INVESTIGATION INTO THE MEMBRANE INTERACTIVE PROPERTIES OF THE *ESCHERICHIA COLI* LOW MOLECULAR WEIGHT PENICILLIN-BINDING PROTEINS

By

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ABBREVIATIONS

CD - Circular dichroism.

CFPs - Channel forming peptides.

DOPC - Dioleoylphosphatidylcholine.

DPPC - Dipalmitoylphosphatidylcholine.

DOPG - Dioleoylphosphatidylglycerol.

EDTA - Ethylenediaminetetraacetic acid.

HPLC - High performance liquid chromatography.

IPTG - Isopropylthiogalactoside.

NMR - Nuclear magnetic resonance.

PBPs - Penicillin-binding proteins.

P4, P5 and P6 - Peptide homologues of the PBP4, PBP5 and PBP6 C-terminal regions.

PEG - Polyethylene glycol.

PIPES - Piperazine-*N*, *N*'-bis (2-ethane-sulphonic acid).

PMSF - Phenylmethylsulphonylfluoride.

SDS - Sodium dodecylsulphate.

SUVs - Small unilamellar vesicles.

TFE - 2, 2, 2-Trifluoroethanol.

μ - Hydrophobic moment.

ABSTRACT

Various results have suggested that in *Escherichia coli* murein assembly may involve a protein complex(es) which could include low molecular mass penicillin-binding proteins (PBPs). These proteins include PBP4, PBP5 and PBP6 which are penicillin sensitive enzymes associated with the periplasmic face of the inner membrane. The levels of these associations have been linked to enzymic activity and elucidation of the mechanism(s) involved in these associations may help identify and understand the regulation of this putative protein complex. It is currently accepted that the membrane associations of PBP5 and PBP6 involve C-terminal amphiphilic α-helices and such helices are ubiquitously employed in the lipid associations of membrane interactive protein molecules. Whether such helical structure features in the membrane associations of PBP4 or indeed if this protein is membrane bound or soluble, are, as yet, open questions. The focus of this research has been to investigate the lipid and membrane interactions of PBP4, PBP5 and PBP6 and in particular, to investigate the role played by these interactions of the C-terminal region of these proteins.

Haemolytic analysis has shown that peptide homologues of the PBP5 and PBP6 C-terminal regions, P5 and P6, are active at the membrane interface and CD analysis has shown that these peptides possess a capacity for α-helix formation. CD and pressure - area isotherm analysis of monolayers formed from P5 and P6 have shown that these peptides are able to adopt α-helical structure at an air - water interface. Monolayer studies have shown that P5 and P6 are able to interact with lipids and that these interactions are characterised by minor requirements for anionic lipid and the involvement of predominantly hydrophobic forces which are enhanced by low pH. Similar characteristics were revealed when perturbant washes and Western blotting were used to

investigate the interactions of PBP5 with membranes derived from a mutant *E. coli* strain, HDL11, in which the level of anionic lipid can be controlled. Overall, these results strongly support the hypothesis that C-terminal amphiphilic α-helices feature in PBP5 and PBP6 membrane anchoring.

Molecular area determinations have implied that a peptide homologue of the PBP4 C-terminal region, P4 is able to adopt α-helical structure and this was confirmed by CD analysis. P4 showed no haemolytic activity but the peptide was found to interact generally with lipid monolayers. These monolayer interactions were characterised by a requirement for anionic lipid and involved predominantly electrostatic forces, which were enhanced by low pH. Similar characteristics but with no detectable requirement for anionic lipid were revealed when perturbant washes and chemiluminesence were used to investigate the interactions of PBP4 with membranes of the overproducing strain HB101/pBK4 and those of HDL11. It is suggested that the PBP4 C-terminal region may play a role in PBP4 - membrane anchoring. Using chemiluminesence, no soluble form of PBP4 could be detected in the wild type *E. coli*, MRE600, suggesting that in wild type strains, PBP4 is exclusively membrane bound. It is suggested that PBP4 - membrane anchoring occurs at a specific binding site and overall, may differ fundamentally from that of PBP5 and PBP6.

CHAPTER 1.

INTRODUCTION

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1.1 THE BACTERIAL CELL WALL

In their natural habitat, most prokaryotes must compete to survive in a hostile environment. These organisms may have to contend for the limited nutrients available or may have to withstand extremes of temperature, pH or other adverse physical and chemical conditions. The first line of resistance to these conditions is the bacterial cell envelope. The content of this envelope varies with genus and may include capsules, external slimes, sheaths or S layers but with the possible exception of some parasitic cells such as *Mycoplasma*, all prokaryotic cell envelopes have in common a cell wall.

