Characterization of derivatives of the high-molecular-mass penicillin-binding protein (PBP) 1 of *Mycobacterium leprae*

Sebabrata MAHAPATRA, Sanjib BHAKTA, Jasimuddin AHAMED and Joyoti BASU[1]

Department of Chemistry, Bose Institute, 93/1 Acharya Prafulla Chandra Road, 700 009 Calcutta, India

Mycobacterium leprae has two high-molecular-mass multimodular penicillin-binding proteins (PBPs) of class A, termed PBP1 and PBP1* [Lepage, Dubois, Ghosh, Joris, Mahapatra, Kundu, Basu, Chakrabarti, Cole, Nguyen-Disteche and Ghuysen (1997) J. Bacteriol. **179**, 4627–4630]. PBP1-Xaa–β-lactamase fusions generated periplasmic β-lactamase activity when Xaa (the amino acid of PBP1 at the fusion junction) was residue 314, 363, 407, 450 or 480. Truncation of the N-terminal part of the protein up to residue Leu-147 generated a penicillin-binding polypeptide which could still associate with the plasma membrane, whereas [ΔM1–R314]PBP1 (PBP1 lacking residues Met-1 to Arg-314) failed to associate with the membrane, suggesting that the region between residues Leu-147 and Arg-314 harbours an additional plasma membrane association site for PBP1. Truncation of the C-terminus up to 42 residues downstream of the KTG (Lys-Thr-Gly) motif also generated a polypeptide that retained penicillin-binding activity. [ΔM1–R314]PBP1 could be extracted from inclusion bodies and refolded under appropriate conditions to give a form capable of binding penicillin with the same efficiency as full-length PBP1. This is, to the best of our knowledge, the first report of a soluble derivative of a penicillin-resistant high-molecular-mass PBP of class A that is capable of binding penicillin. A chimaeric PBP in which the penicillin-binding (PB) module of PBP1 was fused at its N-terminal end with the non-penicillin-binding (n-PB) module of PBP1* retained pencillin-binding activity similar to that of PBP1, corroborating the finding that the n-PB module of PBP1 is dispensable for its penicillin-binding activity.

Key words: β-lactam target, membrane topology, penicilloyl serine transferase.

INTRODUCTION

Penicillin-binding proteins (PBPs) are membrane-bound enzymes that catalyse the final steps of bacterial cell wall synthesis [1,2]. The PBPs are members of the penicilloyl serine transferase family of enzymes, which carry a serine residue at the active site and are characterized by structural motifs unique to each category [2]. The high-molecular-mass PBPs are multimodular proteins that have both a penicillin-binding (PB) module and a non-penicillin-binding (n-PB) module. These can be further divided into two groups based on sequence similarity searches [3,4]. The high-molecular-mass PBPs of class A are bifunctional enzymes that catalyse both the polymerization of the glycan chains (transglycosylation) and the cross-linking of the peptide chains (transpeptidation) during cell wall synthesis [5–10]. The transglycosylation function rests within the N-terminal n-PB module, while the transpeptidation function rests within the C-terminal PB module [11]. In general, the high-molecular-mass PBPs contain near their N-terminus a single hydrophobic transmembrane anchor, which functions as a non-cleavable signal sequence that directs the rest of the protein into the periplasm [12].

The availability of an ordered cosmid library has allowed the overexpression and purification of two high-molecular-mass PBPs of class A from the pathogen *Mycobacterium leprae* [13,14]. The two PBPs, PBP1 and PBP1*, differ markedly in terms of their thermostability, penicillin sensitivity and detergent solubility. PBP1 is characterized by its low affinity towards a range of β-lactams. It appears to be a unique, low-affinity [13], high-molecular-mass PBP of molecular class A. Computational analysis of the sequence does not predict any hydrophobic transmembrane stretches other than the Met-1–Ile-39 pseudo-signal peptide [13]. In an effort to generate a functional, truncated but water-soluble form of the PBP, and to test whether the PB module can function as a penicillin-binding entity in the absence of the n-PB module, genetically manipulated versions of the PBP were expressed in *Escherichia coli*. Here we present evidence that a membrane-associating domain, in addition to the non-cleavable pseudo-signal peptide, exists between residues 147 and 314, a feature common to two other high-molecular-mass PBPs of class A, namely PBP1b of *E. coli* [15,16] and PBP2a of *Streptococcus pneumoniae* [17]. Unlike PBP1b of *E. coli* [11], the C-terminal PB module of PBP1 can function as a soluble, penicillin-binding entity independent of the N-terminal non-PB module.

