Domain organization of penicillin-binding protein 5 from *Escherichia coli* analysed by C-terminal truncation

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The structural organization of penicillin-binding protein (PBP) 5 was investigated by C-terminal truncation. Compared with other low-M_r penicillin-interacting proteins, PBP5 carries a C-terminal extension of about 100 amino acids. The sites for introduction of stop codons were chosen on the basis of the established three-dimensional structure of the *Streptomyces albus* G β-lactamase [Dideberg, Charlier, Wéry, Dehottay, Dusart, Erpicum, Frère and Ghuysen (1987) Biochem. J. **245**, 911–913] and comparative hydrophobic cluster analysis [Gaboriaud, Bissery, Bencheritt and Mornon (1987) FEBS Lett. **224**, 149–155]. Two stop codons were introduced at positions Ile-354 or Val-348 to construct an optimized soluble form of PBP5 for crystallization purposes. The newly constructed soluble and enzymically active form ($PBP5^{s353}$) was isolated by dye-affinity chromatography and gave rise to small crystals. Another two stop codons were introduced at positions Arg-261 or Ala-276 to determine the minimal enzymically active 'core protein'. The truncated form ($PBP5^{s275}$), missing the entire C-terminal extension, showed unaltered penicillin-binding characteristics and a catalytic-centre activity 40 % that of $PBP5^{s353+9}$ using bisacetyl-L-Lys-D-Ala-D-Ala as substrate. This protein, however was more susceptible to proteolytic degradation, which might indicate a role of the C-terminal portion in stabilizing the protein.

INTRODUCTION

The major DD-carboxypeptidase from *Escherichia coli*, also known as penicillin-binding protein 5 (PBP5), is a member of the family of the active-site-serine penicillin-interacting enzymes (Joris et al., 1988). This protein family consists of the low-M_r penicillin-sensitive DD-peptidases, the C-terminal penicillin-interacting domains of the bifunctional high-M_r penicillin-binding proteins and the β-lactamases of classes A, C and D. Within the sequences of all these proteins, several highly conserved short fingerprints, with approximately conserved spacing along the primary sequence, have been identified. Furthermore a striking similarity in the spatial arrangements of secondary-structure elements between the β-lactamase of *Bacillus licheniformis* and the DD-peptidase of *Streptomyces* R61 is apparent from a comparison of their three-dimensional structures (Kelly et al., 1986). Sequence alignment of PBP5 (374 amino acids) and the *Streptomyces albus* G β-lactamase (273 amino acids) revealed that PBP5 possesses a C-terminal extension of about 100 amino acids. Three conserved fingerprints (SxxK, SDN and KTG) could be identified within the N-terminal 275 residues of PBP5 and showed very similar spacing to that found for the β-lactamase (Joris et al., 1988). On the basis of the three-dimensional structure of the β-lactamase of *Streptomyces albus* G (Dideberg et al., 1987), sequence alignment and the structural similarity between DD-peptidases and β-lactamases we developed a working model for the structural organization of PBP5 (Figure 1). The most obvious difference between PBP5 and the β-lactamase is the C-terminal extension of about 100 amino acids. It is well established that the 21 residues at the extreme C-terminus of PBP5 form an amphiphilic α-helix anchoring the protein to the membrane. The construction of a soluble form of PBP5 ($PBP5^{s}$; here referred to as $PBP5^{s353+9}$), which retained full enzymic activity, was described previously by Ferreira et al. (1988). $PBP5^{s353+9}$ lacks the C-terminal 21 amino acids that are responsible for membrane anchoring, but it contains an extra nine amino acid C-terminal extension that was introduced during the construction of the mutant. Crystallization trials using purified $PBP5^{s353+9}$ gave rise to well-formed prismatic crystals of X-ray quality (Ferreira et al., 1988). However, subsequent crystallization of the protein resulted in two distinct crystal forms which might be caused by the additional, possibly flexible, C-terminal segment. No information is available on the structural and functional properties of the remaining part of the C-terminal extension which, however, is large enough to be folded as an independent domain. To gain more insight into the role of this additional domain with respect to enzymic activity, stability and the general organization of PBP5, we decided to delete the complete C-terminal part. Information on the overall distribution and shape of hydrophobic clusters was used to determine potential sites for optimal C-terminal truncation of PBP5 (Gaboriaud et al., 1987). We report here on the construction of a 100 amino acid C-terminal deletion in PBP5 ($PBP5^{s275}$), which yields a protein that retains both DD-carboxypeptidase activity and penicillin-binding capacity. Furthermore we describe the construction of a new soluble form of PBP5 ($PBP5^{s353}$) for crystallization purposes.

