Localization of a putative second membrane association site in penicillin-binding protein 1B of *Escherichia coli*

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We have shown previously that the periplasmic domain of penicillin-binding protein 1B (PBP $1B_{per}$; residues 90–844) from *Escherichia coli* is insoluble in the absence of detergents, and can be reconstituted into liposomes [Nicholas, Lamson and Schultz (1993) J. Biol. Chem. **268**, 5632–5641]. These data suggested that native PBP 1B contains a membrane association site in addition to its N-terminal transmembrane anchor. We have studied the membrane topology of PBP 1B in greater detail by assessing detergent binding and solubility in the absence of detergents for PBP $1B_{per}$ and a set of proteolytic fragments of PBP 1B. PBP $1B_{per}$ was shown by three independent methods to bind to detergent micelles, which strongly suggests that the periplasmic domain interacts with the hydrophobic milieu of membrane bilayers. Digestion with high weight ratios of thrombin of purified PBP 1B containing an engineered thrombin cleavage site on the periplasmic side of the transmembrane anchor generated four fragments in addition to PBP $1B_{per}$ that varied in size from 71 to 48 kDa. In contrast to PBP $1B_{per}$, all fragments of 67 kDa and smaller were eluted from a gel-filtration column in the absence of detergents and did not bind to detergent micelles. The N-terminal sequences of the four fragments were determined, allowing the cleavage sites to be located in the primary sequence of PBP 1B. These data localize the membrane association site of PBP 1B to a region comprising the first 163 amino acids of the periplasmic domain, which falls within the putative transglycosylase domain. Lipid modification does not appear to be the mechanism by which PBP $1B_{per}$ associates with membranes.

INTRODUCTION

Penicillin-binding proteins (PBPs) are membrane-bound enzymes that catalyse the final stages of bacterial cell wall synthesis [1,2]. Penicillin and other β-lactam antibiotics exert their lethal action by reacting covalently with PBPs to form a stable acyl-enzyme complex. This inhibition prevents the cross-linking of the peptide chains and ultimately leads to cell death [3]. *Escherichia coli* contains eight PBPs, which can be divided into two groups based on biochemical and genetic evidence. The high-molecular-mass PBPs (PBPs 1A, 1B, 2 and 3) are essential for cell viability, and are thought to be bifunctional enzymes that catalyse both the polymerization of the glycan chains (transglycosylation) and the cross-linking of the peptide chains (transpeptidation) during cell wall synthesis [4–7]. The low-molecular-mass PBPs (PBPs 4, 5, 6 and 7) are not essential for cell viability and appear to play a lesser role in peptidoglycan synthesis (reviewed in [1]).

Because of the interest in obtaining the three-dimensional structures of PBPs, the membrane topologies of these enzymes have been studied extensively in order to express soluble forms that can be readily crystallized. It is well documented that PBPs 5 and 6 have a C-terminal membrane anchor, since removal of the last 15–20 amino acids at the C-terminus results in the formation of a soluble PBP [8–10]. Conversely, the high-molecular-mass PBPs all contain near their N-terminus a single hydrophobic transmembrane anchor, which also functions as a non-cleavable signal sequence that directs the rest of the protein into the periplasm. In PBPs 2 and 3, replacement of this transmembrane region with a cleavable signal sequence from another protein, or its removal altogether, results in the formation of a soluble form of the PBP [11,12]. These results fit the generally accepted model that most PBPs are soluble proteins that are anchored to the membrane by a single stretch of hydrophobic amino acids.

The membrane topology of PBP 1B, however, appears to be more complex. PBP 1B has been shown to contain a 63-amino-acid N-terminal cytoplasmic tail, followed by a 24-amino-acid transmembrane domain and a 757-amino-acid periplasmic domain [13]. Initial attempts to construct a soluble version of PBP 1B either by fusing the cleavable signal sequence from PBP 5 to residues 90–844 or by removing the transmembrane domain were unsuccessful [14,15]. We therefore constructed a modified PBP 1B containing a genetically engineered thrombin protease cleavage site between residues 89 and 90, which is situated on the periplasmic side of the transmembrane anchor segment [15]. Thrombin digestion of the modified protein resulted in efficient cleavage of the cytoplasmic tail and transmembrane anchor regions of PBP 1B, and the properties of the purified periplasmic domain of PBP 1B (PBP $1B_{per}$) were investigated. Unexpectedly, PBP $1B_{per}$ did not elute from a gel filtration column in the absence of detergents, and it co-migrated with sealed liposomes on a sucrose gradient following detergent dialysis in the presence of asolectin lipid. These data strongly suggest that PBP $1B_{per}$ contains an additional membrane association site that is not apparent by hydropathy analysis. In this paper we show that PBP $1B_{per}$ binds detergent micelles in a manner similar to the full-length protein, which further supports the conclusion that PBP $1B_{per}$ contains a hydrophobic region that interacts with lipid bilayers. Furthermore, using both detergent binding and insolubility in the absence of detergents as indications of the

Abbreviations used: PBP, penicillin-binding protein; PBP 1B-GT/H_6, PBP 1B containing an inserted thrombin-cleavage site and a hexahistidine tag; PBP $1B_{per}$, periplasmic domain of PBP 1B (residues 90–844); CTAB, cetyltrimethylammonium bromide; sPBP, soluble PBP 5.

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presence of this hydrophobic region, we show that the region of PBP $1B_{per}$ mediating membrane association is located within a region 163 amino acids in length in the N-terminal portion of the periplasmic domain.

EXPERIMENTAL

Materials

All restriction and modifying enzymes were from Pharmacia LKB Biotechnology. [^{14}C]Penicillin G was purchased from Amersham. [^{125}I]Penicillin V was synthesized from p-trimethylstannylpenicillin V (a gift from Dr. Larry Blaszczak of Eli Lilly) as previously described [16]. Thrombin was a gift from Dr. Frank Church, Department of Pathology, University of North Carolina at Chapel Hill. The strains of *E. coli* used were MC1061 for subcloning and NK5830 (F′ *lacI*^{q} *pro* /*arg* Δlac pro_{XIII} *NalA rif*^{r} *recA*-56 *su*^{o} *ara thy*) for the expression of PBP 1B constructs.

