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Multiple mechanisms of membrane anchoring of *Escherichia coli* penicillin-binding proteins

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Abstract: The major penicillin-binding proteins (PBPs) of *Escherichia coli* play vital roles in cell wall biosynthesis and are located in the inner membrane. The high M_r PBPs 1A, 1B, 2 and 3 are essential bifunctional transglycosylases/transpeptidases which are thought to be type II integral inner membrane proteins with their C-terminal enzymatic domains projecting into the periplasm. The low M_r PBP4 is a DD-carboxypeptidase/endopeptidase, whereas PBPs 5 and 6 are DD-carboxypeptidases. All three low M_r PBPs act in the modification of peptidoglycan to allow expansion of the sacculus and are thought to be periplasmic proteins attached with varying affinities to the inner membrane via C-terminal amphiphilic α-helices. It is possible that the PBPs and other inner membrane proteins form a peptidoglycan synthesizing complex to coordinate their activities.

Key words: Penicillin-binding proteins; *Escherichia coli*; Membrane proteins; Anchoring mechanisms

Introduction

Gram-negative bacteria such as *Escherichia coli* are able to maintain their rod shape in changing osmotic environments because they have a rigid cell wall. This is a cylindrical sacculus of highly cross-linked peptidoglycan (murein; mucopeptide) that lies between the cytoplasmic (inner) and outer membranes. To facilitate cell growth and division this wall has to be continually expanded with periodic formation of septa at the old cell centres which delimit daughter cells. The peptidoglycan network is synthesized by the addition of GlcNAc-MurNAc-pentapeptide subunits within the polymeric structure. This process necessitates the continual formation and dissolution of peptide cross-bridges linking the growing glycan chains [1]. Formation of peptide cross-bridges by transpeptidation is catalysed by a set of active-site-serine-DD-peptidases [2]. β-Lactam antibiotics exert their activity by behaving as substrate analogues and covalently binding to the active sites of the DD-peptidases [3]. Thus, these enzymes are also penicillin-binding proteins (PBPs). They are ectoproteins located on the periplasmic face of the inner membrane and the purpose of this article is to review information concerning their modes of membrane association.

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Character and function of *E. coli* PBPs

Polyacrylamide gel analysis after treatment of *E. coli* cells with radiolabelled β-lactams initially identified six major PBPs: 1, 2, 3, 4, 5, and 6 [4]. PBP1 was later resolved into separate components PBP1A and PBP1B [5–7]. The PBPs interact with β-lactams at their active sites which share primary sequence motifs (SXXK, SXN and KTG) with β-lactamases, suggesting a common evolutionary origin [2,8]. The properties of the *E. coli* PBPs, and the genes which encode them, are summarised in Table 1.

The higher molecular mass penicillin-binding proteins of *E. coli*, PBP1A, 1B, 2 and 3 catalyse essential steps in peptidoglycan biosynthesis. Defects in these proteins affect cell growth and they are the killing targets of β-lactam antibiotics [9]. Information on the enzymic activities of these high-molecular mass PBPs has been reviewed in detail elsewhere [2,8]. Briefly, all four proteins are believed to be bifunctional transglycosylase/transpeptidases, with the penicillin-sensitive DD-peptidase domain near the C-terminus. They function to insert GlcNAc-MurNAc-pentapeptide subunits into the peptidoglycan structure and also catalyse the cross-linking of the growing strands via their peptide side chains. Despite only limited sequence homology, the enzymic activity shared by PBP1A and 1B is interchangeable. This is confirmed by the ability of one protein to compensate for a deficiency in the other in *ponA* or *ponB* mutants [10,11]. The essential nature of this shared PBP1 activity is demonstrated by the bacteriolytic effects of a *ponA/ponB* double deletion [11], and the similar rapid lethal effect of β-lactams such as cefsulodine which binds with high affinity to the PBP1s [12].

PBP2 activity, in catalysing peptidoglycan synthesis at the point(s) of cell elongation, is vital to maintaining the cell's rod-shaped morphology. This is demonstrated by the formation of spherical cells by a *pbpA*(Ts) mutant at the non-permissive temperature, or when *E. coli* is treated with the β-lactam mecillinam which specifically inhibits PBP2 [12,13]. Unlike PBP1A, 1B and 3, PBP2 also requires the presence of the *rodA* gene product in the inner membrane for its transglycosylase activity [14]; however, the functioning of this partnership is not fully understood. Recent data derived from analysis of mecillinam-resistant mutant strains indicate a further important role for PBP2 in coordinating cell wall synthesis and ribosomal activity [15].

