# CHAPTER 3

## Purification of Penicillin-Binding Protein 4 of Escherichia coli as a Soluble Protein by Dye-Affinity Chromatography

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## SUMMARY

The *dac*B gene of *E. coli*, coding for penicillin-binding protein 4 (PBP4) was cloned under the control of the phage lambda $p_R$ promoter and *cro* gene translation signals. Derepression of the phage lambda promoter for two hours at 42°C in *E. coli* led to the maximum overproduction of PBP4 to 3.8% of total soluble protein. Expression at 42°C but not at 40°C or 37°C led to incomplete processing and aggregation of the preform of PBP4. From a collection of triazine dyes Cibacron Navyblue 2G-E was selected as having high affinity for PBP4. The immobilised dye was used in a two step procedure to isolate 374 mg of PBP4 from the soluble fraction of 125 g (wet mass) cells of the overproducing strain with a recovery of 63.2% and a final purity of 99 % as determined by active-site titration with radiolabelled penicillin. Saturation of PBP4 with various ß-lactam derivatives did not abolish binding to the dye material, nor was PBP4 eluted by addition of ß-lactams from the dye-matrix.

PBP4 behaved as a soluble protein throughout the purification, that was performed in the complete absence of detergents. Furthermore, in flotation experiments on sucrose density gradients and in Triton X-114 fractionation experiments, it showed the characteristics of a soluble protein. Cibacron Navyblue 2G-E showed class specificity for all *E. coli* PBPs except PBP3 and could be used for the isolation of these PBPs from membrane extracts.

## INTRODUCTION

The structural gene *dac*B of the *Escherichia coli* DD-carboxy-peptidase-endopeptidase referred to as PBP4 was recently cloned and sequenced [1, 2]. Analysis of the deduced amino acid sequence of the protein revealed an unusual primary structure of this enzyme when compared to other members of the family of penicillin-interacting enzymes. We have proposed that PBP4 and ß-lactamases of class A share a common evolutionary origin and that PBP4 has acquired an additional domain of 188 amino acids being located between the conserved active-site fingerprints SXXK and SDN [2]. When overexpressed from a runaway replication plasmid, using its own promoter, 80% of PBP4 was detected in the soluble fraction after disrupting the cells [1]. Analysis of the primary structure of PBP4 did not, in contrast to the other *Escherichia coli* PBPs, reveal any evidence for the presence of a transmembrane helix, an amphiphilic membrane anchor or an attachment site for a lipid anchor [2]. Nevertheless, PBP4 is associated with inner membrane vesicles after cell fractionation when expressed at wild-type level [3, 4].

The difficulties to obtain X-ray quality crystals from membrane-derived PBPs and the availability of only one structure for a water-soluble PBP, the *Streptomyces* R61 DD-carboxypeptidase [5], stimulated us to exploit the unique features of PBP4 for protein crystallization studies by developing a purification procedure for PBP4 in the absence of any detergent. We have

previously described the purification of PBP4 using covalent affinity chromatography [1]. In order to avoid the harsh elution conditions necessary to break the covalent ß-lactam-PBP4 bond we decided to develop a purification protocol based on a general affinity principle. A prerequisite was that the method to be developed should also be applicable to mutated forms of PBP4 and therefore does not rely on a functional active-site. Dye chromatography was the method of our choice, since it allows the preparation and screening of a large variety of affinity matrices of different specificities with comparatively little cost and effort. PBP4 is one of the minor PBPs and has therefore not yet been isolated in quantities sufficient for protein crystallization studies. To obtain large scale preparations we optimized the overproduction of PBP4 by making use of the temperature regulated phage lambda $p_R$ promoter and the lambda *cro* gene translation signals [6].

## MATERIALS AND METHODS

**DNA manipulation**
The construction of plasmids is described in the legend to Fig.1. Standard recombinant DNA-methods were used [7]. *E. coli* strain MC1061 was used as a host for subcloning and expression of protein [8]. JM101 was used as a host for M13 phages [9]. Strain BW313 was used in the site-directed mutagenesis protocol [10]. The mutagenesis and sequencing primers were synthesized by Eurosequence, Groningen. The sequence of the mutagenesis primer is given in the legend to Fig.1. Enzymes were obtained from Boehringer Mannheim, except for Mung Bean Nuclease, which was obtained from Stratagene. The enzymes were used according to the manufacturer's instructions. Site-directed mutagenesis was performed exactly as described by Kunkel et al. [10]. Mutated M13 clones were selected by restriction endonuclease digests of phage DNA. They were sequenced completely using T7 polymerase (Sequenase from United States Biochemicals Ohio) and a set of sequencing primers which covered the whole gene. PBP4 overexpressing pROFI2AB clones were identified by colony blotting with a PBP4 specific antiserum [1, 11].

