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Purification and Partial Characterization of a Penicillin-Binding Protein from *Mycobacterium smegmatis*

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Penicillin-binding proteins (PBPs), although characterized from several organisms, have so far not been studied in mycobacteria. The present study is the first characterization of a PBP from *Mycobacterium smegmatis*. The PBP was purified by solubilization of the membranes with Triton X-100 and successive chromatography of the solubilized proteins on ampicillin-linked CH Sepharose 4B and DE-52. The purified PBP (M_r, 49,500) catalyzed a model transpeptidase reaction with the tripeptide acetyl$_2$-L-Lys-D-Ala-D-Ala as the substrate and Gly-Gly as the acceptor. The transpeptidase activity was inhibited by 50% at a benzylpenicillin concentration of 1.8×10^{-7} M, which was similar to the concentration (1.1×10^{-7} M) of benzylpenicillin required to saturate to 50% this PBP. Of several antibiotics tested, the concentration of antibiotic required to inhibit [^{35}S]penicillin binding by 90% was found to be the lowest for cefoxitin and Sch 34343.

The biosynthesis of bacterial cell wall peptidoglycan involves several cytoplasmic and membrane-bound enzymes, which include the penicillin-binding proteins (PBPs) (19). The PBPs are the target enzymes for beta-lactam antibiotics. The PBPs catalyze rupture of the lactam amide bond of penicillin and form a covalent penicilloyl complex via the active serine residue.

The membrane-bound PBPs can be broadly classified into the high-molecular-weight (HMW) and the low-molecular-weight (LMW) PBPs. The HMW PBPs appear to be important in cell elongation, septation, or shape determination (11, 20). The LMW PBPs usually catalyze acyl transfer reactions from D-alanyl-D-alanine-terminated peptides and depsipeptide analogs (6–8). Rupture of the carboxy-terminal peptide bond of acetyl$_2$ (Ac_2)-L-Lys-D-Ala-D-Ala involves transfer of the Ac_2-L-Lys-D-alanyl moiety to the active-site serine with formation of a serine-ester-linked acyl enzyme, and from this to an exogenous acceptor.

Biochemical and genetic studies have shown that resistance to beta-lactam antibiotics involves alteration in the structural genes of several of the PBPs (3, 4, 10, 16) and formation of low-affinity PBPs (9, 13, 14, 17).

Mycobacteria are in general resistant to beta-lactam antibiotics. However, no effort has so far been made to purify and characterize any of the PBPs of mycobacteria. DD-Carboxypeptidase activity in membrane fragments of *Mycobacterium smegmatis* was first reported by Eun et al. (5). In view of the persistent need to develop new drugs for the chemotherapy of mycobacterial diseases, the detailed knowledge of the PBPs of mycobacteria may serve as the first step towards the effective use of beta-lactams against mycobacteria. The present paper describes for the first time the purification of a penicillin-binding protein with an M_r of 49,500 from *M. smegmatis*.

MATERIALS AND METHODS

Antibiotics. Sch 34343 was a gift from the Schering Plough Corporation; other antibiotics were gifts from J.-M. Ghuysen and J.-M. Frere, University of Liege, Liege, Belgium.

Bacterial strain. *M. smegmatis* SN_2 was obtained from the Indian Institute of Science, Bangalore, India.

Growth conditions. *M. smegmatis* was grown in nutrient broth containing 10 g of peptone, 10 g of beef extract, and 5 g of NaCl per liter on a rotary shaker at 200 rpm and 37°C.

Preparation of bacterial membrane fractions. Strains grown to the logarithmic phase were harvested by centrifugation at 7,000 × *g* for 10 min at 4°C and washed twice in 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$. The pellet was suspended in the same buffer containing DNase (2 μg/ml) and disrupted in a sonicator (Labsonic 2000; B. Braun, Melsungen, Germany) for 15 min in bursts of 3 min each. Unbroken cells were removed by centrifugation at 7,000 × *g* for 10 min, walls were removed by centrifugation at 20,000 × *g* for 20 min, and membrane fractions were collected by centrifugation at 100,000 × *g* for 90 min at 4°C, washed twice, and suspended in 10 mM Tris-HCl (pH 7.5).

