Characterization of a 38 kDa penicillin-binding protein and its possible involvement in maintaining stationary-phase cells of *Shigella dysenteriae*

Sebabrata Mahapatra, Joyoti Basu, Josef van Beeumen and Manikuntala Kundu

Author for correspondence: Manikuntala Kundu. Fax: +91 33 350 6790.

INTRODUCTION

Penicillin-binding proteins (PBPs), so named because of their affinity for penicillins and other beta-lactam antibiotics, are important membrane-bound enzymes involved in bacterial peptidoglycan synthesis (Ghuysen, 1991). The PBPs fall into three broad classes: the class A high molecular mass (HMM) PBPs, which possess transpeptidase and transglycosylase activities; the class B HMM PBPs, which possess transpeptidase and possibly other activities; and the low molecular mass (LMM) PBPs (Ghuysen, 1991). The HMM PBPs appear to be important in cell elongation, septation or shape determination (Matsuhashi et al., 1990; Wientjes & Nanninga, 1991). The LMM PBPs usually catalyse acyl transfer reactions from d-alanyl-d-alanine terminated peptides and depsipeptide analogues (Ghuysen, 1991; Waxman & Strominger, 1979) to suitable acceptors. Biochemical and genetic studies have shown that resistance to beta-lactam antibiotics may involve alteration in the structural genes of several of the PBPs (Dowson et al., 1989; Laible et al., 1989; Spratt, 1994) and formation of low-affinity PBPs (Hakenbeck et al., 1991; Piras et al., 1990; Reynolds & Fuller, 1986; Utsui & Yokota, 1985).

The PBPs of *Escherichia coli* were initially characterized by binding of radioactive penicillin to the cell membranes. Subsequently, an enormous amount of knowledge has been generated on the physiological roles of several PBPs of *E. coli* (Ghuysen, 1991; Georgopapadakou, 1993), and their genes have been cloned and sequenced. In contrast, there has been no study on the related human pathogen *Shigella*, which is one of the major causes of diarrhoea related to morbidity and mortality, especially among children (Bennish & Salam, 1992). This paper describes the first attempt to identify the PBPs of *Shigella dysenteriae* and is also the first report on the purification and characterization of one of the PBPs of *S. dysenteriae*.

METHODS

Antibiotics and chemicals. Benzyl[14C]penicillin was purchased from Amersham; flucloxacillin, temocillin, ticarcillin and cloxacillin were gifts from SmithKline Beecham; SCH 34343 was a gift from the Schering Plough Corporation; all other antibiotics were purchased from Sigma.

Growth of organism and determination of minimum inhibitory concentrations (MICs) of different antibiotics. *S. dysenteriae* type 1 strain PB-10 was obtained from the stock culture of the National Institute of Cholera and Enteric Diseases, WHO Collaborating Centre for Diarrhoeal Diseases Research and Training, Calcutta, India. Growth was routinely carried out in Tryptic soy broth (Difco) at 37 °C. MICs of antibiotics were determined by growing *S. dysenteriae* in serial dilutions of the antibiotics on Mueller–Hinton agar plates.

Preparation of membranes. Harvested cells were washed with
0.01 M HEPES (pH 7.5) and disrupted by sonication (Labsonic 2000, Braun) employing four 1 min pulses with an interval of 1 min between the pulses. After removal of cell debris by centrifugation, membranes were pelleted by centrifugation at 16000 g for 30 min. This pellet was washed with 0.01 M HEPES (pH 7.5) and stored at -70 °C.

**PBP assays.** Samples (100 μg) of membrane proteins were incubated at 30 °C for 10 min with different concentrations of benzyl[14C]penicillin (0.5 μCi mol⁻¹, 18.5 MBq mol⁻¹). The reaction was stopped by addition of an excess of non-radioactive penicillin and sodium lauroyl sarcosinate (final concentration, 1%, w/v). The samples were allowed to stand at room temperature for 60 min, centrifuged and SDS gel denaturing buffer was added to the supernatants and boiled immediately for 3 min. Samples were applied to 10% SDS-polyacrylamide gels. For the purified PBP, 0.5 μg protein was used in each assay. In competition experiments, membranes were 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 (Pierre et al., 1990); the reaction was terminated in the usual manner, followed by SDS-PAGE, fluorography and densitometric scanning of the fluorograms.

