1A are reported in this paper.

Penicillin-binding proteins (PBPs)[1] in bacteria play essential roles in duplication of the peptidoglycan sacculus, the basal structure of the bacterial cell wall (1). In *Escherichia coli* six major PBPs, 1A and 2 to 6, and one group of PBPs, numbered 1Bs, have been found (1-3). Among them, the highest molecular weight PBPs, 1A and 1Bs, are supposed to function in formation of the peptidoglycan sacculus in its elongation process. Defect in the cell of one of these two kinds of PBPs by mutation or inhibition is supposed to be compensated for by the other (2,3). Recently Nakagawa, Tamaki and Matsuhashi (4) isolated purified PBP-1Bs practically free from contaminating proteins from *E. coli* membranes and found that this preparation has activities of both peptidoglycan transglycosylase and penicillin-sensitive transpeptidase, enzymes catalyzing two successive reactions in the formation of crosslinked peptidoglycan. These activities are identical with the peptidoglycan synthetase activities demonstrated

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[1]The abbreviations used are: A_2pm, 2,6-diaminopimelic acid; GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; PBP, penicillin-binding protein; SDS, sodium lauryl sulfate.

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many years ago by Izaki, Matsuhashi and Strominger (5) in the *E. coli* membrane. On the contrary, the enzymatic activities of PBP-1A, the supposed detour enzyme for PBP-1B(s) (2,3), were not detected at all using currently available assay procedures. Therefore we were interested to know if the 1A protein actually has enzyme activities. Many β-lactam antibiotics have different affinities to these two kinds of PBPs, and therefore, if an enzyme activity of PBP-1A could be demonstrated, it should be inhibited specifically by β-lactam antibiotics that have specific affinities to PBP-1A.

MATERIALS AND METHODS

Strains used. A plasmid pLC29-47 which putatively carries the *E. coli* chromosomal part covering the structural gene of PBP-1A was obtained from the plasmid bank of Clarke and Carbon (6,7) and introduced into *E. coli* K12 strain JST975srev61, a *recA* derivative strain from strain JST975srev6, which lacks PBP-1Bs and produces an excessively large amount of PBP-1A (2). Strains from Clarke and Carbon's plasmid bank were kindly provided by Dr. K. Ueda, Kyoto University Medical School. Strains with and without the plasmid were both used for preparation of PBP-1A.

Isolation of PBP-1A. *E. coli* membranes were extracted with 1% Triton X-100 for 30 min at room temperature, and the extract was chromatographed on DEAE-cellulose column with an NaCl gradient (0 to 0.4 M) and then material was subjected to affinity chromatography on a 6-aminopenicillanic acid-Sepharose 4B column. The fraction containing PBP-1A was eluted by 10 min treatment with 1 M hydroxylamine solution in 0.5 M Tris-HCl buffer, pH 8.2, containing 0.2% Triton X-100 and was dialyzed overnight against 0.025 M Tris-HCl, pH 7.6, containing 0.2% Triton X-100 and 20% glycerol. Sometimes Bio-Beads SM-2 (BIO RAD Laboratories, USA) were used to remove Triton X-100 from the enzyme solution.

Penicillin-binding assay. Assay of binding of [^{14}C]-labeled penicillin G (60 Ci per mol, Radiochemical Centre, Amersham, England) was carried out using SDS / polyacrylamide gel electrophoresis and fluorography as described previously (2,4).

Peptidoglycan synthetase assay. The method used was essentially that of Nakagawa et al. (4) with the lipid-linked precursor, GlcNAc-MurNAc(-L-Ala-D-Glu-meso-A_2pm-D-Ala-D-Ala)-diphosphate-undecaprenol labeled with either meso-[^{14}C]-diaminopimelic acid (44 Ci per mol) or D-[^{14}C]alanyl-D-[^{14}C]alanine (90 Ci per mol) as substrate. The methods of preparation and utilization of the lipid-linked precursor were originally those of Anderson et al. (8) and have been applied for the system of *E. coli* in our laboratory for many years (unpublished experiments). In the present study the method was modified as described in the legend to Fig. 2.

Chemicals and reagents. Penicillin G potassium salt (Takeda Chemical Industries Co., Osaka) was a commercial product; cephalexin was obtained from Shionogi & Co., Osaka; nocardicin A was from Fujisawa Pharmaceutical Co., Osaka, and macarbomycin was from Meiji Seika Co., Tokyo.