PBP3 activity is specifically involved in peptidoglycan synthesis at the point of septation and is essential for cell viability [12]. Inhibition of PBP3 by growing a *pbpB*(Ts) mutant at the non-permis-

Table 1

The major penicillin-binding proteins of *Escherichia coli*

	Gene	M_r on SDS-PAGE (kDa)	Cleaved Signal peptide	Membrane anchor	Enzymic activity	Essential/ Dispensable
PBP1A	*ponA* (*mrcA*)	91	No	N-term hydrophobic	Transpeptidase/ transglycosylase	Essential
PBP1B	*ponB* (*mrcB*)	90	No	N-term hydrophobic	Transpeptidase/ transglycosylase	Essential
PBP2	*pbpA* (*mrdA*)	66	No	N-term hydrophobic	Transpeptidase/ transglycosylase	Essential
PBP3	*pbpB* (*ftsI*)	60	No	N-term hydrophobic	Transpeptidase/ transglycosylase	Essential
PBP4	*dacB*	49	Yes	C-terminal amphiphilic	DD-carboxypeptidase endopeptidase	Dispensable
PBP5	*dacA*	42	Yes	C-terminal amphiphilic	D-alanine carboxypeptidase	Dispensable
PBP6	*dacC*	40	Yes	C-terminal amphiphilic	D-alanine carboxypeptidase	Dispensable

sive temperature or by antibiotics such as cephalexin leads to cessation of cell division and hence filamentation [12,16]. PBP3 is thought to be the most important killing target for β-lactams in common use [17].

There are a number of lines of evidence which suggest that PBP3 may require interaction with other proteins to function correctly in cell division [1]. In experiments where the 5′ end of the *lacZ* gene was fused to the *pbpB* gene minus the first 40 codons, the cytoplasmic β-galactosidase/PBP3 hybrid bound penicillin and was shown to be concentrated at septum sites unless an *ftsZ* mutant host was used [18]. This was interpreted to suggest a role for cytoplasmic FtsZ protein in the integration of PBP3. Mutations in the *ftsA* gene increase the resistance of cells to lysis by β-lactams, they affect binding of these antibiotics to PBP3, and *ftsA*2 cells filament at 42°C [19]. These data suggest that the cytoplasmic inner membrane-associated FtsA protein and PBP3 may also interact. Lastly, it has been suggested that PBP3 interacts with FtsW, a homologue of RodA, during cell division, although there is as yet no supporting experimental evidence [20].

The low-molecular mass PBPs 4, 5 and 6 together account for 85% of the penicillin-binding capacity of cells [4]. However, their physiological role is still uncertain. All three have D-alanine carboxypeptidase activity which may control the level and nature of peptidoglycan cross-linking catalysed by high-molecular mass PBP transpeptidase activity [3,9,21]. In addition PBP4 is thought to have DD-endopeptidase activity which can cleave peptide cross-links [9,22], and may be required for insertion of new peptidoglycan within the sacculus during elongation.

PBP5 and PBP6 show 62% homology in their primary sequences [23], but there is some evidence to suggest that these two D-alanine carboxypeptidases have distinct roles. Early data on the carboxypeptidase activity of purified PBP5 and PBP6 suggested that their substrate specificities were identical but the activity of PBP6 was about four-fold lower than PBP5 [24,25]. Additional evidence that they have similar activities was the finding that overexpression of either PBP6 or PBP5 could restore cell division in a *pbpB* mutant [21]. However, a recent study using purified PBP5 and PBP6 failed to identify carboxypeptidase activity associated with PBP6 [26]. Elevated levels of PBP6 are tolerated by *E. coli*, whereas overexpression of PBP5 is lethal, possibly because of its higher activity [26–28]. Also, in *dacC* mutants lacking PBP6 there is no increase in the level of pentapeptide peptidoglycan side chains as is seen in PBP5-deficient *dacA* cells [26]. Lastly, the levels of PBP6 but not PBP5 increase during stationary phase at a time when no new peptidoglycan pentapeptide side chains are present [29,30]. These findings suggest that PBP5 and 6 have subtly different activities which are not yet understood. Possibly they act on slightly different peptidoglycan substrates.

