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Mode of membrane insertion and sequence of a 32-amino acid peptide stretch of the penicillin-binding protein 4 of *Enterococcus hirae*

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1. SUMMARY

Analysis of water-soluble derivatives of the *Enterococcus hirae* 75-kDa membrane-bound penicillin-binding protein 4 (PBP4) has yielded the amino acid sequence of a 32-amino acid polypeptide stretch. This peptide is similar to peptide segments known to occur in the N-terminal domain of high-M_r PBPs of class B. The *E. hirae* PBP4 probably belongs to the same class. It is anchored in the membrane at the N-terminus of the polypeptide chain.

2. INTRODUCTION

The monofunctional low-M_r penicillin-binding proteins (PBPs) and bifunctional high-M_r PBPs of class A and B are involved, in one way or another, in the assembly of the bacterial-wall peptidoglycan [1]. These serine DD-peptidases are the targets of penicillin which immobilizes their essential serine in the form of a stable penicilloyl derivative. The low-M_r PBPs are synthesized with a cleavable signal peptide. Some of them do not possess transmembrane segments: they are excreted in the culture medium or they interact with the membrane surface. Most of the low-M_r PBPs possess a 50- to 100-amino acid C-terminal extension, the end of which contains a signal-like peptide segment that serves as membrane anchor. The high-M_r PBPs of class A and B have an N-terminal domain several hundred amino acids long that is linked on its carbonyl side to the penicillin-binding domain per se. These PBPs are not synthesized as preproteins but have a highly hydrophobic region close to the N-terminus of the N-terminal domain, that acts as a non-cleaved signal-like segment both to translocate the bulk of the protein and to anchor it in the plasma membrane. The N-terminal domain of the *Es-*

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cherichia coli high-M_r PBPs 1A and 1B is a penicillin-insensitive transglycosylase that catalyses glycan chain elongation and the C-terminal penicillin-binding domain is a transpeptidase that catalyses peptidoglycan crosslinking. The high-M_r PBPs of class B also catalyse transpeptidation reactions but the question of which enzymatic function their N-terminal domain performs is still a matter of controversy. These domains possess several conserved amino acid groupings not found in the transglycosylase domain of the *E. coli* PBPs 1A and 1B of class A and these groupings might be the stamp of an active site whose function would be different from that of a transglycosylase [2].

Enterococcus hirae possesses, bound to the membrane, one PBP1 of approx. 120 kDa, a cluster of PBPs, 2–5 of approx. 87–70 kDa and one PBP6 of 43 kDa [3]. On the basis of their varying susceptibility to trypsin, PBPs 3, 5 and 6 were selectively converted into water-soluble derivatives and studied extensively [2,4,5]. In the course of these studies, it was also observed that (i) the 75-kDa PBP4 had a short half-life and spontaneously underwent conversion into a water-soluble 99-kDa PBP4* [3]; and (ii) trypsin considerably increased the rate of breakdown of PBP4 giving rise to a reaction product, t-PBP4*, having the same apparent molecular mass as PBP4* [5]. As shown below, PBP4* and t-PBP4* have been purified to almost protein homogeneity. Establishment of their N-terminal amino acid sequence has lead to the conclusion that PBP4 is another member of the class B high-M_r PBPs.

3. MATERIALS AND METHODS

E. hirae strains ATCC 9790 and R40 were described before [4,5]. *E. hirae* strain S197 (isolated from horse intestine) was a gift from Dr. L. Devriese, University of Ghent, Belgium. They were grown in SB medium. The isolated membranes (10–15 mg total protein ml^{-1}) were stored frozen in 40 mM-sodium phosphate pH 7.0 containing 1 mM $MgCl_2$ and 5% (v/v) glycerol. The PBPs were radioactively labelled by benzyl[^{14}C]penicillin under saturation conditions (50 μM penicillin; 30 min; 37 °C) and estimated by SDS-PAGE and fluorography of the gels, using the 37 500-M_r *Streptomyces* R61 low-M_r PBP as standard. The penicillin concentration required to achieve half-saturation was determined by a 5-min incubation at 37 °C. The techniques used were described previously [2–5].

