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# Properties and crystallization of a genetically engineered, water-soluble derivative of penicillin-binding protein 5 of *Escherichia coli* K12

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Derivatives of the *Escherichia coli* penicillin-binding protein 5 (PBP5) with truncated carboxyl terminals were obtained by altering the carboxyl-coding end of the *dacA* gene. After cloning the modified *dacA* gene into a runaway-replication-control plasmid, one clone that overproduced and excreted the desired protein into the periplasm was used as a source for the isolation of a water-soluble PBP5 (i.e. PBP5S). In PBP5S the carboxyl-terminal 21-amino-acid region of the wild-type protein was replaced by a short 9-amino-acid segment. Milligram amounts of PBP5S were purified by penicillin affinity chromatography in the absence of detergents or of chaotropic agents. PBP5S was stable and possessed DD-carboxypeptidase activity without added Triton X-100. Upon reaction with [$^{14}$C]benzylpenicillin it was converted into a rather short-lived acyl-enzyme complex, as observed with PBP5. Both PBP5 and PBP5S were crystallized. In contrast to PBP5, PBP5S yielded enzymatically active, well-formed prismatic crystals suitable for X-ray analysis.

The penicillin-binding proteins (PBPs) [1] and the $\beta$-lactamases [2] catalyse different reactions and fulfill different biological functions. Yet they share remarkable similarities. They all accomodate $\beta$-lactam antibiotics in their active sites (for review see [3]). The penicillin-binding proteins and the majority of the $\beta$-lactamases are active-site-serine enzymes that operate by a common acyl-enzyme mechanism [4, 5]. The protein sequences around the active-site serine show high homology [6 – 9]. Finally several $\beta$-lactamases and the extracellular DD-peptidase/PBP from *Streptomyces* R61 show great similarity in their three-dimensional structure [10 – 13]. Therefore, a possible evolutionary relationship between these different proteins has been postulated [3, 12, 14]. This relationship may be general and apply to all penicilling-binding and penicillin-hydrolysing enzymes with a serine in their active site. As a prerequisite for such a generalization, however, the three-dimensional structure of membrane-bound penicillin-binding proteins has to be established.

As a model compound for the crystallization and for X-ray analysis, the penicillin-binding protein 5 from *Escherichia coli* K12 was chosen for several reasons. It is the major DD-carboxypeptidase in the *E. coli* envelope [15] where it is anchored within the cytoplasmic membrane through a short sequence at the carboxyl end [16]. A weak $\beta$-lactam-hydrolysing activity is associated with the protein [17]. With a molecular mass of only 42 kDa, X-ray studies of its crystal form should be relatively easy. The protein can be purified in an enzymatically active form which binds penicillin stoichiometrically. Finally gene cloning gives access to large quantities of the desired protein [18].

## MATERIALS AND METHODS

### *Reagents and enzymes*

The restriction enzymes, *Bal*31 and T4 ligase were from Boehringer Mannheim (Mannheim, FRG). The depsipeptide bisacetyl-L-lysyl-D-alanyl-D-lactate was from Serva (Heidelberg, FRG) and 6-aminopenicillanic acid from Sigma (Deisenhofen, FRG). CH-Sepharose 4B and heparin-Sepharose were from Pharmacia (Freiburg, FRG).

### *Bacterial strains and plasmids*

*E. coli* HB101 was used as the host strain throughout the work. *E. coli* SP430, a PBP5S overproducer, was kindly provided by Dr. B. G. Spratt. The plasmids pOU-61 [19], pKK232-8 [20], and pBS59 [18] have been described previously and were donated by the authors. pWK-61 was constructed by insertion of a *Hin*dIII-*Sal*I fragment containing the kanamycin-resistance gene of Tn5, into the partially *Bal*31-digested ampicillin-resistance gene of pOU-61.

### *Gene manipulation techniques*

Plasmids were isolated following the alkaline lysis procedure [21]. All other recombinant DNA techniques were based on Maniatis et al. [22].

### *Western blots and immunochemical tests*

Proteins were transferred from acrylamide gels to nitrocellulose sheets and PBP5-related proteins were identified with

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*Abbreviation.* PBP, penicillin-binding protein.

*Enzymes.* Lysozyme (EC 3.2.1.17); T4 DNA ligase (EC 6.5.1.1); DNA polymerase I (Klenow fragment) (EC 2.7.7.7); restriction endonucleases: *Bam*HI, *Eco*RI (EC 3.1.21.4); horseradish peroxidase (EC 1.11.1.7).

rabbit PBP5 antiserum and horseradish-peroxidase-conjugated goat anti-(rabbit antibody) as described by Towbin et al. [23]. 4-Chloro-1-naphthol at 0.5 mg/ml was used as a substrate for peroxidase.