Mutations in PBPs 4, 5 and 6 have no apparent effect on cell growth in laboratory conditions and a 5, 6 double deletion mutant is viable [31,32]. To date no one has constructed a 4, 5, 6 triple deletion mutant.

Additional low-molecular mass PBPs of between 30 and 34 kDa have been identified by binding radiolabelled β-lactams [4,33–36]. Little is known about their function or their mechanism of membrane association and their sequences have not yet been published.

The location of penicillin-binding proteins in *E. coli*

Although their solubility in the non-ionic detergent sarkosyl suggests that the PBPs are all inner membrane proteins [4,37], alternative locations have been proposed based on the use of a variety of techniques. Density gradient centrifugation suggests distribution in both the inner and outer membranes and in an intermediate density fraction [34,36], while immunoelectron microscopy located PBP1B to the cytoplasm, inner membrane and zones of adhesion between the two membranes [38]. Studying strains which are overproducing one or more of the PBPs may cause some of these discrepancies. Perhaps the most reliable evidence confirming the inner membrane location of the PBPs comes from a recent study using a mercury-penicillin derivative as an

ultrastructural probe [39]. Electron dense mercury atoms covalently attached to penicillin V were visualised by high resolution electron microscopy of fixed *E. coli* cells which had been treated with the antibiotic during the exponential phase of growth. This technique does not rely upon maintenance of the structural integrity of the proteins during the procedures to prepare the cells for electron microscopy because the mercury-penicillin is covalently bound as an acylated complex with the PBPs. The mercury carried by the penicillin derivative was found to be predominantly located on the inner membrane with some protrusion into the periplasm.

Accepting the inner membrane location of the *E. coli* PBPs, one group has performed complementary studies on PBP-inner membrane association using sucrose gradient centrifugation [40], agarose gel electrophoresis and sizing chromatography [41]. By these means, different populations of inner membrane vesicles have been separated and their PBP composition shown to be distinct. The non-random distribution of PBPs in separate fractions of the inner membrane may indicate discrete membrane regions containing sets of proteins which perform different functions.

Targeting and anchoring of the PBPs to the inner membrane

Generally, targeting of proteins with exported domains is effected by a signal sequence located at, or near, the N-terminus of the protein [42]. In a type I membrane protein the N-terminal signal sequence is removed by the proteolytic action of a signal peptidase during or some time after export is completed. Anchoring to the membrane is normally via a hydrophobic (apolar) stop-transfer sequence [43]. The cleavable signal sequence has distinctive properties, including positively charged amino acids towards the N-terminus, a hydrophobic (apolar) middle section of 7–13 residues and a signal peptidase cleavage site [42]. In contrast, the membrane anchor sequence is generally longer (22–26 residues) and contains sufficient apolar residues to span the membrane as an α-helix [42]. Normally a couple of positively charged residues are found at the cytoplasmic end of the anchor and the orientation is N-terminus$_{out}$: C-terminus$_{in}$ [43]. In a type II membrane protein an uncleaved N-terminal signal-like sequence is present which acts as an anchor while the C-terminus is extruded across the membrane.