Purification of PBP 1B-GT/H_6

The expression and purification of PBP 1B-GT/H_6 (PBP 1B containing an inserted thrombin cleavage site and a hexahistidine tag) was carried out as previously described [15], with the exception of the expression plasmid. The 5′-end of PBP 1B-GT/H_6 DNA (34 bp) was fused in-frame with the β-galactosidase gene present in pTTQ18K [17], which resulted in a 3–5-fold increase in the level of expression of the protein. PBP 1B has two translation start sites: PBP 1B-α starts at amino acid 1, whereas PBP 1B-γ starts at amino acid 46 [18]. Fusion to the vector translation start site results in the addition of 14 amino acids to the start of PBP 1B-GT/H_6; the first three amino acids, including the initiating Met, are from the β-galactosidase gene, and the remaining 11 amino acids correspond to residues 35–45 of PBP 1B-α. Because the construct has two translation initiation sites, two bands are seen in the purified protein when analysed on SDS/PAGE (see Figure 6a). The larger band corresponds to the fusion protein and the smaller band corresponds to the normal translation product starting at amino acid 46. The two bands merge into one faster migrating band on SDS/PAGE after cleavage with low weight ratios of thrombin, confirming that the heterogeneity is present at the N-terminus (results not shown).

Purification and sequencing of the thrombin fragments of PBP 1B

The thrombin fragments were isolated by HPLC on a Vydac C_4 reverse-phase column for sequencing. Following acetone precipitation, the fragments were dissolved in 88 % formic acid and loaded on to the column equilibrated in 0.1 % trifluoroacetic acid. The fragments were eluted with a linear gradient of 0–70 % acetonitrile (containing 0.1 % trifluoroacetic acid) over 60 min at a flow rate of 1 ml/min. SDS/PAGE of fractions from an analytical run indicated the composition of the eluting peaks of 214 nm absorbance. The peak fractions containing the specified fragments were pooled from several runs, dried in a Speedvac concentrator, and sequenced by Dr. David Klapper at the Protein Sequencing Core Facility, University of North Carolina at Chapel Hill.

Charge-shift electrophoresis

Protein samples (0.5–2.5 μg) were submitted to electrophoresis on a 1 % agarose gel containing 0.5 % Triton X-100 alone or in combination with 0.25 % sodium deoxycholate or 0.05 % cetyltrimethylammonium bromide (CTAB) in 50 mM glycine, 100 mM NaCl, pH 9, buffer as described by Helenius and Simons [19]. PBP 1B proteins in 0.7 % CHAPS were prepared for electrophoresis by diluting them 10-fold into the various detergent buffer combinations and concentrating the protein solution to its original volume using a Microcon-30 apparatus (Amicon). Aliquots of the reconcentrated samples were diluted into the same buffer as before, containing in addition 0.005 % Bromphenol Blue and 10 % glycerol to aid in loading the samples. The gels (20 cm × 25 cm) were run for 3 h at 110 V in a buffer-cooled apparatus set at 18 °C. Following electrophoresis, the gels were placed in fixing solution (10 % methanol/7.5 % acetic acid) for 30 min and then dried on a gel dryer under vacuum without heat for 30 min. The thin gel wafer was then stained with 0.05 % Coomassie Blue R-250 in fixing solution for 30 min and destained overnight in fixing solution. The shifts in the migration positions of each protein in the detergent mixtures were calculated using the equation:

$$\text{Shift } (\%) = [(d_x - d_t)/d_t] \times 100$$

where d_x is the distance travelled in the detergent mixture and d_t is the distance travelled in Triton X-100 alone (both relative to the origin).

Triton X-114 phase partitioning

The procedure of Bordier [20] was followed with a few modifications. Triton X-114 was precondensed with buffer containing 25 mM Tris/HCl, 500 mM NaCl, pH 8.0, after which the final concentration of the detergent was determined by measuring the absorbance at 275 nm. Protein samples (in 0.7 % CHAPS) were exchanged into 0.7 % Triton X-114 in the above buffer by diluting them 10-fold into Triton X-114 buffer and concentrating the protein solution to its original volume using a Microcon-30 apparatus. Samples (300 μl) were incubated on ice for 5 min and then transferred to 37 °C for 5 min. The cloudy sample was separated into two phases by centrifugation through a sucrose cushion. The two phases were partitioned two more times, the phases from each partitioning combined, and proteins from these samples were precipitated with acetone and submitted to SDS/PAGE as described below.

Gel filtration

To determine whether PBP $1B_{per}$ is capable of binding Triton X-100 micelles, PBP 1B-H_6 or PBP $1B_{per}$ (40 μg) was labelled with [^{125}I]penicillin V for 20 min at 30 °C. The amount of free radioligand was decreased by two cycles of dilution/concentration on a Microcon 30 concentrator and the labelled proteins (0.2 ml) were loaded on to a Sephacryl S-300 HR column (1.4 cm × 95 cm) and eluted in 50 mM MOPS, pH 7.0, 500 mM NaCl, 0.5 % Triton X-100 (MNT). Fractions of 0.75 ml were collected and counted in a γ-radiation counter. To calibrate the column, 1 mg each of thyroglobulin (660 kDa), alcohol dehydrogenase (150 kDa), BSA (70 kDa) and carbonic anhydrase (29 kDa) was dissolved in 0.2 ml of MNT and submitted to gel filtration under identical conditions as described above. An aliquot of each fraction (200 μl) was precipitated by the addition of 5 vol. of acetone, and the amount of protein was quantified by the method of Lowry et al. [21]. The identity of each protein peak was confirmed by SDS/PAGE. The void and total volumes of the column were determined by the elution positions of Blue Dextran and $K_3Fe(CN)_6$ respectively.