The prokaryotic cell wall is a bag shaped macromolecule, or sacculus, which encompasses the whole cell. This structure forms the bacterial exoskeleton which gives the cell its mechanical strength and determines the bacterial cell shape. It functions to help maintain the integrity of the cell both by protecting it from external hazards and enabling the cytoplasmic membrane to withstand the large osmotic pressures caused by the high internal concentrations of metabolites (Holtje, 1995).

Based on chemical composition, the cell walls of prokaryotes fall into two fundamentally different groups; those of the eubacteria which contain murein and those of the archaebacteria which contain no murein (Beveridge and Graham, 1991).

1.2 MUREIN

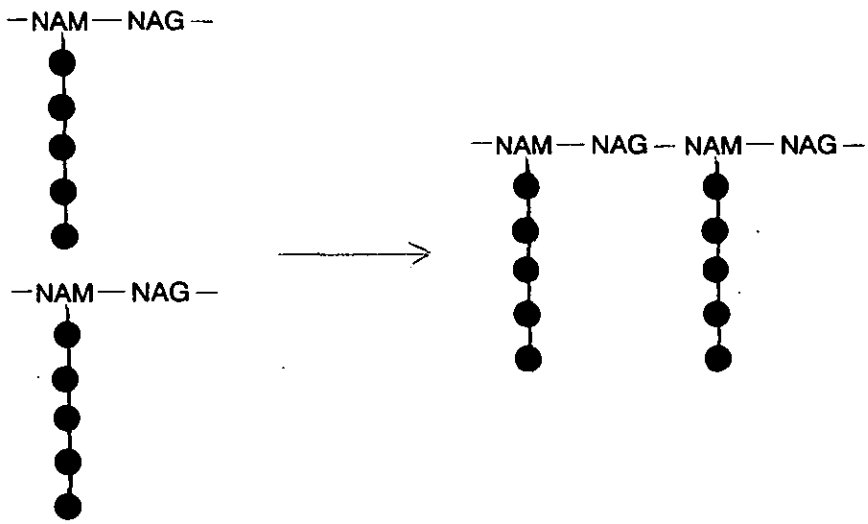
Murein or peptidoglycan, is a biopolymer found only in eubacteria. The murein found in Gram positive and Gram negative organisms differ slightly in their chemical composition and organisation. However, the production of each type of murein proceeds along similar synthetic routes and in each case, results in a covalently closed macromolecule formed from linear strands of the polysaccharide glycan which are cross-linked by peptides.

Glycan monomers are formed in the cytoplasm, each consisting of a residue of N-acetylglucosamine linked to a residue of N-acetylmuramic acid which carries a pentapeptide side chain (figure 1.1). When constructed these units are transported across the plasma membrane by a lipid carrier.

Upon translocation, the translocated glycan monomers are assembled to form murein by a series of enzyme catalysed reactions (Ehlert and Holtje, 1996; Holtje, 1996a;. Holtje, 1996b; Holtje, 1995). In the terminal stages of this process, a transglycosylase catalyses the polymerisation of glycan monomers to form linear chains, linked by β-1, 4 glycosidic bonds (figure 1.1).

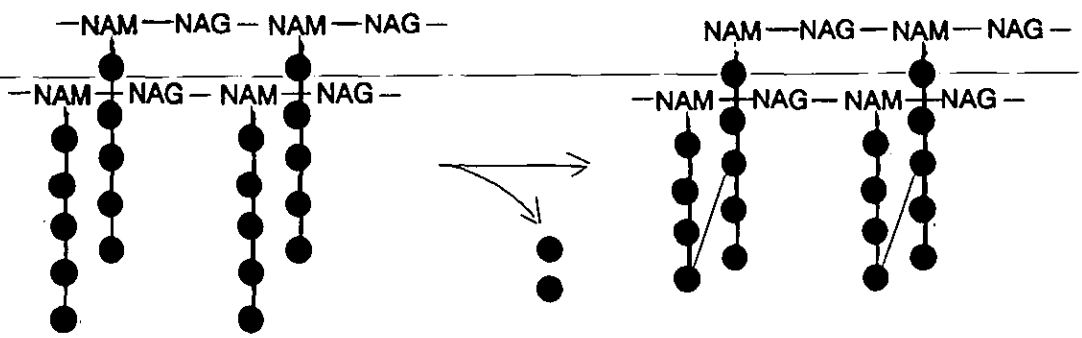
A transpeptidase then catalyses the formation of cross-bridges between the pentapeptide side chains of adjacent peptidoglycan strands (figure 1.2). The nature of the cross-linkage and the amino acid residues comprising the pentapeptide side chains differ for Gram positive and Gram negative organisms. However, in both cases the side chains terminate with two D-alanine residues. In the course of the transpeptidation reaction one side chain acts as a donor polypeptide and that adjacent as an acceptor. The terminal D-alanine of the donor chain is enzymatically cleaved and this provides the free energy necessary to drive the cross-linking of the remaining D-alanine of the donor chain to the central residue in the neighbouring acceptor

Figure 1.1 The transglycosylation reaction of peptidoglycan assembly.