**Solubilization of the membranes with Triton X-100.** Membranes (1 g protein) were solubilized in 10 mM Tris/HCl (pH 8) containing 0.1 mM dithioerythritol and 1% (v/v) Triton X-100 (buffer A) on ice for 60 min. The supernatant was collected after centrifugation at 100000 g for 90 min. This supernatant is referred to as the Triton X-100 extract.

**Purification of the 38 kDa PBP.** The pH of the Triton X-100 extract was adjusted to 5–8 with HCl and the extract was loaded on a Q-Sepharose Fast Flow (Pharmacia) column (20 ml) equilibrated in 25 mM Bis-Tris, pH 5.8, containing 10% (v/v) glycerol and 0.5 mM dithioerythritol (buffer B). Bound proteins were then eluted with a gradient of 0–1 M NaCl in buffer B. Fractions containing penicillin-binding activity were checked for homogeneity by SDS-PAGE. Active fractions were neutralized with 0.1 M NaOH and stored at -20 °C.

**Monitoring the purification.** This was done as described by Basu et al. (1992). Protein samples (50 μl) were incubated with 5 μl benzyl[14C]penicillin (50 μCi mmol⁻¹, 1850 MBq mmol⁻¹; final concentration 34 μg ml⁻¹) for 10 min at 30 °C and then precipitated with 0.5 ml 5% (w/v) trichloroacetic acid and 5 μl 10% Triton X-100. Samples were kept on ice for 5 min, and the precipitate was collected on Whatman GF/C filter circles with a 120A analyser (Applied Biosystems). Automated sequencing was performed on a 477A pulsed liquid sequenator with on-line analysis of the amino acid phenylthiohydantoin derivatives with a 120A analyser (Applied Biosystems).

**Determination of enzyme activities.** The ββ-carboxypeptidase activity was estimated by measuring the hydrolysis of the tripeptide diacetyl-L-Lys-D-Ala-D-Ala (Frere et al., 1976). Briefly, the substrate was incubated with purified PBP (4–10 μg) at 37 °C for 30 min. The N-α-acylase was released by addition of a mixture of v-dianisidine, floravine dinucleotide, peroxidase and β-amino acid oxidase, and incubation for 10 min at 37 °C, followed by addition of methanol/sulfuric acid/water (5:6:5, by vol.). Spectrophotometric readings were recorded at 535 nm. Standard curves were prepared by using D-Ala alone. Membranes (75 μg) of E. coli and S. dysenteriae were also assayed as described by Waxman & Strominger (1979).

**Raising of antibodies against the 38 kDa PBP.** Purified 38 kDa PBP (75 μg) was injected subcutaneously with complete Freund’s adjuvant into one male rabbit. Successive injections of 40 μg each with incomplete Freund’s adjuvant were repeated at the end of the 2nd, 3rd and 4th weeks, followed by bleeding at the end of the 5th week.

**Western blotting and immunoprecipitation.** Membranes of S. dysenteriae or E. coli K12 were run on 10% SDS-polyacrylamide gels. Western blotting using anti-38 kDa PBP antibodies at a dilution of 1:250 was performed according to the method of Towbin et al. (1979) with horseradish-peroxidase-linked anti-rabbit IgG as the second antibody and 4-chloro-1-naphthol as the colour development reagent. Immunoprecipitation was performed on Triton X-100 extracts of membranes using anti-38 kDa antibody (1:250) and protein A-bearing Staphylococcus aureus cells as described by Firestone & Winguth (1990). Prior to electrophoresis, the complex was boiled for 5 min with SDS gel denaturing buffer.