MATERIALS AND METHODS

Bacterial strains and plasmids

All the subcloning and expression of proteins were performed using *E. coli* strain MC1061 as a host (Meissner et al., 1987). Mutagenesis was performed using *E. coli* strains CJ236 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and JM101 and phage M13mp19 (Norrander et al., 1983) as described by Kunkel et al. (1987). The plasmids $pROFIT\text{-}5C^{n}$ and $pROFIT\text{-}5C^{s}$ were described previously (van der Linden et al., 1992). The plasmid

Abbreviations used: PBP, penicillin-binding protein; LB, Luria–Bertani; h.c.a., hydrophobic cluster analysis; PVDF, poly(vinylidene difluoride).
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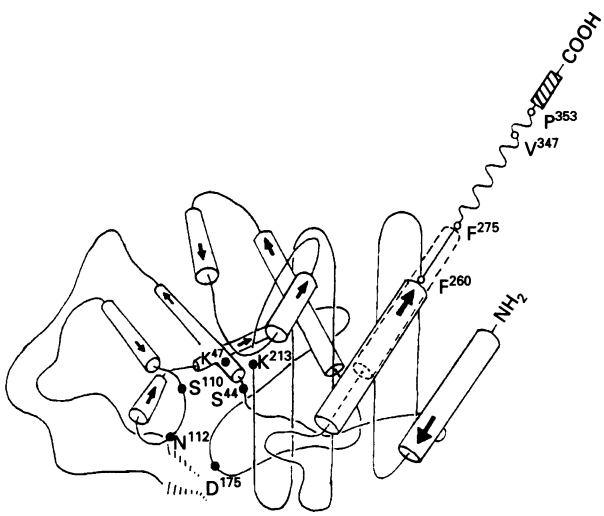

Figure 1 Three-dimensional model structure for PBP5

The Figure shows a working model for PBP5 based on the three-dimensional structure of the β-lactamase of *Streptomyces albus* G (Dideberg et al., 1987). Filled circles indicate the positions of the conserved active-site fingerprints; open circles give the positions of the introduced stop codons. The C-terminal membrane anchor is indicated by a hatched box. Residues 275–374 represent the C-terminal extension present in PBP5.

pLF1-5 containing the *dac*A^s gene was from our collection (Ferreira et al., 1988). Bacteria were grown with aeration at 30 °C in Luria–Bertani (LB) medium, which was supplemented with kanamycin (50 $\mu g \cdot ml^{-1}$) for antibiotic-resistant strains. To induce the lambda-p_R promoter the temperature was shifted to 42 °C at an A_{600} of 0.6.

Enzymes and radiochemicals

Restriction enzymes, Klenow polymerase, T4-DNA ligase and T4-polynucleotide kinase were obtained from Boehringer and used as recommended by the supplier. For penicillin-binding assays, [^{14}C]benzylpenicillin (specific radioactivity 1.97 GBq·mmol^{-1}) was used.

Deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate was used for DNA-sequencing. Both radiochemicals were obtained from Amersham.