**Materials**
Reactive triazine dyes were obtained from Ciba Geigy, Basel and used without further purification. The following 9 dyes were used in this study: Cibacron blue 3G-A, Cibacron blue 3R, Cibacron blue 2R, Cibacron Navyblue 2G-E, Cibacron brown 4GR, Cibacron red 3BA, Cibacron orange 2R, Cibacron yellow R, Cibacron yellow 6G.

**Coupling of dyes to Fractogel TSK HW-65F** After equilibration of 2 ml (settled volume) of TSK HW65-F Fractogel (Merck) with coupling buffer (2% $Na_2CO_3$, 5% NaCl), 2ml of a 0,4 % solution of the dye was added and the slurry was agitated for 2 h in a shaking waterbath at 37°C. The supernatant was removed and 2 ml fresh coupling buffer containing 0,4% dye were added. The coupling reaction was continued for another two hours at 80°C. Uncoupled excess dye was removed by sequentially washing the Fractogel with 0.1 M NaOH, 0.1 M HCl and methanol (technical grade). The coupled resin was stored in 0.02% $NaN_3$. Larger amounts of dye matrix were prepared by scaling up this protocol.

**Screening of dyes for affinity to PBP4**
Dye substituted Fractogel (25 µl settled volume) was equilibrated with 50 mM Glycine-NaOH, pH 10.5 and incubated with 100 µl soluble extract from the PBP4 overproducer (10 min, 4°C). The supernatant was removed and the resin washed two times with 1 ml of equilibration buffer. The bound protein was eluted with 50 µl of equilibration buffer containing 1 M NaCL. 7 µl of the eluate was analysed by SDS/PAGE.

**Optimization of PBP4 expression**
Luria broth (4 x 500 ml) supplemented with 0.5% glucose and kanamycin (50 µg/ml) was inoculated with 1/50 volume of an overnight culture of MC1061/pROFI2AB and grown with aeration at 30°C. Growth was monitored by measuring the absorbance at

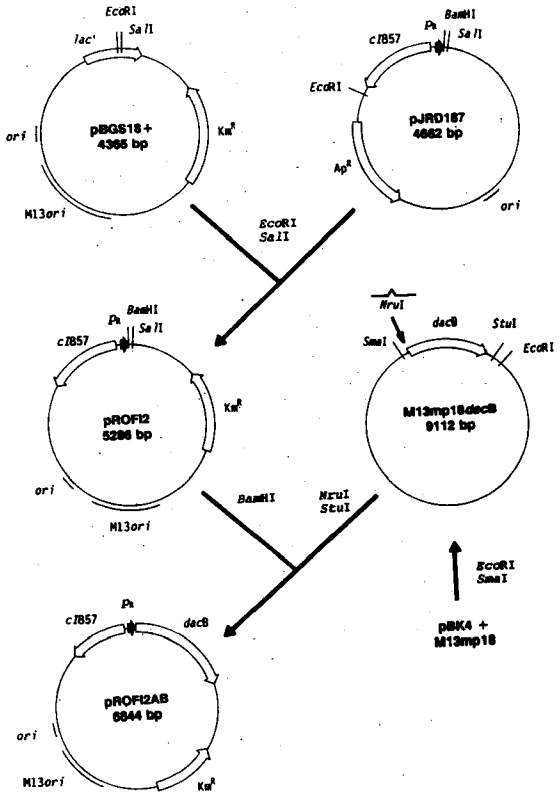


FIG. 1. Construction of plasmids.
Expression vector pROFI2 was constructed by replacing the small *Eco*RI-*Sal*I fragment of pBGS18+ [24] with the 950 bp *Eco*RI-*Sal*I fragment of pJRD187 [6]. The *Eco*RI site was destroyed by the combined action of *Eco*RI and Klenow polymerase. The *dac*B carrying M13 vector M13mp18dacB was constructed by replacing the *Eco*RI-*Sma*I fragment of M13mp18 [25] with the 1884 bp *Eco*RI-*Sma*I fragment of pBK4 [1]. A *Nru*I site was introduced downstream of the ATG initiation codon of the *dac*B gene by site directed mutagenesis [10]. The sequence of the mutagenic primer was CGCGAGATTTCG/CGATTTTCC. The first codons ATG CGA of the *dac*B gene were mutated to TCG/CGA (mutated bases are underlined, the *Nru*I cleavage site is indicated by a slash). The PBP4 expression vector pROFI2AB was constructed by linearizing the plasmid pROFI2 with *Bam*HI removing the protruding 5'-ends with Mung-bean nuclease and ligating with the *Nru*I-*Stu*I fragment of the mutagenized M13mp18dacB. This operation led to a translational fusion in which the original sequence ATG CGA of *dac*B remained unchanged while it is under control of the phage lambda $p_R$ promotor and the translation signals of the phage lambda *cro* gene [6].

