Purification of Four Penicillin-binding Proteins from *Bacillus megaterium*

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Four of the five penicillin-binding proteins in the cytoplasmic membranes of *Bacillus megaterium* have been purified to protein homogeneity. The method used involved the solubilization of the penicillin-binding proteins from the membranes by treatment with non-ionic detergent, followed by partial separation of the proteins by ion-exchange chromatography on DEAE-Sephadex CL-6B. Each protein was then purified to protein homogeneity by covalent affinity chromatography on ampicillin-afinose. The protein with the lowest molecular weight is a DD-carboxypeptidase. The other three proteins have previously been postulated to be peptidoglycan transpeptidases, endopeptidases or DD-carboxypeptidases *in vivo*, but it was not possible to demonstrate any of these activities with the purified proteins in various *in vitro* systems. Possible reasons for the observed lack of enzymic activity *in vitro* are discussed.

INTRODUCTION

The most generally accepted hypothesis for the lethal action of β -lactam antibiotics on bacterial cells is that they inhibit enzymes that catalyse the terminal stages of peptidoglycan synthesis (Blumberg & Strominger, 1974). These penicillin-sensitive enzymes have been postulated to be peptidoglycan transpeptidases, endopeptidases and D-alanyl-D-alanine carboxypeptidases (DD-carboxypeptidases, EC 3.4.12.6; these catalyse the hydrolysis of the terminal D-alanine residue from peptide substrates with a C-terminal sequence of D-alanyl-D-alanine). The initial inhibitory action of β -lactam antibiotics may then be followed by an increase in autolytic activity in the cells which results in lysis and death (Tomasz & Höltje, 1977). Hence, an understanding of the molecular mechanism of penicillin action requires knowledge of the interaction of penicillins with the penicillin-sensitive enzymes and of how this interaction leads to the death of the cell. However, the identification, purification and characterization of the proteins that act as receptors for the lethal action of β -lactams has proved elusive for a number of years.

The cytoplasmic membranes of bacteria contain a number of proteins that bind ^{14}C -labelled benzylpenicillin covalently (penicillin-binding proteins; PBPs) and it is assumed that at least one of these proteins is the enzyme whose inhibition by β -lactam antibiotics leads eventually to the death of the cell (Blumberg & Strominger, 1974). Two complementary approaches have been used to elucidate the functions of the penicillin-binding proteins. The principle behind the genetic approach is to isolate mutants lacking one or more of the penicillin-binding proteins and then to discover what effect these mutations have on the growth or morphology of the cell; this is followed by an investigation of the loss of any biochemical activities in the mutants. This approach has allowed functions to be assigned to some of the penicillin-binding proteins of *Escherichia coli* (Spratt, 1975; Spratt, 1977;

Iwaya & Strominger, 1977; Tamaki *et al.*, 1977) but has not been used for any other micro-organism. The second, biochemical, approach to determine the functions of the penicillin-binding proteins involves their purification followed by an investigation of their enzymic activities. This is the approach used in the work described in this report. In all the organisms examined to date, the penicillin-binding protein with the lowest molecular weight has been found to possess DD-carboxypeptidase activity. Thus in *Bacillus subtilis*, PBP5 is a DD-carboxypeptidase (Blumberg & Strominger, 1972), as is PBP5/6 of *E. coli* (Spratt & Strominger, 1976), PBP5 of *Salmonella typhimurium* (Shepherd *et al.*, 1977) and PBP3 of *Bacillus stearothermophilus* (Yocum *et al.*, 1974). However, comparable studies have not been carried out with the penicillin-binding proteins of higher molecular weight from any organism although these proteins are more likely to be closely involved with the lethal effects of β -lactams than are the DD-carboxypeptidases.

Covalent affinity chromatography has enabled the purification of the DD-carboxypeptidases in high yield in a single-step process. In this technique, first described by Blumberg & Strominger (1972), the penicillin-binding proteins are bound covalently to a derivative of penicillin attached to an inert support and are separated from other proteins which do not bind to the affinity resin. The penicillin-binding proteins are removed from the affinity resin by treatment with hydroxylamine, a reagent that has been shown to break the covalent bond between penicillin and the DD-carboxypeptidases (Lawrence & Strominger, 1970).

One of the major problems in the study of the lethal action of penicillin has been the development of a suitable *in vitro* system for assaying the complex peptidoglycan transpeptidation reactions. The natural model transpeptidation reaction, in which two molecules of the pentapeptide substrate L-alanyl- γ -D-glutamyl-meso-2,6-diaminopimelyl-D-alanyl-D-alanine [L-Ala-D-Glu-(*ms*-A₂pm-D-Ala-D-Ala)] acting both as donor and acceptor are linked by transpeptidation to form a nonapeptide, is assumed to be a good model of the presumed *in vivo* transpeptidation reaction in organisms that have a cell wall structure that is cross-linked by a bond formed between a D-alanine residue of one side-chain and the D centre of a meso-diaminopimelyl residue in another (Nguyen-Distèche *et al.*, 1974). This model reaction is catalysed by PBPs 4 and 5 of *S. typhimurium* (Shepherd *et al.*, 1977), material solubilized from the membranes of *E. coli* (Nguyen-Distèche *et al.*, 1974) and the purified DD-carboxypeptidase of the L-form of *Proteus mirabilis* (Martin *et al.*, 1976). The structure of the cell wall of *Bacillus megaterium* is such that a similar transpeptidation reaction *in vivo* would be expected.