### *[$^{14}$C]Benzylpenicillin-binding assays*

The reaction with [$^{14}$C]benzylpenicillin (58.9 Ci/mol, Amersham, UK) was carried out under saturating conditions [17]. Samples were incubated for 10 min at 30°C with 0.1 mM radioactive penicillin, followed by addition of a 100-fold excess of non-radioactive penicillin. Release of the bound [$^{14}$C]benzylpenicilloyl residue from the acyl-enzymes was determined from time-course experiments. The reactions were stopped with buffer containing sodium dodecyl sulfate (SDS) [24] and by maintaining the samples for 3 min in a boiling-water bath. The radioactivity labelled PBPs were separated by SDS/polyacrylamide gel electrophoresis and estimated by fluorography.

### *Growth conditions and preparation of cell envelopes*

Cells were grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) containing kanamycin (50 μg/ml) at 28°C, shifted to 42°C at an absorbance of 0.3 (574 nm) and incubated for an additional period of 3 h. The cells were collected by centrifugation, suspended in 50 mM phosphate buffer pH 7.0 containing 2 mM dithioerythritol and opened with a French press (18000 lb in $^{-2}$; 124 MPa) or a sonic disruptor. Membranes were isolated from cell extracts by centrifugation at 35000 × *g* for 30 min at 4°C.

### *Solubilization of the membrane-bound PBP5*

For the purification of native PBP5 the cell envelopes isolated from the PBP5-overproducing *E. coli* K12 SP430 were suspended in 300 ml 50 mM sodium phosphate buffer pH 8.0 containing 2 mM mercaptoethanol and 2% Triton X-100, and stirred for 1 h at 4°C. The insoluble material was eliminated by centrifugation at 50000 × *g* for 45 min at 4°C.

### *Purification of membrane-bound PBP5*

Activated CH-Sepharose 4B (15 g) was suspended in 100 ml 0.1 M $NaHCO_3$ pH 8.0 containing 0.5 M NaCl and 800 mg 6-aminopenicillanic acid. After coupling, the 6-aminopenicillanic-acid – CH-Sepharose 4B was washed and added to the Triton X-100 membrane extract corresponding to 300 g (wet weight) of *E. coli* K12 SP430. The suspension was kept at 4°C with gentle stirring for 1 h. The resin was collected on a sintered glass funnel, washed at 4°C with 100 ml 50 mM Tris/HCl buffer pH 8.0 containing 2 mM mercaptoethanol and 1% Triton X-100 and then with 100 ml of the same Tris/HCl buffer containing 1 M NaCl. This double washing was repeated two more times except that, for the final step, buffer without sodium chloride but with 0.2% Triton X-100 was used. The covalently bound PBP5 was eluted with freshly prepared 25 mM Tris/HCl pH 8.0 containing 1 mM dithioerythritol, 0.2% Triton X-100 and 1 M hydroxylamine. The resin was treated several times (4 – 6 cycles) with 15 ml elution buffer at room temperature for 30 min, then with another 15 ml at 30°C and finally with 40 ml at 37°C for 1 h. Each eluate was immediately dialysed against 3 × 500 ml 25 mM Tris/HCl pH 8.0 containing 1 mM dithioerythritol, 0.2% Triton X-100 and 0.5 mM NaCl. A sodium chloride concentration above 0.3 M was necessary to avoid protein precipitation. Using this procedure, PBP5 and PBP6 were recovered unseparated but almost completely free of any other proteins in the eluates. The eluates were pooled, dialysed and loaded at a flow rate of 3 ml/h on a (0.9 × 10-cm) heparin-Sepharose column previously equilibrated with 25 mM Tris/HCl buffer pH 8.0 containing 1 mM dithioerythritol and 0.2% Triton X-100. The column was washed with 50 ml of the same buffer and eluted with a linear NaCl gradient (500 mM NaCl in 25 mM Tris/HCl, pH 8.0, 1 mM dithioerythritol, 0.2% Triton X-100; 3 ml/h). PBP5 and PBP6 were eluted at 180 mM and 250 mM NaCl concentrations respectively.

