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Membrane interaction of *Escherichia coli* penicillin binding protein 5 is modulated by the ectomembranous domain

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E. coli penicillin binding protein (PBP) 5 is anchored to the periplasmic face of the inner membrane by a C-terminal domain which is predicted to form an amphipihilic α-helix. Here we show that the presence of a substrate analogue, benzyl penicillin, causes the protein to be converted from a membrane bound urea inaccessible form to a urea extractable form. If the anchor region is fused to the periplasmic protein, β-lactamase, the fusion protein becomes membrane bound but is unable to exhibit the changes in urea extractability which are observed with PBP5. We therefore conclude that although the C-terminus of PBP5 is sufficient to anchor the protein to the membrane surface the ectomembranous domain can affect the state of the anchor and in vivo changes in the state of anchoring may be related to enzyme activity.

Penicillin binding protein; Membrane anchor; Amphiphilic helix

1. INTRODUCTION

Internal deletions within *E. coli* penicillin binding protein (PBP) 5 have defined the C-terminal 18 residues of the protein as essential for anchoring PBP5 to the periplasmic face of the inner membrane [1]. This region has been predicted to be capable of forming a strongly amphiphilic, surface active α-helix [2,3]. In addition it has been demonstrated that when the PBP5 anchor region is fused to the periplasmic protein β-lactamase the fusion protein is efficiently anchored to the inner membrane implying that this region is solely responsible for membrane anchoring [4]. We wished to address the question of whether this C-terminal domain was indeed exclusively responsible for anchoring PBP5 to the membrane and we have therefore characterised the interaction of the β-lactamase fusion protein with the membrane and compared the results with those obtained for wild type PBP5.

In addition, the interaction of PBP5 with a substrate analogue has been investigated with respect to the effect substrate binding has on membrane interaction. These data have been compared to the effect the substrate analogue has on the β-lactamase fusion protein in an attempt to determine whether the large ectomembranous domain can affect the level of membrane interaction.

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Abbreviations: PBP-penicillin binding protein.

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

The bacterial strains were the *E. coli* K12 derivative SP1048 *his, tsx, sup*F. *Δlac*x74, *srl*::Tn10, *Δdac*C1, *Δdac*A::Km^R[5] and CSH26DF6 *ara, thi, Δ(lac,pro), Δ(rec*A,*srl)*F6, *rps*L [2]. In strain JM103 dacA (*thi, rps*L, *sup*E, *end*A, *sbc*B15, *hsd*R44, *Δ(lac,pro*A,B)/F′ *tra*D36, *pro*A,B, *lac*I^q, Z*Δ*M15) the chromosomal *dacA* gene of JM103 has been inactivated by the insertion of a kanamycin resistance gene [2]. Plasmid pLG364 carries the *dac*A 11191 mutation of PBP5 on a *Bam*HI-*Eco*RI fragment in pBR322 [6]. Plasmid pBS42 encodes wild type PBP5 [4]. Plasmid pMJ214 contains a β-lactamase fusion in pLG339. The fusion consists of the C-terminal 18 amino acids of PBP5 (res. 356–374) which have been fused to the C-terminus of β-lactamase [4]. Two PBP5 deletions (115$\Delta_{308-363}$, 116$\Delta_{308-354}$) were encoded by plasmids pMJ115 and pMJ116 [2]. Bacteria were grown under aeration at 37°C in nutrient broth. Where necessary the medium was supplemented with ampicillin (25 mg/ml), tetracycline (10 μg/ml), kanamycin (12.5 μg/ml) and spectinomycin (100 μg/ml).

2.2. *Preparation of membrane fractions*

Envelope fractions were prepared from 1 litre cultures (A_{450} 0.7–0.9) either by sonication [7] or by osmotic lysis [8]. SP1048 was grown to A_{450} 0.7, harvested and sonicated in 10 mM sodium phosphate buffer pH 7.2. This was stored at −20°C and added to supernatant fractions to ensure efficient recovery of proteins from supernatants upon trichloroacetic acid precipitation [9]. Pelleted envelopes were resuspended in 1 ml of extraction buffer and were incubated on ice for 1 h during which time envelopes prepared by sonication were subjected to three rounds of freeze-thawing (freezing for 1 min in liquid nitrogen and thawing on ice to ensure access to the luminal face of the membrane). After centrifugation at 38,000 rpm for 1 h at 4°C trichloroacetic acid was added to the supernatants to a final concentration of 10% and 10 μl (0.2 A_{450} units) of sonicated SP1048 cells were added to aid precipitation. After 1 h on ice the precipitates were harvested, resuspended in 10 mM sodium phosphate buffer, pH 7.2 containing 0.6 mM phenylmethylsulphonyl fluoride. To test for aggregates membrane samples were resuspended to a final concentration of 60% w/v sucrose, 5 mM EDTA, pH 7.5 and floated up through a sucrose flotation gradient as previously described [2]