600 nm in 15 min intervals. When the cultures had reached the mid-exponential growth phase ($A_{600}$ = 0.4), growth was continued either at 37°C, 40°C, or 42°C. The control culture was left at 30°C. Samples of 50 ml were withdrawn at O, 30, 60, 90 and 120 min after temperature shift, cooled on ice and cells were collected by centrifugation (20 min, 3000 x g, 4°C). Cells were treated with glucose and lysozyme exactly as described below in a final volume of 4 ml and disrupted by ultrasonifying two times for 20 seconds with a microtip at 100 W output. The soluble and membrane fractions were separated by centrifugation (30 min, 150 000 x g, 4°C). The PBP4 concentration was determined as described below.

**Purification of PBP4**

**i) Fermentation and preparation of crude extract**

E. coli strain MC1061 transformed with plasmid pROFI2AB was grown in a 65 l mobile pilot plant fermentor (New Brunswick) equipped with pH- and oxygen-electrodes. All media were supplemented with 0.5 % glucose, plasmid pROFI2AB was selected for with 50 $\mu$g kanamycin sulphate/ml in the precultures. Kanamycin was omitted during large scale fermentations. The bacteria were agitated at 55 rpm and aerated (30 l/min). Non induced bacteria were grown at 28° C and at an $A_{600}$ of 0.5 the temperature was shifted to 42°C and growth was continued for 90 min to the final $A_{600}$ of 0.8. Cells were harvested by centrifugation in a cooled continuous centrifuge. The yield from two fermentations was 125 g of wet mass cells. The cells were stored at -20°C. Frozen cell paste (125 g) was thawed and suspended homogeneously in 500 ml 0.2 M Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 1 $\mu$g/ml DNAse. After addition of 500 ml 0.2 M Tris-Cl, pH 8.0, 12 mM EDTA, 1 M sucrose, 120 $\mu$g/ml lysozyme and stirring for 10 min another 1000 ml of water, 20 ml 1 M $MgCl_2$ and 2 mg DNase were added and stirring was continued for 15 min [12]. Finally phenylmethane-sulphonyl-fluoride (PMSF) in ethanol was added to a final concentration of 40 $\mu$g/ml. Lysis was completed by passing the cell suspension at a velocity of 25 ml/min through a continuous flow cell connected to an ultrasonifier at 400 W (Sonics & Materials). The homogenate was cleared by centrifugation (60 min 20,000 x g, 4°C) and the supernatant was used for purification of PBP4. All manipulations were carried out on ice.

**ii) Dye affinity chromatography**

The supernatant (1900 ml) was applied onto a column (195 x 110 mm) of Cibacron Navyblue 2G-E substituted TSK HW65F Fractogel at a flow rate of 25 ml/min. The column had previously been equilibrated with 50 mM Tris-Cl pH 8.9. Unbound material was washed off at a flow rate of 10 ml/min, with a linear gradient of 1300 ml equilibration buffer and 1300 ml 50 mM Glycine-NaOH, pH 10.5, followed by 5000 ml 50 mM Glycine-NaOH, pH 10.5. Elution of PBP4 was achieved with a linear gradient of 2000 ml 50 mM Glycine-NaOH, pH 10.5 and 2000 ml of the same buffer containing 2 M NaCl. PBP4 containing fractions as detected by immunoblotting were pooled (710 ml) and dialysed overnight against 3 x 2 l of 50 mM Glycine-NaOH, pH 10.5.

**iii) Ion exchange chromatography**

The dialysate after dye affinity chromatography column was further purified and concentrated by anion exchange chromatography on a Fractogel TSK DEAE 650-S column (100 x 10 mm) connected to a Pharmacia FPLC system. The PBP4-pool was applied on the column in 100 ml portions with a flow rate of 2 ml/min, washed with 50 mM Glycine-NaOH until the $A_{280}$ was constant and eluted with a linear gradient from 140 mM NaCl to 190 mM NaCl at a flow rate of 4ml/min. PBP4 containing fractions were pooled, dialysed as above and rechromatographed to concentrate the enzyme. The pooled fractions were finally dialysed against 50 mM Tris-Cl, pH 8.0, 500 mM NaCl, 5 mM $MgSO_4$, 0.02 % $NaN_3$ and stored at 4°C.

