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Active-site and membrane topology of the DD-peptidase/penicillin-binding protein no. 6 of *Enterococcus hirae* (*Streptococcus faecium*) A.T.C.C. 9790

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The membrane-bound 43000-M_r penicillin-binding protein no. 6 (PBP6) of *Enterococcus hirae* consists of a 30000-M_r DD-peptidase/penicillin-binding domain and a ~ 130-residue *C*-terminal appendage. Removal of this appendage by trypsin proteolysis has no marked effect on the catalytic activity and penicillin-binding capacity of the PBP. Anchorage of the PBP in the membrane appears to be mediated by a short 15–20-residue stretch at the *C*-terminal end of the appendage. The sequence of the 50-residue *N*-terminal region of the PBP shows high degree of homology with the sequences of the corresponding regions of the PBPs5 of *Escherichia coli* and *Bacillus subtilis*. On this basis the active-site serine residue occurs at position 35 in the enterococcal PBP.

INTRODUCTION

All the bacteria possess, bound to the plasma membrane, proteins that upon reaction with β-lactam antibiotics form stable serine ester-linked acyl-enzymes and, for this reason, are called penicillin-binding proteins or PBPs [1]. On the basis of various criteria, M_r, amino acid sequence, nature of the catalysed reaction, membrane topology and physiological function, several groups of PBPs are distinguished [1]. The low-M_r (25000–49000) PBPs/DD-peptidases (i) catalyse acyl-transfer tractions on well-defined simple peptides such as Ac_2-L-Lys-D-Ala-D-Ala [2], (ii) have the active-site serine residue located close to the *N*-terminal of the protein, usually in the position region 40–70 [3], and (iii) are inserted into the plasma membrane by a short non-cleaved *C*-terminal signal-like peptide while the bulk of the polypeptide chain is on the periplasmic side of the membrane. This type of membrane topology has been established for the PBPs5 of *Escherichia coli* [4,5], *Bacillus subtilis* and *Bacillus stearothermophilus* [6]. It probably applies to PBP2b and PBP3 of *Streptococcus pneumoniae* [7]. Mention should also be made of *Streptomyces* R61, which excretes a low-M_r PBP-DD-peptidase during growth. Excretion involves cleavage of a 26-residue *C*-terminal stretch from the protein precursor [8].

Enterococcus hirae (formerly *Streptococcus faecium*) A.T.C.C. 9790 possesses seven PBPs, the M_r values of which range from 119000 to 43000 [9,10]. The 43000-M_r PBP6 performs DD-peptidase activity on Ac_2-L-Lys-D-Ala-D-Ala [11]. As described below, it also fulfils the two other criteria that define the low-M_r PBPs/DD-peptidases: it is anchored in the membrane by a small *C*-terminal peptide segment and its active-site serine residue is located close to the *N*-terminal end of the protein.

MATERIALS AND METHODS

Growth conditions and membranes

Enterococcus hirae was grown at 37 °C in unshaken 1-litre flasks containing 500 ml of SB medium [12]. Cells were collected at the late exponential phase (A_{550} = 6.0) and the membranes were prepared essentially as described previously [13]. Cells from 500 ml cultures were suspended in 20 ml of 5 mM-sodium phosphate buffer, pH 7, containing 1 mM-$MgCl_2$ and lysed with a mixture of 2 mg of lysozyme, 40 μg of DNAase, 20 μg of RNAase and 0.2 mg of muramidase I (10 μg/ml). Muramidase I from *Streptomyces globisporus* 1828 [14] was a gift from Dr.K. Yokogawa, Dainippon Pharmaceutical Co., Osaka, Japan. It was further purified by f.p.l.c. (P. Jacques, unpublished work). The membranes were suspended in 40 mM-sodium phosphate buffer, pH 7, containing 1 mM-$MgCl_2$ and 5% (v/v) glycerol.

DD-Carboxypeptidase activity

The catalysed reaction was:

$$Ac_2\text{-L-Lys-D-Ala-D-Ala} + H_2O \rightarrow \text{D-Ala} + Ac_2\text{-L-Lys-D-Ala}$$

The released D-alanine was measured as described previously [11].