2.3. *Analysis of wash experiments*

Fractions were analysed by sodium dodecylsulphate-polyacrylamide gel electrophoresis [10] and Western blotting [11]. The methods were similar to those previously published except that the substrate used in blot development was 3,4,3′,4′-tetra-aminobiphenyl hydrochloride. Quantification of PBP5 within the preparations was carried out by densitometric analysis of the blots using a Zeiss chromatographic scanner at a wavelength of 500 nm.

3. RESULTS AND DISCUSSION

3.1. *Comparison of PBP5 and the β-lactamase fusion protein with respect to membrane interaction*

Envelopes prepared from *E. coli* carrying a plasmid specifying either the fusion protein or wild type PBP5 were incubated over a range of conditions and the binding characteristics of the two proteins were compared by using Western blotting to monitor the amount of protein released from the membrane into the supernatant. The results shown in Fig. 1A indicate that over the pH range of 6–10 the fusion protein exhibits similar binding characteristics to PBP5 and flotation gradients indicated that both proteins were legitimately bound to the membrane (data not shown). These data imply that the PBP5 anchor region is capable of binding at least one periplasmic protein to the inner membrane and hence is presumably solely responsible for anchoring PBP5 to the membrane.

It has previously been shown that changing the pH can affect the urea accessibility of PBP5 [9]. It has been speculated that the urea inaccessibility of the anchor at low pH is due either to membrane insertion of the anchor thus placing it in a non-aqueous environment [12] or due to a stronger level of receptor binding. The urea extractability of the fusion protein was compared to that of wild type PBP5 by incubating the membranes over varying pH in the presence of 4 M urea. Fig. 1B indicates that the β-lactamase fusion protein is released from the membrane to the same extent over the whole pH range tested whilst in contrast the release of wild type PBP5 is only seen under alkaline conditions. This indicates that although the fusion protein is anchored to the membrane, as shown by flotation gradients [4], the anchor is unable to enter the urea inaccessible state without the presence of the PBP5 ectomembranous domain. The degree to which urea can extract PBP5 can be seen to change with pH where minimal extraction occurs below pH 7 and gradually increases above pH 7. If the ectomembranous domain is truly involved in mediating urea extraction, it would be expected to undergo some degree of conformational change over this pH range. Purified PBP5 was therefore subjected to digestion by thermolysin at pH 6 and pH 7. The rate of degradation at pH 6 was increased by 2–3-fold compared to digestion at pH 7 but the digestion pattern appeared identical at both pHs (data not shown). Since the thermolysin activity showed no change over this pH range [13] the kinetics of proteolysis imply that although