To determine the solubility of the thrombin fragments generated from PBP 1B-GT/H_6, the protein digest was chromatographed on a Sephacryl S-200 HR gel filtration column (1 cm × 45 cm) in 20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 10 % glycerol, with and without 0.7 % CHAPS [15]. Aliquots (100 μl)

of the indicated fractions from the gel filtration column were prepared for SDS/PAGE by precipitating with 10 % trichloroacetic acid, followed by an acetone wash to remove traces of acid. The pellets were solubilized in SDS/PAGE loading buffer, heated at 85 °C for 3 min, and submitted to electrophoresis on an SDS/8 %-polyacrylamide gel as described below.

Gel electrophoresis and fluorography

Proteins were incubated with 40 μg/ml [^{14}C]penicillin G or [^{125}I]penicillin V for 10 min at 30 °C. Following this incubation, 0.5 vol of 3 × sample buffer was added, and the mixtures were heated at 85 °C for 3 min and then submitted to electrophoresis on an SDS/8 %-polyacrylamide gel [22]. Gels submitted to fluorography were treated with En^{3}Hance (Dupont NEN), dried and exposed to Fuji X-AR film for 1–5 days. Iodinated proteins were identified after staining with Coomassie Brilliant Blue R-250, drying and exposure to Fuji X-AR film. Non-radioactive gels were stained with either Coomassie Brilliant Blue R-250 or silver [23].

RESULTS

PBP 1B$_{per}$ binds to detergent micelles

We have shown previously that PBP 1B$_{per}$ is not eluted from gel filtration columns in the absence of detergents, and can be reconstituted into lipid vesicles formed from asolectin [15]. These data strongly suggest that native PBP 1B associates with membranes *in vivo* in a bipartite manner via its transmembrane region (amino acids 64–87) and a region within its periplasmic domain. However, hydropathy analysis of PBP 1B$_{per}$ does not reveal any obvious regions that would serve to mediate membrane association. Because of the unusual nature of this association, we carried out a series of experiments to further characterize this region and to localize it in the primary sequence of the protein. We reasoned that if the interaction of PBP 1B$_{per}$ with membranes was hydrophobic in nature, then purified PBP 1B$_{per}$ should bind to detergent micelles. Thus the binding of PBP 1B$_{per}$ to detergent micelles was investigated by three independent methods.

The technique of charge-shift electrophoresis was originally developed to identify proteins that bind to detergent micelles [19]. In this method, proteins are submitted to electrophoresis in 1 % agarose gels in the presence of Triton X-100 alone (non-ionic) or Triton X-100 in combination with either sodium deoxycholate (anionic) or CTAB (cationic). Proteins that bind to detergent micelles migrate with different mobilities depending upon the ionic composition of the detergents, whereas the migration of soluble proteins that do not bind to detergent micelles is unaffected. Figure 1 shows that both PBP 1B-H$_6$ and PBP 1B$_{per}$ were charge-shifted to a significant degree in a manner expected of a detergent-binding protein. The migration position of PBP 1B-H$_6$ in the anionic and cationic detergent mixtures was shifted 165 % and −125 % respectively compared with that in the non-ionic detergent alone, whereas the same mobility shifts for PBP 1B$_{per}$ were 150 % and −215 % respectively. In contrast, BSA (a soluble protein control) was charge-shifted only slightly in the two ionic detergent mixtures (5 % and −15 % respectively). The mobility shifts for BSA may be due to ionic interactions with the detergents or detergent monomer binding [19,24].

Inspection of Figure 1 shows that whereas PBP 1B-H$_6$ and PBP 1B$_{per}$ have mobilities similar to one another in the Triton X-100 and Triton X-100/deoxycholate detergent mixtures, their migration positions in the Triton X-100/CTAB detergent mixture were significantly different. This difference can be explained by examining the difference in charge between the two proteins. The

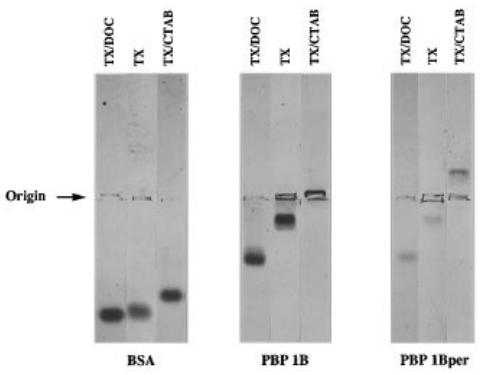

Figure 1 Charge-shift electrophoresis of PBP 1B-GT/H$_6$ and PBP 1B$_{per}$

Charge-shift electrophoresis of PBP 1B-GT/H$_6$, PBP 1B$_{per}$ and BSA on 1 % agarose gels in the presence of Triton/deoxycholate (TX/DOC; anionic), Triton (TX; non-ionic) and TX/CTAB (cationic) detergent mixtures was performed as described in the Experimental section. Aliquots (0.5–2.0 μg) of the indicated proteins were prepared in the appropriate detergent mixture, and following electrophoresis the gel was fixed and stained with Coomassie Brilliant Blue R-250. The large shifts in the mobilities of PBP 1B-GT/H$_6$ and PBP 1B$_{per}$ are indicative of an interaction of the proteins with detergent micelles, whereas the small shifts observed with BSA can be accounted for by ionic interactions or detergent monomer binding.