### *Purification of water-soluble PBP5S*

Exponential-phase cells of *E. coli* HB 101 transformed with pLF1-5 were grown, harvested by centrifugation, suspended in phosphate buffer and disrupted as described above. Purification of PBP5S from the supernatant was achieved in a single step by covalent penicillin affinity chromatography on 6-aminopenicillanic-acid – CH-Sepharose. The procedure described above for the purification of PBP5 was used except that Triton X-100 was omitted. After a minimum of six washing cycles, PBP5S was eluted from the resin using a 25 mM Tris/HCl buffer pH 8.0 containing 1 mM dithioerythritol and 1 M hydroxylamine. The samples were immediately dialysed against 3 × 500 ml 25 mM Tris/HCl pH 8.0 containing 1 mM dithioerythritol. Chromatography on heparin-Sepharose was used as a means to concentrate the purified PBP5S (up to 13 mg/ml).

### *Assay for carboxypeptidase activity*

Carboxypeptidase activity was measured following an unpublished procedure developed by Dr J. Höltje. The purified PBP5 and PBP5S (1 μM) and $Ac_2$-Lys-D-Ala-D-lactate (140 μM) were incubated together for 30 min at 37°C in 25 mM Tris/HCl pH 8.0 containing 0.2 mM dithioerythritol. The samples were maintained in a boiling water bath for 5 min and supplemented with 10% sodium phosphate buffer pH 2.5. The reaction product was separated and quantified by high-pressure liquid chromatography (Waters Associates, USA) using an ODS-Hypersil column (125 × 4.6 mm; 5 μm particle size) (Bischoff GmbH, FRG) and a ultraviolet monitor (205 nm; model 481, Waters Associates, USA). For the elution, a gradient formed with 50 mM $NaOH/H_3PO_4$, pH 4.5, and 40% methanol in 50 mM $NaOH/H_3PO_4$ pH 4.5 was used at room temperature. The flow rate was 1 ml/min and the running time 25 min.

### *Preparation of periplasmic fractions*

Spheroplasts from exponential-phase cells harboring pLF1-5 (after a temperature shift to 42°C) were prepared with lysozyme as described by Osborn et al. [25]. To avoid cell lysis, suspensions containing a maximum of 50% spheroplasts (as monitored by phase-contrast microscopy) were centrifuged at 5000 × *g* for 10 min at 4°C. The proteins in the supernatant fractions were precipitated with trichloroacetic acid and analysed by SDS-PAGE.

### *Polyacrylamide gel electrophoresis and electrofocusing*

The techniques of Lugtenberg et al. [24] (8% acrylamide running gel) and O'Farrell [26] (5% slab gels with 2% ampholines pH 3.5 – 10.0) were used.

### *DNA sequencing*

The dideoxy-nucleotide sequencing method [27] was used (Amersham DNA sequencing kit no.4502).

### *Hydropathy plots*

The program of Pustell and Kafatos [28] was used. It gives the average hydropathy of 9-amino-acid segments as it proceeds to the carboxyl terminus of the polypeptide.

### *Other methods*

Protein contents were measured using the Lowry method [29].

### *Crystallization of PBP5 and PBP5S*

The purified membrane-bound PBP5 and the water-soluble PBP5S were crystallized using the vapour diffusion technique of McPherson [30]. A solution containing the purified membrane-bound PBP5 (4.7 mg protein $ml^{-1}$ in 25 mM Tris/HCl pH 8.0 containing 1 mM dithiothreitol, 0.2% Triton X-100 and 0.5 mM NaCl) was dialysed against 0.1 M sodium cacodylate pH 6.0 containing 0.5% octylpolyoxyethylene. After centrifugation (in order to remove any possible material in suspension), 5-µl drops of the protein solution were (a) mixed with 5 µl cacodylate/octylpolyoxyethylene buffer supplemented with 1 M NaCl and 18% 4-kDa poly(ethyleneglycol); (b) suspended on silanized microscope coverslips over wells filled up with 1 ml cacodylate/octylpolyoxyethylene buffer containing varying concentrations of NaCl (above 1 M) and 4-kDa poly(ethyleneglycol) (above 18%); and (c) allowed to equilibrate at 18°C.

For the purified, water-soluble PBP5S, more than 1000 crystallization trials were conducted in order to test the effects of the protein concentration, pH value, buffer composition, nature of the precipitant and presence of additives. The addition of 1–5% glycerol, ethyleneglycol, methyl-2,4-pentanediol and mesoerythrol had a favorable effect on the crystal habit. The following conditions were eventually adopted. Drops (10 µl) of a freshly centrifuged solution of PBP5S (10 mg protein $ml^{-1}$ in 50 mM Tris/HCl pH 7.8 containing 10 mM $NaN_3$ and 1 mM dithiothreitol) were mixed with 3 µl Tris/$NaN_3$/dithiothreitol buffer supplemented with 3% $(NH_4)_2SO_4$, 1 M NaCl, 16% 10-kDa poly(ethyleneglycol) and 3% methyl-2,4-pentanediol. A four-day equilibration at 18°C against 1 ml Tris buffer containing 10-kDa poly(ethyleneglycol) (16%) yielded well-formed crystals.