**Preparation of spheroplasts.** Cells were pelleted from exponentially growing cultures and resuspended in 15 mM Tris/HCl buffer (pH 8) containing 12.5% (w/v) sucrose (buffer C) at a concentration of 2×10⁶ cells ml⁻¹ as described by Broome-Smith & Spratt (1986). Cells were then treated with lysozyme (2 mg ml⁻¹) and EDTA (5 mM) in buffer C for 25 min at 37 °C. Under these conditions all the cells were converted to spheroplasts.

**Protease digestion of the 38 kDa PBP in spheroplasts.** Spheroplasts were incubated at room temperature with proteinase K at final concentrations ranging from 0.1 to 2 mg ml⁻¹. The digestion was stopped by 100 mM PMSF, then samples were denatured and fractionated on a 10% SDS-polyacrylamide gel. The proteins were electrophoretically transferred to a nitrocellulose sheet and probed with anti-38 kDa antibody as described by Towbin et al. (1979).

**N-terminal sequence analysis.** Automated sequencing was performed on a 477A pulsed liquid sequenator with on-line analysis of the amino acid phenylthiohydantoin derivatives with a 120A analyser (Applied Biosystems).

### RESULTS

**MICs of different beta-lactam antibiotics against S. dysenteriae**

The MICs of different beta-lactam antibiotics against S. dysenteriae are shown in Table 1. The organism was resistant to all the penicillins tested. Of the other beta-lactams tested, the most effective were ceftriaxone, the penem SCH34343 developed by Schering Plough Corporation (Das et al., 1988), the cephazolin cefoxitin and the oxacephem moxalactam.

The organism was found to elaborate an intracellular constitutive beta-lactamase. The contribution of this beta-lactamase activity towards the antibiotic susceptibility/resistance profile of the organism is the subject of a separate study.

**PBP pattern of the membranes of S. dysenteriae**

The membranes of S. dysenteriae PB-10 contained seven major PBPs, of molecular masses 84, 68, 63, 52, 46, 39 and 38 kDa (Fig. 1). A comparison with the PBPs of E. coli K12 is presented in Fig. 1. PBP 1 of S. dysenteriae could be
Penicillin-binding protein from \textit{S. dysenteriae}

\textbf{Table 1. Efficacy of different beta-lactam antibiotics against PBP 7 and other PBPs of \textit{S. dysenteriae}}

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>ID_{50} (M)</th>
<th>MIC (\textmu g ml^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBP 1</td>
<td>PBP 2</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>$8 \times 10^{-4}$</td>
<td>$8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Flucloxacillin</td>
<td>$8 \times 10^{-4}$</td>
<td>$8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>$8 \times 10^{-4}$</td>
<td>$8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>$8 \times 10^{-4}$</td>
<td>$8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>$8 \times 10^{-5}$</td>
<td>$8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>$8 \times 10^{-5}$</td>
<td>$8 \times 10^{-5}$</td>
</tr>
<tr>
<td>Moxalactam</td>
<td>$8 \times 10^{-4}$</td>
<td>$&gt; 8 \times 10^{-4}$</td>
</tr>
<tr>
<td>SCH34343</td>
<td>$8 \times 10^{-6}$</td>
<td>&lt; $8 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

*ND, Not determined.

\textbf{Fig. 1.} PBP pattern of the isolated plasma membranes of \textit{E. coli} (lane A) and \textit{S. dysenteriae} (lane B). The molecular masses (kDa) of the PBPs are as follows. \textit{E. coli}: PBP 1(a, b), 92; PBP 2, 68; PBP 3, 65; PBP 4, 52; PBP 5, 63; PBP 6, 39; PBP 7, 38; \textit{S. dysenteriae}: PBP 1(a, b), 84; PBP 2, 68; PBP 3, 63; PBP 4, 52; PBP 5, 46; PBP 6, 39; and PBP 7, 38.