Trypsin (type XI) and trypsin soybean inhibitor (type I-S) were from Sigma Chemie, Deisenhofen, F.R.G. Proteins were estimated as described before [3]. Isoelectrofocusing was performed on a FBE-3000 LKB apparatus at pH 4–6.5 Pharmalyte according to Pharmacia's instructions. PBP4* and t-PBP4* (separated on SDS-PAGE and electroblotted on Millipore Immobilon membranes) were submitted to automatic amino acid microsequencing on a 477A pulsed liquid sequenator with on-line analysis of the amino acid phenylthiohydantoin derivatives using a 120A analyser (Applied Biosystems, Forster City, CA) [2,4].

4. RESULTS AND DISCUSSION

E. hirae ATCC 9790, S197 and R40 (minimum penicillin inhibitory concentrations: 3.6 μM, 80 μM and 250 μM, respectively) were grown to late-exponential phase. All the culture supernatants (concentrated 50-fold by ultrafiltration) contained one single PBP (yields: 6–30 μg (culture 1)$^{-1}$). This PBP was indistinguishable from the PBP4* and t-PBP4* described below with respect to molecular mass, isoelectric point and sensitivity to penicillin. Half-saturation was achieved by 2–3 μM penicillin after 5-min incubation at 37 °C.

Membranes from *E. hirae* ATCC 9790 (1 g total protein; 1.6 mg PBP4) suspended in 80 ml of 1% (v/v) glycerol, 1 mM $MgCl_2$, 40 mM sodium phosphate, pH 7.0, were supplemented with 60 ml of 60 mM Tris · HCl, pH 8.0, incubated for 2 h at 37 °C and centrifuged at 105 000 × g for 1 h. The released PBP4* was purified in two steps using a FPLC system (Pharmacia) on the basis of its benzyl[^{14}C]penicillin-binding capacity: (1) the supernatant fraction was filtered on a 60-ml Q-Sepharose FF column equilibrated against 25-mM

Bistris · HCl, pH 6.3, and the adsorbed PBP4* was eluted between 80–100 mM NaCl using a 0–1 M-NaCl gradient in the Bistris · HCl buffer; (2) PBP4* (after concentration of the active fractions by ultrafiltration and dialysis against the Bistris · HCl buffer) was submitted to chromatofocusing (2 runs of 4 ml each) using a 4-ml MonoP HR 5/20 column and a pH gradient made with Polybuffer 74, pH 4.0 (Pharmacia). The pH of the collected fractions was readjusted by adding an equal volume of 50 mM sodium phosphate, pH 7.0 PBP4* eluted as an almost perfectly symmetrical peak: p*I*, 4.9; estimated purity, 95–100%; total yield, 350 μg; enrichment, 600-fold. Fig. 1 illustrates the purification procedure.

Membranes of *E. hirae* R40 (1 g total protein; 1.1 mg PBP4) suspended in 70 ml of 1% (v/v) glycerol, 1 mM $MgCl_2$, 40 mM sodium phosphate, pH 6.5, were incubated with 2.2 μg trypsin for 15 min at 37 °C, supplemented with 4,4 μg soybean inhibitor, and centrifuged at 105000 × *g* for 1 h. The released tPBP4* was purified in three steps: (1) the supernatant fraction was filtered on the 60-ml Q-Sepharose FF column as before and the adsorbed tPBP4* was eluted between 0.14 and 0.18 M NaCl concentrations using a 0–1 M NaCl concentration gradient made in 25