EXPERIMENTAL

Materials

All restriction and modifying enzymes were from Life Technologies or from Roche Molecular Biochemicals. [^{14}C]-Benzylpenicillin was from Amersham Pharmacia Biotech. Synthetic oligonucleotides were purchased from Life Technologies. Transformations were routinely carried out in *E. coli* DH5α. The expression of the modified *pon1* genes was carried out in *E. coli*

Abbreviations used: PBP, penicillin-binding protein; PB module, penicillin-binding module; n-PB module, non-penicillin-binding module; MIC, minimum inhibitory concentration; LB, Luria–Bertani; IPTG, isopropyl 1-thio-β-D-galactopyranoside; Gdn-HCl, guanidine hydrochloride; truncated proteins are designated as follows: [ΔM1–R314]PBP1 represents PBP1 lacking residues Met-1 to Arg-314, and so on.

[1]To whom correspondence should be addressed (e-mail joyoti@bic.boseinst.ernet.in).

BL21(DE3). In-frame fusions of PBP1 to TEM β-lactamase were expressed in *E. coli* JM105 or MI1443 (a strain lacking the chromosomal β-lactamase). Luria–Bertani (LB) broth was used for growth, and was supplemented with kanamycin (50 μg/ml) where necessary. DNA sequencing of the constructs was performed using the Thermosequenase Cycle Sequencing Kit from Amersham Pharmacia Biotech.

Construction of β-lactamase fusions with truncated PBP1 derivatives

pJBS633 (carrying the mature TEM β-lactamase encoding blaM) [18] was used for constructing in-frame fusions of TEM β-lactamase with the C-terminal ends of truncated PBP1 derivatives. These derivatives were generated by PCR using the forward primer 5′ TTGGATCCAAATGTCAGAGCGCCTCCCAGCC 3′ containing a *Bam*HI site (underlined) and the sequence corresponding to the N-terminus of PBP1, and reverse primers encoding portions of the PBP1 gene. After amplification by PCR using *Pwo* DNA polymerase (Roche Molecular Biochemicals), the product was digested with *Bam*HI and ligated to pJBS633 digested with *Bam*HI and *Pvu*II. *E. coli* JM105 was transformed with the ligation mixture, and transformants growing on LB agar plates containing 50 μg/ml kanamycin were chosen for further analysis. Transformants containing in-frame PBP–β-lactamase fusions were detected by their ability to grow when patched with toothpicks on to agar containing 200 μg/ml ampicillin [19]. The nucleotide sequences across the β-lactamase fusion junctions were determined.

Ampicillin resistance of β-lactamase fusion proteins

The minimum inhibitory concentrations (MICs) of ampicillin for individual cells of *E. coli* JM105 containing PBP1-Xaa–TEM β-lactamase fusions were determined by spotting 4 μl of a $1:10^5$ dilution of an overnight culture (approx. 40 bacteria) on LB agar plates containing a range of doubling concentrations of ampicillin. The single-cell MIC was the lowest concentration of ampicillin that prevented the growth of bacterial colonies.

Growth and assay of β-lactamase activity of *E. coli* transformants carrying in-frame β-lactamase fusion proteins

E. coli JM105 or MI1443 (kindly provided by Professor Bernd Weidemann, University of Bonn, Germany) harbouring plasmids with in-frame β-lactamase fusions were grown at 37 °C in LB medium containing 50 μg/ml kanamycin. β-Lactamase activity in the different cellular fractions was assayed using nitrocefin as the substrate [20].

Construction of truncated [ΔS2–F82]PBP1 (PBP1 lacking residues Ser-2 to Phe-82)

pET28a was digested with *Nde*I and *Bam*HI to eliminate a 40 bp fragment. This was replaced by oligonucleotides 5′ TGGAGGTACCCCGCTCGATCGGTCCGAGACTAA 3′ and its reverse complement strand, with overhangs of AT at the 5′ end and GATC at the 3′ end, to complement *Nde*I and *Bam*HI respectively. The resulting plasmid is termed pJS201. *Kpn*I and *Rsr*II sites (underlined) were present in the oligonucleotide. Plasmid pDML905 [13] was digested with *Kpn*I (in order to remove the N-terminal 82 amino acids) and *Rsr*II. The resulting 2209 bp *Kpn*I–*Rsr*II fragment of the *pon1* gene was inserted between the *Kpn*I and *Rsr*II sites of pJS201 to yield pJS202, encoding the truncated PBP1 devoid of N-terminal amino acids 2–82.

Construction of C-terminally truncated [ΔN658–D821]PBP1 (PBP1 lacking residues Asn-658 to Asp-821)

pDML905 was digested with *Pst*I and *Rsr*II to remove a 485 bp fragment encoding the C-terminus of PBP1 (starting from Asn-658). This was replaced by the oligonucleotide 5′ GCTAAGGCGCGCC 3′ and its complementary strand, with an overhang of TGCA at the 5′ end and GAC at the 3′ end to complement *Pst*I and *Rsr*II respectively, with a stop codon (underlined) after the *Pst*I site. The resulting construct (pJS203) was used to express a protein comprising residues Met-1 to Ser-657 of PBP1.