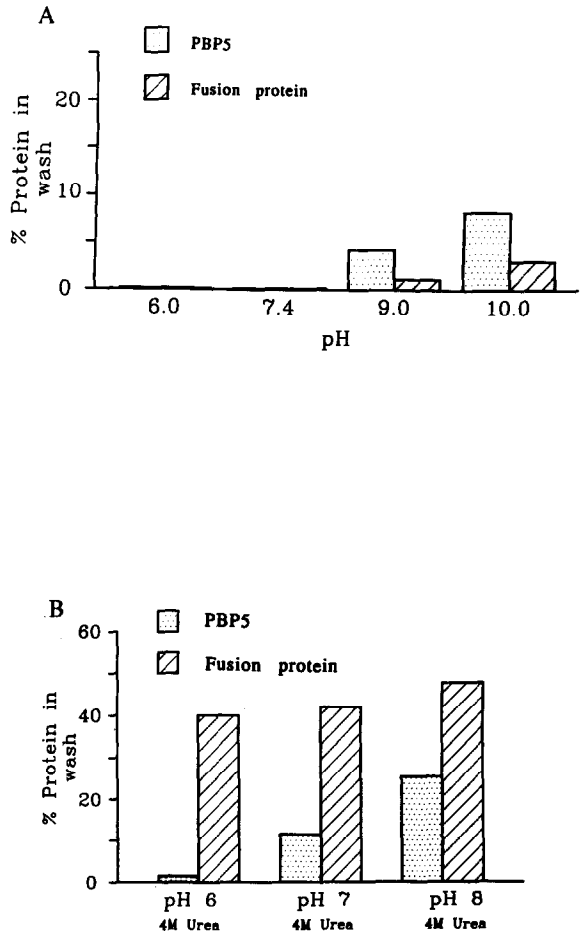

Fig. 1. Envelope fractions were prepared by osmotic lysis from *E. coli* containing either wild type PBP5 or the β-lactamase fusion protein. The envelope fractions were resuspended in 10 mM sodium phosphate buffer at varying pH (Fig. 1A) or in phosphate buffer containg 4 M urea at varying pH (Fig. 1B). After incubation the level of protein released into the supernatants was quantified via western blot and densitometric analysis.

there has been no detectable conformational change the protein is probably in a more 'relaxed form' at pH 6.

In an attempt to investigate the possibility that the anchor was interacting with the ectomembranous domain we examined the binding characteristics of mutants with internal deletions near the C-terminus. PBP5 is 374 amino acids in length and one of the mutants had lost 55 residues including half of the proposed anchor region (115Δ308–363) and the 116 mutant had lost 46 residues but preserved the anchor region (116Δ308–354)[2]. The mutant PBPs were very unstable but the pH washes indicated that in both cases anchoring had been destabilized and washing at pH 8 with 4 M urea released 50% of both mutants. It therefore seems likely that both of the deletions have affected the ability of PBP5 to interact with the membrane. Whether this is a direct effect due to the deletion or whether this is a secondary effect caused by changes in protein conformation is presently being investigated.

3.2. *Comparison of the effect of a substrate analogue on the membrane binding properties of PBP5 and the β-lactamase fusion protein*

If the ectomembranous domain affects the state of anchoring it may follow that in vivo this interaction has a function. It is known that in some cases a substrate or co-factor can affect the anchored state of a membrane bound enzyme. For example the enzyme levan sucrase is released from the membrane by interaction with iron (Fe^{2+}) [14].We therefore studied the effect of the substrate analogue, benzyl penicillin, on urea accessibility. Penicillin has been shown to acetylate the active site residue of penicillin binding proteins thus forming a reasonably stable penicilloyl–enzyme complex which is analogous to the transient acyl intermediate formed with the natural peptidoglycan substrate [15]. In addition the 'wild type' PBP5 used in this study contained an amino acid substitution near the active site which allows the acylation of the enzyme but which prevents the deacylation of the acyl enzyme intermediate. This provides a long lived species that ensures that the protein is held in an active conformation for a significant length of time [6]. It can be seen from Fig. 2A that in the presence of penicillin, PBP5 shows 40–50% urea extraction over the whole pH range tested. The β-lactamase fusion protein which is also able to bind penicillin still gives 50% urea extractability (Fig. 2B). These data therefore imply that upon entering an active conformation PBP5 becomes urea accessible.