**Active-site-labelling with [$^{14}$C]-benzylpenicillin**

To determine PBP4 concentrations in the purification and expression experiments 20 µl of PBP4 containing solution was incubated with 1,85 kBq of [$^{14}$C]benzylpenicillin (15 min at 37°C). To stop the reaction 1 ml of bovine serum albumin (1 mg/ml) and 0.2 ml 3 M trichloroacetic acid were added. After 5 min at room temperature the precipitate was collected by centrifugation (5 min, 16 000 x g, 4°C). The supernatant was carefully removed and the precipitate was resuspended in 1 ml of acetone containing 20 mM HCl and centrifuged again as above. The supernatant was removed and residual solvent was evaporated by 2 min incubation at 80°C. The protein pellet was dissolved in 100 µl 50 mM Tris-Cl, pH 8.0, 5 mM MgSO4, 0.5 M NaCl and radioactivity was counted after addition of 1 ml of scintillation cocktail (Packard emulsifying scintillator 299).

**SDS/PAGE and autoradiography**

Discontinuous SDS/PAGE was performed according to Laemmli [13]. The composition of the separating gel was 10%T, 2.7%C and the composition of the stacking gel was 4%T, 2.7%C. The size of the gels was 100 x 100 x 0.5 mm. Labelling of PBPs was performed in 20 µl fractions with varying amounts of [$^{35}$S]-benzylpenicillin as indicated in the figure legends. After incubation for 10 min at 37°C the reaction was stopped by boiling 3 min in SDS sample buffer. SDS/PAGE was performed and the labelled PBP-ß-lactam complexes were detected by autoradiography with Kodak X-OMAT AR film at room temperature.

**Affinity of E. coli PBPs to immobilized Cibacron Navyblue 2G-E**

A 2000 ml culture of E. coli strain HB101 in Luria broth supplemented with 0.1 % glucose was grown at 37°C with aeration to late log phase ($A_{600}$ = 0.9). Cells were harvested (15 min, 10 000 x g, 4°C), the cell pellets were resuspended in 35 ml 100 mM Tris-Cl, pH 8.0, 10 mM $MgSO_4$, 100 µg/ml lysozyme, 40 µg/ml PMSF, 1 µg/ml DNAse and 2 mM ß-mercaptoethanol and passed two times at 10,000 psi through a French pressure cell (Aminco). After removal of the cell debris (5 min, 3000 x g, 4°C) the membranes were pelleted (220 000 x g, 1 h, 4°C). The membrane pellet was resuspended in 8 ml 50 mM Tris-Cl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 2 % Triton X-100 and membrane proteins were solubilized by agitation on a rotating wheel overnight. Insoluble material was spun down (10 min, 16 000 x g, 4°C) and the supernatant was loaded on a 2 ml column (disposable syringe) of Cibacron Navyblue TSK Fractogel at a flow rate of 1 ml/min. The column was washed with 40 ml of 50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.1 % Triton X-100 and eluted with 4 ml of wash buffer containing 1 M NaCl.

**Other Techniques**

Protein concentrations were determined by the Biuret method [14]. The amino-terminal sequence of isolated PBP4 was determined exactly as described before [2]. Triton X-114 fractionations were done as described by Bordier with slight modifications [15]. Sucrose density gradient flotation experiments with purified PBP4 were performed as described by Hirst *et al.* [16].

**Radiolabelled compounds**

[$^{35}$S]benzylpenicillin (0.41 TBq/mmol) was obtained from New England Nuclear. The amounts necessary for labelling the PBPs were dried in a speed vac and the protein solution was added to the dried sample. [$^{14}$C]benzylpenicillin was obtained from Amersham at a specific activity of 2.16 GBq/mmol.