Hydrophobic cluster analysis and comparison of amino acid sequences

Hydrophobic cluster analysis (h.c.a.) was performed as described by Gaboriaud et al. (1987). The method is based on a two-dimensional representation of the distribution of hydrophobic clusters along the protein sequence. Clusters of hydrophobic residues are taken as indicators of the presence of secondary-structure elements used for the alignment of weakly related primary structures.

Site-specific mutagenesis

The 1.7-kb *Bam*HI–*Eco*RI fragment from pLF1-5 was cloned into the *Bam*HI and *Eco*RI sites of M13mp19. The method described by Kunkel et al. (1987) was applied for oligonucleotide-directed mutagenesis. The mismatched oligonucleotides were synthesized by Eurosequence (University of Groningen, Groningen, The Netherlands) on an Applied Biosystems model-380 B DNA synthesizer. The sequences of the primers used to introduce stop codons are 3′-CGTTCTTTAGGGCATTCGA-ACTGGACGTCGG-5′ for PBP5^{s353}; 3′-CGGGCGACCAA-ATCAACGTTCTTT-5′ for PBP5^{s347}; 3′-TTTCTCAAG-ATTAGACTTGGC-5′ for PBP5^{s275} and 3′-CCCCGAAG-ATTAAGAAACTT-5′ for PBP5^{s260} (mutated bases are underlined). Mutagenesis was verified by DNA sequencing using the dideoxy chain-termination method (Sanger et al., 1977).

Protein purification

The native, membrane-bound cytoplasmically overproduced form of PBP5 (PBP5^{nc}) and the different cytoplasmically overproduced soluble forms of PBP5 (PBP5^{s353+9}, PBP5^{s353} and PBP5^{s275}) were purified using the dye–ligand chromatography method exactly as described previously (van der Linden et al., 1992).

Kinetic measurements

The DD-carboxypeptidase activity of PBP5 and its various mutated forms was determined by incubating purified protein (10 μg in 100 mM Tris, pH 8.0, at 37 °C) with various concentrations of substrate (6–25 mM; bisacetyl-L-Lys-D-Ala-D-Ala; Sigma). Aliquots were removed every 2 min up to 10 min, and the amount of released D-alanine was determined using D-amino acid oxidase and *o*-dianisidine chloride as described by Frère et al. (1976).

SDS/PAGE and Western blotting

SDS/PAGE was performed as described by Lugtenberg et al. (1975), using a 10% (w/v) running gel and a 4.5% (w/v) stacking gel with an acrylamide monomer/dimer ratio of 60:1 (w/w). Proteins were transferred electrophoretically from acrylamide gels on to nitrocellulose as described by Bittner et al. (1980). Rabbit antisera (1000 × diluted) obtained in this laboratory from rabbits immunized with purified PBP5^s or PBP5^{s275} were used for immunochemical detection of PBP5^{nc} and its different truncated forms in combination with goat anti-(rabbit IgG)–horseradish peroxidase (Boehringer) and chloronaphthol/H_2O_2 (Talbot et al., 1984).

Penicillin-binding assays

Membrane-bound and soluble forms of PBP5 (25 pmol) were assayed for their penicillin-binding activity by incubation with [^{14}C]benzylpenicillin (200 pmol) for 10 min at 37 °C. SDS/PAGE sample buffer was added to the incubation mix; samples were boiled for 3 min and applied to a polyacrylamide gel. Fluorography was performed by the procedure of Chamberlain (1979). The binding stoichiometry of the different forms of PBP5 was determined by incubating protein samples of 25 pmol with 200 pmol of [^{14}C]benzylpenicillin at 37 °C for 10 min. The molar ratio of bound [^{41}C]benzylpenicillin and the time course of the release of the [^{14}C]benzylpenicilloyl moiety from PBP5 were determined as described previously (van der Linden et al., 1992).

N-terminal determination

N-terminal determination was performed by means of Edman degradation of protein immobilized on a poly(vinylidene difluoride) (PVDF) filter (Matsudaira, 1987).