The cytoplasmic membranes of *B. megaterium* contain five penicillin-binding proteins which have been numbered 1 to 5 in order of decreasing molecular weight (Chase *et al.*, 1977). The characteristics of the interaction of benzylpenicillin with PBP1 are consistent with this protein being a peptidoglycan transpeptidase whose inhibition by benzylpenicillin results in death of the cell (Reynolds *et al.*, 1978). It is possible to demonstrate peptidoglycan transpeptidase activity catalysed by cell wall plus membrane fragments of *B. megaterium* (Reynolds, 1971) and in an attempt to demonstrate that this activity is catalysed by PBP1, this protein has been solubilized from the membranes and purified to protein homogeneity (Chase *et al.*, 1978). Some preliminary work on the enzymic activities of PBPs 1, 3 and 4, when studied in partially purified preparations (Chase *et al.*, 1977), suggested that these proteins are devoid of DD-carboxypeptidase and transpeptidase activities. PBP5 has also been purified to protein homogeneity (Chase *et al.*, 1977) and has been shown to be a DD-carboxypeptidase but the characteristics of the binding of benzylpenicillin to this protein exclude it from consideration as the lethal target for β -lactams.

The aim of the work described here was to purify as many as possible of the penicillin-binding proteins of *B. megaterium* and to determine their enzymic activities.

METHODS

Bacterial strain and growth conditions. *Bacillus megaterium* strain KM was used throughout this study. The growth medium contained (per litre) 10 g tryptone (Difco Bacto), 5 g yeast extract (Difco Bacto), 2.5 g K_2HPO_4 and 1 g glucose; the pH was adjusted to 7.2. The organisms were cultured in 2 l Erlenmeyer flasks containing 800 ml growth medium in a Gallenkamp orbital incubator (200 rev. min⁻¹) at 37 °C. Growth was followed turbidimetrically using a Hilger 'Spekker' absorptiometer with neutral filters.

Preparation of membranes. Batches of cells were harvested in the exponential phase of growth at a culture density of 0.4 mg dry wt ml⁻¹ by centrifuging at 8500 rev. min⁻¹ (12000 g approx.) for 2 min in a Sorvall RC-5 centrifuge (GS-3 rotor). Lysed protoplast membranes were prepared by treatment of cells with lysozyme (EC 3.2.1.17; Sigma) and the membranes were obtained as described by Chase *et al.* (1978); they were stored at a density of 10 mg protein ml⁻¹ in 50 mM-Tris/HCl buffer, pH 7.2 at -25 °C.

Solubilization of membrane-bound penicillin-binding proteins. Membranes were sedimented by centrifuging at 50000 g for 20 min and resuspended at a density of 10 mg protein ml⁻¹ in 50 mM-Tris/HCl buffer, pH 8.0 containing various amounts of the non-ionic detergent isotridecanol polyglycol ether (trivial name: Genapol X-100; the kind gift of Farbwerke Hoechst, Frankfurt, Germany) and NaCl. Solubilization was achieved by incubation at 37 °C for 10 min with constant stirring. Insoluble material was removed by centrifuging at 4 °C (165000 g, 1 h).

Ion-exchange chromatography. Membranes (20 ml, 200 mg protein) from *B. megaterium* were solubilized with 1 M-NaCl/1 % (w/v) Genapol X-100/50 mM-Tris/HCl buffer, pH 8.0, as described above. The solubilized material was dialysed against 5 l 50 mM-2-amino-2-methylpropane-1,3-diol/HCl buffer, pH 9.5, containing 0.1 % (w/v) Genapol X-100 and applied to a 200 ml column of DEAE-Sephacel CL-6B (Pharmacia) equilibrated in similar buffer. The column was eluted at 4 °C with a linear gradient of NaCl (0 to 0.5 M) in 2 l of the above buffer at a flow rate of 80 ml h⁻¹ and 20 ml fractions were collected.

Covalent affinity chromatography. Covalent affinity chromatography was performed using ampicillin-affinose prepared from Affi-Gel 202 (Bio-Rad) to which ampicillin had been coupled by the method described by Pollock *et al.* (1974). For all experiments, a 4 cm × 1 cm column of ampicillin-affinose was used and the affinity resin was equilibrated against 10 mM-sodium cacodylate/HCl buffer, pH 6.0 containing 0.1 % (w/v) Genapol X-100. The material to be chromatographed was incubated with the ampicillin-affinose for 10 min at 37 °C in order that the penicillin-binding proteins should bind to the resin, after which the column was poured. Proteins that did not bind covalently to the resin were removed by washing at 4 °C with 50 ml 1 M-NaCl in 50 mM-Tris/HCl buffer, pH 8.8 containing 0.1 % (w/v) Genapol X-100 (flow-rate 150 ml h⁻¹). Proteins that were bound covalently to the column were eluted by incubation of the affinity resin with successive 2 ml portions of neutralized 1 M-hydroxylamine hydrochloride in 0.5 M-Tris/HCl buffer, pH 8.8 containing 0.1 % (v/v) Genapol X-100. Hydroxylamine was removed by dialysis at 4 °C against two 5 l quantities of 25 mM-Tris/HCl buffer, pH 8.0.