RESULTS

Purification of PBP-1A. Fig. 1 shows the SDS / polyacrylamide gel electrophoretic pattern of PBP-1A purified from the membranes of *E. coli* K12 strain

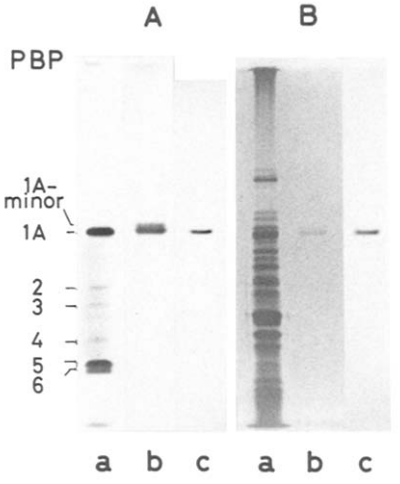

FIGURE 1. SDS / polyacrylamide gel electrophoretic pattern of purified PBP-1A. Samples were treated with [^{14}C]penicillin G and subjected to SDS / polyacrylamide gel electrophoresis. A Fluorogram. B Coomassie brilliant blue staining. a Membrane of E. coli K12 strain JST975srev61(pLC29-47); b PBP-1A purified from the membrane of strain JST975srev61(pLC29-47); c PBP-1A purified from the membrane of strain JST975srev6.

JST975srev61(pLC29-47) and strain JST975srev6 in comparison with the pattern of whole membranes of the former strain. The left half of Fig. 1 is a fluorogram showing the position of the radioactive penicillin G - protein complex and the right half shows similar electrophoregrams stained with Coomassie brilliant blue. The [^{14}C]penicillin G complex of the PBP-1A purified from the plasmid-carrying strain gave a doublet band on electrophoresis, a major band corresponding to normal PBP-1A, and a minor band with slightly lower mobility, whereas the crude membrane gave only the normal, major band of PBP-1A. When PBP-1A was purified from strain JST975srev6, no minor band of PBP-1A was obtained. Another band(s) of the PBP - [^{14}C]penicillin G complex with a molecular weight of about 150 to 200 K dalton was also seen after DEAE-cellulose fractionation in both cases. This band(s) was eluted from the affinity column a little later than the PBP-1A doublet and is not seen in the purified sample shown in the figure. Pure preparations without bound [^{14}C]penicillin G also showed similar electrophoretic patterns.

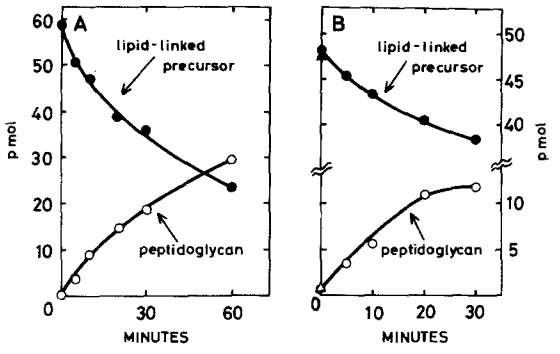

FIGURE 2. Time course of peptidoglycan formation by purified PBP-1A. The reaction mixture in the normal test tube assay (A) consisting of 14 mM Tris-HCl buffer, pH 7.6, 0.9 mM $MgCl_2$, 11% glycerol, 14% methanol, 0.15% Triton X-100, 60 p mol lipid-linked precursor labeled in meso-A_2pm and purified PBP-1A from E. coli K12 strain JST975srev61(pLC29-47) (ca. 0.2 ug protein judging by Coomassie brilliant blue staining) in a final volume of 35 ul, was incubated at 37°C for the indicated times. The reaction was stopped by adding 10 ul of isobutyric acid. After paper chromatography of the reaction mixture in isobutyric acid - 1 M ammonia (1:0.6), radioactivity in the positions of peptidoglycan (origin, open circles) and lipid-linked precursor (Rf 0.9, closed circles) was counted. In the filter paper assay (B), the reaction mixture, consisting of 21 mM Tris HCl-buffer, pH 7.6, 0.9 mM $MgCl_2$, 0.04 mM sodium ethylenediamine tetraacetate, 8.6% glycerol, 14% methanol, 0.43% Triton X-100, 50 p mol lipid-linked precursor labeled in meso-[^{14}C]A_2pm and purified PBP-1A (ca. 0.2 ug protein) in a final volume of 35 ul, was applied directly (circles) or after incubation in a test tube for 10 min at 37°C (triangles) to Whatman No. 3MM filter paper, half dried under a stream of cold air, and incubated in a humid chamber at 20°C for indicated times. The reaction was terminated by spotting a drop of isobutyric acid and the paper was subjected to chromatography and following procedures were as described above.

The purified PBP-1A doublet could bind about 3.5 n mol of [^{14}C]penicillin G per mg protein (assayed by densitometrical tracing of Coomassie brilliant blue-stained gel, with bovine serum albumin as standard), or about 0.35 mol [^{14}C]-penicillin G per mol protein, assuming that its molecular weight was 100 K dalton. About 360 fold purification was achieved on the basis of the [^{14}C]penicillin G-binding capacity per unit of protein. The activity of peptidoglycan transglycosylase at 37°C was 5 n mol peptidoglycan repeating units per mg protein per min, or 0.5 mol repeating unit per mol protein per min.