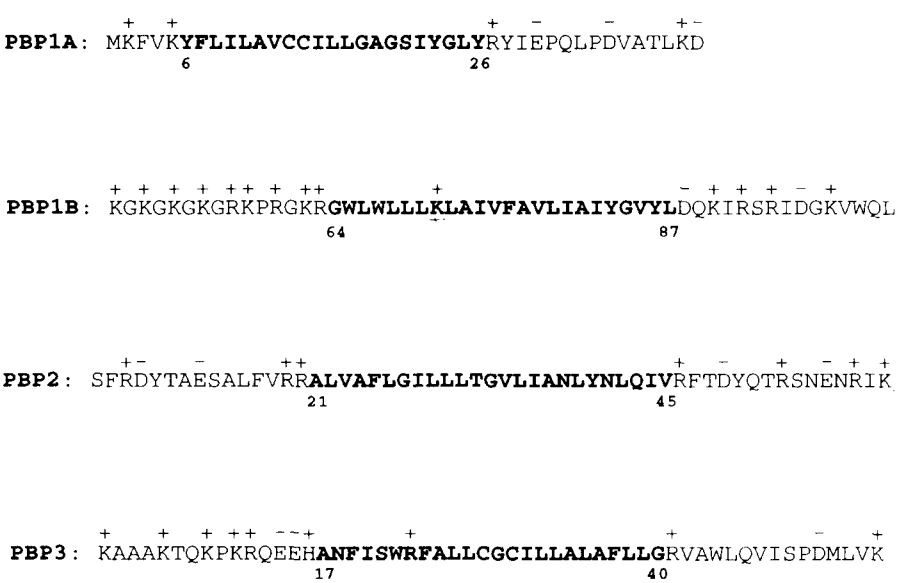

Fig. 1. Primary structure of potential membrane anchor domains of high M_r *E. coli* PBPs. Predicted transmembrane sequences are shown in bold and their terminal residues are numbered. Charged residues are indicated above each sequence. The sequences of PBP1A and PBP1B, PBP2 and PBP3 are taken from [46], [47] and [55], respectively.

Such signal/anchors are characterized by having an apolar region of hydrophobicity like that of a type I membrane protein anchor [43]. They also generally obey the positive inside rule, that is the distribution of positive charges flanking the anchor is biased towards the cytoplasmic side [43–45].

Penicillin-binding proteins 1A, 1B, 2 and 3

Once the nucleotide sequences for PBP1A, 1B and 2 genes had been determined [46,47], analysis of hydropathy profiles of the predicted polypeptide sequences showed that there is only one region of each protein with the potential to act as a membrane spanning domain [46–48].

In PBP1A the potential membrane spanning domain occurs at the N-terminus and resembles a conventional signal sequence. However, there is no evidence that this sequence is removed because the size of the *ponA* gene product synthesized in vitro and in vivo is identical (Pratt, J.M. and Spratt, B.G. unpublished). It has been concluded that the uncleaved signal peptide facilitates export of the catalytic domain of PBP1A and then anchors the protein to the inner membrane (Fig. 1). PBP1A is therefore predicted to be a type II membrane protein although this has not been confirmed experimentally.

Three forms of PBP1B have been recognised. The two major forms, α and γ appear to arise from translation initiation at two different in frame start codons, which produces a full size polypeptide (α) and a species (γ) lacking the 45 N-terminal residues [46]. A third form, β, is apparently a break down product of α, that has lost the 24 N-terminal residues through proteolytic cleavage [49]. The extremely hydrophilic and basic PBP1B N-terminus, that varies in size between the three forms, is followed by a hydrophobic sequence between residues 64 and 87 that is predicted to be able to span a membrane in an α-helical form and comprise the anchor in this type II membrane protein [48,50]. It is interesting that the proposed hydrophobic transmembrane sequence is broken by a lysine residue at position 71 (Fig. 1).

Evidence supporting this topology model for PBP1B is the pattern of protease sensitivity of PBP1B in spheroplasts and inverted membrane vesicles and the levels of ampicillin resistance conferred by a panel of in-frame *ponB–bla* gene fusions [50]. Despite these data supporting the role of an N-terminal hydrophobic signal in the membrane association of PBP1B, another finding suggests that there may be other interactions involved in binding PBP1B to the inner membrane. A fusion protein in which the PBP1B N-terminus (residues 1–87) is replaced by the cleavable signal sequence of PBP5 has been generated by genetic manipulation [50,51]. This would be predicted to mediate complete translocation of the PBP1B polypeptide into the periplasm. However, it was found that the hybrid protein was distributed partly in the periplasm and partly in the membrane fraction, indicating that there is another region(s) of the protein involved in membrane association. Recently, it has been shown that PBP1B minus its N-terminal membrane anchor associates with phospholipid vesicles [52]. Furthermore, cross-linking data support the idea that PBP1B is in close association with other PBPs [53], possibly as part of a peptidoglycan synthesizing complex. Thus, it appears that in addition to anchoring via the N-terminal transmembrane domain, the translocated domain of PBP1B may associate with the inner membrane via interaction with other membrane proteins and/or by direct binding to membrane phospholipid.