Construction of truncated [ΔM1–L147]PBP1 (PBP1 lacking residues Met-1 to Leu-147)

pJS202 was digested with *Nco*I. The 5′ overhangs were filled in with Klenow polymerase and dNTPs, followed by digestion with *Pst*I. A 1779 bp fragment encoding an N-terminal portion (Met-1 to Leu-147) of PBP1 was eliminated, leaving a derivative of pJS202 with one blunt and one staggered (*Pst*I-compatible) end. pDML905 was digested with *Nde*I and *Pst*I, and the resulting *pon1*-derived *Nde*I–*Pst*I fragment was cloned into the *Nde*I–*Pst*I sites of pUC19, yielding pJS204. pJS204 was digested with *Bst*EII and the 5′ overhang was filled in with Klenow polymerase and dNTPs, followed by digestion with *Pst*I, yielding a 1528 bp fragment of *pon1* (encoding an N-terminal portion of PBP1 after the second conserved module) with one blunt and one staggered (*Pst*I-compatible) end. Ligation of this fragment with the pJS202 derivative generated above gave a construct pJS205 for the expression of [ΔM1–L147]PBP1.

Construction of truncated [ΔM1–R314]PBP1 (PBP1 lacking residues Met-1 to Arg-314)

The N-terminal 314 amino acids, encompassing the four conserved boxes of the n-PB module of PBP1, were removed through this construction, generating the gene encoding a truncated PBP1 bearing the PB module and the C-terminal 22 amino acids of the predicted n-PB module. pJS202 was digested with *Nco*I and *Nhe*I to remove a 696 bp fragment coding for the N-terminal 314 amino acids of PBP1. The 5′ overhangs were filled in with Klenow polymerase and dNTPs to generate blunt ends, which were then circularized by ligation. The resulting construct (pJS206) was used for the expression of [ΔM1–R314]PBP1.

Construction of chimaeric PBP

pDML909 [14] was digested with *Bam*HI and *Xho*I, and the 146 bp fragment encoding amino acid residues 315–367 of PBP1* was ligated into pUC19 digested with *Bam*HI and *Sal*I to give the plasmid pJS261. Nucleotides 1105–1562 were amplified by PCR from pDML905 using primers 5′ ATCGATCCGGGCAAAGATGCCCATCGCGTG 3′ (sense; *Cla*I site underlined) and 5′ TCTAGAAAGCTTTCGTTGCTGTCCGGATTATA 3′ (antisense; *Hin*dIII site underlined). The 451 bp PCR product, encoding amino acids 357–510 of PBP1 [13], was cloned into pJS261 digested with *Cla*I and *Hin*dIII using asymmetric *Cla*I and *Hin*dIII sites present in the PCR product, to give pJS262, which was sequenced. pDML909 was digested with *Bgl*II and *Bam*HI, and the resulting 1170 bp fragment encoding amino acids 1–316 of PBP1* [14] was ligated into pJS262 digested with *Bam*HI. Recombinants encoding the first 326 amino acids of PBP1* fused to amino acids 357–510 of PBP1 in the correct orientation in pUC19 were checked by nucleotide sequencing. The resulting plasmid was designated pJS263. pDML905 was digested with *Nde*I and *Hin*dIII, and the 6647 bp fragment

containing nucleotides 1562–2770 of *pon1* in pET28a (fragment A) was isolated. pJS263 was digested with *Nde*I and *Hin*dIII, and the 1447 bp fragment (fragment B) harbouring nucleotides 1–1020 of *ponA* (the gene encoding PBP1*) and nucleotides 1105–1562 of *pon1* (the gene encoding PBP1) was isolated. Fragment B was ligated with fragment A to give pJS273, encoding amino acids 1–326 of PBP1* fused at the C-terminal end to amino acids 357–821 of PBP1.

Expression of recombinant PBP1 constructs

E. coli transformants grown to an absorbance of 0.5 at 600 nm were induced with isopropyl β-D-thiogalactopyranoside (IPTG) at concentrations of 50–200 μM at 37 °C for 2 h. Cells were harvested and sonicated at 200 W for 2 min. Unbroken cells and debris were removed by centrifugation at 600 ***g*** for 10 min. Inclusion bodies were precipitated at 5000 ***g*** for 10 min, and plasma membranes were precipitated at 100000 ***g*** for 30 min. Both inclusion bodies and plasma membranes were washed twice and stored at −20 °C until further use.

Western blotting

Western blotting using anti-PBP1 antibodies [13] at a dilution of 1:2500 was performed using the method of Towbin et al. [21], with horseradish peroxidase-conjugated anti-rabbit antibody as the secondary antibody and 4-chloro-1-naphthol as the colour development reagent.