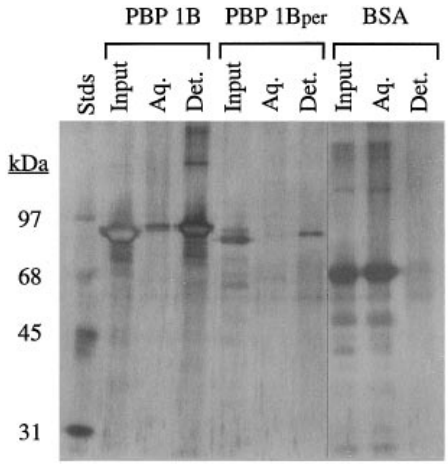

Figure 2 Triton X-114 phase partitioning of PBP 1B-H$_6$ and PBP 1B$_{per}$

The indicated proteins were transferred into Triton X-114 detergent-containing buffer and submitted to phase separation as described in the Experimental section. Aliquots of the starting material (Input), the detergent-poor aqueous phase (Aq.) and the detergent-rich phase (Det.) of each preparation were then separated on SDS/PAGE and the bands visualized by silver staining. This experiment was repeated three times with similar results. The left-hand lane contains molecular mass standards (Stds).

17-residue N-terminal cytoplasmic tail of PBP 1B-H$_6$ adjacent to the transmembrane anchor contains 10 lysine or arginine residues (in addition to six histidine residues from the hexahistidine tag). The high density of positive charges in this area may have decreased the amount of the positively charged CTAB detergent in the mixed micelle by charge repulsion, resulting in a decrease in the mobility of detergent-bound PBP 1B-H$_6$. Since PBP 1B$_{per}$ lacks these amino acids, the mixed micelle would contain a higher percentage of CTAB, resulting in a greater mobility of the protein towards the cathode.

The detergent-binding properties of PBP 1B$_{per}$ were also assessed by Triton X-114 phase partitioning, as described by

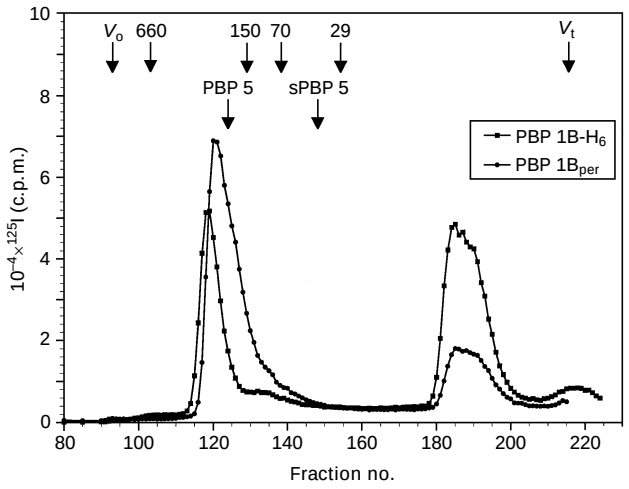

Figure 3 Gel filtration of PBP 1B-H$_6$ and PBP 1B$_{per}$ on Sephacryl S-300 in the presence of 0.5% Triton X-100

Purified PBP 1B-H$_6$ and PBP 1B$_{per}$ (40 μg each) in 0.7% CHAPS were labelled with [^{125}I]penicillin V, and the free label was removed by successive dilution/concentration cycles using a Microcon 30 ultrafiltration device. The proteins were diluted to 0.2 ml in 50 mM MOPS, 500 mM NaCl, 0.5% Triton X-100, pH 7.5 (MNT), loaded on to a Sephacryl S-300 HR column (1.4 cm × 95 cm) and eluted in MNT. Fractions of 0.75 ml were collected, and ^{125}I radioactivity was determined for each fraction in a γ-radiation counter. The column was calibrated with the standard proteins thyroglobulin (660 kDa), alcohol dehydrogenase (150 kDa), BSA (70 kDa) and carbonic anhydrase (29 kDa) under identical conditions, as shown. The elution positions of PBP 5 and sPBP 5 are also indicated (see Table 1).

Table 1 Apparent molecular masses of PBPs calculated from gel filtration in 0.5% Triton X-100

The molecular masses were calculated from the deduced amino acid sequences of the cloned genes. Apparent molecular mass values were calculated from elution positions on a calibrated Sephacryl S-300 HR column run in the presence of 0.5% Triton X-100/0.5 M NaCl as shown in Figure 3. sPBP 5 represents a soluble form of PBP 5 in which the last 17 amino acids have been replaced by six amino acids (GDPVID) added on during vector construction [8].

	Molecular mass (Da)	
Protein	Calculated	Apparent
PBP 1B-H$_6$	91216/89499*	250000
PBP 1B$_{per}$	84205	235000
sPBP 5	39774	42000
PBP 5	41311	184000

* The two values represent the sizes of the protein products from two translation initiation sites in the expression construct; see the Experimental section for details.

Bordier [20]. Triton X-114 solutions are homogeneous at low temperatures, but at higher temperatures (i.e. 37 °C) the detergent solution separates into detergent-rich and detergent-poor phases. Proteins that bind to detergent micelles partition preferentially into the detergent-rich phase, whereas soluble proteins partition into the detergent-poor phase. Consistent with their behaviour in charge-shift electrophoresis, both PBP 1B-H$_6$ and PBP 1B$_{per}$ partitioned into the detergent-rich phase (Figure 2). In contrast, BSA partitioned exclusively into the detergent-poor phase. These data again indicate that PBP 1B$_{per}$ binds to detergent micelles.