### *X-ray diffraction*

The crystallized native PBP5 was studied using a synchroton radiation source and an oscillation camera (LURE, Orsay, France, and DESY, EMBL station, Hamburg, FRG). Crystals of the PBP5S were analysed by precession photography and the unit-cell dimensions were refined on a Stoe-Siemens, four-circle X-ray diffractometer.

## RESULTS

### *Construction and characterization of recombinant plasmids coding for water-soluble PBP5S*

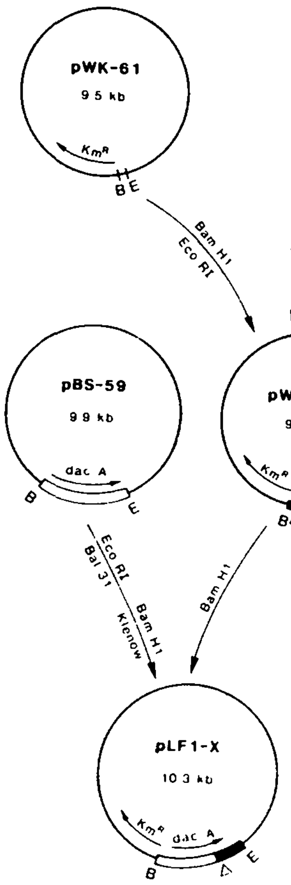

Fig. 1. *Construction of replication-control plasmids coding for the truncated, water-soluble PBP5S.* The *dacA* gene coding for PBP5 was isolated from plasmid pBS59 [18], shortened and introduced into the runaway replication control plasmid pWKL-61. For this purpose, pBS59 was cleaved with *Eco*RI, partially digested with *Bal*31, cleaved with *Bam*HI and filled-in with Klenow enzyme. Fragments ranging from 1.2 kb to 1.3 kb were isolated and finally cloned into the filled-in *Bam*HI sites of pWKL-61 yielding the collection of pLF1-X plasmids. Plasmid pKW-61 is a derivative of pOU-61 [19], a runaway replication-control plasmid carrying a kanamycin-resistant gene. To obtain pWKL-61, a 0.3-kb *Bam*HI-*Eco*RI fragment from pKK232-8 was introduced into pWK-61. This 0.3-kb fragment of known sequence [20] carries stop codons (bold type) in all three possible reading frames: GATCCGTCGACCTGCAGCCAAGCT**TGA**G**TAG**GACAAATCCCGCCGAGCTTCGACGAGATTTTCAGGAGC**TAA**. Depending on the reading frame used, peptide tails of 8 (TGA), 9 (TAG) or 23 (TAA) amino acids were fused to the truncated PBP5 derivative. Abbreviations used: $Ap^R$ = ampicillin resistance; $Km^R$ = kanamycin resistance; $Cm^R$ = chloramphenicol resistance. Restriction sites: B, *Bam*HI; E, *Eco*RI. The position of translation stop codons is indicated by an arrowhead

Attempts to crystallize the native, membrane-bound penicillin-binding protein 5 in the presence of detergent yielded crystals not suitable for X-ray analysis (see below). Therefore, it was decided to construct a water-soluble derivative of PBP5. For this purpose the plasmid pBS-59 [18] was used as a source of the PBP5-encoding gene. The excised *dacA* gene was shortened at the carboxyl-coding end and the truncated gene was cloned into the runaway replication control plasmid pWKL-61 in such a way that it was adjacent to a sequence containing stop codons in all three possible reading frames, replacing the original translation stop codon (for details see legend of Fig. 1). The plasmid pWKL-61 (a kanamycin-resistance-coding derivative of plasmid pOU-61; [19]) allows gene amplification and overproduction of the