## RESULTS

**Overexpression of PBP4**

The expression vector pROFI2AB was constructed which allows temperature regulated high-level expression of PBP4 (Fig.1), using the phage lambda $p_R$ promoter, which is controlled by the

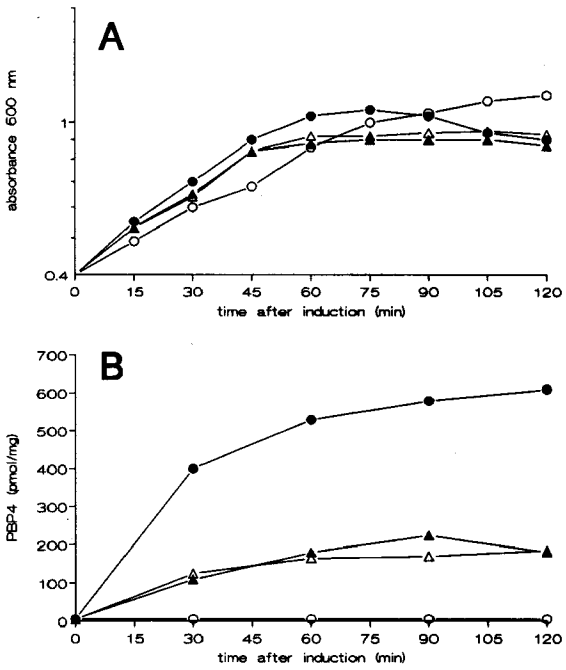


**FIG. 2A,B.** Growth of *E. coli* and overexpression of PBP4 at various induction temperatures.
**(A)** *E. coli* strain MC1061, harbouring PBP4 expression plasmid pROFI2AB was grown at 30°C to mid-exponential phase ($A_{600}$ = 0.4) and growth was continued either at 30°C (-○-) as control or at the induction temperatures of 37°C (-▵-), 40°C (-▲-), 42°C (-●-). Absorbance was measured at 15 min intervals at 600 nm.
**(B)** The amount of active PBP4 in the soluble fraction of the cells was measured by affinity labelling with [$^{14}$C]-benzyl-penicillin and related to the overall protein content of the soluble fraction. Samples were taken at the time of induction and at 30 min intervals.

temperature sensitive *c*I857 repressor [6]. The *dac*B gene was cloned downstream and in frame with the ATG codon of the *cro* gene, without changing its coding sequence. The *cro* gene fragment of phage lambda provides the translation initiation signals for optimal expression [6]. The expression of PBP4 with this system was found to be stable and reproducible. In the repressed state we could not detect any expression as determined by active-site labelling using [$^{14}$C]-benzylpenicillin (Fig.2A,B). To determine the optimal conditions for expression we analysed the amount of PBP4 by active-site titration with radiolabelled benzylpenicillin as a function of induction temperature, period of induction and growth of the strain MC1061, harbouring the plasmid pROFI2AB. The results are summarized in Fig.2A,B. The expression of PBP4 was not linearly correlated with the induction temperature. Expression at 37°C or 40°C resulted in the same relative amount of PBP4, whereas at 42°C the relative amount of PBP4 was threefold the amount produced at 37°C or 40°C. The maximum overproduction in these analytical experiments was 3 % of PBP4 relative to total soluble protein. The data derived from active-site titration were in accordance with the amount of PBP4 being detected on poly-acrylamide gels. Furthermore we observed at 42°C, but not at 37°C or 40°C on

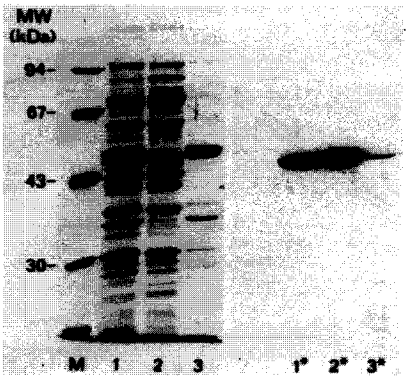


FIG. 3. Cell fractionation and detection of PBP4 expressed at 42°C. *E. coli* cells from strain MC1061 carrying pROFI2AB were separated after sonication into soluble fraction and particulate fraction by centrifugation (150 000 x g, 30 min). Samples (40 $\mu$g each) were labelled with 8 kBq [$^{35}$S]-benzylpenicillin, submitted to SDS/PAGE, stained with Coomassie R250 (lane 1-3) and autoradiographed for 5 days (lane 1* to 3*). Lane 1 and 1*: total cell protein; lane 2 and 2*: 100 000 x g supernatant; lane 3 and 3*: particulate fraction. M denotes the molecular mass marker.

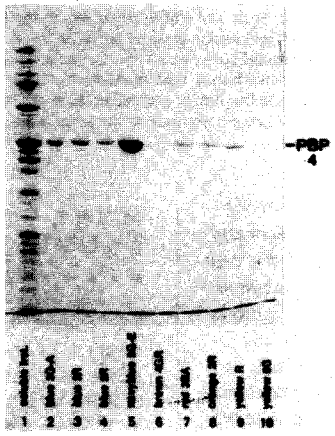


FIG. 4. Screening of Cibacron dyes for affinity to PBP4. SDS/PAGE of eluates from different dye matrices which have been incubated with the soluble protein fraction from PBP4 overproducing strain MC1061/pROFI2AB. Lane 1: 10 $\mu$g soluble protein fraction; lane 2 - 10: eluates from dyes after treatment with 1 M NaCl.