Assay of penicillin-binding proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The presence of penicillin-binding proteins was assayed by treatment of a sample with [¹⁴C]benzylpenicillin followed by preparation of the sample for sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. Samples were incubated with [¹⁴C]benzylpenicillin [potassium 6-phenyl[1-¹⁴C]acetamidopenicillanate, 15 µg ml⁻¹; sp.act. 52 Ci mol⁻¹ (1.92 TBq mol⁻¹); The Radiochemical Centre, Amersham] for 10 min at 37 °C followed by addition of unlabelled benzylpenicillin (10 mg ml⁻¹) and 4 vol. acetone to precipitate the protein. The samples were stored at -30 °C for 20 min and the precipitate was collected by centrifuging at 10000 g for 20 min, dried *in vacuo*, and redissolved by boiling for 2 min in 10 mM-Tris/HCl buffer, pH 7.2 containing 10 % (w/v) glycerol, 1 % (w/v) SDS, 1 % (v/v) 2-mercaptoethanol and 0.002 % (w/v) bromophenol blue. Discontinuous electrophoresis in polyacrylamide slab gels containing SDS was carried out essentially as described by Laemmli & Favre (1973), but at a constant voltage of 100 V until the samples had penetrated the stacking gel, and thereafter at a constant voltage of 450 V with cooling of the apparatus. The composition of the running gel (15 cm × 15 cm × 2 mm) was 10 % (w/v) acrylamide and 0.13 % (w/v) methylene bisacrylamide. The running gel was topped by a 1 cm layer of stacking gel. Gels were stained with Coomassie brilliant blue according to the method of Fairbanks *et al.* (1971) and destained in 10 % (v/v) acetic acid. In order to detect the radioactive bands, the gels were prepared for fluorography by impregnation with the scintillant 2,5-diphenyloxazole as described by Bonner & Laskey (1974) and Laskey & Mills (1975). In order to relate accurately the image densities obtained on the X-ray film to the amount of radioactivity in the gel, the X-ray film was sensitized by controlled partial pre-fogging (Laskey & Mills, 1975). The exposure time for fluorography was 14 d.

Enzymic activities of the isolated proteins. The purified penicillin-binding proteins were assayed for transpeptidase, carboxypeptidase and endopeptidase activities (see Table 1 for details) by incubating 20 µl samples with 5 µl of the relevant substrate. For some incubations 2 µl 150 mM-MgCl₂ (final concentration 10 mM)

Table 1. *In vitro* systems for study of the enzymic activities of the purified penicillin-binding proteins

Activity studied	Substrates	Products	Method of following reaction
DD-Carboxypeptidase	UDP-MurNAC-L-Ala-D-Glu- (<i>ms</i> A ₂ pm-D-Ala-D-Ala) + H ₂ O	UDP-MurNAC-L-Ala-D-Glu- (<i>ms</i> A ₂ pm-D-Ala) + D-Ala	Colorimetric determination of D-alanine released
DD-Carboxypeptidase	L-Ala-D-Glu-(<i>ms</i> A ₂ pm-D-Ala-D-Ala) + H ₂ O	L-Ala-D-Glu-(<i>ms</i> A ₂ pm-D-Ala) + D-Ala	Colorimetric determination of D-alanine released
Model transpeptidase	L-Ala-D-Glu-(<i>ms</i> [¹⁴ C]-A ₂ pm-D-Ala-D-Ala) acting both as acceptor and donor	L-Ala-D-Glu(OH) $\left[\frac{L}{\frac{L}{\frac{[^{14}C] A_2pm}{D}} - D-Ala-D-Ala(OH)} \right]$ $\left[\frac{L}{\frac{L}{\frac{[^{14}C] A_2pm}{D}} - D-Ala - \frac{D}{D}} - (OH) \right]$ + D-Ala	Separation of reactants and products by paper electrophoresis at pH 6.5
Model transpeptidase	MurNAC-L-Ala-D-Glu- (<i>ms</i> [¹⁴ C]A ₂ pm-D-Ala-D-Ala) acting both as acceptor and donor	MurNAC-L-Ala-D-Glu(OH) $\left[\frac{L}{\frac{L}{\frac{[^{14}C] A_2pm}{D}} - D-Ala-D-Ala(OH)} \right]$ $\left[\frac{L}{\frac{L}{\frac{[^{14}C] A_2pm}{D}} - D-Ala - \frac{D}{D}} - (OH) \right]$ $\left[\frac{L}{\frac{L}{\frac{[^{14}C] A_2pm}{D}} - (OH)} \right]$ + D-Ala	Separation of reactants and products by paper electrophoresis at pH 6.5
Endopeptidase	GlcNAC-MurNAC-L-Ala-D-Glu(OH) + H ₂ O	GlcNAC-MurNAC-L-Ala-D-Glu(OH) $\left[\frac{L}{\frac{L}{\frac{[^{14}C] A_2pm}{D}} - D-Ala(OH)} \right]$ $\left[\frac{L}{\frac{L}{\frac{[^{14}C] A_2pm}{D}} - (OH)} \right]$	Paper chromatography using solvent A