Other techniques

The concentration of proteins was routinely determined by the method of Bradford (1976), which was calibrated by a PBP5 standard subjected to an amino acid determination on h.p.l.c. after hydrolysis.

RESULTS AND DISCUSSION

Construction of a new soluble form of PBP5

The previously constructed soluble and crystallizable form of PBP5 ($PBP5^{s353+9}$) turned out to cause problems in the reproducibility of the crystallization experiments (Ferreira et al., 1988). The protein crystallized in two distinct forms. Therefore we constructed a new soluble and enzymically active form of PBP5 which does not possess a flexible C-terminal segment. To obtain this soluble form of PBP5 stop codons were introduced into the *dac*A^{s353+9} sequence after either Pro-353 or Val-347. The gene coding for $PBP5^{s353+9}$ (*dac*A^{s353+9}), was recloned from pLF1-5 into M13mp19 using the *Bam*HI and *Eco*RI restriction sites and subjected to site-directed mutagenesis.

The first stop codon resulted in a protein truncated after Pro-353 ($PBP5^{s353}$) thus deleting the additional nine amino acids of the $PBP5^{s353+9}$ construct. In addition to this mutation the primer introduced a *Hin*dIII site just downstream of the new stop codon. This restriction site was used to remove a 17 bp *Hin*dIII fragment containing part of the information for the extra residues of $PBP5^{s353+9}$. Since the reading frame was also altered eventual expression of the extra amino acids by read-through of the stop codon was avoided.

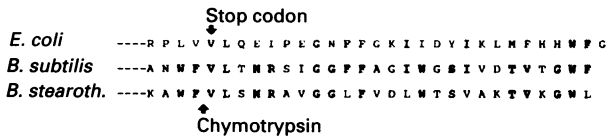

Figure 2 Amino-acid-sequence comparison of the C-terminal regions of the D-alanine carboxypeptidases of *E. coli* (374 amino acids), *B. subtilis* (396 amino acids) and *B. stearothermophilus* (*B. stearoth.*)

Identical residues identified by sequence alignment are shaded. The chymotrypsin-cleavage site yielding a soluble form of *B. stearothermophilus* is indicated by an arrow. The site for introduction of a stop codon to create a soluble form of the *E. coli* enzyme is indicated by an arrow.

By analogy to the chymotrypsin-solubilized forms of the DD-carboxypeptidases of *Bacillus subtilis* and *Bacillus stearothermophilus*, another stop codon was introduced at Val-348, yielding $PBP5^{s347}$ (Figure 2; Waxman and Strominger, 1979, 1981a,b).

Construction of C-terminal deletions in $PBP5^{s353+9}$

To study the domain organization of PBP5 and to determine the minimal 'core structure' that is essential for enzymic and penicillin-binding activity, the C-terminal extension of about 100 amino acids was deleted. Using hydrophobic cluster analysis for predictions of secondary-structure elements in the sequence of PBP5, a putative α-helix corresponding to the C-terminal α-helix in the β-lactamase was positioned between amino acid-255 and -271. Stop codons introduced at two sites possibly located at the end of this secondary-structure element yielded the truncated forms $PBP5^{s275}$ and $PBP5^{s260}$ (Figure 3).

Expression of the truncated proteins

The mutated genes were recloned into the vector pROFIT5C using the *Sma*I and *Eco*RI restriction sites, and expressed in the host strain *E. coli* MC1061 (Figure 4). All four truncated proteins were overexpressed at levels comparable with that of $PBNP5^{s353+9}$ (van der Linden et al., 1992). The solubility of the different proteins was determined by disrupting the cells in an Aminco French press at 60.89 MPa (10000 lbf/in^2), followed by ultracentrifugation (45 min; 100000 *g*; 4 °C), after which the soluble fraction and the particulate fraction were separated and applied to an SDS/polyacrylamide gel. $PBP5^{s353+9}$, $PBP5^{s353}$, $PBP5^{s275}$ and $PBP5^{s260}$ were found in the soluble fraction, whereas $PBP5^{s347}$, although completely missing the membrane anchor, was found quantitatively in the particulate fraction, as was the wild-type protein, $PBP5^{nc}$, which was used as a control (Figure 5).