Labelling with radioactive benzylpenicillin

All the PBPs, except the highly resistant PBP5, were quantitatively labelled by reaction with 50 μM-benzyl[^{14}C]penicillin (54 Ci/mol; The Radiochemical Centre, Amersham, Bucks., U.K.) for 30 min at 37 °C. The second-order rate constant of PBP acylation was derived from the benzyl[^{14}C]penicillin concentration required to cause half-saturation [15,16].

Abbreviations used: PBP(s), penicillin-binding protein(s); tPBP(s), tryptic penicillin-binding protein derivative(s).

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SDS/polyacrylamide-gel electrophoresis and fluorography

Discontinuous gel electrophoresis [17] was carried out at constant voltage, in an LKB 2001 apparatus (Pharmacia, Uppsala, Sweden). The acrylamide/bisacrylamide ratio was 37.5:1 for the stacking gel and 60:1 for the separating gel. Depending on the M_r of the proteins, 7.2%, 10% and 12% acrylamide separating gels (16 cm × 18 cm or 32 cm × 18 cm) were used. The Coomassie Blue-stained gels were submitted to fluorography [9] and exposed for 2–7 days at −70 °C.

Hydrophobicity

Partitioning between the upper aqueous phase and the lower detergent phase in the presence of Triton X-114 [18] was used as an index of the hydrophilic versus hydrophobic character of the PBPs.

Amino acid sequencing

Automated sequence analysis was carried out on a 477 A pulsed liquid Sequenator with on-line analysis of the amino acid phenylthiohydantoin derivatives with a 120 A analyser (Applied Biosystems, Foster City, CA, U.S.A.). Two samples of soluble purified tPBP6* (3 and 4.5 nmol respectively) were applied to glass-fibre filters carrying 3 mg of twice-precycled Polybrene. A sample of the native PBP6 (600 pmol) was sequenced after SDS/12%-polyacrylamide-gel electrophoresis and electroblotting on a Millipore Immobilon [poly(vinylidene difluoride)] membrane by using a Bio-Rad Mini Trans-Blot cell. The initial yields were about 30% for the two tPBP6* runs and about 10% for the electroblotted PBP6 run. The blotting experiment was carried out according to the procedure described in ref. [19].

Analytical polyacrylamide-gel isoelectric focusing

Experiments were carried out on a 111 Mini IEF cell following the manufacturer's (Bio-Rad Laboratories) instructions and with pH 2–pH 5.5 Pharmalyte (Pharmacia, Uppsala, Sweden).

RESULTS

Each of the seven PBPs in *Enterococcus hirae* A.T.C.C. 9790 represented about 0.2% of the total membrane proteins. Aeration conditions during bacterial growth had little effect on their absolute and relative abundance. Cells were grown and collected, and the membranes were prepared as described above.

Proteolytic conversion of PBP6 into water-soluble tPBP6a and tPBP6*

Membranes, previously labelled with benzyl[^{14}C]-penicillin, were extracted with Triton X-100, and the extracts containing the radioactively labelled PBPs were submitted to partitioning with the two-phase system with Triton X-114. SDS/polyacrylamide-gel electrophoresis and fluorography of samples of the lower detergent phase and the upper aqueous phase showed that the PBPs, including PBP6, occurred only in the detergent phase, i.e. were hydrophobic.

It was known from previous studies [9,10] that (i) the membrane-bound PBPs of *Enterococcus hirae* had differing susceptibilities to proteolysis by trypsin, PBP6 being the most resistant one, and (ii) the end product of trypsin degradation of PBP6 was a water-soluble catalytically active 30000-M_r tPBP6* derivative. In order to check whether conversion of PBP6 into tPBP6* might involve the transitory formation of a water-soluble intermediate, benzyl[^{14}C]penicillin-labelled membranes (total proteins 3.6 mg) were incubated for 30 min at 37 °C in 300 μl of 10 mM-Tris/HCl buffer, pH 7.8, containing 150 mM-NaCl and 0.75% (v/v) Triton X-100 and then centrifuged at 40000 *g* for 1 h. Portions of the supernatant fraction (50 μl; total proteins 480 μg) were incubated as such and with 0.2%, 0.5% and 2% (w/w) trypsin for 10 min at 37 °C and then extracted twice with Triton X-114. Analysis of the aqueous and detergent phases by SDS/polyacrylamide-gel electrophoresis and fluorography (Fig. 1) revealed the transitory occurrence of a radioactive 41000-M_r hydrophilic tPBP6a. On the basis of radioactivity measurements, the sum of the amounts of the 43000-M_r hydrophobic PBP6, 41000-M_r