Refolding of the Leu-315–Asp-821 module of PBP1 from inclusion bodies

Inclusion bodies were washed once with 10 mM Tris/HCl, pH 8, containing 0.1 mM PMSF and once with 10 vol. of 10 mM Tris/HCl, pH 8, containing 1% (w/v) Triton X-100. The remaining pellet contained mainly the recombinant, truncated PBP1. The pellet was extracted with 20 vol. of 150 mM Tris/HCl, pH 8, containing 1 mM Na_2EDTA, 1 mM dithiothreitol and 5 M guanidine hydrochloride (Gdn-HCl) at room temperature for 30 min. After centrifugation at 40000 ***g*** for 30 min, the unfolded recombinant protein was obtained in the supernatant (Gdn-HCl extract). This was dialysed against 100 vol. of different buffers at different temperatures for 16 h in order to determine the ideal conditions for obtaining a refolded protein.

Fluorimetric analysis of purified [ΔM1–R314]PBP1

The concentrations of the denatured and the refolded proteins were determined using the Pierce Micro BCA Protein Assay Reagent Kit and by measuring absorbance at 280 nm. The proteins were then reconstituted in the appropriate buffers to a concentration of 1.5 μM, and their emission spectra were analysed on a Hitachi spectrofluorimeter in 1-cm cuvettes after excitation at 280 nm with a 5-nm slit width. Fluorescence spectra are represented after subtracting the background light intensity measured with buffer alone.

Penicillin-binding assays

Isolated membranes and purified derivatives were analysed by SDS/PAGE, Coomassie Blue staining and fluorography of the gels after labelling of the protein with different concentrations of [^{14}C]benzylpenicillin for 30 min at 37 °C. The values of the second-order rate constant of enzyme penicilloylation (k_2/K) and the first-order rate constant of acyl-enzyme breakdown (k_3) were estimated by previously published methods [22].

RESULTS

M. leprae PBP1 sequence and topology

From the hydropathy plot and from sequence alignments [3,13], PBP1 has been proposed to consist of a possible Met-1–Ile-39 pseudo-signal peptide fused to an n-PB module bearing the conserved modules characteristic of the high-molecular-mass PBPs of class A. The n-PB module is fused to a PB module, which is followed by a C-terminal extension of 205 amino acid residues downstream of the KTG motif. The different modules of both PBP1 and PBP1* are depicted in [3] (as Mle1 and Mel1* respectively). Previous studies have shown that [ΔMet-1–Ile-39]PBP1 remained associated with the plasma membrane [13]. This suggested that PBP1 of *M. leprae* probably contains a membrane association domain downstream of residue 39.

Analyses of PBP1–β-lactamase fusions

Random fusions of PBP1 with the N-terminal end of the TEM β-lactamase reporter were generated. *E. coli* JM105 had a single-cell MIC of 4 μg/ml for ampicillin. The transformants T1–T5 [in which Xaa (the amino acid of PBP1 at the fusion junction) was Arg-314, His-363, Ala-407, Asn-450 and Arg-480 respectively] all had single-cell MICs of 200 μg/ml for ampicillin, consistent with the view that these fusion sites were each in the periplasm, since β-lactamase fusion proteins can provide single cells of *E. coli* with ampicillin resistance only if the β-lactamase moiety is translocated to the periplasm. Several in-frame β-lactamase fusions obtained at postions where Xaa represented an amino acid between residues 90 and 280 were obtained. These PBP-Xaa–β-lactamase fusion proteins were expressed in *E. coli* lacking the chromosomal β-lactamase. Using a calculation method that sets the normalized activity of the most active fusion protein (T5) at 100 and gives values for the other fusions relative to this highest value, it was observed that the β-lactamase activities present in the cell-free extracts of these transformants were between 10 and 20, whereas transformants T1–T4 showed values between 90 and 100. This suggested that the lack of protection against ampicillin was due to these β-lactamase fusions giving rising to inactive proteins. Thus a detailed analysis of membrane topology using PBP-Xaa–β-lactamase fusion proteins was not possible. Attempts were therefore made to progressively truncate PBP1 at its N-terminal end in an effort to generate a soluble PBP retaining its penicillin-binding function.

Truncation of the N-terminal domain of PBP1

[ΔS2–F82]PBP1, although lacking the pseudo-signal peptide, nevertheless associated with the membrane (Figure 1). This suggested that there is a membrane association site for PBP1 that is present downstream of the N-terminal 82 amino acids. The truncated PBP was able to bind benzylpenicillin with the same efficiency as the full-length PBP1, with values for k_2/K of 5–6 $M^{-1} \cdot s^{-1}$ and k_3 of 2.3×10^{-4} s^{-1}.