Murein monomers, composed of residues of N-acetylglucosamine (NAG) attached to residues of N-acetylmuramic acid (NAM) which carry pentapeptide side chains, are polymerised to form long strands of peptidoglycan

Figure 1.2 The transpeptidation reaction of peptidoglycan assembly



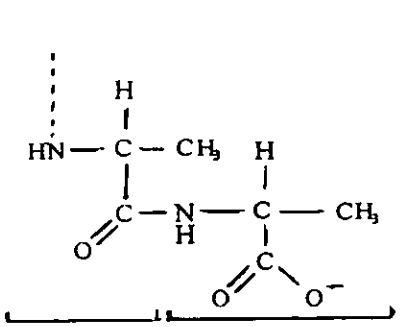
The final two residues of the glycan pentapeptide side chains are D-alanines. In the transpeptidation reaction, the terminal D-alanine of a donor pentapeptide is cleaved and the terminal D-alanine of the resulting tetrapeptide is then linked to the central residue of an adjacent acceptor pentapeptide. These acceptor residues are either L-lysine for Gram positive or meso-diaminopimelic acid for Gram negative organisms.

chain (figure 1.2). Murein is a dynamic structure and must be continually expanded to accommodate cell growth and the periodic formation of septa in cell division. This expansion involves the insertion of glycan monomers and the continual formation and dissolution of peptide cross-links. The level of this cross-linking is believed to be regulated by at least two further enzyme catalysed reactions. In the first of these reactions the terminal D-alanine of a pentapeptide chain is removed by a carboxpeptidase but no with no concomitant formation of new peptide cross-bridges and in the second, existing cross-bridges are cleaved by an endopeptidase. All of the above reactions involved in peptidoglycan manufacture are catalysed by a series of penicillin sensitive murein hydrolases, carboxypeptidases and synthases - the penicillin-binding proteins (PBPs) (Ehlert and Holtje, 1996; Holtje, 1996a; Holtje, 1996b; Phoenix and Harris, 1995; Holtje, 1995; Gittins *et al.* 1993; Nanninga, 1991).

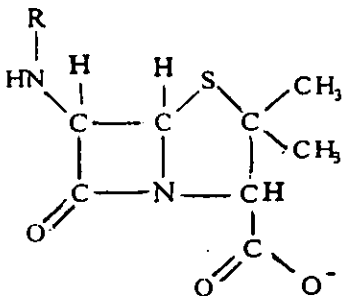
1.3 PENICILLIN-BINDING PROTEINS

PBPs are found in all eubacteria but vary from species to species in number, size and amount produced in the cell (Gittins *et al.* 1993, Ghuysen, 1991; Reynolds, 1988, Reynolds and Brown, 1985). These proteins have been shown to be associated with the cytoplasmic membrane in both Gram negative (Paul *et al.* 1992) cells and Gram positive cells (Paul *et al.*. 1995) and in both cell types the presence of specific PBPs are essential for cell viability (Georgopapadakou, 1993; Spratt and Cromie, 1988; Reynolds, 1988). These PBPs usually possess a high molecular mass (60 kDa-120 kDa) and vary from two to four in number according to the organism (Georgopapadakou, 1993). In *E. coli*, members of the high molecular mass subset of the PBPs (section 1.4; section 1.5.1) have been identified as being essential for cell growth and division,

Figure 1.3. Penicillin and the β-lactam ring as a substrate analogue for PBPs

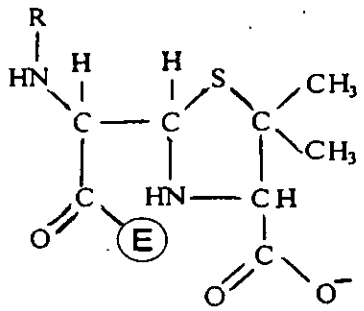


(a)



Penicillin

(b)



Penicillin PBP complex

(c)

Figure 1.3a shows the D-alanyl-D-alanine substrate of the PBPs. It can be seen by comparison to figure 1.3b that penicillin resembles this substrate. The resemblance allows penicillin and other beta-lactam ring compounds to bind to the target PBP (figure 1.3c) and inhibit enzymic activity.