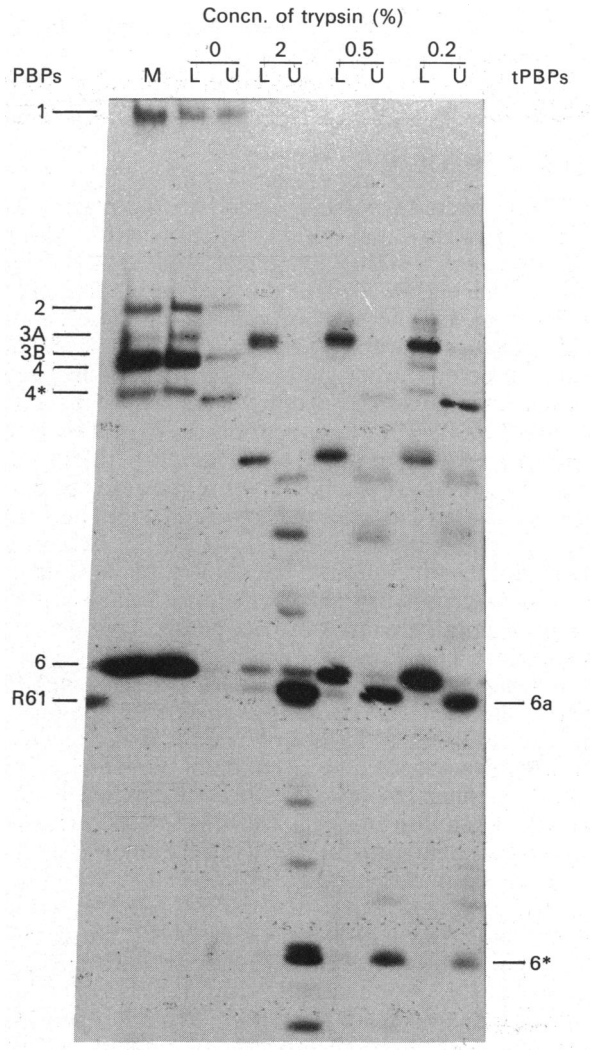

Fig. 1. Tryptic conversion of the membrane-bound benzyl[^{14}C]-penicilloyl-PBP6 of *Enterococcus hirae* into radioactive water-soluble tPBP6a and tPBP6*

All samples, except sample M (for membrane control), were submitted to partitioning with the Triton X-114 two-phase system. Key: L, lower, lipophilic, phase; U, upper, hydrophilic, phase. For conditions see the text.

hydrophilic tPBP6a and 30000-M_r hydrophilic tPBP6* remained constant irrespective of the condition of trypsin treatment. Hence loss of a ~ 20-residue peptide stretch was sufficient to release PBP6 from the membranes in the form of tPBP6a.

Quantitative conversion of PBP6 into tPBP6* and purification of tPBP6*

On the basis of preliminary studies of the effects of pH, buffer nature, trypsin concentration and incubation time, the following optimal conditions were used [10]. Each step of the procedure described below was monitored by estimating the amounts of tPBP6* on the basis of its benzyl[^{14}C]penicillin-binding capacity and DD-carboxypeptidase activity.