PBP2 is also predicted to be a type II membrane protein [47]. There is a stretch of hydrophobic sequence between residues 21–45 that is thought to constitute a signal/anchor (Fig. 1). Removal of this region results in the accumulation of the mutant PBP2 as a cytoplasmically located soluble protein which is still able to bind β-lactams [54]. As well as confirming the dual function of the hydrophobic region as both a signal and an anchor, the soluble PBP2 may prove extremely useful in preparing crystals for X-ray diffraction analysis and the determination of the three-dimensional structure of this enzyme.

From the derived amino acid sequence of PBP3 [55] it appeared that a hydrophobic sequence (residues 17–40) following the highly hydrophilic and basic N-terminus would function as a signal/

anchor sequence capable of initiating translocation of the bulk of the protein and then anchoring it in the membrane (Fig. 1). As for PBP1B, the proposed apolar transmembrane sequence is broken by a basic residue (Arg) at position 23.

However, comparison of in vivo and in vitro generated PBP3 showed that it is initially synthesized as a precursor which is processed to the mature form in the presence of cytoplasmic membranes; the most likely explanation being the cleavage of an N-terminal signal sequence [55]. Since no additional hydrophobic sequence was detected this did not explain the mechanism of PBP3 anchoring to the membrane. The sequence of PBP3 residues 26–30 Leu-Leu-Cys-Gly-Cys bears similarity to a site for lipid modification [56]. It was postulated that PBP3 is a lipoprotein with a signal sequence which is cleaved by signal peptidase II after lipid modification at the Cys residue within the approximate consensus sequence [57]. Membrane anchoring would therefore be via the N-terminal lipid modification. Unlike other lipoproteins, however, processing was not inhibited by globomycin and was much slower than similar lipoprotein processing events [57]. In addition, when cells were incubated with [^{3}H]palmitate or [^{3}H]glycerol, both were incorporated but only a small proportion of PBP3 molecules were found to be lipid modified although most were proteolytically processed [57]. These data, and the lack of another potential hydrophobic anchor sequence suggested that the N-terminal signal may not be cleaved and may in fact be a signal/anchor, tethering the N-terminus to the cytoplasmic membrane while the rest of the protein was free to fold in the periplasm (like PBP1A, 1B, and 2). The possibility that the apparent precursor–product relationship is due to a processing reaction occurring at the C-terminus was therefore investigated. Hybrid and truncated forms of PBP3 were produced and used to demonstrate that a C-terminal processing event did occur [58]. The precise site of cleavage was then identified by peptide mapping and amino acid sequence analysis [59]. It was found that a slow processing event at Val-577 resulted in the removal of the 11 C-terminal residues of PBP3 thus explaining the larger size seen in vitro and supporting the hypothesis that the N-terminal region acts as a signal/anchor sequence. Processing was absent in a *prc* (processing involving C-terminal cleavage) mutant; however, almost wild-type growth characteristics were seen and therefore the C-terminal cleavage event does not appear to be essential [60]. Indeed, when pulse–chase experiments are performed on maxi-cells synthesizing PBP3, no processing event is visible despite extending the chase for an hour, perhaps an indication that the *prc* activity may be strain-dependent or labile (Peters S. and Pratt, J.M., unpublished data).

The hypothesis that PBP3 is an N-terminally anchored type II membrane protein in the inner membrane has been confirmed in a number of ways. First, protease sensitivity experiments showed PBP3 to be totally sensitive to proteases applied to the periplasmic face, but resistant to digestion when applied from the cytoplasmic face [61]. Second, Bowler and Spratt (1989) generated a panel of in-frame *pbpB–bla* gene fusions (of varying lengths of PBP3 with β-lactamase at the C-terminus) and showed that the first 36 amino acids of PBP3 contained the signal/anchor sequence which could direct export of β-lactamase to the periplasm. Third, it was found that if residues 1–52 of PBP3 were replaced with a cleavable signal sequence (that of PBP5) the resulting PBP3* was released into the periplasm and still bound penicillin [51,61]. It has been demonstrated in this laboratory that this 'periplasmic' PBP3* is able to complement a temperature-sensitive mutation in PBP3. When a *recA*, *pbpB*(Ts) cephalexin^R strain was transformed with plasmids encoding wild-type PBP3 or PBP3*, both restored the ability of the strain to grow at the non-permissive temperature and also strains became sensitive to cephalexin (Peters S. and Pratt, J.M., unpublished data). Therefore, it may not be necessary for PBP3 to be anchored to the inner membrane for it to perform its essential roles.