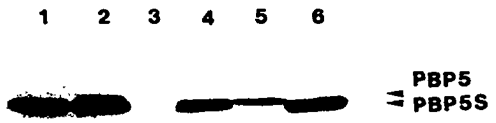

Fig. 2. *Comparison of PBP5 and PBP5S by western blots.* To ensure identical overexpression and processing of the proteins, two plasmids from the pLF1-X collection were chosen. One of them, pLF1-5, expressed PBP5S, the other carried the intact *dacA* gene including the original translation stop codon (sequencing data not shown). Both plasmids were overexpressed in *E. coli* HB101 by growth at 42°C. The membrane and soluble fractions prepared from cell extracts were analysed by western blots (Materials and Methods). Tracks 1, 2 and 3: total extract, membrane and soluble fraction of *E. coli* cells expressing the wild-type PBP5. Tracks 4, 5 and 6: total extract, membrane and soluble fraction of *E. coli* cells expressing PBP5S. The non-plasmid-coded PBP5 of the host cell does not appear because of its low amount compared with the overexpressed protein. The gels were overloaded to reveal possible degradation products; because of the overloading the small difference in molecular masses of PBP5 and PBP5S is not apparent, the bands are not doublets

coded proteins after raising the growth temperature of the host above 35°C. The introduction of the shortened *dacA* gene into pWKL-61 yielded several plasmids (pLF1-X). One of them, pLF1-5, coded for a water-soluble form of PBP5 which was highly stable, not associated with the cytoplasmic membrane (Fig. 2) and enzymatically fully active. Analysis of the supernatant fraction from lysozyme-induced spheroplasts demonstrated that the overproduced water-soluble PBP5 derivative was localized in the periplasm (data not shown). This truncated protein was called PBP5S.

### *Isolation and properties of PBP5S*

After a temperature-shift to 42°C of *E. coli* HB101, transformed with pLF1-5, PBP5S was isolated in milligram amounts from cell extracts by single-step affinity chromatography on CH-Sepharose 4B coupled with 6-aminopenicillanic acid (Fig. 3). The purified protein was concentrated up to 13 mg protein $ml^{-1}$ by an absorption/elution cycle on heparin-Sepharose (for details, see Materials and Methods). No membrane solubilization was necessary and no chaotropic agents or detergents had to be used at any step of the isolation, purification and concentration procedure. The final yield was about 0.3 mg PBP5S/g, wet mass, of cells. PBP5S represented 0.5–1% of the total water-soluble proteins present in the combined cytoplasmic and periplasmic fractions. PBP5S was stable upon storage at −20°C for at least four months, even after several freezing and thawing cycles.

PBP5S was compared to the wild-type, membrane-bound PBP5 purified to protein homogeneity (as described in Materials and Methods). Triton X-100 had to be present at each step of the purification procedure in order to avoid precipitation and to ensure enzyme activity of PBP5. The DD-carboxypeptidase activity of PBP5S (with the depsipeptide carbonyl donor $Ac_2$-L-Lys-D-Ala-D-lactate as a substrate) was Triton-independent (Fig. 4). As expected, the detergent enhanced the enzymic activity of PBP5 but, even, with detergent, PBP5 was less active than PBP5S [specific activities: 23 pmol and 50 pmol product formed $min^{-1}$ (µg PBP5 and PBP5S)$^{-1}$ respectively]. Derivatization of PBP5S and PBP5 with [$^{14}$C]-benzylpenicillin gave rise to acyl-([$^{14}$C]penicilloyl)enzymes with comparable, rather short lives (8 and 5 min respectively; data not shown).

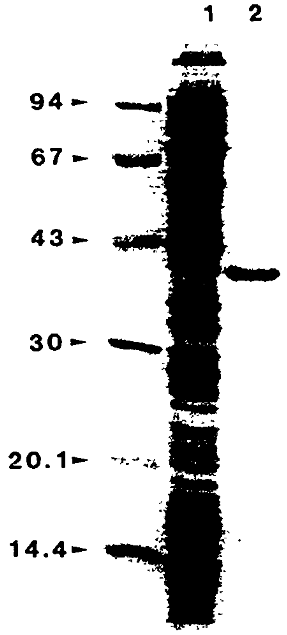

Fig. 3. *Purification of PBP5S by affinity chromatography.* Coomassie-blue-stained SDS-PAGE of the soluble fraction of cell extracts (300 µg protein; track 1) and of the first hydroxylamine eluate obtained at room temperature (4 µg protein; track 2:). For details see Materials and Methods. Molecular mass markers are indicated

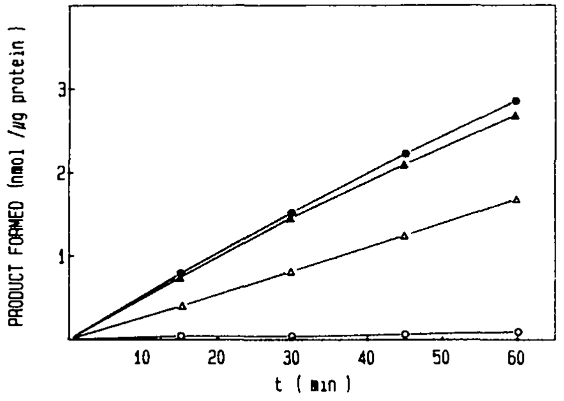

Fig. 4. *Time course of hydrolysis of the depsipeptide $Ac_2$-Lys-D-Ala-D-Lactate (140 µM) by equimolar amounts of PBP5 and PBP5S (1 µM).* For details see Materials and Methods. PBP5S (●, ▲) and PBP5 (○, △) were assayed with (▲, △) or without (●, ○) 0.2% Triton X-100