Lastly, we submitted both PBP 1B-H$_6$ and PBP 1B$_{per}$ to gel filtration on a Sephacryl S300-HR column in the presence of 0.5% Triton X-100. Because of the large size of the Triton X-100 micelle, a protein that binds a micelle of detergent will elute with an apparent molecular mass that is much larger than the molecular mass of the protein portion alone. Figure 3 shows that PBP 1B-H$_6$ and PBP 1B$_{per}$ eluted within one fraction of each other from a Sephacryl S-300 gel filtration column in the presence of 0.5% Triton X-100. The apparent molecular masses of the two proteins, which were derived from a standard curve of the elution positions of a series of soluble proteins, were 250 and 235 kDa respectively (Figure 3; Table 1). Since the molecular masses of PBP 1B-H$_6$ and PBP 1B$_{per}$ are 91216/89499 Da (due to two translation start sites; see Table 1 and the Experimental section) and 84205 Da respectively, these results indicate that both PBP 1B-H$_6$ and PBP 1B$_{per}$ bind to detergent micelles, which shifts their apparent molecular mass to a higher value. The slight difference in the elution positions of PBP 1B-H$_6$ and PBP 1B$_{per}$ can be accounted for by the presence of the transmembrane anchor and cytoplasmic tail regions of PBP 1B-H$_6$, which as mentioned above is highly charged and therefore probably in an extended conformation. This extra protein sequence, although of low molecular mass (7011 or 5294 Da for the two different translated species of PBP 1B-H$_6$), would effectively increase the Stokes radius of the detergent–PBP 1B-H$_6$ complex over that of the detergent–PBP 1B$_{per}$ complex and cause it to elute earlier from the gel filtration column.

The ability of this approach to distinguish a detergent-binding protein from a soluble protein is illustrated by the elution behaviour of PBP 5 and sPBP 5 determined under identical conditions (Figure 3; Table 1). sPBP 5 is a soluble, truncated, form of PBP 5 in which the last 17 amino acids at its C-terminus have been replaced with six non-hydrophobic vector-derived amino acids [8]. sPBP 5 (molecular mass 39774 Da) was eluted with an apparent molecular mass of 42200 Da, indicating that it does not interact with Triton X-100 micelles. In contrast, PBP 5 (molecular mass 41311 Da) migrated with an apparent molecular mass of 184000 Da, i.e. approx. 140000 Da larger than the molecular mass of the protein (Table 1). The molecular mass of the Triton X-100 micelle alone was determined by gel filtration to be 110000 Da (results not shown). All of these data strongly suggest that PBP 1B contains a detergent-binding region within the periplasmic domain that mediates membrane association.

Properties of proteolytic fragments of PBP 1B generated by thrombin

In order to localize the membrane association site within the primary sequence of the protein, we generated several proteolytic fragments of PBP 1B-GT/H$_6$ and characterized their detergent-binding and solubility properties. As shown in Figure 4(a), PBP 1B-GT/H$_6$ is cleaved into several smaller fragments at high ratios (1:5 or 1:1, w/w) of thrombin to protein. The apparent molecular masses of these fragments calculated from SDS/PAGE are 71, 67, 64 and 48 kDa respectively. Incubation of the protein with a saturating concentration of penicillin G before digestion had no effect on the fragmentation pattern or on the relative amounts of each fragment produced (results not shown), indicating that the regions around the cleavage sites were not significantly perturbed by the binding of antibiotic. When the thrombin fragments of PBP 1B-GT/H$_6$ were incubated with [^{14}C]penicillin G and submitted to SDS/PAGE and fluorography, all of the fragments were labelled (Figure 4b). Because binding activity is localized to the C-terminus, these data predict that the proteolytic clips occurred within the N-terminal region and that the fragments comprise most if not all of the C-terminal region of PBP 1B.

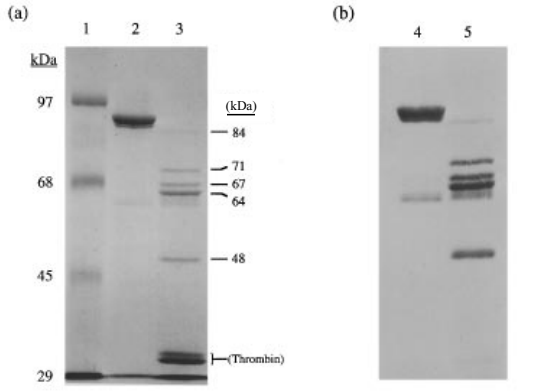

Figure 4 Cleavage of PBP 1B-GT/H_6 at a 1:1 (w/w) ratio of thrombin/protein

Purified PBP 1B-GT/H_6 (4 μg) was incubated with or without 4 μg of thrombin in 25 mM Tris/HCl, pH 8.0, 500 mM NaCl, 0.7% CHAPS for 1 h at 37 °C, and the samples were either submitted to electrophoresis directly or incubated with 10 μg/ml [^{14}C]penicillin G for an additional 10 min before electrophoresis. Prelabelling of PBP 1B-GT/H_6 prior to digestion with thrombin had no effect on the banding pattern. (**a**) Coomassie Brilliant Blue staining; (**b**) fluorography. Lane 1, molecular mass standards (values in kDa), lanes 2 and 4, no thrombin added; lanes 3 and 5, thrombin digestion. The mobilities of molecular mass standards are indicated: phosphorylase B, 97 kDa; BSA, 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa.

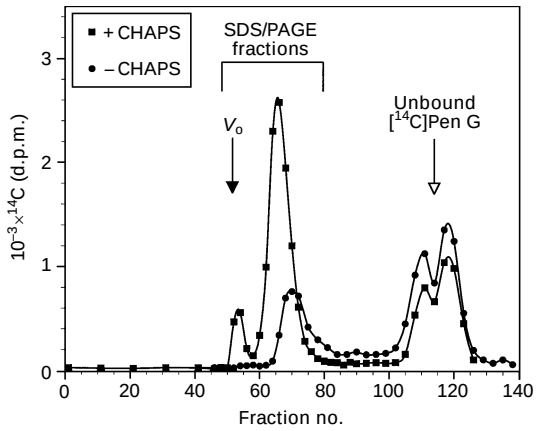

Figure 5 Gel filtration of thrombin cleavage fragments in the presence and absence of 0.7% CHAPS

PBP 1B-GT/H_6 was digested with a 1:1 (w/w) ratio of thrombin for 1 h at 37 °C. The digest was then incubated with [^{14}C]penicillin G ([^{14}C]Pen G) for 10 min at 30 °C, and identical aliquots were submitted to gel filtration in the presence and absence of detergent as described in the Experimental section. Equal aliquots (200 μl) of the indicated fractions were either assayed for radioactivity by scintillation counting or precipitated with trichloroacetic acid in preparation for SDS/PAGE, as shown in Figure 6.