Coomassie stained polyacrylamide gels a strong additional band about 2 kD larger than PBP4 (Fig.3). This band crossreacted with anti-PBP4 antiserum on Western blots. However, this band did not bind penicillin and after cell fractionation with Triton X-114 was detected in the insoluble debris of the membrane fraction. These findings indicate that a significant portion of the overexpressed PBP4 accumulated in the unprocessed preform and formed insoluble aggregates. High-level expression of PBP4 in the periplasmic space is expected, due to its DD-endopeptidase activity, to destabilize the murein-polymer layer by reducing the degree of D-Ala-γ-$A_2$pm cross-bridges. We observed that as a consequence of increasing amounts of PBP4 the cells became very sensitive to shear forces, and changes in the osmotic pressure of the medium. However, microscopically we could not detect a specific phenotype resulting from PBP4 overproduction.

**Screening of Cibacron dyes for affinity to PBP4**

A set of nine Cibacron dyes was coupled to a chromatographic support (Fractogel HW65-F) and screened in batch for enrichment of PBP4 from the soluble fraction of the overproducer. The results are summarized in Fig.4. All dye-matrices except Cibacron brown 4GR and Cibacron yellow 6G showed some affinity to PBP4. Cibacron Navyblue 2G-E with its outstanding high binding-capacity was selected for further use in preparative isolation of PBP4 (Fig.4).

**Purification of PBP4**

The purification of PBP4 is summarized in Table 1 and Fig.5. Only two steps were necessary to purify the enzyme from the overproducing strain. The application of the dye chromatography step is very effective since it allows enrichment of PBP4 to 93 % purity with a recovery of 71 %, directly from the soluble fraction after cell disintegration, in one step. The anion-exchange chromatography step adds little to the purification as far as protein is concerned, but it removes material which absorbs at 260 nm. The $A_{280/260}$ ratio of the pooled PBP4 containing fractions after dye chromatography was 1.02 and increased to 2.07 after anion exchange chromatography, indicating the removal of nucleotide containing material. With the final preparation of the enzyme a 1:1 stoichiometry for the penicillin-protein complex was found after active-site titration using [$^{14}$C]-benzylpenicillin, which indicates that no denaturation of PBP4 occurred during this purification protocol.

**Interaction of *Escherichia coli* PBPs with Cibacron Navyblue 2G-E**

In order to analyse whether the affinity of Cibacron Navyblue 2G-E to PBP4 reflects a general behaviour of this dye towards PBPs, membranes were isolated, solubilized with Triton X-100 and dye chromatography was performed as described in the material and methods section. We found, that not only PBP4, but also the other PBPs with the exception of PBP3 can be enriched by this procedure (Fig.6). This result was confirmed with purified PBPs 1A, 1B, 3, 5 and 6. The question whether the interaction of the dye with the PBPs proceeds via the active-site cavity of the proteins was addressed. We found, that PBP4 was neither eluted from the dye-matrix by incubation with various ß-lactams, nor was binding of the protein to the dye-matrix influenced by preincubation with various ß-lactams.

**Solubility of PBP4**

In the wild-type cell PBP4 is found to be associated with the membrane [3,4]. No information whatsoever is available on the kind of membrane-protein interaction. We showed earlier, that most of the overproduced PBP4 is detected in the soluble fraction and that the primary structure of PBP4 does not show any characteristics of a membrane bound protein [1,2]. In this report we demonstrate, that enzymatically active PBP4 can indeed be isolated in the absence of detergents and behaves as a soluble protein. We performed a Triton X-114 fractionation and a sucrose density gradient floatation experiment with the

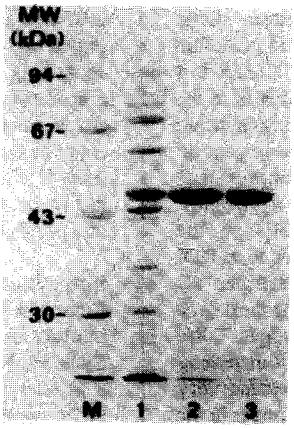


FIG. 5. Purification of PBP4. SDS/PAGE of the different steps of the purification procedure. Lane 1: molecular mass marker; lane 2: 30 $\mu$g of soluble protein fraction; lane 3: 3 $\mu$g of pooled fractions after chromatography on Cibacron Navyblue 2G-E; lane 4: 3 $\mu$g of pooled fractions after chromatography on DEAE-Fractogel.