with defects in these enzymes usually leading to cell death (Spratt and Cromie, 1988). These *E. coli* high molecular mass PBPs are the killing targets of β-lactams (Spratt, 1983) and in general, because of their role as the target sites of penicillins and other β-lactam antibiotics the PBP's are of primary importance in antimicrobial therapy (Georgopapadakou, 1993). Tipper and Strominger (1965) suggested that β-lactam compounds exert their antimicrobial action by acting as substrate analogues for the PBPs. It is now known that these antibiotics are able to mimic the acyl-D-alanyl-D-alanine substrate of the PBPs involved in the transpeptidase and

carboxypeptidase reactions (figures 1.3a and 1.3b). They bind covalently to the active site responsible for the DD-peptidase activities of the enzyme (figure 1.3c) and form a stable inactive, penicilloyl-enzyme complex. This structure is analogous to the acyl-enzyme transition state formed by the natural substrate and the target PBP and consequently prevents the final stages of murein assembly (Waxman and Strominger, 1983). PBPs are unique to prokaryotes and have no eukaryotic counterparts. Therefore apart from allergic reactions, β-lactam drugs have minimal side effects when administered to humans.

As a consequence of this β-lactam antibiotics are amongst the most widely used drugs in clinical practice. However, mutations in the genes that code for the PBPs can lead to resistance to these antibiotics. These are usually missense mutations that alter the structure of the target PBP by a single amino acid. This can lead to a decreased affinity for the target PBP by the antibiotic without affecting the catalytic activity of the PBP. This form of resistance is more common in Gram positive bacteria than in Gram negative bacteria (Harris *et al.* 1995a).

Because of their efficacy and minimal risk to man, β-lactam antibiotics are more often, the first choice of drug in combatting bacterial infection. However, the spread of microbial drug resistance has become a general problem and there is a need for new antimicrobial agents and identification of new sites of action for such agents. There is some evidence that murein biosynthesis in *Escherichia coli* may involve a protein complex which includes PBPs (Holtje, 1996a; Holtje, 1996b; Ehlert and Holtje, 1996). It has been suggested that an understanding of how the activities of this putative complex are regulated could lead to the identification of new targets for future antibacterial compounds (Harris and Phoenix, 1997a). To contribute towards the identification of this putative protein complex, this research has focused on the membrane interactions of the low molecular mass sub-group of the *Escherichia coli* penicillin-binding proteins in which enzymic

activity has been linked to the level of membrane association (Harris and Phoenix, 1995; Gittins *et al.*, 1993; Phoenix and Pratt, 1993).

1.4 THE *E. coli* PENICILLIN-BINDING PROTEINS

In *E. coli* seven major PBPs have been well characterised and these fall into two groups. The first of these groups contains the high molecular mass PBPs 1a, 1b, 2, 3, which are encoded by the *ponA*, *ponB pbpA* and *pbpB* genes respectively and have molecular masses ranging from 60 kDa to 91 kDa (table 1.1). The second of these groups is formed by the low molecular mass PBPs 4, 5 and 6 which are encoded by the *dacB*, *dacA* and *dacC* genes respectively. It appears that the recently reported PBP6b (Baquero *et al.*. 1996) is also a low molecular mass PBP and together the molecular masses of these four proteins range from 40 kDa to 49 kDa (table 1.1). Other PBP s which have been reported are the very low molecular mass PBP7 and PBP8 (Henderson *et al.*, 1994) which have molecular masses estimated at 31.2 kDa and 29.5 kDa respectively (table 1.1). It appears that PBP8 is a proteolytic degradation product of PBP7 and a potential site near the latter protein's C-terminus was identified where proteolytic cleavage would create a peptide of approximately the size of PBP8 (Henderson *et al.* 1995).

It is generally accepted that the major *E. coli* PBPs are ectoproteins associated with the periplasmic face of the inner membrane (Gittins *et al.* 1993). Techniques used to investigate the topography of the PBP s have included assays with radiolabeled penicillin (Spratt and Pardee, 1975), electron microscopy and low temperature immunogold labelling (Bayer *et al.* 1990) with the most recent evidence for a periplasmic location coming from the use of high resolution

electron microscopy and a penicillin-mercury probe (Paul *et al.* 1992). *E. coli* cells were treated with the labelled antibiotic, prepared for electron microscopy and examined. When visualised, the majority of the electron dense mercury atoms were sited at the inner membrane with intrusions