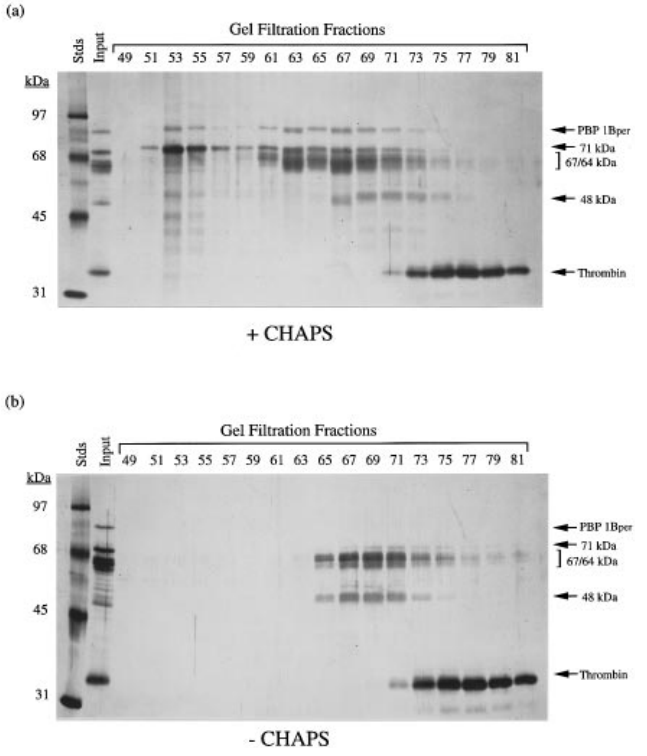

Figure 6 SDS/PAGE analysis of thrombin fragments eluted from gel filtration in the presence and absence of 0.7% CHAPS

Aliquots of the indicated fractions from the gel filtration runs in Figure 5 were submitted to SDS/PAGE on an 8% polyacrylamide gel and silver stained. (**a**) Fractions eluted in the presence of CHAPS; (**b**) fractions eluted in the absence of CHAPS. The lanes labelled 'Input' contain the starting material before gel filtration. The molecular masses of standard proteins (Stds) are shown in kDa.

The production of proteolytic fragments from purified PBP 1B-GT/H_6 provided a means to further localize the membrane association site to a region within the periplasmic domain. The thrombin fragments were labelled with [^{14}C]penicillin G, divided into two equal aliquots and chromatographed on a Sephacryl S-200 column in the presence or absence of 0.7% CHAPS. As shown in Figure 5, two peaks of radioactivity were observed in the fractions eluted in the presence of 0.7% CHAPS, whereas a single peak of reduced yield was observed when the digest was eluted in the absence of detergent. SDS/PAGE analysis of the fractions eluted in 0.7% CHAPS indicated that the first peak was composed almost entirely of the 71 kDa fragment (Figure 6a). The position of this peak corresponded to the void volume, and thus represented a higher aggregate of the 71 kDa fragment. When fractions from the main peak of radioactivity were analysed, all of the thrombin fragments were observed. However, when the same fractions from the gel filtration column eluted in the absence of detergents were analysed by SDS/PAGE, only the 67, 64 and 48 kDa fragments were clearly observed (Figure 6b). A very small amount of the 71 kDa fragment was also observed. PBP $1B_{per}$ present in the digest did not elute in the absence of CHAPS, as was observed previously [15]. These results indicate that the membrane association site is not located within the region of PBP 1B encompassing the 67, 64, or 48 kDa fragments.

To characterize the detergent-binding properties of the thrombin fragments, Triton X-114 phase partitioning of the digest was carried out. Analysis of the detergent-rich and detergent-poor phases indicated that the 67, 64 and 48 kDa fragments were fractionated entirely into the detergent-poor phase, again suggesting that these proteolytic fragments do not interact with detergent micelles (Figure 7). In contrast to the clear partitioning of the other fragments, the 71 kDa fragment was found in both phases. Since PBP $1B_{per}$ is found completely in the detergent-rich phase and the 67, 64 and 48 kDa fragments of PBP 1B are found entirely in the detergent-poor phase, these results suggest that the 71 kDa fragment may contain at least a part of the membrane

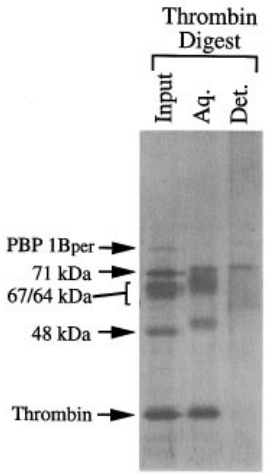

Figure 7 Triton X-114 phase partitioning of the thrombin digest of PBP 1B-GT/H_6

PBP 1B-GT/H_6 was digested for 1 h with a 1:1 (w/w) ratio of thrombin and submitted to Triton X-114 phase partitioning as described in the Experimental section and in the legend to Figure 2.

association site, which imparts partial hydrophobicity to the fragment and results in dual partitioning.