Purification of the truncated proteins

Purification of the different truncated proteins was performed using the immobilized dye Procion Rubine MX-B as described previously for $PBP5^{nc}$ and $PBP5^{s353+9}$ (van der Linden et al.,

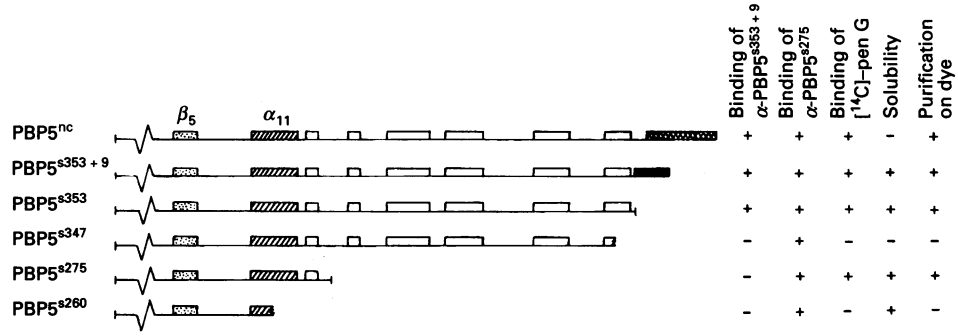

Figure 3 C-terminal truncations in PBP5 of *E. coli*

Secondary-structure elements in the C-terminus of the native membrane-bound PBP5 ($PBP5^{nc}$) were predicted from h.c.a. In combination with sequence alignments using the active-site serine residue as internal marker, the predicted secondary-structure elements identified to be counterparts of the elements β5 and α11 in the *Streptomyces albus* G β-lactamase structure are indicated (Joris et al., 1988). The predicted C-terminal amphiphilic helix (Jackson and Pratt, 1987) is indicated by a double hatched box. The extra nine amino acids in $PBP5^{s353+9}$ are indicated by a filled box. Some of the characteristics of the different forms of the protein are presented.

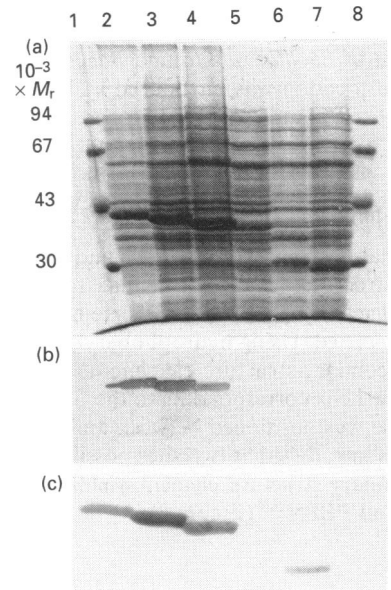

Figure 4 Overexpression of the C-terminally truncated forms of PBP5

(**a**) Coomassie Blue-stained SDS/polyacrylamide gel. (**b**) Western blot after immunostaining using antibodies directed against PBP5^{s353+9}. (**c**) Fluorogram after incubation with [^{14}C]benzylpenicillin. After 2 h growth at 42 °C the total cell protein was applied to the gel. Lanes 1 and 8, M_r marker; lane 2, PBP5^{nc}; lane 3, PBP5^{s353+9}; lane 4, PBP5^{s353}; lane 5, PBP5^{s347}; lane 6, PBP5^{s275}; lane 7, PBP5^{s260}.