[ΔM1–L147]PBP1 also associated with the membrane (Figure 1). It bound benzylpenicillin with a k_2/K value of 6–8 $M^{-1} \cdot s^{-1}$ and a k_3 value of 2×10^{-4} s^{-1}. A deletion including the first two conserved modules present in the transglycosylase domain therefore did not affect the penicillin-binding activity of PBP1.

Truncation of the C-terminal domain

E. coli PBP1a and PBP1b and *M. leprae* PBP1 each have different C-terminal extensions. The extensions are possibly exposed at the surfaces of the proteins. *M. leprae* PBP1 contains a 205-amino-acid C-terminal extension after the final conserved

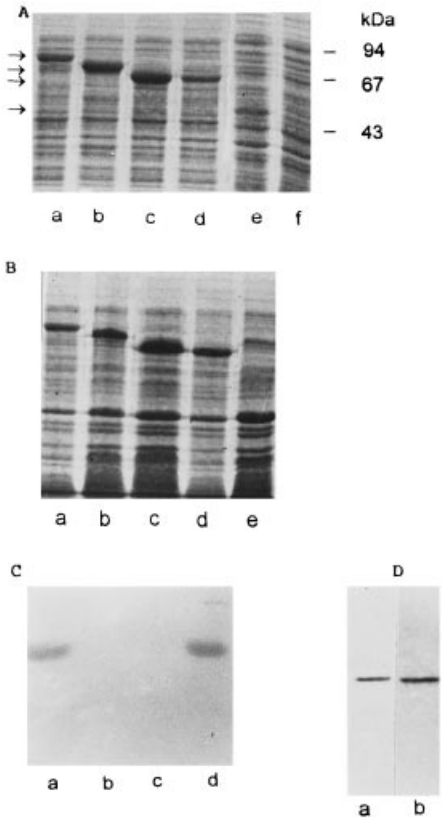

Figure 1 Overproduction of *M. leprae* PBP1 constructs in *E. coli* BL21(DE3)

Analysis was performed by SDS/PAGE and Coomassie Blue staining. (**A**) Expression of PBPs in crude cell extracts; (**B**) expression of PBPs in membrane fractions. Cells expressed the following proteins after induction: lane a, PBP1; lane b, [ΔS2–F82]PBP1; lane c, [ΔM1–L147]PBP1; lane d, [ΔN658–D821]PBP1; lane e, [ΔM1–R314]PBP1. Lane f represents uninduced cells. Arrows indicate the positions of the overexpressed proteins. The identity of each protein was verified by Western blotting using anti-PBP1 antibody. (**C**) Western blot analysis of fractions from *E. coli* BL21(DE3)/pJS206 after induction with IPTG. Following SDS/PAGE, proteins were transferred to nitrocellulose and probed with anti-PBP1 antibody. Fractions: lane a, crude cell extract; lane b, cytosol; lane c, membranes; lane d, inclusion bodies. (**D**) Soluble, refolded [ΔM1–R314]PBP1 was labelled after incubation with 1 mM [^{14}C]benzylpenicillin for 30 min at 37 °C before electrophoresis. Lane a, Coomassie Blue staining; lane b, fluorography.

motif (KTG) of the PB module [13]. The C-terminal 164 amino acids were deleted, generating a truncated PBP, [ΔN658–D821]PBP1, which associated with the membrane (Figure 1) and bound benzylpenicillin with an efficiency equal to that of full-length PBP1 ($k_2/K = 5$–$7\ M^{-1}\cdot s^{-1}$; $k_3 = 2 \times 10^{-4}\ s^{-1}$). The presence of 41 amino acid residues downstream of the KTG motif was therefore sufficient to maintain the penicillin-binding activity of PBP1.

Construction and characteristics of [ΔM1–R314]PBP1

To establish whether the PB module of PBP1 could function as an independent penicillin-binding entity in the absence of the n-PB module, a truncated PBP1 containing residues Leu-315 to Asp-821 was produced in *E. coli*. [ΔM1–R314]PBP1 was present exclusively in inclusion bodies. This was verified using a polycolonal antibody raised against PBP1 of *M. leprae* (Figure 1).

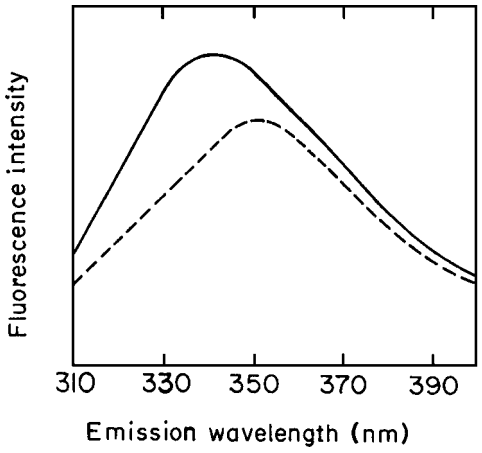

Figure 2 Emission spectra of unfolded and refolded [ΔM1–R314]PBP1

A sample of 1.5 μM of each protein was excited at 280 nm and then scanned for the emission spectra. The solid line indicates the emission spectrum of the refolded protein and the broken line that of the unfolded protein in 5 M Gdn-HCl.