*ms*A₂pm, *meso*-diaminopimelic acid; MurNAC, *N*-acetyl-muramic acid; GlcNAC, β -1,4-*N*-acetylglucosaminyl-L-*N*-acetylmuramic acid.

was added, and in order to study the effect of alteration of the pH of the incubation, in some experiments 2 μ l 1 M-sodium acetate buffer, pH 6.0 or 2 μ l 1 M-Tris/HCl buffer, pH 9.0 was added. All incubations were in a final volume of 30 μ l. The samples were incubated for at least 3 h at 37 °C and the reactions were terminated by immersing the tubes in boiling water for 1 min. The extent of the reaction was determined by the methods indicated in Table 1. When using the substrates that were not radioactively labelled, the reactions were followed by measuring the release of D-alanine. The reactions involving radioactively labelled substrates were followed by separating the reactants from the radioactive products by paper electrophoresis or paper chromatography. The location of radioactivity on the paper was determined by autoradiography, the radioactive areas were cut out and the amount of radioactivity present was determined by liquid scintillation counting using 0.4 % (w/v) 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene in toluene as scintillant.

Preparation of substrates. UDP-*N*-acetylmuramoyl-L-alanyl- γ -D-glutamyl-*meso*-2,6-diaminopimelyl-D-alanyl-D-alanine [UDP-MurNAc-L-Ala-D-Glu-(*ms*-A₂pm-D-Ala-D-Ala)] was prepared as described by Barnett (1973). L-Ala-D-Glu-(*ms*A₂pm-D-Ala-D-Ala) was prepared as described by Martin *et al.* (1976) but with the modifications described by Chase *et al.* (1978). L-Ala-D-Glu-(*ms*[1,7-¹⁴C]A₂pm-D-Ala-D-Ala) was prepared as described by Shepherd *et al.* (1977).

[¹⁴C]Bisdisaccharide peptide dimer (see Table 1 for structure) was prepared from the isolated peptidoglycan of a strain of *Escherichia coli* that requires diaminopimelic acid for growth. *Escherichia coli* W7 (Braun *et al.*, 1974) was grown overnight at 37 °C with vigorous shaking in the medium used for the growth of *B. megaterium* supplemented with diaminopimelic acid (6 μ g ml⁻¹). In the morning, 3 l of similar medium was inoculated with 50 ml of the overnight culture. When the culture density had reached 0.07 mg dry wt ml⁻¹, 2,6-diamino-1,7-¹⁴C]pimelic acid [50 μ Ci; sp.act. 44 Ci mol⁻¹ (1.6 TBq mol⁻¹); The Radiochemical Centre, Amersham] was added and the cells were harvested when a culture density of 0.9 mg dry wt ml⁻¹ had been reached. The cell pellet was washed once in distilled water and resuspended in 50 ml water. Cell disintegration was achieved by sonication; a Dawe 'Soniprobe' type 7532A fitted with a Branson Converter was used with the output control set to position 5. The cell suspension was kept at 4 °C and 30 s bursts of sonication were used. A total of 4 min sonication resulted in breakage of more than 90 % of the cells. EDTA (0.1 M, pH 7.4) was added to a final concentration of 0.01 M and the cell envelope preparation was washed three times with water by centrifuging (50000 g, 20 min) and finally resuspended in 8 ml water. Peptidoglycan with attached lipoprotein was isolated from the cell envelope preparation by a procedure modified from that described by Braun & Rehn (1969). The envelope preparation was added dropwise to 50 ml boiling 4 % (w/v) SDS with constant stirring and was then boiled for a further 10 min. Peptidoglycan/lipoprotein was sedimented by centrifuging at 40000 rev. min⁻¹ for 30 min in the 50Ti rotor of a Beckman ultracentrifuge (105000 g approx.) and washed three times with water. The pellet was resuspended in 15 ml 0.01 M-Tris/HCl buffer, pH 8.0 containing 10 mM-CaCl₂. Then 100 μ g trypsin (BDH) was added and the suspension was shaken vigorously at 37 °C for 3 h. EDTA was added to a final concentration of 10 mM followed by 400 μ g trypsin inhibitor (BDH) and the peptidoglycan was collected by centrifuging (100000 g, 20 min). The peptidoglycan was washed once in water, resuspended in 15 ml 0.01 M-Tris/HCl buffer, pH 7.2 and 300 mg lysozyme was added. The preparation was incubated for 24 h at 37 °C; then a further 100 mg lysozyme was added and incubation was continued for 36 h at 20 °C. The lysozyme-degraded peptidoglycan was filtered in 0.1 M-LiCl on two columns of Sephadex G-50 and Sephadex G-25 connected in series (Van Heijenoort *et al.*, 1969) which separated disaccharide peptide monomer from bisdisaccharide peptide dimer. Each fraction was desalted by filtration in water on a column of Sephadex G-10 (85 cm $\times$ 3 cm; flow rate 80 ml h⁻¹). The volume of the fraction containing bisdisaccharide peptide dimer was reduced to 1.6 ml and the radioactivity was determined to be 3500 d.p.m. μ l⁻¹.

