Analysis of the membrane-anchoring properties of the putative amphiphilic α-helical anchor at the C-terminus of *Escherichia coli* PBP 6

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Penicillin-binding protein (PBP) 6 is anchored to the periplasmic face of the *Escherichia coli* inner membrane. Analysis of the C-terminal 20 amino acids of PBP 6 implies the presence of a C-terminal amphiphilic α-helical anchor comparable to that of PBP 5. A C-terminal deletion of PBP 6 was constructed; it resulted in the release of the protein from the inner membrane into the periplasm, thus confirming that this region is essential for anchoring. Treatment of *E. coli* K12 membrane vesicles with various reagents was used to probe the membrane-binding characteristics of both PBP 5 and PBP 6. The results indicate that, although the strength of membrane anchoring of PBP 6 is weaker than that of PBP 5, both modes of anchoring involve a large hydrophobic element and have similar membrane-binding characteristics. This is in agreement with the hypothesis that both proteins exhibit the same novel method of anchoring.

**Keywords:** penicillin-binding protein, *Escherichia coli*, membrane anchoring, α-helical anchor

**INTRODUCTION**

Membrane anchoring of proteins is usually achieved via a relatively long sequence of 15–25 hydrophobic amino acids. These anchoring sequences have no apparent sequence specificity, and synthetic amino acid sequences of high hydrophobicity are capable of acting as membrane anchors (Davis & Model, 1985). *Escherichia coli* penicillin-binding protein (PBP) 5, encoded by *dacA*, and PBP 6, encoded by *dacC*, are involved in peptidoglycan biosynthesis. Analysis of these two proteins has failed to identify either a conventional hydrophobic membrane-anchor sequence or any residues indicative of anchoring via covalent modifications, yet both these proteins are located on the periplasmic face of the inner membrane (Pratt *et al.*, 1986). Deletion experiments previously showed that the C-terminal 18 amino acids of PBP 5 are essential for the anchoring of this protein to the membrane (Jackson & Pratt, 1987, 1988). This membrane-binding domain is predicted to form an amphiphilic α-helix (Fig. 1a) (Jackson & Pratt, 1987) which the hydrophobic-moment plot (Eisenberg *et al.*, 1982, 1984; Fig. 1b) shows to be surface active and capable of membrane interactions (Phoenix, 1990).

Recently, the C-terminal 17 amino acids of PBP 6 have been shown to be essential for membrane interaction (van der Linden *et al.*, 1992). PBP 6 is 60% homologous with PBP 5 and the C-terminus also has the potential to form an amphiphilic α-helix (Fig. 1a). However, the PBP 6 helix possesses a lower level of amphiphilicity than the PBP 5 helical anchor, and the hydrophobic-moment plot indicates that in the case of PBP 6 the C-terminus is only marginally surface active (Phoenix, 1993). This poses the question of whether the interactions involved in the membrane association of PBP 6 are the same as, or different from, those of PBP 5.

In this paper we report the construction of a C-terminal deletion of PBP 6 which is exported to the periplasmic space. In addition, membrane vesicles carrying PBP 6 have been treated with various reagents to establish the nature of the interactions of PBP 6 with the membrane and the degree to which these interactions are comparable.

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with those of wild-type PBP 5 (Phoenix & Pratt, 1990) and with a mutant PBP 5 in which \( \alpha \)-helix formation in the C-terminal anchor has been destabilized by the presence of a proline residue, His\(^{39} \) Pro (PBP 5\(^* \)) (Jackson & Pratt, 1987).

**METHODS**

**Bacterial strains and growth conditions.** The *E. coli* strains used (Table 1) were grown in nutrient broth (Oxoid), K medium or K medium minus Casamino acids at 37 °C with aeration. K medium is M9 salts (6 g anhydrous Na\(_2\)HPO\(_4\) 2\( H \)\(_2\)O, 0.5 g NaCl, 0.5 g KH\(_2\)PO\(_4\), 1.0 g NH\(_4\)Cl) supplemented with 0.8% glucose, 0.2% Casamino acids, 1 mM MgSO\(_4\), 1 mM CaCl\(_2\), 10 μg thiamin ml\(^{-1}\). For plasmid-bearing strains, ampicillin, kanamycin, tetracycline and spectinomycin were added to final concentrations of 50, 25, 10 and 100 μg ml\(^{-1}\), respectively.

**DNA manipulations.** Restriction endonucleases, T4 DNA ligase and dNTPs were supplied by Boehringer and used as instructed by the manufacturer. Routine DNA manipulations were carried out as described by Sambrook *et al.* (1989).

**Expression of genes under the control of the lacUV5 and T7 promoter.** Cells harbouring plasmids or \( \lambda \) lysogens carrying genes under *lac* control were grown overnight in K medium supplemented with the appropriate antibiotic, then diluted 1:50 in K medium minus Casamino acids and grown to OD\(_{660}\) 0.5 at 37 °C with aeration. IPTG was added to a final concentration of 0.4 mM and incubation was continued for 2 h.