In order to definitively localize the membrane association site, the amino acid sequences of the thrombin fragments were determined (Table 2). The thrombin fragments were separated on a C_4 reverse-phase column, and four distinct peaks of 214 nm absorbance were obtained. SDS/PAGE analysis indicated that the four peaks were thrombin, the 48 kDa fragment, a mixture of the 64 and 67 kDa fragments, and the 71 kDa fragment (results not shown). Protein sequencing of the proteins revealed that the N-terminus of the 48 kDa fragment begins at residue 405 of the deduced amino acid sequence of PBP 1B, whereas the N-terminus of the 71 kDa fragment begins at residue 219 (Table 2). When the pool containing the mixture of the 64 and 67 kDa fragments was sequenced, both a major sequence, beginning at amino acid 251, and a minor sequence, beginning at amino acid 261, were obtained. The yields and N-termini of these two sequences were consistent with the staining intensities of the 64 and 67 kDa fragments in both the HPLC pool (results not shown) and the thrombin digest (Figure 4); i.e. the minor sequence was derived from the 67 kDa fragment and the major sequence was derived from the 64 kDa fragment. The predicted and apparent molecular masses of each fragment suggest that all of the fragments extend to the C-terminus (Table 2). Taken together, these data indicate that the membrane association site in PBP 1B resides between amino acids 88 and 250 (the start of the periplasmic domain and the residue preceding the N-terminus of the 67 kDa fragment respectively), a span of 163 amino acids. Since its detergent-binding and solubility properties suggest that the 71 kDa fragment contains at least part of the membrane association site, the region between the 71 and 67 kDa fragments, a span of 32 amino acids, may also be implicated in membrane binding.

Since the region delineated above does not contain any obvious hydrophobic stretches that might mediate membrane association, the possibility that PBP $1B_{per}$ is lipid-modified during transport to the periplasmic space was also investigated. *E. coli* cells constitutively overexpressing PBP 1B were labelled with either [^{3}H]palmitate or [^{3}H]myristate. SDS/PAGE and fluorographic analysis of whole cells labelled with [^{3}H]palmitate identified multiple (approx. 10–15) lower-molecular-mass proteins that presumably correspond to lipoproteins (results not shown). The same bands were labelled at slightly lower efficiency when [^{3}H]myristate was used. Even though PBP 1B was highly overproduced in these cells, labelling of PBP 1B by these lipids was not observed even upon long exposure (results not shown). When the cells were labelled with [^{3}H]penicillin G, a sizeable band corresponding to PBP 1B was observed. Thus these data indicate that lipid modification with palmitate or myristate is not a means by which PBP $1B_{per}$ associates with membranes.

DISCUSSION

We have shown previously that PBP $1B_{per}$ is insoluble in the absence of detergents and can be reconstituted into lipid vesicles [15]. In the present study we have utilized three independent methods, charge-shift electrophoresis [19], Triton X-114 phase partitioning [20] and gel filtration in 0.5% Triton X-100 [24], to show that PBP $1B_{per}$ binds to detergent micelles. Since detergent binding is hydrophobic in nature, these results strongly suggest that PBP $1B_{per}$ interacts with membranes through a hydrophobic region of the protein, and argues against any electrostatic or lipid-specific interactions, such as polar head group or oligosaccharide lipid interactions, in the reconstitution experiments with liposomes formed from asolectin. These data also suggest that the inability of PBP $1B_{per}$ to elute from a gel filtration column in the absence of detergents is due to hydrophobic

Table 2 N-terminal sequences of the thrombin fragments of PBP 1B

The N-terminal sequences of the indicated fragments are shown. X indicates that no single amino acid could be identified at that step; the amino acid in parentheses following an X is the amino acid expected at that position. Residue numbers represent the location of the N-terminal sequence in the amino acid sequence of PBP 1B-α. The 64 and 67 kDa thrombin fragments could not be resolved by reverse-phase HPLC; however, two sequences (one major and one minor sequence) were clearly discerned, and the major sequence represented the fragment (64 kDa) with the highest abundance in the mixture as determined by SDS/PAGE (see the text for details).

Proteolytic fragment	N-terminal sequence	Position in PBP 1B sequence	Calculated molecular mass (Da)
71 kDa	X(S)-G-F-P-D-L-L	219–225	68886
64/67 kDa	X(T)-V-Q-G-A-S-T (major)	261–267	64300
	X(A)-V-L-A-N-L-T (minor)	251–257	65268
48 kDa	G-G-V-I-S-P-Q	405–411	47650

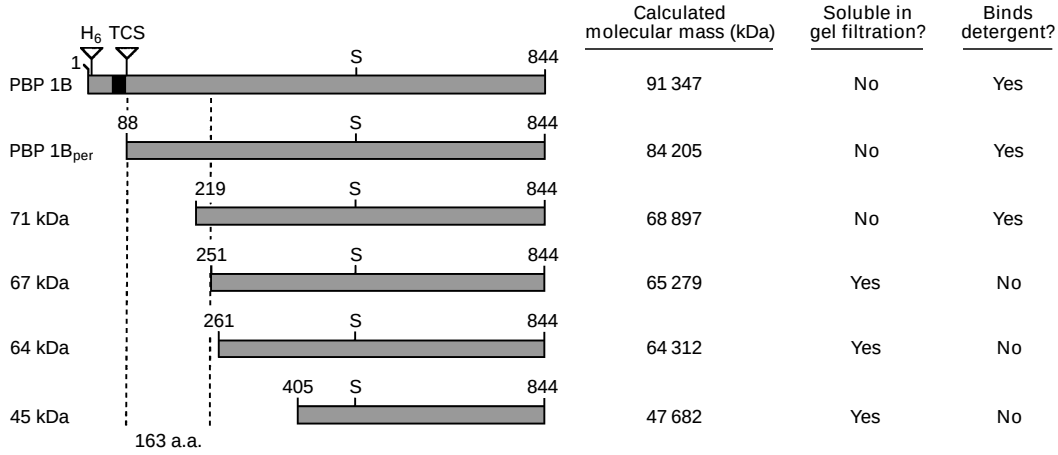

Figure 8 Schematic diagram indicating the localization of the membrane association site of PBP 1B

The lengths of the various PBP 1B proteins and thrombin fragments are aligned by their C-termini. The N-termini of the fragments were determined by amino acid sequencing (Table 2). The black rectangle in PBP 1B-γ represents the signal sequence/transmembrane anchor, and the locations of the hexahistidine tag (H_6) and the thrombin cleavage site (TCS) of PBP 1B-GT/H_6 are indicated. The broken lines demarcate the region of PBP $1B_{per}$ that mediates membrane association and detergent binding. This region corresponds to amino acids 88–250 of the PBP 1B sequence and is 163 amino acids (a.a.) in length. S designates the active-site serine residue at amino acid 510.

aggregation caused by the removal of detergent from the membrane association region. Since we have already shown that PBP $1B_{per}$ can be reconstituted into lipid vesicles, our data predict that PBP 1B exists *in vivo* with two membrane-binding domains, one encompassing the transmembrane anchor (residues 65–87) and the other within the periplasmic region.