A membrane suspension (total proteins 1.35 g; 62.1 nmol of PBP6) made in 90 ml of 40 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-$MgCl_2$ and 5% (v/v) glycerol was incubated with 13.5 mg of trypsin (type XI; Sigma Chemical Co.) for 10 min at 37 °C and then centrifuged at 40000 *g* for 30 min. This pretreatment had virtually no effect on the membrane-bound PBP6 but caused substantial degradation of the other PBPs into water-soluble fragments, and thus yielded a PBP6-enriched membrane pellet. This pellet was resuspended in 40 ml of 100 mM-ammonium bicarbonate buffer, pH 7.8, containing 0.1 mM-$CaCl_2$ and incubated with 130 mg of trypsin for 2 h at 37 °C. Centrifugation yielded a supernatant S1 fraction that contained 59% of the original DD-carboxypeptidase activity. The pellet thus obtained was re-incubated for 2 h at 37 °C in the same trypsin-containing buffer as above. Centrifugation yielded a supernatant S2 fraction that contained 26% of the DD-carboxypeptidase activity.

The combined S1 and S2 fractions (80 ml; total proteins 1.2 g) were supplemented with an equal volume of 50 mM-Tris/HCl/40 mM-sodium phosphate buffer, pH 8.0. The solution was filtered through a Pharmacia Q-Sepharose Fast Flow column (2.6 cm × 40 cm; gel volume 58 ml) and the enzyme was eluted (flow rate 5 ml/min; fraction volume 10 ml) with the help of an NaCl gradient made in 25 mM-Tris/HCl/20 mM-sodium phosphate buffer, pH 8.0 (Fig. 2). Fractions 78–88 (110 ml) contained 67% of the DD-carboxypeptidase activity. They were combined and concentrated to 10 ml by ultrafiltration on an Amicon YM membrane. The resulting preparation was supplemented with 10 ml of 50 mM-sodium phosphate buffer, pH 7.0, containing 1.7 M-$(NH_4)_2SO_4$, and 6 ml portions of the solution were filtered separately through a Pharmacia 1 ml phenyl-Superose HR5/5 column. The enzyme was eluted (flow rate 0.5 ml/min; fraction volume 0.5 ml) with the help of a decreasing $(NH_4)_2SO_4$ gradient made in the same buffer. Fractions 20–25 contained the purified tPBP6* (Fig. 3).

The overall yield of the operation in terms of DD-carboxypeptidase activity was 42.5% with a 490-fold enrichment. Fig. 4 illustrates the efficacy of the purification procedure in terms of benzyl[^{14}C]penicillin-

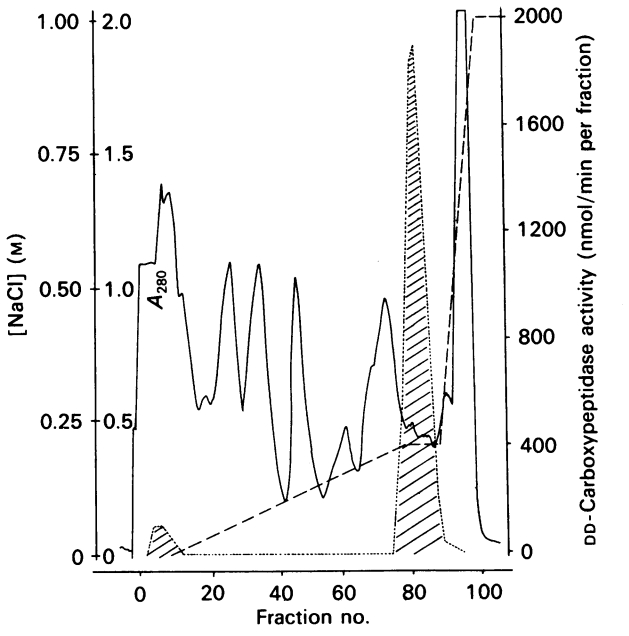

Fig. 2. Purification of the *Enterococcus hirae* tPBP6* by anion-exchange chromatography on a Pharmacia Q-Sepharose Fast Flow column

Samples of each fraction were incubated with 4.5 mM-Ac_2-L-Lys-D-Ala-D-Ala in a final volume of 20 μl for 20 min at 37 °C. Results are expressed in nmol of D-alanine released/min per total fraction. ——, A_{280}; ------, enzyme activity. The gradient (----) was made by mixing buffer A (25 mM-Tris/HCl/20 mM-sodium phosphate buffer, pH 8.0) with buffer B (buffer A containing 1 M-NaCl).