The comparison of the carboxyl-terminal portion of PBP5S with that of PBP5 (as deduced from nucleotide sequencing) (Fig. 5) showed that the last 21 amino acids of PBP5 were replaced in PBP5S by a 9-amino-acid sequence possessing 2 arginine and 1 lysine residues. This modification caused the elimination of the hydrophobic segment Phe$^{358}$-Lys$^{366}$ from PBP5, which anchors the protein in the membrane. The average isoelectric point of the amino acids in the modified sequence of PBP5S is more basic than that in native PBP5. This well explains the increased isoelectric point of PBP5S when compared to that of PBP5 (i.e. 5.9 versus 5.5; data not shown).

### *Crystallization of PBP5 and PBP5S*

PBP5 gave rise to crystals in the form of very thin plates that grew up to 0.1 mm in length and 10 µm in width. All

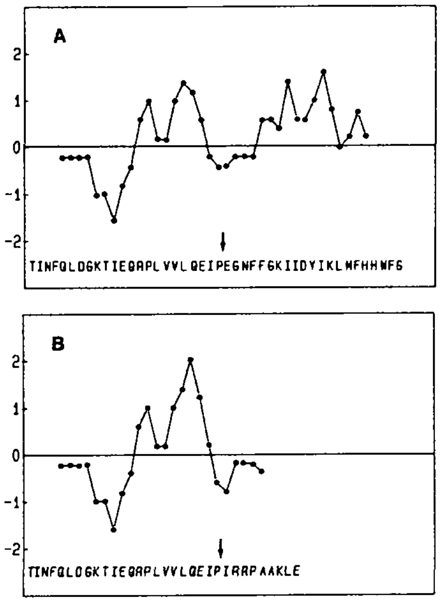

Fig. 5. *Sequence and hydropathy plot of the terminal amino acid segment of PBP5 (A) and PBP5S (B) (as deduced from nucleotide sequencing; see Materials and Methods).* The arrows indicate the points of truncation

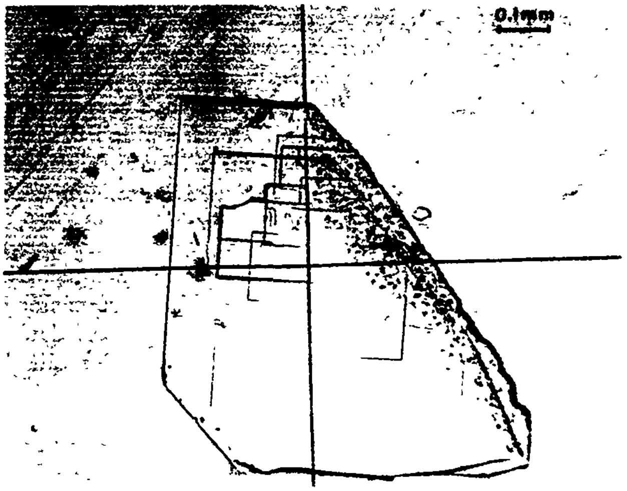

Fig. 6. *Crystallized PBP5S.* For conditions of crystallization, see Materials and Methods

attempts yielded crystals of the same type, not suitable for high-resolution X-ray analysis. Inspection of photographs of PBP5 gave the following approximative unit-cell dimensions: $a \approx 4.8$ nm; $b \approx 5.7$ nm; $c \approx 11.9$ nm. PBP5S gave rise to well-formed prismatic crystals (Fig. 6) which diffracted to at least 0.3 nm. The space group was $P2_1$ and the unit-cell dimensions (using 15 reflections) were: $a = 4.651$ nm; $b = 5.693$ nm; $c = 8.000$ nm; $\beta = 106.5$ °C. Assuming one 42-kDa protein molecule per asymmetric unit, a specific volume of 0.00254 $nm^3$/Da was calculated. This value was within the range found for protein crystals [31]. The crystals were very stable in the X-ray beam. When exposed to the $\beta$-lactam nitrocefin (0.1 mM in mother liquor), they became red after 10 min at 18 °C, as a result of the catalysed hydrolysis of the $\beta$-lactam amide bond.