\textbf{Fig. 2.} Purification of the 38 kDa PBP of \textit{S. dysenteriae} from a Q-Sepharose Fast Flow column as described in Methods. The arrow indicates the penicillin-binding activity corresponding to the 38 kDa PBP. , $A_{280}$; $\bullet$, [\textsuperscript{14}C]penicillin bound.

resolved into two bands, 1a and 1b, under some conditions, as in the case of \textit{E. coli}. PBPs 2, 3 and 4 of \textit{S. dysenteriae} appeared to have molecular masses similar to PBPs 2, 3 and 4 of \textit{E. coli}. PBPs 5 and 6 of \textit{E. coli} could be seen as two distinct bands when shorter exposure times of the gels to the X-ray films were used. However, PBP 5 of \textit{S. dysenteriae} could not be resolved into more than one band. \textit{S. dysenteriae} showed two additional LMM PBPs of 39 and 38 kDa. PBPs with molecular masses of about 30–34 kDa have been reported in \textit{E. coli} (Barbas et al., 1986). The 38 kDa PBP observed by us in \textit{E. coli} probably corresponds to PBP 7 of \textit{E. coli} reported by earlier workers. It may be mentioned that the molecular masses of the \textit{E. coli} PBPs determined by us differed slightly from those reported by Spratt (1977), namely 91 kDa (1), 66 kDa (2), 60 kDa (3), 49 kDa (4), 42 kDa (5) and 40 kDa (6). Such differences have been observed by other workers also. All the PBPs were saturated at concentrations of benzylpenicillin > $10^{-4}$ M (data not shown), indicating that all the PBPs are fairly resistant towards penicillin, and suggesting that this is one of the factors contributing towards the resistance of the organism towards penicillins.

\textbf{Purification of a 38 kDa PBP}

The Triton X-100 extract was adjusted to pH 5.8 with HCl, loaded on a Q-Sepharose column equilibrated in 25 mM Bis-Tris, pH 5.8, containing 10% glycerol and eluted as described in Methods. One of the PBPs eluted from the column at an NaCl concentration close to 1 M (Fig. 2). Its homogeneity was confirmed by [\textsuperscript{14}C]penicillin binding followed by SDS-PAGE and fluorography (Fig. 3). This PBP had a molecular mass of 38 kDa, and corresponds to PBP 7 of \textit{S. dysenteriae}. A total of 200 \mu g of purified PBP 7 was obtained from 1 g of membrane protein.

\textbf{N-terminal sequence analysis}

N-terminal sequence analysis (Fig. 4) showed that the 38 kDa PBP does not have sequence homology with any of the \textit{E. coli} PBPs cloned and sequenced so far. It also
S. MAHAPATRA and OTHERS

**Fig. 3.** SDS-PAGE of the purified 38 kDa PBP of S. dysenteriae. Lane A, Coomassie blue-stained gel; lane B, fluorogram obtained after binding of benzyl[14C]penicillin at saturating concentration.

**Fig. 4.** N-terminal sequence of the purified 38 kDa PBP of S. dysenteriae.

Further confirmed the homogeneity of the purified protein.

**Determination of DD-carboxypeptidase activity of the purified PBP**

DD-Carboxypeptidase activity of the purified PBP was determined in a model reaction using diacetyl-L-Lys-D-Ala-D-Ala as substrate. Under the conditions of our assay, no DD-carboxypeptidase activity could be demonstrated. However, DD-carboxypeptidase activity was detected in positive controls of membranes of both *E. coli* and *S. dysenteriae*.

**Competition experiments**

The relative efficacy of different beta-lactams in binding to the purified 38 kDa PBP was determined by studying their ability to inhibit benzyl[14C]penicillin binding as described in Methods. The 90% inhibitory doses (ID$_{90}$) for the different antibiotics are given in Table 1. The resistance of the PBPs towards the penicillins in general suggests that this is one of the factors contributing towards the resistance of the organism against penicillins. The 38 kDa PBP (PBP 7) was sensitive to cefoxitin, SCH 34343 and moxalactam. Although PBP 7 is not the exclusive target of any of these antibiotics, it may be one of the important targets for each of these antibiotics. Further studies are needed in order to draw conclusions.