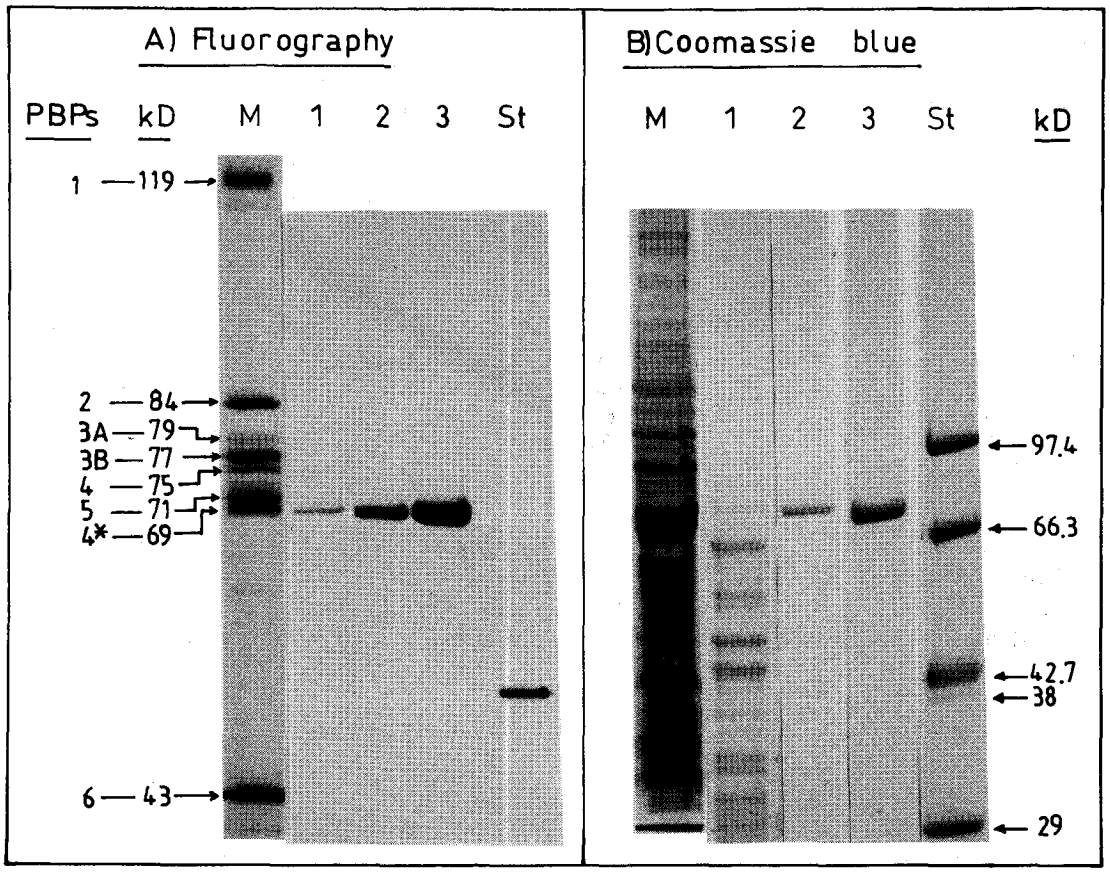

Fig. 1. SDS-PAGE analysis of PBP4* at each step of the purification. **A**), Fluorography; **B**), Coomassie blue staining. **M**, membranes of *E. hirae* ATCC 9790 (200 μg proteins); **1** supernatant fraction (80 μg proteins) of membranes incubated for 2 h at 37 °C and pH 8.0; **2** sample after filtration on Q-Sepharose FF column (15 μg proteins); **3** sample after chromatofocusing (7.5 μg proteins); St, standard proteins of decreasing molecular masses: phosphorylase B, bovine serum albumin, *Streptomyces* R61 low-M_r PBP and carbonic anhydrase. The samples were labelled with benzyl[^{14}C]penicillin under saturating conditions before SDS-PAGE. M samples were run under different conditions than those used for the other samples (7.2% and 8.5% acrylamide, respectively).