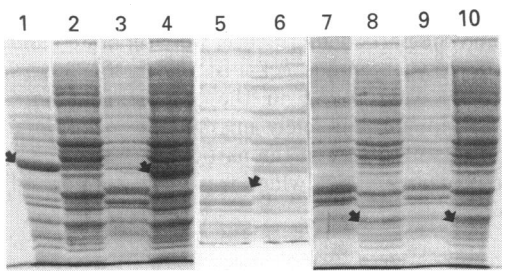

Figure 5 Solubility of overexpressed PBP5 derivatives

Coomassie Blue-stained SDS/polyacrylamide gel showing the solubility of different C-terminally truncated forms of PBP5. Particulate fractions: lane 1, PBP5^{nc}; lane 3, PBP5^{s353}; lane 5, PBP5^{s347}; lane 7, PBP5^{s275}; lane 9, PBP5^{s260}. Soluble fractions: lane 2, PBP5^{nc}; lane 4, PBP5^{s353}; lane 6, PBP5^{s347}; lane 8, PBP5^{s275}; lane 10, PBP5^{s260}. Arrows indicate the positions of the various forms of PBP5.

1992). By using this method, PBP5^{s353} and PBP5^{s275} were purified from the soluble fraction of the overproducing strains. Typically, from 18 g wet weight of cells, between 20 and 40 mg of the mutated proteins could be purified by this one-step dye-chromatography procedure. Since PBP5^{s347} and PBP5^{s260} were not bound by Procion Rubine MX-B, they could not be purified by this method (Figure 6).

Specificity of polyclonal antibodies for different truncated forms of PBP5

Antibodies against PBP5^{s353+9} were raised in rabbits. The serum from these rabbits was used to incubate Western blots of the different truncated forms of PBP5. PBP5^{nc}, PBP5^{s353+9} and PBP5^{s353} showed similar levels of antibody binding. Anti-

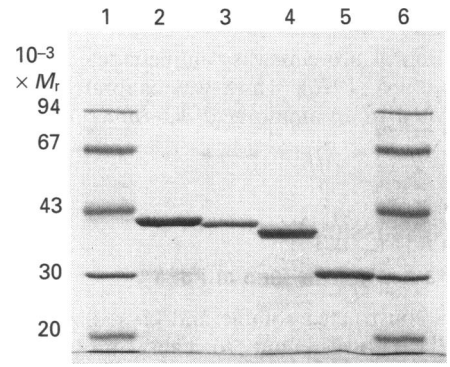

Figure 6 Purification of different forms of PBP5

The Figure shows a Coomassie Blue-stained SDS/polyacrylamide gel of different forms of PBP5 after purification using the immobilized dye Procion Rubine MX-B. Lanes 1 and 6, M_r markers; lane 2, PBP5^{n} (1 μg); lane 3, PBP5^{s353+9} (1 μg); lane 4, PBP5^{s353} (1 μg); lane 5, PBP5^{s275} (1 μg).

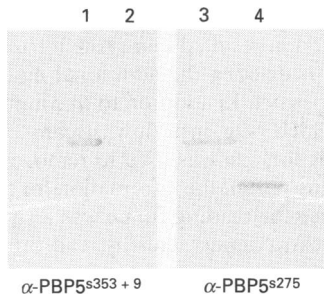

Figure 7 Specificity of polyclonal antibodies directed against PBP5^{s353+9} or PBP5^{s275}

Western blot after immunostaining with rabbit antisera raised against PBP5^{s353+9} (left panel) and PBP5^{s275} (right panel). Lanes 1 and 3, purified PBP5^{s353+9} (1 μg); lanes 2 and 4, purified PBP5^{s275} (1 μg).

PBP5^{s353+9} antibodies showed only very weak affinity for PBP5^{s347}, PBP5^{s275} and PBP5^{s260} (Figure 4b).