Paper chromatography. Paper chromatography was carried out on Whatman no. 1 paper for 24 h at 4 °C using isobutyric acid/1 M-ammonia (5:3, v/v) (solvent A).

Paper electrophoresis. High-voltage paper electrophoresis at pH 6.5 was carried out on Whatman 3MM paper at 60 V cm⁻¹. The buffer used was pyridine/acetic acid/water (100:3:897, by vol.).

Autoradiography. The position of radioactive compounds on chromatograms and electrophoretograms was determined by placing the paper in contact with X-ray film (Kodak Blue Brand) in the dark for 2 weeks, then developing in Ilford Contrast FF developer and fixing in Kodafix fixer.

Estimation of D-alanine. D-Alanine was estimated using a method adapted from that of Johnson *et al.* (1975), with the modifications that water-soluble *o*-dianisidine sulphate was used (allowing methanol to be omitted) and the reactions were terminated by the addition of 4 vol. 0.25 % (w/v) SDS.

Estimation of protein. Protein was estimated using the Lowry method with dried bovine serum albumin as standard. Sodium dodecyl sulphate was added to all solutions to a final concentration of 1 % (w/v) in order to prevent the formation of precipitates in the presence of non-ionic detergent.

Ultrafiltration. Ultrafiltration was carried out at 4 °C in an Amicon model 12 ultrafiltration unit using a UM10 filter.

Antibiotics. Benzylpenicillin (Crystapen) was purchased from Glaxo Laboratories, Greenford, Middx.; ampicillin (Penbritin) from Beecham Research Laboratories, Brentford, Middx., and cephalothin (Keflin) from Eli Lilly & Co., Indianapolis, U.S.A.

RESULTS

Solubilization of the penicillin-binding proteins

The effect of the concentration of NaCl in the presence of the non-ionic detergent Genapol X-100 on the solubilization of the penicillin-binding proteins from the cytoplasmic membranes of *B. megaterium* is shown in Fig. 1. The definition of 'soluble' material used in these studies is material that is not sedimented by centrifuging at 165000 g for 1 h. Similar definitions have been used by other workers (Barnett, 1973), although Penefsky & Tzagoloff (1971) have pointed out that some procedures may lead to the formation of small membrane fragments that do not sediment readily and may thus appear to be soluble. Treatment of the membranes with 1% (w/v) Genapol X-100 in 50 mM-Tris/HCl buffer, pH 8.0 for 10 min at 37 °C resulted in the solubilization of most of PBPs 1, 3, 4 and 5. Complete solubilization of these penicillin-binding proteins was achieved if NaCl was added to a final concentration of between 0.5 and 2 M. The extraction of firmly associated membrane proteins is to some extent dependent on the ratio of detergent to membrane lipid (Helenius & Simons, 1975) and treatment with 0.1% (w/v) Genapol X-100 resulted in only poor solubilization of the penicillin-binding proteins. Solubilization with 5% (w/v) Genapol X-100 was, however, not significantly better than that using 1% detergent. On the basis of these results, treatment with 1% Genapol X-100 and 1 M-NaCl in 50 mM-Tris/HCl buffer, pH 8.0 for 10 min at 37 °C was chosen as the standard condition for the solubilization of PBPs 1, 3, 4 and 5 of *B. megaterium*. PBP2 appeared to be very unstable and lost its penicillin-binding activity under the conditions used for solubilization. After incubation of membranes, even in the absence of detergent, for 10 min at 37 °C followed by centrifuging, this penicillin-binding protein could not be detected in either the pellet or the supernatant.

Isolation of the penicillin-binding proteins

A flow chart of the procedure used to purify the penicillin-binding proteins is given in Table 2. The penicillin-binding proteins were partially separated from each other by ion-exchange chromatography of the material solubilized from the membranes on DEAE-Sephacel CL-6B as described previously (Chase *et al.*, 1978) (Fig. 2). PBP5 and PBP3 were eluted in the same fractions but PBP4 was completely separated from the other penicillin-binding proteins in fractions 33 to 35. As had been observed previously, all fractions that contained PBP1 also contained PBP4.

Penicillin-binding proteins were isolated from the fractions obtained after ion-exchange chromatography by covalent affinity chromatography on ampicillin-*affinose*. The fractions resulting from ion-exchange chromatography that contained PBP4 as the sole penicillin-binding protein (33, 34 and 35) were pooled and concentrated by ultrafiltration. PBP4 was then isolated by covalent affinity chromatography on ampicillin-*affinose*. Examination of the hydroxylamine eluates by SDS-polyacrylamide gel electrophoresis showed that PBP4 had been purified to homogeneity. It could be visualized as a single band after staining with Coomassie brilliant blue (Fig. 3*a*) and on the corresponding fluorogram (Fig. 3*b*).

Although all fractions that contained PBP1 also contained PBP4, it was possible to isolate PBP1 from fractions 37 to 49 by the procedure described previously by Chase *et al.* (1978). This technique exploits the observation that the rates of elution by hydroxylamine of PBP1 and PBP4 are substantially different (Chase *et al.*, 1977) and it is thus possible to obtain fractions after covalent affinity chromatography that contain PBP1 but not PBP4.