Peptidoglycan synthetase activities of PBP-1A. The time courses of utilization of lipid-linked precursor and formation of peptidoglycan are illustrated in Fig. 2A. The peptidoglycan formed was sensitive to lysozyme digestion and contained about 8% crosslinking. Peptidoglycan formation (transglycosylase reaction) and crosslink formation (transpeptidase reaction) both occurred in the

TABLE I. Peptidoglycan Synthesis by Purified PBP-1A and Effect of Antibiotics[a]

Condition	Formation of peptidoglycan (p mol repeating unit)	Crosslinkage (%)
Experiment 1 (test tube assay)		
Complete	24	7.8
+ Penicillin G, 3 μg per ml	23	1.8
+ Cephalexin, 10 μg per ml	23	2.4
+ Nocardicin A, 10 μg per ml	19	3.5
+ Macarbomycin, 0.1 μg per ml	2	-
Experiment 2 (filter paper assay)		
Complete	9.3	4.6
+ Penicillin G, 10 μg per ml	7.9	-
+ Macarbomycin, 1 μg per ml	0	-
Complete, Triton X-100 removed	7.9	8.1

[a] *The reaction systems were practically the same as for Fig. 2 A and B. Incubation was carried out for 30 min (Experiment 1) or 15 min (Experiment 2). The degree of crosslinkage was calculated after paper chromatography of the lysozyme digest from the ratio (radioactivity) of bis(disaccharide-peptide) to [bis(disaccharide-peptide) + disaccharide-peptide]. A ratio of 1 corresponds to 50% crosslinkage. No appreciable release of D-alanine was observed when the lipid-linked precursor labeled with D-[^{14}C]alanyl-D-[^{14}C]alanine was used as substrate.*

absence of magnesium. Crosslink formation was strongly inhibited in the presence of 3 μg per ml of penicillin G, 10 μg per ml of cephalexin or 10 μg per ml of nocardicin A (Table I, experiment 1). The latter two β-lactam antibiotics have much higher affinities to PBP-1A than to PBP-1Bs and inhibit the peptidoglycan crosslinking reaction due to PBP-1B(s) only very weakly. The β-lactam antibiotics shown in Table I slightly reduced the formation of peptidoglycan at concentrations high enough to inhibit the crosslinking reaction due to PBP-1A. Macarbomycin at 0.1 μg per ml almost completely inhibited the peptidoglycan formation.

Peptidoglycan formation on filter paper. When the reaction mixture contained 0.5% Triton X-100, no peptidoglycan formation could be observed in the usual test tube assay. Nevertheless, peptidoglycan formation could be observed when the reaction mixture was spotted on filter paper, half dried and incubated in a humid chamber at 20°C, and then subjected to paper chromatography. The time course of the reaction shown in Fig. 2B indicates that the formation of peptidoglycan was dependent only on the time of incubation on the paper and not on the time in the test tube, and was inhibited by macarbomycin (Table I, ex-

periment 2). The peptidoglycan formed on the paper was sensitive to digestion with lysozyme and was poorly crosslinked. The degree of crosslinkage of the peptidoglycan formed on the filter paper could also be increased by removing Triton X-100 from the reaction mixture.

DISCUSSION

The present results indicate that purified PBP-1A preparations have enzymatic activity of peptidoglycan transglycosylase and penicillin-sensitive transpeptidase, which together form crosslinked peptidoglycan. Probably these two enzyme activities are properties of the single peptide chain of PBP-1A. The properties of the PBP-1A enzyme activities are in strong contrast with those of PBP-1B(s). The transglycosylase reaction due to PBP-1B(s) requires 10 mM $MgCl_2$ for maximal activity and is significantly enhanced by the presence of β-lactam antibiotics such as penicillin G or cephalexin and the transpeptidase reaction due to PBP-1B(s) is 50% inhibited by 1 μg per ml of penicillin G, or 300 μg per ml of nocardicin A, and 25% inhibited by 1000 μg per ml of cephalexin (Matsuhashi, Nakagawa, Ishino, Nakajima-Iijima, Tomioka, Doi and Tamaki, β-Lactam Antibiotics, in press, Japan Scientific Societies Press, Tokyo).

Introduction of a plasmid carrying the *E. coli* chromosomal part covering the putative structural gene for PBP-1A caused formation of an additional minor 1A protein. The results presented in this paper were mostly obtained using a PBP-1A preparation purified from the plasmid-carrying strain of *E. coli*, but a preparation purified from the *E. coli* strain not carrying the plasmid also catalyzed formation of peptidoglycan. Probably the minor proteins are related to the formation of PBP-1A, for instance as "pre-PBP-1A", but more precise work is required on this problem.

The necessity of incubating half dried samples on filter paper to demonstrate peptidoglycan synthesis has also been observed with sonic membrane preparations of *Staphylococcus aureus* and *Micrococcus lysodeikticus* (8,9). This phenomenon suggests that some condition achieved in this way is important for formation of peptidoglycan, such as correct alignment of the growing peptidoglycan strands or the enzyme itself.

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