One possible explanation for the presence of the membrane association region of PBP $1B_{per}$ is that, following thrombin cleavage, the N-terminus becomes unstable and unfolds to expose hydrophobic residues that were buried in the full-length protein. Although such a scenario cannot be conclusively ruled out, it is unlikely for several reasons. Both PBP 1B and PBP $1B_{per}$ are precipitated at identical ammonium sulphate concentrations and have the same affinity for penicillin G, properties that would probably be affected by protein rearrangements. In addition, no change in intrinsic tryptophan fluorescence is observed following thrombin cleavage, suggesting that at least large rearrangements do not occur. Finally, there is no precedent that we are aware of in which removal of an N- or C-terminal transmembrane anchor from a membrane protein causes unfolding or rearrangement of the remaining portion. Thus it is very likely that the membrane association site also exists in PBP 1B.

The presence of an additional membrane association site within PBP 1B was unexpected, especially since the other high-molecular-mass proteins of *E. coli*, PBPs 2 and 3, function with only a single transmembrane anchor. When the transmembrane anchors from these proteins are either removed or replaced with a cleavable signal sequence, the resulting protein is soluble [11,12,25]. This is clearly not the case for PBP 1B, and suggests that this structural feature may be important in its function. Indeed, it is likely that the membrane association site in PBP $1B_{per}$ accounts for the inability of this domain to fold into an active conformation (as determined by [^{14}C]penicillin G binding) when expressed in the cytoplasm [14], whereas an active protein is obtained when a cleavable signal sequence is fused to PBP $1B_{per}$ [14,15].

In order to localize the region in PBP $1B_{per}$ that mediates membrane association, we developed conditions that generated a ladder of fragments from PBP 1B-GT/H_6. We used solubility in the absence of detergents and the interaction with detergent micelles to assess which thrombin fragments contained the hydrophobic detergent-binding region. In contrast to PBP $1B_{per}$, which binds to detergent micelles (Figures 1–3) and is not eluted from a gel filtration column in the absence of detergents [15], the three smallest fragments (67, 64 and 48 kDa) were eluted from a gel filtration column in the absence of detergents (Figure 6) and partitioned entirely into the detergent-poor phase upon phase separation of the Triton X-114 solution (Figure 7). Thus the detergent-binding region (and by extrapolation the membrane association site) must be located between residues 88 (the beginning of PBP $1B_{per}$) and 250 of PBP 1B, a span of 163 amino acids. This region is indicated in Figure 8 by the broken lines.

In contrast to the results with the 67, 64 and 48 kDa fragments, the 71 kDa fragment behaved anomalously. A large proportion of the 71 kDa fragment was aggregated even in the presence of detergent, and was eluted in the void volume of a Sephacryl S-200 column. The anomalous behaviour of the 71 kDa fragment was also observed in Triton X-114 phase partitioning, where it partitioned into both the detergent-rich and detergent-poor phases. There are several possibilities that can explain these results. One possibility is that the detergent-rich and detergent-poor phases contain monomeric and aggregated forms respectively of the 71 kDa fragment. An alternative explanation is that the 71 kDa fragment contains a portion of the membrane association site, making it partially hydrophobic and leading to its partitioning into both phases. Dual partitioning in Triton X-114 solutions has recently been observed with the α subunit of transducin, which is a weakly hydrophobic, fatty acid-acylated protein from rod outer segments in the mammalian visual system [26]. Thus the behaviour of the 71 kDa fragment in Triton X-114 phase partitioning may suggest that the additional 32 amino acids in the 71 kDa fragment that are not present in the 67 kDa fragment make up at least a portion of the membrane association site.

Inspection of the sequence of the protein containing the membrane association site does not reveal any regions of significant hydrophobicity when examined by a hydropathy plot. We considered the possibility that PBP 1B was lipid-modified

within its periplasmic domain, which could have accounted for the hydrophobic properties of PBP $1B_{per}$, but neither [^{3}H]palmitate nor [^{3}H]myristate was able to metabolically label PBP 1B. Although it is possible that other lipids are involved in modifying PBP 1B, this seems unlikely. Thus it would appear that hydrophobic amino acid side chains in the protein are responsible for the membrane association of PBP $1B_{per}$. Although hydropathy analysis is useful for identifying strongly hydrophobic regions that are candidates for membrane-spanning segments, a hydrophobic 'patch' on the protein surface arising from secondary or tertiary structures formed by the three-dimensional folding of the polypeptide chain would be silent in such analyses [27]. We believe that this possibility remains the most plausible explanation of the membrane association site in PBP 1B. The most obvious secondary structures that might be implicated would be either an amphipathic β sheet or an α helix motif. For example, it is thought that the C-terminal regions of several D-alanine carboxypeptidases form amphipathic helices that mediate membrane association [28,29]. Likewise, porins interact with the outer membrane by forming a β-barrel motif [30]. Several candidate regions within the localized area defined in this paper can be identified by protein sequence analysis, but the importance of these motifs in membrane association, if any, awaits further analysis.

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