## DISCUSSION

The attachment of the low-molecular-mass PBPs 5 of *Bacillus stearothermophilus, B. subtilis* and *E. coli* to the plasma membrane (or to lipid vesicles) is mediated by a C-terminal segment [16, 32–34]. After solubilization with a detergent, micelles are supposed to provide an environment that ensures the insertion of the isolated protein, correct polypeptide folding and generation of a functional enzyme's active site [32, 35].

Water-soluble DD-peptidases/PBPs have been obtained from *B. stearothermophilus* and *Streptococcus faecalis* by proteolytic treatment of the corresponding plasma membranes [32, 36]. Moreover, *Streptomyces* R61 excretes a water-soluble DD-peptidase/PBP during growth [37]. This PBP is synthesized as a precursor that possesses both a cleavable amino-terminal extension, which has the characteristics of a signal peptide, and a cleavable C-terminal extension. Should it not be removed during protein maturation, this carboxyl-terminal extension might function as a halting signal through which the enzyme would become membrane-bound [38]. All these experimental data lead to the conclusion that DD-peptidase and penicillin-binding activities do not require membrane insertion and, as a corollary, that the active sites of the membrane-bound enzymes are, in all likelihood, exposed to the periplasm of the cell close to their substrate, i.e. the murein. Membrane insertion, however, might be important for the *in vivo* regulation of the enzyme activity of the PBPs and/or for association with other cell-envelope components.

Recently the PBP5 of *E. coli* has been converted into water-soluble derivatives by removing amino acids at the carboxyl terminus [16] using a cloning strategy similar to that described here. However, some of the resulting truncated proteins were unstable, none of them could be overproduced after cloning into high-copy-number plasmids (J. Pratt, personal communication) and their enzymatic activity and interaction with [$^{14}$C]benzylpenicillin were not analyzed. The observed reduced stability may result from the introduction of hydrophobic amino acids at the truncated ends coded by the inserted oligonucleotide stop fragments. In contrast, PBP5S can be overproduced and is both stable and enzymatically active in the absence of detergent. Its stability may result from the fact that the truncated carboxyl terminus carries several highly hydrophilic amino acids (Fig. 5).

The specific activity of PBP5S in the absence of detergent is twice that of the wild-type protein as measured in the presence of Triton X-100, a property reminiscent of that of the water-soluble proteolytic fragments obtained from the DD-peptidases/PBPs of *B. stearothermophilus* and *S. faecalis* [32, 36]. These fragments also have a higher specific activity than the wild-type parents. Removal of the detergent leaves PBP5S fully active whereas the activity of the wild-type protein is completely abolished. The higher specific activity of the water-soluble PBP5, as compared to the intact PBP5 when measured in the presence of Triton X-100, may be due to the distribution of PBP5 both on the inner and outer sides of the micelles, whereas the substrate has access to the outside only.

A possible mechanism for the emergence of active-site serine $\beta$-lactamases in the bacterial world is that bacteria exposed to $\beta$-lactam antibiotics once excreted one or several (non-essential) PBPs in their environment or the periplasmic compartment [12]. Further improvement of this detoxification mechanism resulted in the conversion of these water-soluble PBPs into $\beta$-lactam-hydrolysing enzymes. The likelihood of this hypothesis rests upon the similarity of the spatial arrangement of secondary structures, shown by several $\beta$-lactamases and the extracellular, water-soluble DD-peptidase/PBP of *Streptomyces* R61 [10–13]. In addition to this naturally occurring water-soluble PBP, the work described here has given access to a genetically engineered, water-soluble PBP5 from *E. coli*. The establishment of the three-dimensional structure of these two PBPs is in progress. They may become models of choice for experiments aimed to reproduce *in vitro* the proposed evolutionary transition from PBPs to $\beta$-lactamases. Since membrane-bound penicillin-binding proteins play a key role in the metabolism of murein [1], which is responsible for the maintenance of bacterial shape [39], structural data on PBPs will also help in understanding problems related to bacterial morphogenesis and cell division.