Table 1.1. Properties of *E. coli* PBPs

PBP	Gene encoding PBP	Molecular mass of PBP (kDa)	PBP enzymic activity	Estimated number of PBP molecules in a log phase cell
PBP1a	*ponA*[a]	91[a]	Transpeptidase / transglycosylase[a]	221±20[d]
PBP1b	*ponB*[a]	90[a]	Transpeptidase / transglycosylase[a]	127±13[d]
PBP2	*pbpA*[a]	66[a]	Transpeptidase / transglycosylase[a]	120±14[d]
PBP3	*pbpB*[a]	60[a]	Transpeptidase / transglycosylase[a]	132±17[d]
PBP4	*dacB*[a]	49[a]	DD-carboxypeptidase / DD- endopeptidase[a]	120±11[d]
PBP5	*dacA*[a]	42[a]	DD-carboxypeptidase[a]	791±105[d]
PBP6	*dacC*[a]	40[a]	DD-carboxypeptidase[a]	221±26[d]
PBP6b	*dacD*[b]	43[b]	DD-carboxypeptidase[b]	[d*]
PBP7	*pbpG*[c]	31.2[d*]	DD- endopeptidase[c]	387±15[d]
PBP8	*pbpG*[c]	29.5[d*]	DD- endopeptidase[c]	362±19[d]

Table 1.1 shows the properties of the E. coli *PBPs and were obtained from [a] (Gittins et al. 1993), [b] (Baquero et al. 1996), [c] (Henderson et al. 1995), [d] (Dougherty et al. 1996) [d*] in this study, the molecular masses of PBP7 and PBP8 were estimated and the number of PBP6b molecules in a log phase* E.coli *were not determined, [e] (Romeis and Holtje, 1994a).*

into the periplasm. this result would appear to suggest a common periplasmic location for the ectomembranous domains of the major *E. coli* PBPs and recent studies show that PBP6b is also associated with the inner membrane (Baquero *et al.* 1996). In addition to their soluble forms, PBP7 and PBP8 also appear to exist in membrane bound forms (Henderson *et al.* 1994) and were found to be associated with the membrane fraction when these fractions and radiolabeled penicillin were used to determine the number of individual PBPs in an *E. coli* cell. The results of these latter studies showed that the overall number of PBPs present in the cell depended upon growth conditions but in logarithmic phase *E. coli* cells the total number of PBP molecules present was approximately 2,500 (Dougherty *et al.*. 1996). The estimated number of each PBP molecular species present in the cell under these conditions is shown in table 1.1.

The high molecular weight PBPs are bifunctional enzymes and possess both transglycosylase and transpeptidase activity. PBP1a is interchangeable with PBP1b and mutants deficient in one can be compensated by the presence of the other (Yousif *et al.* 1985). PBP4, PBP5 and PBP6 are DD-carboxypeptidases, an activity which is also apparently possessed by PBP6b. In addition, PBP4 is a DD-endopeptidase, an activity which appears to be shared by PBP7 and PBP8 (table 1.1). The active sites which govern these activities, share the primary sequence motifs Ser-X-X-Lys, Ser-X-Asn and Lys-Thr-Gly. These motifs are present in the primary structures of the major PBPs and in the predicted amino acid sequences of PBP6b (Baquero *et al.*, 1996), PBP7 and PBP8 (Henderson *et al.* 1995). It is the serine residue in the first of these motifs which is acylated by penicillin and inhibits murein assembly (Ghuysen, 1991). Using site directed mutagenesis, it has been shown for PBP5, that the presence of this serine residue is essential for both catalytic activity and penicillin binding and that changes in the other amino acid residues, comprising these motifs, result in substantial reductions in the enzyme's performance (Van der Linden *et al.*

specific function of the complex in murein synthesis as being either cell wall elongation or septation. These complexes are also proposed to include PBP4 and / or PBP7 (Holtje, 1996a; Holtje, 1996b). Further studies involving the inhibition of phospholipid biosynthesis have suggested that these putative complexes may be assembled at specific membrane sites where integral membrane proteins such as RodA and FtsW facilitate the translocation of lipid-linked murein precursors to the periplasm (Ehlert and Holtje, 1996).

It appears that the various *E. coli* PBPs have in common their association with the periplasmic face of the inner membrane, however the modes of association of these proteins with this membrane are diverse.