Purified PBP5^{s275} was used to immunize rabbits resulting in anti-PBP5^{s275} antiserum. When a Western blot containing PBP5^{s353+9} and PBP5^{s275} was incubated with these antibodies, binding to both proteins was observed (Figure 7). PBP5^{nc}, PBP5^{s353}, PBP5^{s347} and PBP5^{s260} could also be detected with this antiserum. It is concluded that the anti-PBP5^{s353+9} polyclonals are mainly directed against the C-terminal domain, whereas antibodies raised against PBP5^{s275} are essentially directed against the 'core structure' of PBP5 and are thus expected to bind to all forms of PBP5.

Characterization of the truncated proteins

The penicillin-binding activity of the different proteins was studied by incubation with [^{14}C]benzylpenicillin. PBP5^{nc}, PBP5^{s353+9} and PBP5^{s353} showed wild-type penicillin-binding activity at a 1:1 stoichiometry. PBP5^{s275}, although lacking 100 C-terminal residues, still showed wild-type penicillin-binding activity. PBP5^{s347} and PBP5^{s260} no longer bound penicillin (Figure 4c).

PBP5 is known to release bound penicillin with a half-life of the PBP5–penicillin complex of 4 min (van der Linden et al., 1992). Similar values were found for PBP5^{s353} and PBP5^{s275} (Table 1). Apparently the removal of up to 100 C-terminal amino

Table 1 Kinetic constants of PBP5^{nc} and C-terminally truncated forms

The different forms of PBP5 were purified by using the dye-affinity procedure described previously (van der Linden et al., 1992). Assay for the membrane-bound PBP5^{nc} were performed in the presence of 0.2% Triton X-100 using bisacetyl-L-Lys-D-Ala-D-Ala as substrate.

	Hydrolysis of [^{14}C]benzylpenicillin		Carboxypeptidase activity	
	$t_{\frac{1}{2}}$ (s)	$10^3 \times k_3$ (s^{-1})	K_m (mM)	$k_{cat.}$ (s^{-1})
PBP5^{nc}	260 ± 10	2.7 ± 0.1	*	*
PBP5^{s353+9}	210 + 9	3.3 ± 0.13	14 ± 2	1.46 ± 0.1
PBP5^{s353}	200 ± 10	3.5 ± 0.2	18.4 ± 1.8	0.96 ± 0.05
PBP5^{s275}	218 ± 10	3.2 ± 0.15	20 ± 3.5	0.58 ± 0.05

* Different experiments for the determination of K_m and $k_{cat.}$ for PBP5^{nc} did not give consistent values.

acids did not seriously affect the deacylation rate of the PBP5–penicillin complex.

The DD-carboxypeptidase activity of the truncated forms of PBP5 was determined using the artificial substrate bisacetyl-L-Lys-D-Ala-D-Ala. The K_m and $k_{cat.}$ values of the truncated proteins are given in Table 1. Prolonged incubation of a purified sample of PBP5^{s353} at room temperature resulted in quantitative degradation to a protein of slightly lower M_r, probably caused by a co-purified *E. coli* proteinase. This was previously observed for purified mutated forms of PBP5 (M. P. G. van der Linden, L. de Haan and W. Keck, unpublished work). N-terminal sequencing of the lower-M_r protein revealed an N-terminal truncation of eight amino acids, the new N-terminus being:

Met-Ile-Pro-Gly-Val-Pro-Gln-

Due to proteolytic loss of these eight N-terminal amino acids, which in our working model are proposed to form the first α-helix, the purified PBP5^{s353} appeared as a double band after SDS/PAGE (Figure 6). The N-terminally degraded protein was still able to bind and hydrolyse penicillin ($t_{\frac{1}{2}} = 240 \pm 12$ s, $k_3 = 2.87 \pm 0.14 \times 10^{-3}$ s^{-1}). Depending on the degree of degradation the protein showed decreased DD-carboxypeptidase activity, indicating the N-terminal α-helix to be an essential part of the 'core structure'. The degree of degradation was strongly diminished when the temperature was kept at 4 °C and the purification time was as short as possible. The related DD-peptidase from *Streptomyces* K15 is described as lacking the N-terminal as well as the C-terminal α-helix, which might create a hydrophobic patch, possibly involved in membrane association of the protein (Palomeque-Messia et al., 1991). In PBP5^{s353} the loss of the N-terminal α-helix alone does not seem to result in such a hydrophobic patch, since the protein remains completely soluble.