PBP assays. To inhibit the β-lactamase activity, membranes were first incubated with 2×10^{-5} M beta-iodopenicillanic acid for 20 min at 30°C. Samples (100 μg) of membrane proteins were incubated at 30°C for 10 min with different concentrations of [^{35}S]benzylpenicillin (0.5 mCi/mmol) (Du Pont Co., Wilmington, Del.). The reaction was stopped by addition of an excess of nonradioactive penicillin and sodium lauroyl sarcosinate (final concentration, 1%). After the samples were allowed to stand at room temperature for 20 min, sodium dodecyl sulfate (SDS) gel denaturing buffer was added and boiled immediately for 3 min. Samples were applied on 10% SDS gels.

For the purified PBP, 0.5 μg of protein was used in each assay. In competition experiments, purified PBP was first incubated with different concentrations of the competing antibiotic for 10 min at 30°C and then with saturating concentrations of radioactive penicillin for 10 min at 30°C; termination of the reaction was done in the usual manner.

Solubilization of membranes with Triton X-100. Membranes (849 mg) were solubilized in 10 mM Tris-HCl (pH 8.0) containing 1 M LiCl, 0.1 mM DTE (dithioerythrol), and 1% Triton X-100 (buffer A) on ice for 60 min. The supernatant was collected after centrifugation at 100,000 × *g* for 90 min.

Purification of the 49,500-M_r PBP from the Triton X-100 extract by affinity chromatography on ampicillin-linked CH

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Sepharose 4B. The affinity chromatography reagent was prepared as described by Coyette et al. (1). The Triton X-100 extract (60 mg of protein) was mixed with 4 g of ampicillin-linked CH Sepharose 4B in 5.5 ml (final volume). The suspension was stirred gently for 30 min at 4°C and then for 30 min at 30°C. The unbound proteins were removed by filtration through a sintered glass filter.

The bound membrane proteins were eluted by gentle agitation for 30 min at 30°C with 15 ml of 1 M hydroxylamine (pH 8) in buffer A, and the supernatant was collected after centrifugation. The procedure was repeated five times. The extracts containing penicillin-binding activity were pooled, dialyzed against 10 mM Tris-HCl (pH 8.0) containing 0.1% Triton X-100 and 0.1 mM DTE (buffer B), and loaded on a 2-ml DE-52 column equilibrated in buffer B. The bound protein was eluted with buffer B containing 0.1 M NaCl. Fractions containing penicillin-binding activity were pooled (200 μg of protein) and analyzed by SDS gel electrophoresis and fluorography.

Monitoring the purification. Protein samples (50 μl) were incubated with 5 μl of [^{14}C]penicillin (50 mCi/mmol; final concentration, 34 μg/ml) for 10 min at 30°C and then precipitated with 0.5 ml of 5% trichloroacetic acid and 5 μl of 10% Triton X-100 by the method of Waxman and Strominger (18). Samples were kept on ice for 5 min, and the precipitate was collected on Whatman GF/C filter circles with a Millipore filtration apparatus. The filters were washed four times with 2 ml of 5% trichloroacetic acid and then washed four times with 2 ml of 47% ethanol containing 0.01 N HCl. Filters were dried and counted.

Determination of enzymatic activities. The DD-carboxypeptidase activity was estimated by measuring the hydrolysis of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala (a generous gift from J.-M. Ghuysen), and the transpeptidase activity was measured by using Gly-Gly as the acceptor (12). Briefly, Ac_2-L-Lys-D-Ala-D-Ala was incubated with the purified PBP at 37°C for 30 min in the absence or presence of the acceptor Gly-Gly. The D-Ala released was estimated by addition of a mixture of *o*-dianisidine, flavin adenine dinucleotide, peroxidase, and D-amino acid oxidase and incubation for 10 min at 37°C, and then a mixture of methanol-sulfuric acid-water (5:6:5) was added. Spectrophotometric readings were recorded at 535 nm. Standard curves were prepared by using D-Ala alone.