It has not proved possible to separate PBP3 from PBP5 by ion-exchange chromatography so the technique described above to isolate PBP4 could not be used to isolate PBP3. Also,

Fig. 1. Solubilization of the membrane-bound penicillin-binding proteins of *B. megaterium* as visualized by fluorography. Samples of the supernatants (S) and the residual pellets (P) after solubilization were analysed by SDS-polyacrylamide gel electrophoresis and the penicillin-binding proteins (PBP) were detected by fluorography. The concentrations of Genapol X-100 (% w/v) and NaCl (M) used in the solubilization experiments were: (A) 0, 0; (B) 1, 0; (C) 1, 0.2; (D) 1, 0.5; (E) 1, 1; (F) 1, 2; (G) 0.1, 0.5; (H) 5, 0.5. The polarity of electrophoresis is shown on the figure.

Table 2. *Purification of the penicillin-binding proteins of B. megaterium: flow chart*

Fig. 2. Separation of the penicillin-binding proteins of *B. megaterium* by ion-exchange chromatography. Chromatography on DEAE-Sephacrose was carried out as described in the text and in Methods. Samples (1 ml) of alternate fractions were analysed by SDS-polyacrylamide gel electrophoresis. Proteins were revealed by staining with Coomassie brilliant blue (a) and penicillin-binding proteins were revealed by fluorography (b). I indicates the position of the interface between the stacking and the running gels and M marks the position of the bromophenol blue marker.

although PBP3 is eluted from ampicillin-afinose by hydroxylamine more slowly than PBP5 the rates of elution of these two penicillin-binding proteins are not sufficiently different to be able to separate the two proteins, as was possible with PBPs 1 and 4. Hence, in order to separate PBP3 from PBP5 it was necessary to use a technique that exploits the observation that cephalothin is bound to PBP3 at a much lower concentration than results in binding to PBP5 (Chase *et al.*, 1977). Fractions 27, 28 and 29 from the ion-exchange chromatography (see Fig. 2) were pooled and concentrated by ultrafiltration. The concentrated material was incubated with $5 \mu\text{g}$ cephalothin ml^{-1} for 10 min at 37°C under which conditions cephalothin should bind selectively to PBP3 (Chase *et al.*, 1977). The material was then subjected to covalent affinity chromatography on ampicillin-afinose. PBP5 was adsorbed to the ampicillin-afinose as it did not have cephalothin bound to it and could be subsequently eluted with hydroxylamine. PBP3 was not adsorbed to the column as it was already present as a protein- β -lactam complex. Cephalothin was removed from PBP3 by treatment of the unadsorbed material with an equal volume of 1 M-hydroxylamine in 0.5 M-Tris/HCl buffer, pH 8.8 containing 0.1% (w/v) Genapol X-100 for 30 min at 37°C followed by dialysis against 2×51 50 mM-Tris/HCl buffer, pH 8.0 at 4°C . These conditions had been shown previously to result in the removal of cephalothin from PBP3 (Chase *et al.*, 1977). The dialysed material was applied to another column of ampicillin-afinose to which PBP3 and some PBP5 were adsorbed. The PBPs were eluted from ampicillin-afinose by hydroxylamine. Examination of the hydroxylamine eluates by SDS-polyacrylamide gel electrophoresis showed that some PBP3 had been obtained pure in two fractions (Fig. 4, tracks F and G), although four earlier fractions did contain some PBP5. Some fractions contained small amounts of penicillin-binding material that did not correspond to any of the penicillin-binding proteins and these are assumed to have been derived from PBP3 or PBP5 by proteo-

Fig. 3. Purification of PBP4 of *B. megaterium*. Fractions obtained by ion-exchange chromatography containing PBP4 were pooled, concentrated and subjected to covalent affinity chromatography on ampicillin-affinose. Samples of various fractions in the purification procedure were analysed by SDS-polyacrylamide gel electrophoresis. Proteins were revealed by staining with Coomassie brilliant blue (a) and penicillin-binding proteins were detected by fluorography (b). The samples were: (A) pooled, concentrated fractions before affinity chromatography; (B) material not adsorbed to the affinity resin; (C) material solubilized from the membranes; (D to I) material eluted by incubation with hydroxylamine for 0 min (D), 0 to 1 min (E), 1 to 10 min (F), 10 to 30 min (G), 30 to 100 min (H), 100 to 300 min (I). I indicates the position of the interface between the stacking and the running gels and M marks the position of the bromophenol blue marker.

lysis. The presence of PBP5 in these fractions was not expected as it indicated that some PBP5 had not been adsorbed to ampicillin-affinose during the first stage of affinity chromatography. Possibly partial binding of cephalothin to PBP5 occurred under the conditions used.

Samples containing pure PBP5 were obtained as described previously (Chase *et al.*, 1977) by covalent affinity chromatography of material treated with low concentrations of cephalothin after solubilization from protoplast membranes. This procedure does not require ion-exchange chromatography to achieve separation of the penicillin-binding proteins.

By the above procedures, samples of pure PBPs 1, 3, 4 and 5 were obtained (Fig. 5) which could then be used to ascertain the enzymic activity of these penicillin-binding proteins.