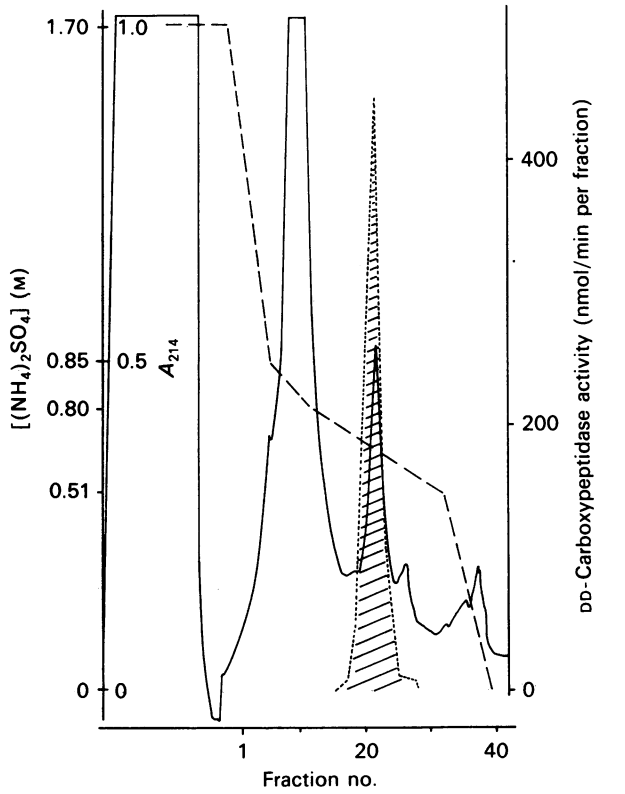

Fig. 3. Purification of the *Enterococcus hirae* tPBP6* by hydrophobic chromatography on a Pharmacia phenyl-Superose HR5/5 column

DD-Carboxypeptidase activity is expressed as indicated in Fig. 2 legend. ——, A_{214}; ------, enzyme activity. The gradient (----) was made by mixing buffer C [50 mM-sodium phosphate buffer, pH 7.0, containing 1.7 M-$(NH_4)_2SO_4$] with buffer D [buffer C without $(NH_4)_2SO_4$].

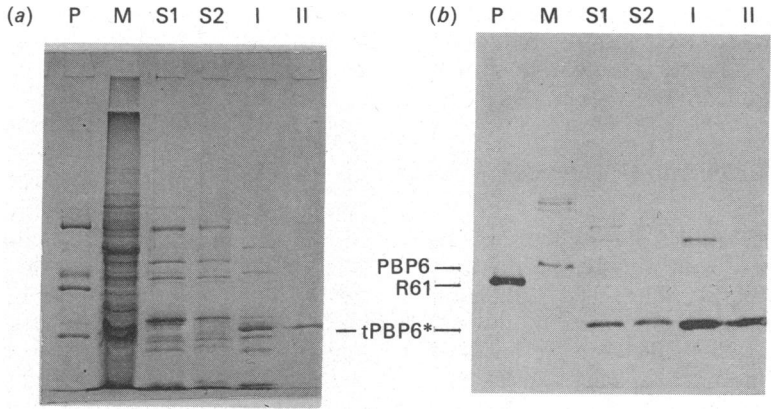

Fig. 4. SDS/polyacrylamide-gel electrophoresis illustrating the purification steps of the *Enterococcus hirae* tPBP6*: (*a*) Coomassie Blue staining; (*b*) fluorography

Key: M, original membranes (120 μg of protein); S1 (48 μg of protein) and S2 (34 μg of protein), supernatant fractions obtained after two successive trypsin treatments of the membranes; I (14.7 μg of protein) and II (0.5 μg of protein), active fractions obtained after Q-Sepharose and phenyl-Superose chromatography respectively; P, standard proteins (bovine serum albumin, ovalbumin, benzyl[^{14}C]penicilloyl-DD-peptidase/PBP of *Streptomyces* R61 and carbonic anhydrase).