Penicillin-binding proteins 4, 5 and 6

Comparison of the amino acid sequences of the low-molecular mass PBPs 4, 5 and 6 with certain classes of serine β-lactamases, suggest that they are distantly related [2,8,62]. In addi-

tion, it is possible to model the three-dimensional structure of these PBPs and other DD-peptidases on the medium resolution three-dimensional structures of β-lactamases of diverse bacterial species [63–65]. The striking coincidence of elements of secondary structure supports the hypothesis of a common evolutionary origin. Interestingly, PBP4 has an extra 188 amino acid residues between the SXXK and SXN active site motifs (residues 59–246) [62]. Within the modelled three-dimensional structure these amino acids are predicted to lie in a loop on the periphery of the structure [64]. It has been speculated that this potentially structurally independent domain has a regulatory function, although it has been shown that at least residues 116–238 can be deleted without loss of penicillin-binding activity [64].

PBPs 4, 5 and 6 are all targeted to the inner membrane via cleavable N-terminal signal sequences and in non-overexpressing strains the mature proteins are quantitatively recovered with the inner membrane [66–68]. However, overproduction of PBP4 to high levels results in over 80% of the PBP4 appearing as a soluble component of the periplasm with no apparent effect on cellular morphology [22]. In contrast, overproduction of PBP5 and 6 results in their quantitative recovery with the inner membrane, and in the case of PBP5 cells become round and lyse [26–28]. Inspection of the amino acid sequences of these PBPs did not reveal a conventional hydrophobic anchor sequence and the mechanism of anchoring was unclear [67]. Studies on PBP5 involving the construction and analysis of both C-terminally and internally deleted proteins demonstrated that the C-terminal 20 amino acid residues of PBP5 were essential for anchoring [67,69]. This region did not, however, contain obvious sites for known covalent modifications, but appeared to have the potential to form an α-helix [69,70]. When the C-terminal 18 amino acid residues are displayed using a Schiffer-Edmundson helical wheel [71], a degree of segregation between the hydrophilic and hydrophobic amino acids is seen, giving the α-helix a strongly amphiphilic character (Fig. 2).

This idea of anchoring via an amphiphilic helix is supported by the demonstration that membrane-binding is destabilised by the incorporation of a helix-breaking proline residue within the proposed anchor sequence [70]. When the C-terminal regions of PBP6 and PBP4 are also analysed, it is apparent that these too can be modelled as an amphiphilic α-helix (Fig. 2). The use of randomly generated helices predicts that the probability of obtaining these levels of amphiphilicity is less than 2% which implies that these regions possess structural significance. Moreover, as would be expected for an anchoring domain, all are predicted to be surface active according to Eisenberg's criteria [72] and have a strong propensity towards a preferred orientation at a membrane interface [73]. In addition, the arrangement of the charged residues within these regions is characteristic of membrane interactive proteins [74]. It, therefore, appears that the low-molecular mass PBPs may be anchored to the membrane by virtue of a novel mechanism, the C-terminal amphiphilic helix.

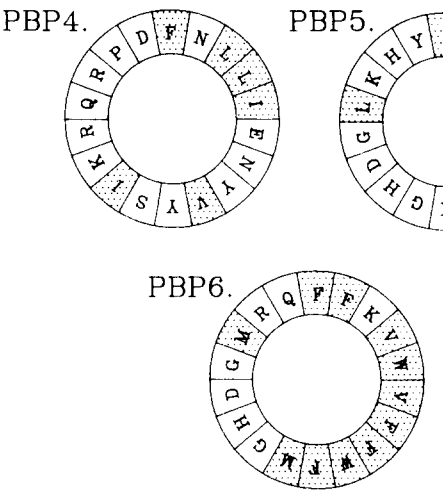

Fig. 2. Residues 440–457, 357–374 and 352–369 of PBPs 4, 5 and 6, respectively, are plotted on a Schiffer and Edmundson helical wheel [71] to demonstrate their amphiphilic α-helix forming potential. Hydrophobic residues are shaded. The sequence of PBP4 is taken from [62] and those of PBP5 and PBP6 from [23].