Enzymic activities of the isolated proteins

Samples of the isolated PBPs 1, 3 and 4 were tested as described in Methods for the enzymic activities specified in Table 1. No release of D-alanine from UDP-MurNAc-L-Ala-D-Glu-(*msA*₂pm-D-Ala-D-Ala) or L-Ala-D-Glu-(*msA*₂pm-D-Ala-D-Ala) was catalysed by purified PBPs 1, 3 or 4 under any of the conditions tested even after prolonged incubation (5 h). The presence of Mg²⁺ (10 mM) and/or an alteration of the pH of the incubation to pH 6 or 9 did not result in the appearance of any activity. This suggests that PBPs 1, 3 and 4 are not DD-carboxypeptidases capable of catalysing the release of D-alanine from the substrates used. Purified PBP5 showed DD-carboxypeptidase activity towards both substrates

Fig. 4. Purification of PBP3 of *B. megaterium*. Fractions obtained by ion-exchange chromatography containing PBP3 and PBP5 were treated with cephalothin and subjected to affinity chromatography. Material not adsorbed to the column (containing PBP3 and non-penicillin-binding proteins) was treated with neutral hydroxylamine, dialysed and re-subjected to affinity chromatography. Samples of various proteins in the purification procedure were analysed by SDS-polyacrylamide gel electrophoresis. Proteins were revealed by staining with Coomassie brilliant blue (a) and penicillin-binding proteins were detected by fluorography (b). The samples were: (A) cytoplasmic membranes of *B. megaterium*; (B to G) material eluted from the second affinity chromatography procedure by incubation with hydroxylamine for 0 min (B), 0 to 1 min (C), 1 to 10 min (D), 10 to 30 min (E), 30 to 100 min (F), 100 to 300 min (G). I indicates the position of the interface between the stacking and the running gels and M marks the position of the bromophenol blue marker.

Fig. 5. The purified penicillin-binding proteins of *B. megaterium*. Samples of each of the purified penicillin-binding proteins were analysed by SDS-polyacrylamide gel electrophoresis. Proteins were revealed by staining with Coomassie brilliant blue (a) and penicillin-binding proteins were detected by fluorography (b). The samples were: (A) PBP1; (B) PBP3; (C) PBP4, (D) PBP5.

under all the above conditions. Samples containing purified PBPs 1, 3 or 4 also showed no enzymic activity towards the substrates L-Ala-D-Glu-([1,7- 14 C]*msA*₂pm-D-Ala-D-Ala) or MurNAC-L-Ala-D-Glu-([1,7- 14 C]*msA*₂pm-D-Ala-D-Ala) even after incubation for 5 h. In all experiments a single radioactive spot that ran in the same position as the original substrate was observed on the autoradiogram of the electrophoretogram. Samples containing PBP5 catalysed the release of D-alanine from these substrates (i.e. the expected DD-carboxypeptidase activity) as evidenced by the formation of a radioactive compound that migrated in the position expected for L-Ala-D-Glu-([1,7- 14 C]*msA*₂pm-D-Ala) or the *N*-acetylmuramoyl derivative. However, there were no spots on the autoradiogram corresponding to the radioactive nonapeptide dimer or the des-Ala octapeptide dimer. Shepherd *et al.* (1977) observed that the amount of dimer formed by PBP4 of *S. typhimurium* reached a maximum after a certain time and then appeared to be hydrolysed. In order to ensure that a similar situation was not occurring with PBP5 of *B. megaterium*, a similar time period (involving incubation at 37 °C for 7, 15, 30, 45 and 60 min) was investigated but no formation of dimer was observed at any time. All experiments were carried out at a variety of pH values (from pH 6 to 9), in the presence or absence of MgCl₂ (10 mM) and at various ionic strengths (10 to 500 mM) but no formation of dimers was observed.

The endopeptidase activity of PBPs 1, 3 and 4 was tested by following the appearance of disaccharide peptide monomer GlcNAC-MurNAC-L-Ala-D-Glu-(*ms*[14 C]*A*₂pm-D-Ala) arising from hydrolysis of the bisdisaccharide peptide dimer. It has not been possible to observe the formation of monomer under any of the conditions of Mg²⁺ concentration or pH described above for transpeptidase activity. This result suggests that none of these penicillin-binding proteins is a peptidoglycan endopeptidase.

DISCUSSION

Purification of PBPs 1, 3, 4 and 5 of *B. megaterium* is an important first step in the understanding of the role of penicillin-binding proteins in the cell and their involvement in the lethal action of β -lactam antibiotics. It should now prove possible to elucidate the biochemical functions of these proteins by finding suitable model systems in which they show biochemical activities. Another approach would be to raise antibodies against the purified proteins and to investigate the effects of such antibodies on the synthesis and morphology of the cell wall. It has not been possible to purify PBP2 of *B. megaterium* as this protein appears to lose its ability to bind benzylpenicillin under the conditions used to solubilize the other penicillin-binding proteins. Hence studies on possible enzymic activities of this protein have not been pursued.