The truncated protein PBP5^{s347} was stably overexpressed, did not bind penicillin, could not be purified from the particulate fraction using the dye-affinity procedure and was not detected by α-PBP5^{s353+9} antibodies. Apparently the C-terminal structural organization of PBP5 differs from the DD-carboxypeptidases of *Bacillus subtilis* and *Bacillus stearothermophilus*. Predictions of the location of secondary-structure elements from h.c.a. are in good agreement with the observed properties of PBP5^{s353} and PBP5^{s347} (Gaboriaud et al., 1987). Removal of the C-terminal outermost 21 residues producing PBP5^{s353} exactly deletes the last predicted secondary-structure element and terminates the protein with a proline residue. Proline is considered a breaker of hydrophobic clusters, thus inducing segmentation between different clusters. Pro-353 is separating two predicted hydrophobic regions. The stop codon introduced at Val-348 resulting in PBP5^{s347} is located in the middle of a predicted secondary-structure element which is apparently disturbing the correct folding of the enzyme, resulting in a completely inactive and aggregated protein. Crystallization trials using PBP5^{s353} have resulted in crystals that so far are too small for X-ray-diffraction analysis. Attempts to grow larger crystals by seeding experiments are in progress.

Deletion of the 100-amino-acid-long additional C-terminal segment of PBP5 by insertion of stop codons at positions Arg-261 or Ala-276 resulted in only one functional protein, PBP5^{s275}. The other truncated form, PBP5^{s260}, was soluble, but since it could not be purified using dye-affinity chromatography, did not bind penicillin and was not detected by anti-PBP5^{s353+9} antibodies, it seems to be present in a non-functional conformation. Most probably the stop codon at Arg-261 affects the core structure of the protein, owing to its localization within an α-helix, which was predicted by h.c.a. (Figure 3). PBP5^{s275}, on the other hand, still bound and hydrolysed penicillin at the same rate as PBP5^{s353+9}. The protein could be purified from the soluble fraction by dye chromatography and showed DD-carboxypeptidase activity towards bisacetyl-L-Lys-D-Ala-D-Ala. The higher K_m of PBP5^{s275} for this substrate (20 ± 3.5 mM) and the lower $k_{cat.}$ value (0.58 ± 0.05 s^{-1}) indicate that the protein was less efficient in performing D-Ala-D-Ala cleavage than PBP5^{s353+9} (Table 1). Prolonged incubation at 37 °C resulted in complete degradation of PBP5^{s275} in about 5 h. Since PBP5^{s353+9} remained unaffected under similar conditions, this indicates a destabilization of PBP5^{s275}. However, it is clear that PBP5^{s275} lacking 100 C-terminal residues was still a functional DD-carboxypeptidase with a $k_{cat.}$ at 40% of PBP5^{s353+9}, while leaving penicillin binding and the rate of penicillin release completely unaffected.

The C-terminal extension of 100 amino acids in PBP5 apparently represents a possibly flexible and very antigenic domain, the last 21 residues of which form the membrane anchor. The domain seems to stabilize the protein, but is not directly involved in the catalytic mechanism. It could serve in orienting the membrane-bound PBP5 properly towards its substrate, the periplasmic murein polymer. This may provide the cell with a means of regulating the DD-carboxypeptidase activity by restricting the free mobility of the protein towards the substrate. Elucidation of the three-dimensional structure of PBP5 by X-ray-diffraction studies will given more insight into these still open questions.

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Received 2 July 1992/3 August 1992; accepted 6 August 1992