**Preparation of periplasmic fractions by osmotic shock.** IPTG-induced cells (1 ml) were harvested in a microcentrifuge, then resuspended in 125 μl of 20% (w/v) sucrose, 30 mM Teis, 1 mM EDTA, pH 8.0, and incubated at room temperature for 3 min. The cells were reharvested, supernatants collected (SN1), and pellets resuspended in 312 μl ice-cold distilled water and shaken on ice for 10 min. After centrifugation in a microcentrifuge, the supernatants (SN2, corresponding to the periplasm) were collected. The pellets were briefly washed in 4 M urea, pH 6.0, and repelleted to give pellet (P) and wash (W) fractions. Proteins in the SN1, SN2 and W fractions were precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 15% (w/v). After 1 h on ice and microcentrifugation, precipitates were resuspended in 10 mM sodium phosphate buffer, pH 7.2, containing 6 mM PMSF. These fractions, together with the pellets (P), were then analysed by SDS-PAGE and Western blotting.

**Preparation of membrane fragments by osmotic lysis.** Membrane fractions were prepared from 11 of culture (OD\(_{660}\) 0.7–0.9) by osmotic lysis (Osborn *et al.*, 1972). Membranes pelleted from 50 ml of the original culture were resuspended in 1 ml of each extraction buffer and incubated on ice for 1 h. Extraction buffers comprised 10 mM sodium phosphate buffer containing the required concentration of urea, NaCl or sodium thiocyanate (NaSCN) and were adjusted to the required pH by the addition of NaOH or HCl. Incubated membranes were then pelleted by centrifugation at 105000 g for 1 h at 4 °C and TCA was added to the supernatants to a final concentration of 10% (w/v); 10 μl (0.2 OD\(_{660}\) units) of sonicated SP1048 cells was added to aid precipitation (Phoenix & Pratt, 1990). Pellets and supernatants were then analysed by SDS-PAGE and Western blotting.

**SDS-PAGE.** The procedure was essentially that of Laemmli (1970), using a 13% (w/v) acrylamide resolving gel with a 7% (w/v) stacking gel. The acrylamide monomer:dimer ratio was 44:0.8.

**Western blotting.** Western blotting using rabbit anti-PBP 6 (or anti-PBP 5) serum was carried out as described by Towbin *et al.* (1979) except that the substrate used was 3,4,3',4',-tetramethylphenyldihydrochloride. Quantification of PBP 5 in

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotype</th>
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<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
</tr>
<tr>
<td>JM103</td>
<td>lac pro thi str.A end.A1 dcmB15 hisR4 supE F'(traD36 proAB lacI(ZAM15)</td>
</tr>
<tr>
<td>SP1048</td>
<td>his tix supF ΔlacX74 rrl::Tn10 ΔlacC1 ΔlacA::Tn5</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>hsd gal ( \gamma ) m( \gamma ) (DE3) ( \lambda ) carrying lac UV5 T7 gene 1</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>pBS102</td>
<td>Kan' PBP 6</td>
</tr>
<tr>
<td>pSP2</td>
<td>Amp' PBP 6</td>
</tr>
<tr>
<td>pLysS</td>
<td>Cm' T7 lysozyme</td>
</tr>
<tr>
<td>pLG517</td>
<td>Tet' PBP 6</td>
</tr>
<tr>
<td>pLG364</td>
<td>Amp' PBP 5</td>
</tr>
<tr>
<td>pMJ117</td>
<td>Amp' PBP 5* (His(^{39} ) Pro)</td>
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Anchoring of *E. coli* PBP 6

**RESULTS**

**Construction of a defined C-terminal deletion of dacC and expression of the PBP 6 C-terminal deletion in vivo**

A defined deletion of *dacC* was constructed by taking advantage of one of the *Pvu*II sites in the coding region of *dacC* in pBS102 (Fig. 2). The *Eco*RI/*Pvu*II fragment from pBS102 was cloned into pGEM3 (Promega Biotech), which had been digested with *Eco*RI and *Sma*I, to place the truncated *dacC* gene under the control of a T7 promoter. The omega fragment of pH45Ω (Prentki & Krisch, 1984) was then cloned as a *Sma*I fragment into the *Hinc*II site of the pGEM3 downstream of *dacC*. The resultant plasmid was designated pSP2 (Fig. 2).