Apart from the purification of PBP4 of *Salmonella typhimurium* (S. T. Shepherd, personal communication), *B. megaterium* is the only organism for which the purification of any of the penicillin-binding proteins except the one of lowest molecular weight (the DD-carboxypeptidase) has been reported. Solubilization of penicillin-binding proteins followed by separation by ion-exchange chromatography and isolation by covalent affinity chromatography should be a technique that could be used generally in the purification of penicillin-binding proteins from other organisms. Such a method has been used recently in this laboratory to purify PBP1, PBP4 and PBP5 from *E. coli* (H. A. Chase, unpublished results).

It has not been possible to show *in vitro* model transpeptidase activity with any of the penicillin-binding proteins of *B. megaterium* by studying the dimerization of L-Ala-D-Glu-(*msA*₂pm-D-Ala-D-Ala). It has also not been possible to show the dimerization of MurNAC-L-Ala-D-Glu-(*msA*₂pm-D-Ala-D-Ala) which is of slightly greater chemical similarity to the presumed substrate in the natural peptidoglycan transpeptidation reaction than the pentapeptide substrate. Although there is circumstantial evidence that PBP1 of *B. megaterium* catalyses a penicillin-sensitive peptidoglycan transpeptidation reaction (Reynolds *et al.*,

1978), it has not been possible to prove this suggestion directly by studying the enzymic activity of the isolated protein. Possibly the natural model transpeptidation reactions are not suitable assays for *in vivo* peptidoglycan transpeptidases, especially as none of the proteins that catalyse these reactions has been shown to be a peptidoglycan transpeptidase *in vivo*. Indeed, although PBP4 of *E. coli* can catalyse these natural model transpeptidation reactions (H. A. Chase, unpublished results), there is various genetic evidence that loss of this protein does not affect the growth or morphology of cells and presumably it is not a peptidoglycan transpeptidase *in vivo* (Iwaya & Strominger, 1977; Matsushashi *et al.*, 1977). Hence, the failure of proteins isolated from *B. megaterium* to catalyse the model reactions does not exclude the possibility that these proteins are peptidoglycan transpeptidases. Isolated protoplast membranes of *B. megaterium* are also incapable of catalysing the model reactions (Chase *et al.*, 1977); this observation rules out the criticism that the isolated proteins fail to catalyse the reactions because they have been removed from the membrane and thus have altered properties.

The natural peptidoglycan transpeptidation reaction observed in cell wall plus membrane preparations of *B. megaterium* (Reynolds, 1971) is coupled to the synthesis of peptidoglycan from nucleotide precursors. These peptidoglycan transpeptidases may require for activity a highly organized spatial configuration between the donor molecule (nascent peptidoglycan) and the acceptor (the cell wall). This spatial configuration may not be reproduced in the model systems and may only be created in the coupled systems in which peptidoglycan is synthesized *in situ* from precursors. The binding sites on the enzyme for the donor and acceptor may require more complicated substrates than those provided in the model reactions. It is possible that the enzyme does not contain a binding site for the cell wall acceptor but relies on the relative positioning of the cell wall to the membrane to provide a suitably placed amino-acid side-chain to act as an acceptor in the transpeptidation reaction.

The endopeptidase reaction studied is just another manifestation of DD-carboxypeptidase activity as the interpeptide bond that is split [a D-Ala-(D)-*meso*-diaminopimelyl linkage in an α position relative to a free carboxyl group] fulfils the substrate requirements for a DD-carboxypeptidase. Indeed, Yocum *et al.* (1974) showed that the isolated DD-carboxypeptidase of *B. stearothermophilus* was capable of catalysing this endopeptidase reaction. However, this reaction is not strictly the reverse of a transpeptidation reaction as the latter requires the cleavage of the dimer by D-alanine rather than by water. Hence, the failure of the penicillin-binding proteins to catalyse the endopeptidation reaction again does not exclude them from being peptidoglycan transpeptidases.

It is unlikely that the isolated penicillin-binding proteins lack enzymic activity as a result of denaturation during the procedures used for solubilization and purification. The purified penicillin-binding proteins still retain the ability to bind [^{14}C]benzylpenicillin and the DD-carboxypeptidase (PBP5) can be isolated by these techniques without loss of enzymic or penicillin-binding activities.

The peptidoglycan transpeptidases may be multi-protein complexes consisting of different polypeptide chains which may be held together in the correct quaternary configuration as a result of being bound to the membrane. The procedures used for the purification of the penicillin-binding proteins may split this multi-protein complex into its constituent subunits and it is possible that one subunit contains the binding site for benzylpenicillin (i.e. this is the subunit that has been isolated as it is a penicillin-binding protein) but is not, by itself, catalytically active. If this were the situation, the isolated penicillin-binding proteins would not be expected to possess enzymic activity; they may act as receptors for penicillins but lack enzymic activities themselves.

Some penicillin-binding proteins may be responsible for the triggering of autolytic activity that occurs on treatment of cells with penicillin (Tomasz & Höltje, 1977). An *in vitro* assay system to investigate the stimulation by penicillins of the autolytic activity in extracts of *Klebsiella pneumoniae* and *E. coli* has been reported by Fontana *et al.* (1977). It may be

possible to devise a similar kind of system to determine whether any of the penicillin-binding proteins can directly trigger autolytic activity in *B. megaterium*.

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