RESULTS

PBP pattern of the isolated membranes. The plasma membranes of *M. smegmatis* contained multiple PBPs, the most important ones having apparent molecular weights of 94,000, 67,000, and a cluster between 56,000 and 46,000 containing three PBPs (PBP 3, 4, and 5) (Fig. 1). Saturation experiments (Fig. 1) showed that the PBP with an M_r of 94,000 (PBP 1) was fully saturated at 3 μM [^{35}S]benzylpenicillin, the PBP with an M_r of 67,000 (PBP 2) was saturated at 30 μM, and PBPs 3, 4, and 5 were saturated at between 3 and 10 μM.

Purification of the 49,500-M_r PBP. The purification of the 49,500-M_r PBP was monitored by assaying penicillin-binding activity. Membranes were solubilized by using 1% Triton X-100 as a detergent. Further purification of the 49,500-M_r PBP was carried out by affinity chromatography on ampicillin-linked CH Sepharose 4B as described above. All the PBPs bound to the affinity matrix under these conditions, and all of them were susceptible to hydroxylaminolysis as studied by [^{35}S]penicillin-binding and then by fluorography (data not shown). The final purification was achieved by ion-exchange chromatography on DEAE-cellulose, in which step only the PBP with an M_r of 49,500 was eluted with 0.1 M NaCl. Two hundred micrograms of purified 49,500-M_r PBP was obtained by starting with 93 g (wet weight) of cells. The homogeneity of the protein was checked and confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography (Fig. 2).

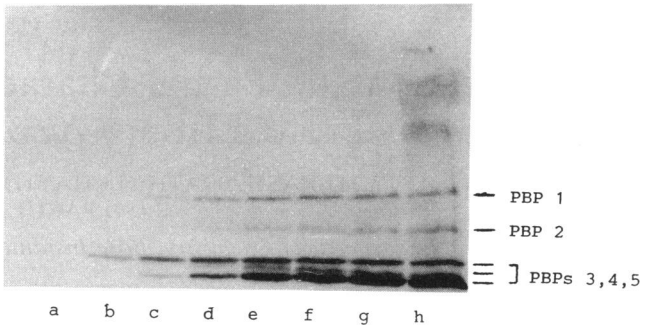

FIG. 1. PBP pattern of the isolated plasma membranes of *M. smegmatis* treated with the following concentrations (molar) of [^{35}S]benzylpenicillin: a, 0.3×10^{-7}; b, 1×10^{-7}; c, 0.3×10^{-6}; d, 1×10^{-6}; e, 0.3×10^{-5}; f, 1×10^{-5}; g, 0.3×10^{-4}; h, 1×10^{-4}. Lanes represent fluorograms obtained after exposure of the gel to X-ray film (Kodak X-Omat AR) at −70°C for 1 week. A total of 100 μg of membrane protein was loaded in each lane.

Properties of the 49,500-M_r PBP. The purified 49,500-M_r PBP demonstrated transpeptidase activity in a model reaction with Ac_2-L-Lys-D-Ala-D-Ala as a substrate and Gly-Gly as an acceptor. Its ability to perform acyl transfer with water as the exogenous acceptor was low, and DD-carboxypeptidase activity was 16.5 nmol of D-Ala released per min per mg of protein. Transpeptidase activity with the same substrate and Gly-Gly as an acceptor was 280 nmol of D-Ala released per min per mg of protein. The transpeptidation/carboxypeptidation ratio was 17 when the two activities were measured in separate experiments. The transpeptidase activity was inhibited by 50% at a benzylpenicillin concentration of 1.8×10^{-7} M, which was similar to the concentration (1.1×10^{-7} M) of benzylpenicillin required to give 50% saturation of the 49,500-M_r PBP (Fig. 3).

Competition of benzylpenicillin binding with other beta-lactams. In experiments aimed at determining the relative

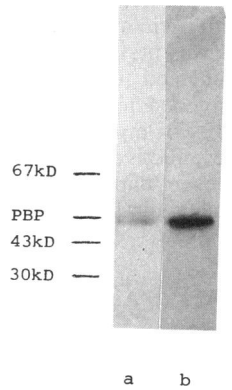

FIG. 2. SDS-PAGE of the purified PBP of *M. smegmatis*. (a) Coomassie blue-stained gel; (b) fluorogram obtained after binding of [^{35}S]benzylpenicillin at a saturating concentration.