1.5 THE MEMBRANE ANCHORING OF THE *E. coli* PENICILLIN-BINDING PROTEINS

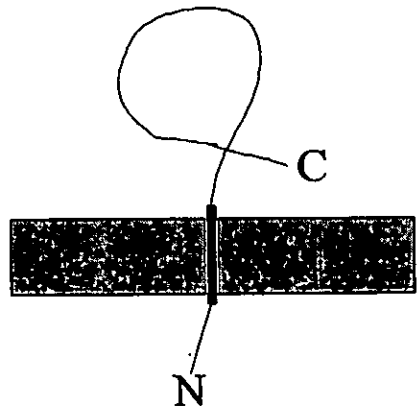
1.5.1 The high molecular mass PBPs

The high molecular mass PBPs are believed to possess N-terminal hydrophobic amino acid sequences which span the bilayer (Gittins *et al.* 1993) and have the topology of class II integral membrane proteins. These are proteins which are targeted to the membrane *via* an N-terminal or internal, uncleaved signal sequence and translocation results in exported C-termini and non-translocated N-termini (figure 1.4.; Von Heijne and Gavel, 1988).

PBP1a and PBP1b are known to occur as monomers and dimers (Zijderfeld *et al.*, 1991). Dimeric PBP1b appears to exist in two forms, one of which associates with murein and a second which, like the monomeric form, associates with the inner membrane (Zijderfeld *et al.* 1995a). Two

major types of PBP1b exist, α and γ. PBP1b-γ lacks the N-terminal 45 residues of PBP1-α (Broome-Smith *et al.* 1985) and experiments involving β-lactams have suggested that the γ form of the protein exists in two kinetically distinct conformations which are in slow equilibrium

Figure 1.4. The anchoring topology of a class II integral membrane protein.



For this class of protein, in the course of translocation the C-terminal domain is exported whilst the N-terminal domain remains non-translocated (Von Heijne and Gavel, 1988).

(Page, 1994). Both forms of PBP1b have the topology of class II integral membrane proteins (Edelman *et al.* 1987). However, it has been suggested that the PBP1b ectomembranous domain may also interact with the inner membrane (Nicholas *et al.* 1993). In support of this suggestion, the use of hybrid proteins has recently shown that the presence of both the transpeptidase and transglycosylase domains of PBP1b are essential for the specific function of the protein (Zijderveld *et al.* 1995b). A region comprising the first 163 amino acid residues of the PBP1b ectomembranous domain and which falls within the protein's transglycosylase domain appears to contain a putative membrane binding site (Wang *et al.*. 1996). Contrary to earlier findings of

Nicholas *et al.* (1993), the membrane association of this putative PBP1b binding site does not appear to involve lipid interactions (Wang *et al.* 1996). Cross-linking studies have indicated that PBP1b may be in close association with PBP3 and PBP5 (Said and Holtje, 1983) and it has been suggested that PBP1b may participate in protein complexes involved in murein manufacture (Harris and Phoenix, 1997a; Holtje, 1996a; Ehlert and Holtje, 1996; Gittins *et al.* 1993).

PBP2 appears to be a class II protein (Asoh *et al.* 1986) and consistent with this idea, it has been shown that when an internal hydrophobic sequence of amino acids, believed to constitute the PBP2 signal - anchor sequence, is removed, a soluble form of PBP2 results which accumulates in the cytoplasm but is able to interact with β-lactam antibiotics (Adachi *et al.* 1987).

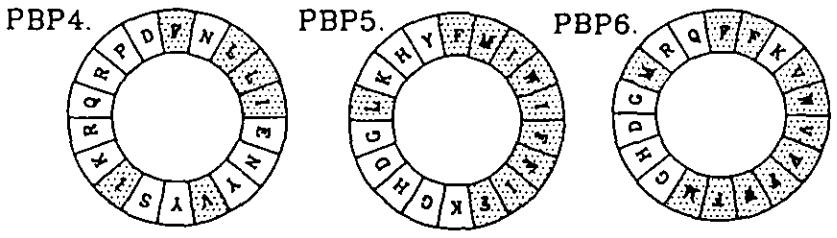
It appears that PBP3 can exist in a dimeric form (Ayala *et al.* 1994b) but differs from the other high molecular weight PBPs in that it undergoes a C-terminal processing event, involving the cleavage of a decapeptide (Nagasawa *et al.* 1989). However, PBP3 possesses a polar, hydrophilic amino terminus followed by a stretch of 24 hydrophobic amino acid residues (Nakamura *et al.* 1983) which has been confirmed to function as the protein's signal - anchor sequence. It has been found that if the N-terminus of PBP3 which contains the signal - anchor sequence is replaced with the OmpA signal peptide, the hybrid protein is exported into the periplasm. These periplasmic forms of PBP3 are soluble, are able to bind penicillin and undergo the PBP3 C-terminal processing event (Fraipont *et al.* 1994). The construction of hybrid proteins has shown that the presence of both the transglycosylase and transpeptidase domains of PBP3 are essential for the specific activity of the protein (Zijderveld *et al.* 1995b). More recently, it has been shown that the presence of key amino acid sequences in the non-penicillin-binding domain of PBP3 are essential for the protein to adopt a stable penicillin-binding conformation. However, although the adoption of such a conformation is necessary for the *in vivo* septation activity of PBP3, this alone

is insufficient and it was found that septation activity is also dependent upon the presence of the N-terminal sequence methionine 1 to glutamic acid 56 which appears to function as a non-cleaved pseudosignal - anchor sequence (Goffin *et al.* 1996).