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## REFERENCES

1. Spratt, B. G. (1975) *Proc. Natl Acad. Sci. USA 72*, 2999–3003.
2. Ambler, R. P. (1980) *Philos. Trans. Soc. Lond. B Biol. Sci. 289*, 321–331.
3. Spratt, B. G. (1983) *J. Gen. Microbiol. 129*, 1247–1260.
4. Frère, J.-M., Duez, C., Ghuysen, J.-M. & Vandekerkhove, J. (1976) *FEBS Lett. 70*, 257–260.
5. Knott-Hunziker, V., Waley, S. G., Orlek, B. S. & Sammes, P. G. (1979) *FEBS Lett. 99*, 59–61.
6. Waxman, D. J. & Strominger, J. L. (1980) *J. Biol. Chem. 255*, 3964–3976.
7. Waxman, D. J., Amanuma, H. & Strominger, J. L. (1982) *FEBS Lett. 139*, 159–163.
8. Glauner, B., Keck, W. & Schwarz, U. (1984) *FEMS Microbiol. Lett. 23*, 151–155.
9. Keck, W., Glauner, B., Schwarz, U., Broome-Smith, J. K. & Spratt, B. G. (1985) *Proc. Natl Acad. Sci. USA 82*, 1999–2003.
10. Dideberg, O., Charlier, P., Wéry, J. P., Dehottay, P., Dusart, J., Erpicum, T., Frère, J.-M. & Ghuysen, J.-M. (1987) *Biochem. J. 245*, 911–913.
11. Herzberg, O. & Moult, J. (1987) *Science (Wash. DC) 236*, 694–701.
12. Kelly, J. A., Dideberg, O., Charlier, P. C., Wery, J. P., Libert, M., Moews P. C., Knox, J. R., Duez, C., Fraipont, Cl., Joris, B., Dusart, J., Frère, J.-M. & Ghuysen, J.-M. (1986) *Science (Wash. DC) 231*, 1429–1431.
13. Samraoui, B., Sutton, B. J., Todd, R. J., Artymiuk, P. J., Waley, S. G. & Phillips, D. C. (1986) *Nature (Lond.) 320*, 378–380.
14. Tipper, D. J. & Strominger, J. L. (1965) Proc. Natl Acad. Sci. USA 54, 1133–1141.
15. Amanuma, H. & Strominger, J. L. (1980) *J. Biol. Chem. 255*, 11173–11180.
16. Pratt, J. M., Jackson, M. E. & Holland, I. B. (1986) *EMBO J. 5*, 2399–2405.
17. Spratt, B. G. (1977) *Eur. J. Biochem. 72*, 341–352.
18. Stoker, N. G., Broome-Smith, J. K., Edelman, A. & Spratt, B. G. (1983) *J. Bacteriol. 155*, 847–853.
19. Larsen, J. E. L., Gerdes, K., Light, J. & Molin, S. (1984) *Gene 28*, 45–54.
20. Brosius, J. (1984) *Gene 27*, 151–160.
21. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acid. Res. 7*, 1513–1523.
22. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular cloning. A laboratory manual*, Cold Spring Harbor Laboratory, New York.
23. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl Acad. Sci. USA 76*, 4350–4354.
24. Lugtenberg, B., Meijers, J., Peters, R., van der Hoek, P. & van Alphen, L. (1975) *FEBS Lett. 58*, 254–258.
25. Osborn, M. J., Gander, J. E. & Parisi, E. (1972) *J. Biol. Chem. 247*, 3973–3986.
26. O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
27. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl Acad. Sci. USA 74*, 5463–5467.
28. Pustell, J. & Kafatos, F. C. (1982) *Nucleic Acid Res. 10*, 51–59.
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
30. McPherson, A. (1976) *Methods Biochem. Anal. 23*, 249–345.
31. Matthews, B. W. (1968) *J. Mol. Biol. 33*, 491–497.
32. Waxman, D. J. & Strominger, J. L. (1979) *J. Biol. Chem. 254*, 4863–4875.
33. Waxman, D. J. & Strominger, J. L. (1981) *J. Biol. Chem. 256*, 2059–2066.
34. Waxman, D. J. & Strominger, J. L. (1981) *J. Biol. Chem. 256*, 2067–2077.
35. Umbreit, J. N. & Strominger, J. L. (1973) *Proc. Natl Acad. Sci. USA 70*, 2997–3001.
36. Coyette, J., Ghuysen, J.-M. & Fontana, R. (1980) *Eur. J. Biochem. 110*, 445–456.
37. Ghuysen, J.-M., Frère, J.-M., Leyh-Bouille, M., Perkins, H. R. & Nieto, N. (1980) *Philos. Trans. Soc. Lond. B Biol. Sci. 289*, 285–301.
38. Duez, C., Piron-Fraipont, Cl., Joris, B., Dusart, J., Urdea, M. S., Martial, J. A., Frère, J.-M. & Ghuysen, J.-M. (1987) *Eur. J. Biochem. 162*, 509–518.
39. Weidel, W. & Pelzer, H. (1964) *Adv. Enzymol. 26*, 193–232.

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