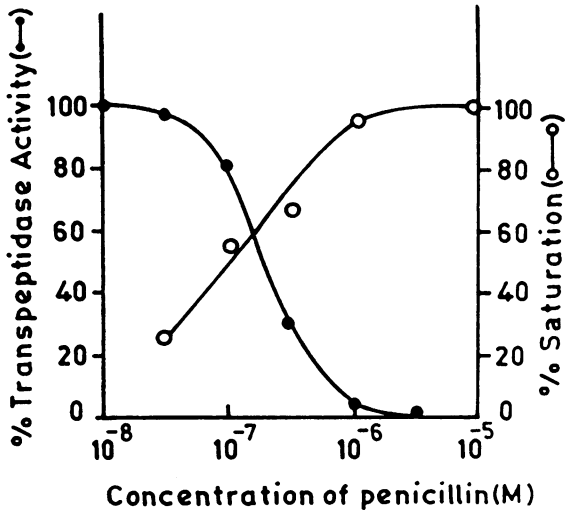

FIG. 3. Saturation of the 49,500-M_r PBP and inhibition of the transpeptidase activity with benzylpenicillin. For the saturation experiment, purified PBP was incubated with [^{35}S]benzylpenicillin at the concentrations indicated, and next fluorography and densitometric scanning of the developed film were done. For inhibition studies, the purified PBP was incubated with benzylpenicillin at 30°C for 10 min at the concentrations indicated, and then transpeptidase activity was determined as described in Materials and Methods.

efficacy of different beta-lactams in binding to the PBP with an M_r of 49,500, competition with benzylpenicillin was carried out as described in Materials and Methods. The 90% inhibitory doses (ID_{90}s) for the different antibiotics tested are shown in Table 1. This was lowest for Sch 34343 manufactured by Schering Plough (2), which belongs to the penem class of beta-lactams and cefoxitin. Of the penicillins tested, ampicillin was found to be the most active.

DISCUSSION

On the basis of the present study, *M. smegmatis* contains two major sets of PBPs, those having M_rs of between 94,000 and 67,000 and those having M_rs of between 56,000 and 46,000. Triton X-100 was found to be an effective detergent for solubilization of all the PBPs from the membranes, and all the PBPs were bound to ampicillin-linked CH Sepharose 4B. The PBP with an M_r of 49,500 was purified by subsequent ion-exchange chromatography. This PBP demonstrated transpeptidase activity with the substrate Ac_2-L-Lys-D-Ala-D-Ala. It was found to be homogeneous on 10% SDS-PAGE.

TABLE 1. Efficacy of different beta-lactam antibiotics against the 49,500-M_r PBP of *M. smegmatis*

Beta-lactam	ID_{90} (M)
Ampicillin	2.5×10^{-7}
Ticarcillin	5×10^{-6}
Cloxacillin	2×10^{-4}
Methicillin	5×10^{-5}
Carbenicillin	1×10^{-6}
Cephalothin	5×10^{-5}
Cephaloridine	5×10^{-5}
Cephalexin	2×10^{-4}
Cefazolin	2×10^{-5}
Cefotaxime	2×10^{-5}
Cefoperazone	2×10^{-5}
Cefoxitin	5×10^{-8}
Moxalactam	1×10^{-6}
Sch 34343	5×10^{-8}

From competition experiments, it was found that of the beta-lactams which can be administered orally, the ID_{90} was lowest in the case of ampicillin. The relatively β-lactamase-resistant cloxacillin and methicillin were much less effective than ampicillin. The β-lactamase-resistant 7-alpha-methoxy-cephalosporin cefoxitin showed maximum inactivating potency against the 49,500-M_r PBP, along with the penem antibiotic Sch 34343. The low ID_{90} of cefoxitin is of particular interest in view of its effectiveness in the mouse foot pad model of leprosy (15).

Although the LMW PBPs have been postulated to be less essential for cell growth than the HMW PBPs, a proper balance between DD-carboxypeptidase and transpeptidase is a prerequisite for functional cell division. The importance of the transpeptidase as a possible lethal target for beta-lactams needs to be investigated in detail, particularly by characterization of the transpeptidase in mutants resistant to cefoxitin or Sch 34343.

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