1.5.2 The low molecular mass PBPs

This group of proteins comprises PBP4, PBP5 and PBP6 and the mature proteins are 457, 374 and 369 amino acid residues in length respectively (Phoenix and Harris, 1995; Gittins *et al.* 1993). These PBPs are targeted to the inner membrane *via* cleavable N-terminal signal sequences (Pratt *et al.* 1981; Pratt *et al.* 1986; Mottl and Keck, 1991) and in non-overproducing systems the translocated proteins are anchored to the periplasmic face of the inner membrane (Phoenix and Harris, 1995; Gittins *et al.* 1993). Nonetheless, when the primary structures of the low molecular mass PBPs are examined, there are no apparent conventional hydrophobic anchor

Figure 1.5 Two dimensional axial projections of the C-terminal regions of the low molecular weight PBPs.



When the C-terminal regions of PBP4 (Mottl et al. 1991), PBP5 and PBP6 (Broome-Smith et al. 1988) are plotted as two dimensional axial projections (Schiffer and Edmundson, 1967) the general segregation of the hydrophobic (shaded) and the hydrophilic residues demonstrate the potential of these regions to form amphiphilic α-helical structures.

sequences and no obvious sites for covalent modifications. The anchoring mechanisms of the low molecular mass PBPs appear unclear but deletion analysis has shown that the C-terminal sequences of PBP5 and PBP6 were essential for efficient membrane interaction. When the C-terminal sequences of PBP4, PBP5 and PBP6 were displayed as two dimensional projections (figure1.5) the potential for α-helix formation was revealed and in the case of PBP5 the involvement of the proteins C-terminal region in membrane anchoring was indirectly supported by the demonstration that when a proline residue, with its ability to distort or disrupt an α-helical conformation, was incorporated into the protein's C-terminal region, the membrane anchoring of PBP5 was greatly destabilised (Jackson and Pratt, 1988). Thus, it has been suggested the membrane associations of the low molecular mass PBPs may involve the participation of a C-terminal amphiphilic α-helix (Phoenix and Harris 1995; Gittins *et al.* 1993).

In membrane dependent processes, interactions between amphiphilic α-helical protein structure and membrane lipids are ubiquitous (Segrest *et al.* 1990) and accordingly, the possibility that such interactions may feature in the putative membrane interactions of the low molecular mass PBP C-terminal regions has been considered.

1.6 THE AMPHIPHILIC α-HELIX

1.6.1 Membrane lipids and amphiphilic interactions

The bilayer phase is inherently an amphiphilic structure which possesses an internal hydrophobic lipid core with external hydrophilic surfaces. This amphiphilicity facilitates the membrane interaction of many protein or peptide molecules which also possess amphiphilic characteristics.

On a structural basis, these protein and peptide molecules may be considered to have either primary, secondary or tertiary amphiphilicity. Primary amphiphilicity is found in integral membrane proteins where the primary structure contains sequences of amino acids distributed such that transmembrane stretches of apolar residues are demarcated at the membrane surface by clusters of hydrophilic residues. Secondary amphiphilicity is characteristic of many membrane interactive polypeptides and is manifested in the polypeptide primary sequence as the periodic occurrence of doublets or triplets of polar or apolar residues. Correct folding of the polypeptide leads to the segregation of hydrophilic and hydrophobic residues and the formation of secondary amphiphilic motifs such as α-helices or β-sheets. Tertiary amphiphilicity can arise when amino acid residues, distal in the primary struture of a protein, are brought together by the tertiary structure to form an amphiphilic site. This form of amphiphilicity is typically found in the structures of snake cardiotoxins (Chien *et al.* 1994). The interaction of all these amphiphilic architectures with the bilayer usually involves some degree of membrane penetration by the protein or peptide concerned and can have a wide range of effects on lipid organisation. Membrane proteins and thus sites of primary amphiphilicity can diffuse in the plane of the membrane and generally induce disorder in the bilayer structure by restricting the diffusion of neighbouring lipids. These restricted lipids are able to rapidly exchange places with other such molecules, indicating that the protein - lipid interactions are weak in nature. Some secondary amphiphiles such as certain apolipoproteins are able to stabilise bilayers whereas others such as some cytolytic peptides are able to destabilise these lipid structures. It has been suggested that the dynamic molecular shapes of these secondary amphiphiles can mimic those of inverted cone shaped and cone shaped phospholipids. Insertion of these geometries into the bilayer can either complement lipid packing and lead to membrane stabilisation or induce non-bilayer structure