Solubilization of [ΔM1–R314]PBP1

Attempts were made to extract a properly folded truncated protein from the inclusion bodies. The Gdn-HCl extract containing mainly unfolded recombinant PBP1 was obtained as described above. The unfolded protein was dialysed against different buffers at different temperatures in order to determine the ideal conditions for obtaining a refolded protein. Among the buffers tested were 0.4 M Tris/HCl, pH 8 (buffer A), buffer A containing 5 mM Na_2EDTA, buffer A containing 20% (v/v) glycerol, buffer A containing 0.5 M NaCl, and buffer A containing 1 mM Na_2EDTA and 0.5 M L-arginine (buffer B). Dialysis against buffer B at 20 °C for 16 h was found to be the best condition for obtaining refolded protein (as defined by affinity for penicillin) (Figure 1). After dialysis, a small amount of aggregated protein was removed by centrifugation. Aggregation could be minimized by limiting the concentration of protein to 10–50 μg/ml. The use of Tris buffer at a concentration of at least 0.4 M was found to be necessary for obtaining the refolded PBP1 derivative. This has been reported for several test proteins expressed in *E. coli* [23]. The effect of L-arginine to increase yields of *in vitro* folding of proteins is also well documented, although not clearly understood. Once folded, the soluble recombinant [ΔM1–R314]PBP1 was stable for at least 2 weeks when stored at −20 °C. The yield of the solubilized recombinant protein was 25–30% of that present in the inclusion bodies.

Fluorescence emission spectra of unfolded and refolded [ΔM1–R314]PBP1

The overall structures of the unfolded Gdn-HCl-containing PBP1 and the refolded PBP1 were compared using intrinsic tryptophan fluorescence. The shift of the emission peak by 10 nm to a lower wavelength and an enhancement of this peak in the refolded protein (Figure 2) suggested that refolding had indeed taken place.

Penicillin-binding assays

The soluble, refolded [ΔM1–R314]PBP1 bound penicillin (Figure 1) with kinetic characteristics similar to those of the intact PBP1

[13]. The value of the second-order rate constant for acylation by benzylpenicillin was 5–8 $M^{-1} \cdot s^{-1}$, and the value of the first-order rate constant for deacylation was 1.8×10^{-4} s^{-1}. The truncated, refolded PBP lacking the n-PB module was thermostable (it retained its activity after a 10 min incubation at 60 °C), just like the intact PBP1 [13]. This suggests that the expressed soluble PBP1 derivative was structurally similar to wild-type PBP1.

Evaluation of the penicillin-binding characteristics of a PBP1*–PBP1 chimaera

A chimaeric protein consisting of N-terminal amino acids 1–326 of PBP1* of *M. leprae* fused at the C-terminal end to amino acids 357–821 of PBP1 of *M. leprae* was expressed in *E. coli*. This protein therefore harboured the n-PB module of PBP1* fused to the PB module of PBP1. The resulting chimaeric PBP localized to the membranes and could bind benzylpenicillin with an efficiency equal to that of PBP1 (results not shown).

DISCUSSION

The high-molecular-mass PBPs all contain near their N-terminus a single hydrophobic transmembrane anchor which also functions as a non-cleavable signal sequence that directs the rest of the protein into the periplasm [3]. In agreement with this, all *M. leprae* PBP1-Xaa–β-lactamase fusion proteins in which Xaa was beyond amino acid residue 314 of PBP1 were periplasmic, supporting a topology in which the bulk of the protein was exposed to the periplasmic side of the membrane. However, a detailed analysis of topology using fusions to the TEM β-lactamase was not possible, since several of the in-frame fusions gave rise to inactive β-lactamases that were most probably not correctly folded forms.

A strategy of generating truncations from the N-terminal end of PBP1 was chosen in order to generate a soluble form of PBP1 capable of binding penicillin. [ΔM1–L147]PBP1 associated with the plasma membrane, whereas [ΔM1–R314]PBP1 could not associate with the membrane, suggesting that a region of the putative transglycosylase domain between residues Leu-147 and Arg-314 is essential for the correct folding of PBP1 from *M. leprae*, and also includes a stretch of amino acids which facilitate association with the plasma membrane. Although not apparent from hydropathy analysis, it would appear that an additional hydrophobic patch on the protein surface results from secondary or tertiary structures formed by the three-dimensional folding of the polypeptide chain. Membrane association domains in addition to the pseudo-signal sequence have been reported for PBP1b of *E. coli* between residues 88 and 251 [15,16] and for PBP2a of *Streptococcus pneumoniae* between residues 78 and 156 [17]. In these cases also, computational analyses did not predict hydrophobic stretches or amphiphilic helices which could account for the membrane association domain.