**Detection of 38 kDa protein(s) in *E. coli* membranes using anti-38 kDa antibody**

The anti-38 kDa antibody of *S. dysenteriae* was cross-reactive with a single protein from *S. dysenteriae* membranes (Fig. 5) as well as from *E. coli* membranes (data not shown). Since Western blots may give positive bands with comigrating species other than PBP(s) when membranes are used, immunoprecipitation was performed after radioactive penicillin binding. Briefly, membranes of *E. coli* and *S. dysenteriae* were solubilized with Triton X-100 (as described before) after labelling with radioactive penicillin. The Triton X-100 extracts were incubated successively with anti-38 kDa antibody and Protein A-containing *S. aureus* cells followed by centrifugation, in order to immunoprecipitate cross-reactive proteins. The pellet was boiled with SDS gel denaturing buffer, followed by SDS-PAGE and fluorography. As evident from Fig. 5, lanes D and E, the immunoprecipitated proteins from *E. coli* and *S. dysenteriae* were both PBPs of Mr 38000, corresponding to PBP 7 of *S. dysenteriae*.

**Protease digestion of the 38 kDa PBP in spheroplasts**

This was done essentially as described by Bowler & Spratt (1989) to establish the membrane topology of PBP 3 of *E. coli*. Western blotting revealed that the 38 kDa PBP is susceptible to proteolysis from the periplasmic side, since incubation of spheroplasts with proteinase K led to a...
concentration as high as 2000 pg ml$^{-1}$ does not destroy the integrity of the spheroplasts.

**Expression of the 38 kDa PBP at different stages of growth**

The level of the 38 kDa PBP at different stages of growth was monitored by studying penicillin-binding activity in membranes at different stages of growth, as well as by Western blotting using the anti-38 kDa antibody. Both these methods showed that the level of this PBP gradually increased, reaching a maximum at 6 h of growth, i.e. when the cells had reached stationary phase. The level thereafter remained steady (Fig. 7). The gradual increase in intensity of the band obtained after penicillin-binding and fluorography, reaching its maximum at the stationary phase, was unique to this PBP (Fig. 7a).

**DISCUSSION**

Penicillin-binding proteins have been extensively studied in *E. coli*. However, the present study is the first attempt to characterize the PBPs of *S. dysenteriae*. The pattern of the PBPs of *S. dysenteriae*, not surprisingly, resembles that of *E. coli*. The general resistance of the PBPs of *S. dysenteriae* towards benzylpenicillin suggests that this may be one of the causes of the resistance of the organism towards penicillins, although the contribution of other factors, such as the beta-lactamase, and outer membrane permeability, cannot be overlooked. The 38 kDa PBP purified by us is the first PBP to have been purified from *S. dysenteriae*. This PBP corresponds to PBP 7 in our present nomenclature of the PBPs of this organism. Since antibody raised against this PBP gives only one positive 38 kDa band in Western blots of membranes, the possibility that this PBP may be a degradation product of some other PBP may be ruled out. This PBP is cross-reactive with a 38 kDa PBP of *E. coli* which is of lower molecular mass than PBP 6 of *E. coli*. Its N-terminal sequence does not bear homology with that of any PBP from *E. coli*. The purification of this PBP therefore opens up the possibility of detailed studies on a hitherto uncharacterized beta-lactam target in enterobacteria. The sensitivity of this PBP towards SCH 34343, cefoxitin and moxalactam suggests that this PBP is one of the targets of these antibiotics, to which the organism itself is susceptible. However, it does not indicate that the 38 kDa PBP is the only target. The increased level of this PBP in particular in stationary-phase cells deserves further investigation into the possible role of this PBP in the stationary phase. Penicillin-binding protein from *S. dysenteriae* was unique to this PBP (Fig. 7a).

...
ACKNOWLEDGEMENTS

This work was supported in part by grants from the Indian Council of Medical Research and the Department of Science and Technology, Government of India to M.K. The authors are grateful to Professor Parul Chakrabarti for her constructive criticism of the manuscript.

REFERENCES


Received 10 May 1994; accepted 8 July 1994.