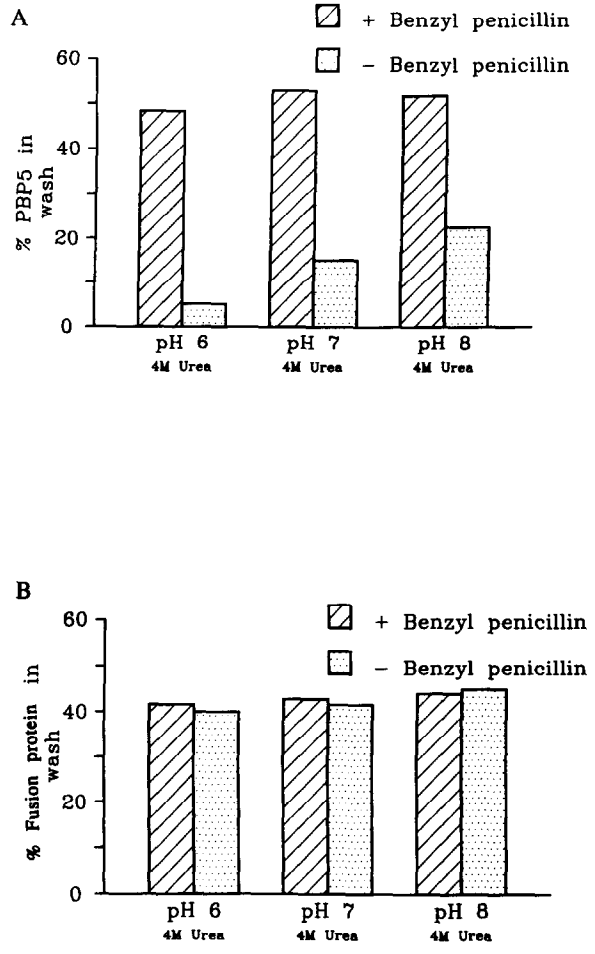

Fig. 2. Envelope fractions were prepared by osmotic lysis from *E. coli* containing either PBP5 (A) or the β-lactamase fusion protein (B). The PBP5 used (pLG364) has a single mutation close to the active site which prevents deacylation of the acyl enzyme intermediate The envelope fractions were resuspended in 100 μl of 10 mM sodium phosphate buffer in the presence or absence of 120 μg/ml of benzyl penicillin. After a 30 min incubation the volume was made up to 1 ml containing 4 M urea at varying pH (Fig. 1B). After further incubation the level of protein released into the supernatants was quantified via Western blot and densitometric analysis.

In summary, it appears that the C-terminal region of PBP5 contains sufficient information to allow PBP5 and at least one periplasmic protein to become membrane associated. Although the ectomembranous domain does not appear to be involved in forming the anchoring domain the deletion experiments imply that the overall protein conformation can affect the strength of receptor binding/level of membrane insertion. This is supported by the inability of the membrane bound β-lactamase fusion protein to enter into a urea inaccessible state. The benzyl penicillin data suggest that in vivo the state of anchoring is related to enzyme activity. It is interesting to note that cross-linking data imply that when PBP5 is in an active form it is able to form a complex with PBP1A/1B and PBP3 [16]. It may well be that upon forming this complex the site of anchoring becomes accessible to the aqueous environment or alternatively the degree of membrane interaction decreases. The simultaneous deletion of PBP1A and PBP1B is lethal, but either can be deleted individually. Mutants lacking either PBP1A, PBP1B or PBP6 were analysed with respect to PBP5 membrane interaction. All the strains were shown to have wild type PBP5 membrane binding characteristics (data not shown). Unfortunately the cross-linking data of Said et al. were unable to distinguish whether PBP1A, PBP1B or both were capable of complex formation. The formation of a complex involving these enzymes would be an attractive proposition as it would contain transglycosylase, transpeptidase and carboxypeptidase activity thus presenting the possibility of antagonistic control of peptidoglycan biosynthesis. If complex formation does occur during times of activity then it seems feasible to assume that the accessibility of the anchor to the aqueous environment may be affected.

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REFERENCES

[1] Pratt, J.M., Jackson, M.E. and Holland, I.B. (1986) EMBO J. 5, 2399–2845.
[2] Jackson, M.E. and Pratt, J.M. (1987) Mol. Microbiol. 1, 23–28.
[3] Phoenix, D.A. (1990) Biochem. Soc. Trans. 18, 948–949
[4] Jackson, M.E. and Pratt, J.M. (1988) Mol. Microbiol. 2, 563–568.

[5] Broome-Smith, J.K. (1985) J. Gen. Microbiol. 131, 2115–2118.
[6] Broome-Smith, J.K. and Spratt, B.G. (1984) FEBS Lett. 165, 185– 189.
[7] Osborn, M.J., Gander, J.E. and Parisi, E. (1972) J. Biol. Chem. 247, 3973–3986.
[8] Boyd. A. and Holland, I.B. (1979) Cell 18, 287–296.
[9] Phoenix, D.A. and Pratt, J.M. (1990) Eur. J. Biochem. 190, 365–369.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Towbin, M., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[12] Gilmore, R. and Blobel, G. (1985) Cell 42, 497–505.
[13] Beynon, R.S. and Bond (eds.) (1991) Proteolytic Enzymes – A Practical Approach, IRL Press, UK.
[14] Chambert, R. and Petit-Glatron, M. (1988) J. Gen. Microbiol. 134, 1205–1214.
[15] Strominger, J.L. (1983) The Target of Penicillin (Hackenbeck, R., Holtje, J. and Labischinski, M., eds.) Walter de Gruyter and Co. New York, pp. 349–359.
[16] Said, I.M. and Holtje, J. (1983) The Target of Penicillin (Hackenbeck, R., Holtje, J. and Labischinski, M., eds.) Walter de Gruyter and Co., New York, pp. 439–444.