![Fig. 2. Plasmids pBS102 and pSP2 encoding *dacC* (PBP 6) and a C-terminal deletion of *dacC* (*dacC*'), respectively. *spc*, *amp* and *kan* represent spectinomycin, ampicillin and kanamycin resistance genes, respectively.](image)

The truncated *dacC* gene encoded by pSP2 was expressed in the T7 polymerase expression system devised by Studier & Moffatt (1986). pSP2 was transformed into *E. coli* BL21(DE3)(pLysS). BL21(DE3) is a λ lysogen containing a single copy of T7 gene 1, which encodes T7 RNA polymerase, under the control of the *lacUV5* promoter. The *lac* promoter may be induced by the addition of IPTG to produce T7 RNA polymerase which in turn initiates high-level expression of the target gene encoded downstream of the T7 promoter. As there may be low-level expression of T7 RNA polymerase from the uninduced *lacUV5* promoter, pLysS was included, as the *lys* gene product (T7 lysozyme) inhibits this low level of T7 RNA polymerase. This precaution was taken in view of the possible lethality of over-expressed PBP 6 and PBP 6 truncates.

*E. coli* JM103(pBS102) and BL21(DE3)(pLysS)(pSP2) were incubated with 0.4 mM IPTG for 2 h to induce expression of wild-type PBP 6 and the truncated PBP 6, respectively. Cells were then subjected to osmotic shock and a brief wash in 4 M urea, pH 6.0, to yield periplasmic and wash fractions, respectively, and a pellet containing membrane and cytoplasmic proteins. The fractions were then analysed by Western blotting and densitometry. In the case of wild-type PBP 6 the expected 40 kDa protein was located exclusively in the pellet fraction (Fig. 3). In the case of the truncated PBP 6 (encoded by pSP2) bands are visible at 33 and 29 kDa. The 33 kDa species could be the precursor (with the N-terminal signal sequence still attached) and the 29 kDa species could be the processed form. Alternatively, the 33 kDa protein could be the mature form and the 29 kDa protein a breakdown product. The predicted size of the truncated PBP 6 lacking the last 74 amino acids and its signal sequence is 32 kDa, and since both proteins are released into the...
Fig. 4. (a) Effect of pH on membrane association. Membrane fragments were produced by osmotic lysis and incubated in 10 mM sodium phosphate buffer at varying pH. (b) Effect of pH and 4M urea on membrane anchoring. Membrane fragments were produced by osmotic lysis and incubated in 4M urea at varying pH. (c) Effect of 2M salt on membrane anchoring. Membrane fragments were produced by osmotic lysis and incubated in either 2M NaCl or 2M sodium thiocyanate at pH 7.0. The percentage of the total protein released from the membrane was determined by Western blot and densitometric analysis in (a-c).

**DISCUSSION**

PBP 5 and PBP 6 have been shown to be approximately 60% homologous, and deletion experiments have localized the membrane-binding domain of both proteins at the C-terminus. These anchoring domains both appear to have the potential to form amphiphilic α-helices (Fig. 1a). The importance of the amphiphilicity in membrane binding is presumably the reason why the amphiphilic nature of these regions has been preserved even where amino acid substitutions have occurred during evolutionary divergence. Although segregation of the hydro-
phobic and hydrophilic amino acids has been preserved, the PBP 6 anchor is only marginally membrane interactive as characterized by the hydrophobic-moment plot of Eisenberg et al. (1984) (Fig. 1b). This predicted lower level of PBP 6 membrane interaction is clearly reflected in the pH wash experiments, where high levels of PBP 6 are extracted in comparison with PBP 5. However, although the levels of extraction varied, the trends observed were similar for PBP 5 and PBP 6, with the protein extractability increasing with increasing pH, thus indicating that both proteins are probably anchored by a similar mechanism.

The accessibility of the anchor to the aqueous environment was determined by washing the membranes with 4 M urea. For both PBP 5 and PBP 6 there was a urea-inaccessible form of the anchor which resisted extraction and a urea-accessible form which was extracted. We predict that the inaccessible form of the PBP 5 and PBP 6 anchors occur due to a stronger level of receptor binding or due to the insertion of the anchor into the bilayer, possibly via the formation of a protein complex (Phoenix & Pratt, 1990). The wash data indicate that compared to PBP 5 a greater proportion of PBP 6 is in a urea-accessible form and, as PBP 6 falls further from the surface-active class of peptides, this may indicate a decreased ability of the protein to insert into the membrane. Indeed, PBP 6 shows a level of extraction comparable to PBP 5*. This mutant protein has been postulated to undergo decreased levels of membrane interaction due to the structural changes induced by the His\textsuperscript{187}Pro change within the anchor region (Phoenix & Pratt, 1990).

From the above data we conclude that PBP 5 and PBP 6 are both likely to be anchored by C-terminal amphiphilic \(\alpha\)-helices and both possess similar membrane-binding characteristics. The decreased surface activity of PBP 6 is reflected by an increase in the urea-accessible form of this protein and may indicate that in comparison with PBP 5 this anchor has less ability to insert into the bilayer.

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REFERENCES


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