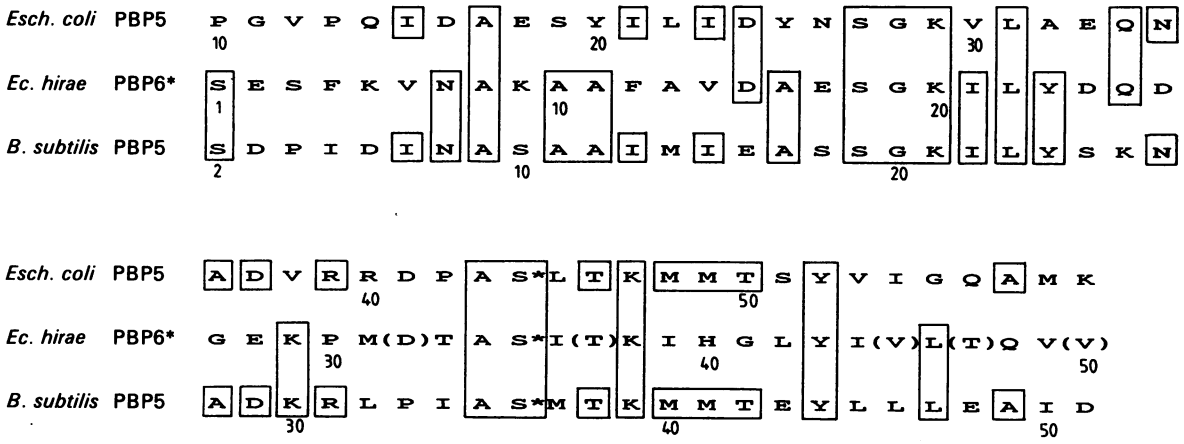

Fig. 5. Amino acid sequences of the *N*-terminal regions of the *Enterococcus hirae* PBP6 and the *Escherichia coli* [19] and *Bacillus subtilis* [20] PBPs5

There are 11 identities between the pair *Enterococcus hirae* PBP6–*Escherichia coli* PBP5, 18 between the pair *Enterococcus hirae* PBP6–*Bacillus subtilis* PBP5 and 21 between the pair *Escherichia coli* PBP5–*Bacillus subtilis* PBP5. The *Enterococcus hirae N*-terminal sequence shown in the Figure is that of the water-soluble derivative tPBP6*. The 20-residue *N*-terminal region of the purified membrane-bound PBP6 has exactly the same sequence. Amino acids in parentheses were not determined with certainty.

binding activity. The absolute amount of purified tPBP6* was 0.8 mg with a 98% estimated degree of purity.

tPBP6* migrated as a single compound on SDS/polyacrylamide-gel electrophoresis and exhibited an apparent 30000 M_r. When compared with PBP6, tPBP6* was slightly less sensitive to inactivation by benzylpenicillin (second-order rate constant of enzyme acylation 445 $M^{-1} \cdot s^{-1}$ compared with 155 $M^{-1} \cdot s^{-1}$) and slightly more sensitive to *p*-chloromercuribenzoate, a 5.5 μM instead of 30 μM concentration being sufficient to inhibit the enzyme activity by 50%.

Preparation of benzyl[^{14}C]penicilloyl-PBP6 for amino acid sequencing

PBP6-enriched membranes (total proteins 1.92 g) obtained by the trypsin pretreatment described above were extracted with 60 ml of 25 mM-Bistris/HCl buffer, pH 6.3, containing 1% (v/v) Triton X-100. All the steps described below, except step 4, were carried out in buffers containing 0.05% (v/v) Triton X-100. The extracted PBP6 was isolated from the supernatant fraction by the following procedure: (1) filtration on a Pharmacia Q-Sepharose Fast Flow column, elution with an NaCl concentration gradient in 25 mM-Bistris/HCl buffer, pH 6.3, and concentration by ultrafiltration on an Amicon YM membrane; (2) filtration on an ampicillin-Sepharose column [11], elution with a 0.5 M-Tris/HCl buffer, pH 8.0, containing 1 M-hydroxylamine and dialysis against 5 mM-sodium phosphate buffer, pH 7.0; (3) reaction of the PBPs with radioactive penicillin, filtration on a Mono Q HR5/5 column, elution with an NaCl concentration gradient in 25 mM-Bistris/HCl buffer, pH 6.3, and concentration by filtration on a Centricon 10 filter; (4) separation of the radioactive

PBPs by SDS/polyacrylamide-gel electrophoresis and electroblotting of the gel on a Millipore Immobilon transfer membrane as described above. The benzyl[^{14}C]-penicilloyl-PBP6 band, lightly stained with Coomassie Blue, was cut and used for amino acid sequencing.