The deletion of 164 amino acids from the C-terminus of PBP1 did not have any effect on the membrane localization or the penicillin-binding efficiency of the truncated protein, suggesting that this domain probably does not hinder the conformation of the penicillin-binding site. On the other hand, deletion downstream of Asp-762, i.e. in the putative C-terminal extension of PBP1b of *E. coli*, resulted in a protein devoid of penicillin-binding activity [24]. Through construction of a series of deletion mutants of high-molecular-mass PBPs, such as PBP1b [11] and PBP3 [10] of *E.coli*, PBP2′ of *Staphylococcus aureus* [25] and PBP5 of *Enterococcus hirae* [26], it has been postulated that acquisition of a conformation of the PB module that is competent to bind benzylpenicillin requires the presence of the n-PB module in the case of the multimodular high-molecular-mass PBPs. Our studies with PBP1 of *M. leprae* demonstrated that the PB module is capable of binding benzylpenicillin independently of the n-PB module. The expressed protein, when refolded, displayed the same penicillin-binding characteristics as intact PBP1, and was judged to be $> 90\%$ pure by SDS/PAGE. The conclusion that the n-PB module of PBP1 is dispensable for the penicillin-binding function of the PB module was also supported by the observation that replacement of the n-PB module of PBP1 with the n-PB module of PBP1* resulted in a chimaeric protein with penicillin-binding characteristics similar to those of PBP1.

The present studies mark attempts to probe the functional role of stretches of amino acids in the N-terminal n-PB module, or the C-terminal domain, with regard to the maintenance of penicillin-binding function. It may be stressed that, while these studies have evaluated the effects of deletions of stretches of amino acids on the penicillin-binding functions of PBP1, the consequences of such deletions on the peptidoglycan-synthesizing activity of this PBP *in vivo* remain to be explored. Nevertheless, the ability of the PB module to function as an independent low-affinity penicillin-binding entity opens up interesting possibilities for exploiting this truncated version of PBP1 as a soluble PBP of low affinity for future analysis of the three-dimensional structure of active-site-serine enzymes of this class. This is, to the best of our knowledge, the first report of a soluble, overexpressed derivative of a high-molecular-mass PBP of class A that is capable of binding penicillin with the same affinity as the full-length PBP.

This work was supported in part by a grant to J. B. from the Council of Scientific and Industrial Research (CSIR), Government of India. S. M. was supported by a fellowship from the CSIR.

REFERENCES

1 Waxman, D. and Strominger, J. L. (1983) Penicillin binding proteins and the mechanism of action of beta-lactam antibiotics. Annu. Rev. Biochem. **52**, 825–869
2 Ghuysen, J. M. (1994) Molecular structures of penicillin-binding proteins and beta-lactamases. Trends Microbiol. **2**, 374–380
3 Goffin, C. and Ghuysen, J. M. (1998) The multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiol. Mol. Biol. Rev. **62**, 1079–1093
4 Massova, I. and Mobashery, S. (1997) Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. Antimicrob. Agents Chemother. **42**, 1–17
5 Nakagawa, J., Tamaki, S., Tamioka, S. and Matsuhashi, M. (1984) Functional biosyntheis of cell wall peptidoglycan by polymorphic bifunctional polypeptides: penicillin-binding protein 1Bs of *Escherichia coli* with activities of transglycosylase and transpeptidase. J. Biol. Chem. **259**, 13937–13946
6 van Heijenoort, Y., Derrien, M. and van Heijenoort, J. (1978) Polymerization by transglycosylation in the biosynthesis of the peptidoglycan of *Escherichia coli* K12 and its inhibition by antibiotics. FEBS Lett. **89**, 141–144
7 Suzuki, H., van Heijenoort, Y., Tamura, T., Mizoguchi, J., Hirota, Y. and van Heijenoort, J. (1980) In vitro peptidoglycan polymerisation catalysed by penicillin-binding protein 1b of *Escherichia coli* K12. FEBS Lett. **110**, 245–249
8 Ishino, F., Mitsui, K., Tamaki, S. and Matsuhashi, M. (1980) Dual enzyme activities of cell wall peptidoglycan synthesis, peptidoglycan transglycosylase and penicillin sensitive transpeptidase, in purified preparations of *Escherichia coli* penicillin binding protein 1A. Biochem. Biophys. Res. Commun. **97**, 287–293
9 Matsuhashi, M. (1994) Utilization of lipid-linked precursors and the formation of peptidoglycan in the process of cell growth and division: membrane enzymes involved in the final steps of peptidoglycan synthesis and the mechanism of their regulation. In Bacterial Cell Wall (Ghuysen, J. M. and Hakenbeck, R., eds), pp. 55–71, Elsevier Science Publishers, Amsterdam
10 Ghuysen, J. M. and Dive, G. (1994) Biochemistry of the penicilloyl serine transferases. In Bacterial Cell Wall (Ghuysen, J. M. and Hakenbeck, R., eds), pp. 103–130, Elsevier Science Publishers, Amsterdam
11 Terrak, M, Ghosh, T. K., van Heijenoort, J., Van Beeumen, J., Lampilas, M., Aszodi, J., Ayala, J. A., Ghuysen, J. M. and Nguyen-Disteche, M. (1999) The catalytic, glycosyl transferase and acyl transferase modules of the cell wall peptidoglycan-polymerising penicillin-binding protein 1b of *Escherichia coli*. Mol. Microbiol. **34**, 350–364
12 Bowler, L. D. and Spratt, B. G. (1989) Membrane topology of penicillin-binding protein 3 of *Escherichia coli*. Mol. Microbiol. **3**, 1277–1286