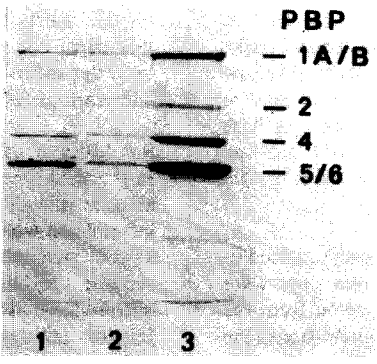


FIG. 6. Affinity of *E. coli* PBPs to Cibacron Navyblue 2G-E Autoradiography of SDS/PAGE exposed for 24 h after labelling with 48 kBq of [$^{35}$S]-benzylpenicillin per sample. Lane 1: membranes solubilized in 2 % Triton X-100; lane 2: non absorbed proteins after dye chromatography; lane 3: proteins eluted from dye-column with 1 M NaCl. 40 $\mu$g protein were applied per lane.

**Table 1.** Purification of PBP4

| Purification step | Total protein | | | Protein-bound [$^{14}$C]Pen.G | PBP4 enzyme | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | volume | concentration | amount | | concentration | amount | purity | purification | recovery |
| | ml | mg/ml | mg | pmol/mg | mg/ml | mg | % | -fold | % |
| Soluble extract | 1900 | 7.46 | 14170 | 771 | 0.285 | 542 | 3.8 | 0 | 100 |
| Cibacron navyblue | 710 | 0.58 | 412 | 18690 | 0.538 | 382 | 93 | 24 | 71 |
| DEAE Fractogel | 24 | 15.6 | 374.4 | 19949 | 15.44 | 370.5 | 99.0 | 1.1 | 68 |

Protein concentration was determined by the Biuret method. The purity of the enzyme was calculated by relating the molar amount of PBP4 in the fraction as determined by active-site titration with [$^{14}$C]-benzylpenicillin ([$^{14}$C]-Pen.G) to the total protein in the fraction.

isolated PBP4 and observed that the enzyme behaved with both methods as an ordinary soluble protein (data not shown).
Amino-terminal sequencing confirmed, that the signal peptide was cleaved off at the predicted position as described earlier [2].

## DISCUSSION

High-level overexpression of PBP4 has been achieved by cloning the structural gene *dac*B downstream of the tightly regulated phage lambda $p_R$ promoter. Although overproduction of PBP4 is, due to its DD-carboxypeptidase and DD-endopeptidase activities, detrimental and finally lethal to the producing cells conditions could be established for the stable expression of the enzyme. PBP4 accumulated in the cells to 3.8% of the soluble protein fraction. On the basis of the estimate that there are about 110 molecules of PBP4 per wild-type cell [17] and under the assumption, that the soluble protein fraction is equivalent to 50 % of the dry mass of the *Escherichia coli* cell [18], the overproduction of PBP4 was calculated to be approximately 1000-fold compared to the wild-type expression level.
Glauner has shown that in the murein sacculus of *Escherichia coli* besides the classical type of DD-crosslinks between the D-alanine residue and the D-centre of meso-diaminopimelic acid (D-Ala-γ-$A_2$pm) another type of crosslink exists with DL stereochemistry ($A_2$pm-$A_2$pm) [19]. As we have described earlier, Expression of PBP4 at higher than wild-type levels causes a drastic decrease in the degree of D-Ala-γ-$A_2$pm cross-bridges in the murein-polymer, that is partially compensated for by the increase in the degree of the DL-crosslinks $A_2$pm-$A_2$pm [1]. This indicates that the cells are able to compensate for the loss of stability of the murein-polymer being caused by the cleavage of DD-cross-bridges, by increasing the level of DL-transpeptidation activity. By this regulative mechanism the cells are able to tolerate such immense overproduction of enzymatically active PBP4. The same conclusion can be drawn, when the growth curves and the kinetics of production of PBP4 during the three different induction temperatures are compared. During one generation time after induction ($A_{600}$ = 0.4 to $A_{600}$ = 0.8) the overproducing cells divide at normal rate. In fact the cells which produce the largest amounts of PBP4 (42 °C) still double the fastest. We may speculate, that the conditions prevalent in the periplasmic space of the cell do not allow optimal functioning of the DD-endopeptidase activity, possibly due to inaccessibility of

its substrate. Another possible explanation comes from the behaviour of the isolated enzyme. We found that PBP4 shows the precipitation characteristics of a globin type enzyme. It precipitates reversibly at neutral or slightly alkaline pH in the absence of salt. Upon overproduction, only a small portion of the overproduced PBP4 might be in an active form, which would explain that overproduction does not immediately affect the growth of the producing bacteria.