It is not known at what stage in the membrane assembly of the low-molecular mass PBPs the amphiphilic helix assumes a membrane interactive conformation. After translocation and signal

sequence cleavage, the polypeptide may exist as a transient periplasmic intermediate where it will experience a decrease in pH due to the proton gradient and the cationic effect of the membrane. This pH drop may be sufficient to stabilize the amphiphilic α-helical secondary structure at the C-terminus required for insertion into the inner membrane (Fig. 3). Alternatively, amphiphilic helix formation at a late stage of translocation may prevent complete release and anchor the protein in the membrane (Fig. 3).

The amphiphilic helix form of anchor proposed for low-molecular mass PBPs does not appear to be limited to *E. coli*, since studies of carboxypeptidases of *B. subtilis* and *B. stearothermophilus* indicate that they have similar C-terminal domains [75]. This would imply a ubiquitous method of anchoring for carboxypeptidases involving C-terminal membrane-interactive helices.

The anchoring of PBPs 5 and 6 via an amphiphilic helix is generally accepted and the C-terminal anchor of PBP6 has also been confirmed by deletion analysis [26]. However, the anchoring of PBP4 by a similar mechanism is not accepted by Keck and co-workers who, using the method of Eisenberg [72], were unable to detect an amphiphilic helix in the C-terminal region [62]. In the cases of PBP5 and 6 there are 159 and 163 residues, respectively, after the KTG motif, ensuring a good spacing between the penicillin-binding domain and the anchor. PBP4, in comparison, is significantly truncated in this region with only 58 residues after the KTG motif, similar to soluble β-lactamases [62]. The finding that nearly

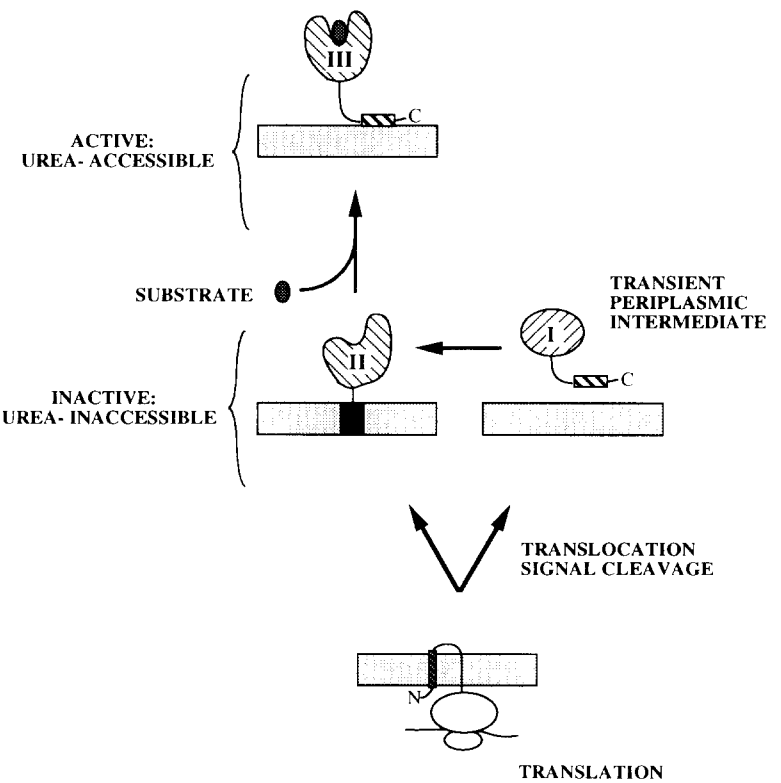

Fig. 3. Hypothetical scheme for PBP5 membrane anchoring and modulation of the anchor in response to changes in enzymic activity. After translocation and signal sequence cleavage, PBP5 may either be completely translocated and exist as a transient periplasmic intermediate (I) before binding to the membrane (II). Alternatively, amphiphilic α-helix formation late in translocation may prevent complete release (II). The membrane-bound PBP is then thought to be converted from a urea-inaccessible to a urea-accessible form upon binding of substrate (III), i.e. when switching from an inactive to an active state [77].

all PBP4 in overproducing cells was in a soluble form may be interpreted as additional evidence for the anchoring of PBP4 to be different from that of 5 and 6 [22].