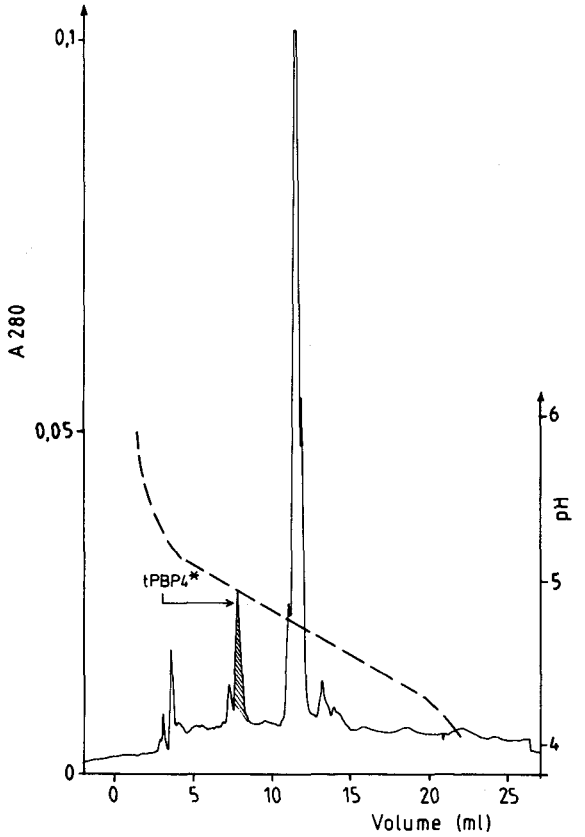

Fig. 2. Final purification of t-PBP4* of E. hirae R40 by chromatofocusing on MonoP HR 5/20. The column was equilibrated against 25 mM Bistris·HCl pH 6.3. ------, pH gradient made with polybuffer 74 pH 4.0 at a flow rate of 0.5 ml min^{-1}; ———, A280; shaded peak, t-PBP4*.

mM Tris-borate, pH 8.0, (the borate buffer protected t-PBP4* against further proteolytic degradation); (2) the t-PBP4*-containing fractions were concentrated by ultrafiltration and filtered on a 100-ml Superose 12 prep-grade column in 25 mM Tris-borate, pH 8.0; (3) the tPBP4* was finally purified by chromatofocusing as described above (Fig. 2): p*I*, 4.85; estimated purity, 90%; yield, 180 μg; enrichment, 820-fold.

The sequences of the 24- and 25-amino acid N-terminal region of t-PBP4* and PBP4* overlapped (Fig. 3) so that, altogether, they gave the sequence of a 32-amino acid peptide stretch. These partial structural data led to the following conclusions: t-PBP4* originates by trypsin cleavage of a K-S peptide bond which occurs at positions 8–9 in PBP4*. The 32-amino acid peptide is similar to (Fig. 3) peptide segments known to occur within the N-terminal domain of high-M_r PBPs of class B, particularly with the L58I87 peptide segment of *Streptococcus pneumoniae* PBP2X (13 strict identities) [6]. Note that the 12-amino acid C-terminal region of the peptides under comparison (Fig. 3) is one of the conserved, typical boxes of the high-M_r PBPs of class B [1,2]. Finally, conversion of the *E. hirae* membrane-bound, 75-kDa, high-M_r PBP4 into the water-soluble 69-kDa PBP4* or 69-kDa t-PBP4*, whether it occurs spontaneously during cell growth and storage of the membranes or by trypsin cleavage, is made by elimination of an approx 60-amino acid amino-terminal segment, containing the membrane anchor of the protein.

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E.hirae	PBP4*	:	-GAMEAKVKSTLTLTIQTSXPRGMIY-			
	t-PBP4*	:	-STLTLTIQTSXPRGMIYDNTGAPL-			
S.pneumoniae	PBP2X	:	58-LAKEAKKVHQTTRTVPAK--RGTIYDRNGVPI-	87	[6]	
S.aureus	PBP2'	:	133-IPGMQKDQSIHIENLKSE--RGKILDRNNVEL-	162	[7]	
E.hirae	PBP5	:	155-FPGMEDTDKVSLTSEEAK--RGDILDRNGKKL-	184	[*]	
E.coli	PBP2	:	50-YQTRSNENRIKLVPIAPS--RGIIYDRNGIPL-	79	[8]	
E.coli	PBP3	:	53-LVKEGDMRSLRVQQVSTS--RGMITDRSGRPL-	82	[9]	

Fig. 3. N-terminal amino acid sequence of PBP4* and t-PBP4* and comparison with peptide segments of several high-M_r PBPs of class B. •, strict identities. The boxed dodecapeptide is a highly conserved amino acid grouping characteristic of the high-M_r PBPs of class B.

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