At the highest rate of overexpression at 42°C, about half of the PBP4 accumulated as unprocessed precursor in an insoluble and inactive form, whereas the other half was detected as mature and active PBP4 in the soluble fraction. The same phenomenon has been reported to be associated with high-level overproduction of the TEM ß-lactamase of *E. coli*, when a certain threshold level of protein synthesis was exceeded [20]. The insoluble ß-lactamase precursor was shown to be located in the cytoplasm. We were not able to determine the cellular location of the aggregated preform of PBP4, since our efforts to fractionate the cells led immediately to lysis of the cells and scrambled the location of the precursor.

The occurrence of a precipitated precursor of PBP4 and of the TEM ß-lactamase, both depending on high-level expression, might be based on the same principle. Bowden and Gregoriou have proposed that the TEM ß-lactamase precipitates when its cytoplasmic concentration exceeds the concentration of the chaperonins GroEL and GroES which keep the unprocessed precursor in a translocation competent state [20]. Another interpretation of our findings could be that the folding and translocation of PBP4 shows a strong temperature dependence and is negatively affected above 40 °C. This could be corroborated by expression studies with chemically inducible promoters.

Our experiments prove that PBP4 is indeed a water-soluble protein as we predicted earlier [1,2]. PBP4 was isolated in the absence of detergents and behaved as a soluble protein throughout the isolation procedure. The isolated protein is at pH 10.5 completely soluble at a concentration of at least 15.4 mg/ml. At lower pH a precipitate is formed that can be disolved completely by the addition of sodium chloride. This behaviour supports our view that PBP4 is associated with the membrane not by hydrophobic patches on the surface of the protein, since such hydrophobic interactions would be strengthened by an increase in the salt concentration, but that it might indirectly be bound to the membrane via another membrane integrated protein. This would also explain why the major portion of the overproduced PBP4 is detected in the soluble fraction. In cells with wild-type level expression PBP4 has been localized in the inner membrane fraction [3,4,17] and we ourselves have purified PBP4 from the membrane fraction of *E. coli* [1]. *Proteus vulgaris* P18, an enterobacterium closely related to *E. coli* shows the same PBP-pattern as *E. coli* and its corresponding PBP4 behaves also as a soluble protein [21].

The application of dye chromatography led to a very simple protocol for the isolation of PBP4. We developed this method mainly to be able to purify mutated forms of PBP4, which show altered penicillin-binding behaviour and can therefore not be isolated by traditional covalent penicillin affinity chromatography. We found indeed, that the binding and release of PBP4 to and from the Cibacron Navyblue 2G-E matrix is not influenced by ß-lactams. Rather unexpected was the finding, that all PBPs of *E. coli* with the exception of PBP3 bound to the dye matrix and could be purified from membrane extracts. It remains to be shown whether this behaviour is unspecific or reflects the occurence of a common structural element of all these PBPs, like the dinucleotide fold in NAD-binding proteins to which Cibacron blue F3GA binds [22].

The purity of the PBP4 preparations that were obtained by dye-chromatography was directly correlated to the degree of overproduction being achieved. This might be explained by a slightly higher binding affinity of PBP4 to the dye compared to other proteins and is therefore able to displace them from the dye ligand. One drawback of the described method is the rather low

capacity of the dye matrix, which was found to be 200 $\mu$g PBP4 per ml of gelbed volume. This low capacity can easily be compensated for by increasing the size of the column. Recently, screening of a larger collection of dyes (about 100) in our laboratory revealed, that affinity to PBP4 is not restricted to Cibacron dyes. The screening approach described by us is a simplified version of the one described by Scopes [23]. Rather than performing the preliminary screening of the dyes by column chromatography, we found it convenient to carry out the first screening in batch and to judge the quality of the dye by performing SDS/PAGE or more rapidly by dot blot analysis with a specific antiserum. Screening of larger collections of dyes can conveniently be done using microtiter plates. This is the first report on the purification of a penicillin-binding protein by dye chromatography. From our recent work on the purification of other PBPs from *E. coli* and other organisms we learned (unpublished work), that the chance of finding a selective dye is rather good as long as the collection of dyes being screened is large enough (50-100 dyes). Overexpression of the protein in question will help to set up a simple and rapid purification protocol with this method.

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