Sequence of the *N*-terminal region of PBP6 and tPBP6*

The amino acid sequence was determined up to residue 50 for the purified tPBP6* (two runs) and up to residue 20 for the purified electroblotted PBP6. Both PBPs had exactly the same 20-residue *N*-terminal region (Fig. 5). Electrofocusing of tPBP6* (pH gradient from 2 to 5.5) yielded two polypeptides with isoelectric points of 4.08 and 4.15 occurring in a molar ratio of 1:2. Since tPBP6* had one single *N*-terminal amino acid, it was concluded that these two polypeptides had slightly different *C*-termini.

DISCUSSION

Conversion of the membrane-bound 43000-M_r PBP6 of *Enterococcus hirae* into the water-soluble 30000-M_r tPBP6* by trypsin action is made by loss of a 13000-M_r polypeptide segment without alteration of the *N*-terminal region of the protein but with generation of a ragged *C*-terminal, as evidenced by the occurrence of two tPBP6* species with slightly different isoelectric points. Since tPBP6* performs DD-carboxypeptidase activity and binds penicillin, it is concluded that the ~ 130-residue segment that is eliminated from the *C*-terminal region of the protein exerts little influence, if any, on the polypeptide scaffolding of the protein and the conformation of the active site. The role of this long *C*-terminal extension is unknown except that the last 15–20-residue stretch anchors the protein into the plasma membrane. Loss of this latter small segment appears to be sufficient to convert PBP6 into a 41000-M_r water-soluble derivative tPBP6a.

It has been proposed [3] that the penicillin-binding domain of the active-site-serine penicillin-recognizing enzymes (β-lactamases and DD-peptidases/PBPs) starts approx. 60 residues upstream of the conserved tetrad Ser-Xaa-Xaa-Lys, where Ser is the active-site serine residue, and terminates approx. 60 residues downstream of the conserved triad His-Thr-Gly, Lys-Thr-Gly or Lys-Ser-Gly. On the basis of this definition, the *Escherichia coli* and *Bacillus subtilis* DD-peptidases/PBPs5 also possess downstream of their penicillin-binding domain a long *C*-terminal stretch of about 105 and 125 residues respectively, the last 15–20 residues of which serve as membrane-anchoring device [4–6].

The active site of the *Enterococcus hirae* PBP6 has not been identified chemically. But on the basis of the high degree of homology that exists between the amino terminal region of PBP6 and the corresponding region of both *Escherichia coli* [20] and *Bacillus subtilis* [21] PBPs5, it can be safely concluded that Ser-35 of the tetrad Ser^{35}-Ile-(Thr)-Lys^{38} is the active-site serine residue of the enterococcal PBP. The 50-residue *N*-terminal region of the *Enterococcus hirae* PBP6 possesses five lysine residues, at positions 5, 9, 20, 29 and 38. The fact that the adjacent peptide bonds escape trypsin cleavage suggests that these residues belong to highly organized structured elements. The corresponding *N*-terminal region of the β-lactamases of class A consists of one α-helix followed by two β-strands and then by a second α-helix. The active-site serine residue is located at the *N*-terminal end of this latter α-helix [22,23]. The same disposition of secondary structures is thought to occur in the low-M_r DD-peptidases/PBPs [3,24].

Both the *Enterococcus hirae* PBP6 [9] and the *Escherichia coli* PBP5 [25] are susceptible to inactivation by thiol-blocking reagents. Inactivation of the *Escherichia coli* PBP5 is by blocking of the thiol group of Cys-115, which is located 70 residues downstream of the active-site serine residue. The position of the susceptible cysteine residue in the enterococcal PBP6 remains unknown, but it is located downstream of the 50-residue *N*-terminal region.

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