The nature of the anchoring of PBP5 and PBP6 has been further investigated by membrane washing experiments and shown to involve a large element of hydrophobic interaction ([76]; Phoenix D.A. and Pratt J.M., unpublished data). In addition, it has been shown that a change in pH from acid to alkaline converts the PBP5 anchor from a urea-inaccessible to a urea-accessible state. Also, upon interaction with a β-lactam antibiotic, the protein tends towards the urea-accessible form, thus implying that conversion of the enzyme to an active conformation may cause a decrease in the degree of membrane interaction (Fig. 3) [77].

Further evidence for this relationship was obtained in experiments where the PBP5 anchor domain was attached to the soluble periplasmic protein β-lactamase [70]. The anchor was able to bind the periplasmic protein to the inner membrane in what appeared to be a wild-type manner, yet the fusion protein was unable to enter a urea-inaccessible form [77].

Anchoring of PBP5 could occur by virtue of an interaction between the C-terminal anchor and either a lipid or proteinaceous element in the inner membrane. The amphiphilic moment and charge distribution of the anchor sequence indicate that direct insertion into the lipid bilayer would be unlikely unless complex formation occurred with other membrane proteins. Indeed the Gibbs free energy of membrane association indicates that only the last 10 amino acids of PBP5 could possibly insert perpendicularly into a lipid environment [78].

Unfortunately, no washing experiments have been carried out with PBP4 to determine the properties of its membrane interaction at wild-type levels and it is not known whether PBP4 is interacting with the membrane phospholipid or proteinaceous components, possibly including other PBPs, to form a complex.

The idea of PBP complex formation becomes quite attractive when it is considered that cross-linking experiments have identified a possible interaction between PBP5, PBP1A or 1B and PBP3 [53]. This would be a favourable combination, since it unites the transglycosylase/transpeptidase activities of the higher-molecular mass PBPs with the carboxypeptidase activity of the low-molecular mass PBPs, which provides an opportunity for antagonistic control of peptidoglycan biosynthesis. It could be envisaged that within such a situation, alteration of the complex activity

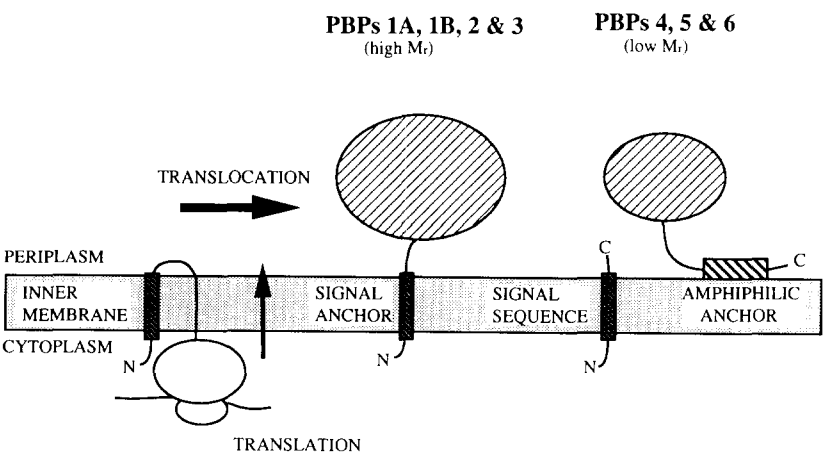

Fig. 4. Summary of proposed membrane anchoring mechanisms of the *E. coli* PBPs. Targeting to the inner membrane and translocation are directed by signal anchors for the high M_r PBPs (1A, 1B, 2, 3) and by cleaved N-terminal signal sequences for the low M_r PBPs (4, 5, 6). After translocation, the high M_r PBPs are anchored to the membrane via their signal anchor sequences while the low M_r PBPs use C-terminal amphiphilic α-helices.

could affect the state of the anchor of the low-molecular mass PBPs either by decreasing the strength of receptor binding, altering the level of lipid interaction available or by allowing the exposure of the anchor site to the aqueous environment.

In the light of available data, Fig. 4 shows a proposed model for the targeting and anchoring of the *E. coli* PBPs.

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