13 Basu, J., Mahapatra, S., Kundu, M., Mukhopadhyay, S., Nguyen-Disteche, M., Dubois, P., Joris, B., Van Beeumen, J., Cole, S. T., Chakrabarti, P. and Ghuysen, J. M. (1996) Identification and overexpression in *Escherichia coli* of a *Mycobacterium leprae* gene pon1, encoding a high-molecular-mass class A penicillin-binding protein, PBP1. J. Bacteriol. **178**, 1707–1711
14 Lepage, S., Dubois, P., Ghosh, T. K., Joris, B., Mahapatra, S., Kundu, M., Basu, J., Chakrabarti, P., Cole, S. T., Nguyen-Disteche, M. and Ghuysen, J. M. (1997) Dual multimodular class A penicillin-binding proteins in *Mycobacterium leprae*. J. Bacteriol. **179**, 4627–4630
15 Nicholas, R. A., Lamson, D. R. and Schultz, D. E. (1993) Penicillin-binding protein 1B of *Escherichia coli* contains a membrane association site in addition to the transmembrane anchor. J. Biol. Chem. **268**, 5632–5641
16 Wang, C. C., Schultz, D. E. and Nicholas, R. A. (1996) Location of a putative second membrane association site in penicillin-binding protein 1B of *Escherichia coli*. Biochem. J. **316**, 149–156
17 Di Guilmi, A. M., Mouz, N., Martin, L., Hoskins, J., Jaskunas, S. R., Dideberg, O. and Vernet, T. (1999) Glycosyl transferase domain of penicillin-binding protein 2a from *Streptococcus pneumoniae* is membrane associated. J. Bacteriol. **181**, 2773–2781
18 Broome-Smith, J. K., Tadayyon, M. and Zhang, Y. (1990) Beta-lactamase as a probe of membrane protein assembly and protein export. Mol. Microbiol. **4**, 1637–1644
19 Broome-Smith, J. K. and Spratt, B. G. (1986) A vector for the construction of translational fusion to TEM beta-lactamase and the analysis of protein export signals and membrane protein topology. Gene **49**, 341–349
20 O'Callaghan, C. H., Morris, A., Kirby, S. M. and Shingler, A. H. (1972) Novel method for detection of beta-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1**, 283–288
21 Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
22 Granier, B., Jamin, M., Adam, M., Galleni, M., Lakaye, B., Zorzi, W., Grandchamps, J., Wilkin, J. M., Fraipont, C., Joris, B., et al. (1994) Serine type D-Ala-D-Ala peptidases and penicillin-binding proteins. Methods Enzymol. **244**, 249–266
23 Rudolph, R. and Lilie, H. (1996) In vitro folding of inclusion body proteins. FASEB J. **10**, 49–56
24 Lefevre, F., Remy, M.-H. and Masson, J. M. (1997) Topological and functional investigation of *Escherichia coli* penicillin-binding protein 1b by alanine stretch scanning mutagenesis. J. Bacteriol. **179**, 4761–4767
25 Wu, E. C. Y., Alborn, Jr, W. E., Flokowitsch, J. E., Hoskins, J., Unal, S., Blaszczak, L. C., Preston, D. A. and Skatrud, P. L. (1994) Site-directed mutagenesis of the mecA gene from methicillin-resistant strains of *Staphycoccus aureus*. J. Bacteriol. **176**, 443–449
26 Mollerach, M. E., Partoune, P., Coyette, J. and Ghuysen, J. M. (1996) Importance of the E46-D160 polypeptide segment of the non-penicillin binding module for the stability of the low-affinity, multimodular class B penicillin-binding protein 5 of *Enterococcus hirae*. J. Bacteriol. **178**, 1774–1775

Received 17 February 2000/3 May 2000; accepted 24 May 2000