such as the hexagonal phase H_{II} and lead to membrane destabilisation (Tytler *et al.* 1993; Cornut *et al.* 1993). Cytolytic secondary amphiphiles also share properties with some tertiary amphiphiles such as the snake venom cardiotoxins. Both groups of amphiphiles have been reported to be able to influence the ordering and dynamic motion of lipid acyl chains, the dynamics and orientation of phospholipid headgroups, and lipid phase transitions. These amphiphiles are also able to induce membrane fusion, phase separation, lipid domain formation and non-bilayer structures (Cornut *et al.* 1993; Vernon and Bell, 1992). Amphiphilic motifs are commonly employed in biological protein / peptide - lipid interactions but of these, the most ubiquitous is probably the amphiphilic α-helix. These helices are found in polypeptide secondary structure and protein secondary and supersecondary structure (Rao and Rossman, 1973; Clothia and Finkelstein, 1990). A particular form of this helical motif is the amphiphilic α-helix which may be defined as an α-helix with opposing polar and non-polar faces orientated along the long axis of the helix (Segrest *et al.* 1990). The molecular architecture of these helices is close to that of the classical or ideal α-helix, with differences in detailed geometries arising from variation in amino acid composition and environment (Kabasch and Sander, 1983; Barlow and Thornton, 1988). The inherent amphiphilicity of this helical arrangement gives these structures a role as an interface in a wide range of biological activities (Taylor and Osapay, 1990; Kaiser, 1984). Amphiphilic α-helices feature in protein - protein interactions with roles ranging from G protein recognition and activation by an intracellular helical region of the muscarinic acetylcholine receptor (Wess *et al.* 1995) to antibody recognition by an amphiphilic immunodominant loop of a foot and mouth viral protein (France *et al.* 1994). However, it is in the protein - lipid interactions of membrane dependent processes that the amphiphilicity of these α-helical segments achieves particular prominence (Cserhati and Szogyi, 1992; Cserhati and Szogyi, 1990). The ability of

amphiphilic segments to self associate permits the formation of hydrophilic channels. This is manifested in the action of the fish toxin paradaxin. Amphiphilic domains at the C-termini of the cytotoxic peptide associate to form membrane pores and cell lysis results (Shai, 1994). Amphiphilic α-helices are able to interact directly with phospholipids. This is illustrated by the action of the cytolysin melittin, where insertion of amphiphilic α-helical regions of the monomeric peptide into the bilayer causes perturbations leading to cell lysis (Dempsey, 1990). Recognition of the fundamental importance of the amphiphilic α-helix in bioactivity has led to the incorporation of such helices into proteins and peptides designed to probe membrane associated processes. Peptides homologous to amphiphilic α-helical regions of parathyroid hormone (Neugebauer *et al.* 1995) and penicillin-binding proteins (chapter 2) have been used to study the membrane interactive properties of the parent proteins. Peptides which do not occur naturally have been designed to probe the role of hydrophobicity in antimicrobial activity (Zhong *et al.* 1995) and investigate ion channel formation in lipid membranes (Tomich, 1993; DeGrado *et. al.* 1989). However, the synthesis peptides which do not occur naturally and possess sequences with the capacity to form α-helical regions or indeed the identification of these regions in naturally occurring proteins and peptides, requires techniques which can predict the potential of a given amino acid sequence to adopt an amphiphilic α-helical conformation.

1.6.2 The prediction of α-helical sequences

A variety of methods exist for the identification and characterisation of amphiphilic α-helical segments but may be generally classified as either graphical, those based on the Fourier transform or statistical (Auger, 1993). Probably the most commonly used graphical technique is the

Schiffer-Edmundson helical wheel diagram (Schiffer and Edmundson, 1967). These are two dimensional projections where the side chains of residues forming the α-helix are projected onto a unit circle, in a plane perpendicular to the long axis of the helix. Consecutive amino acid residues are offset by 100^{o} and amphiphilicity is revealed by the clustering of hydrophobic residues on one side of the unit circle and hydrophilic residues on the opposing side. In figure 1.5, typical helical wheel projections reveal the potential amphiphilicity of the C-terminal domains of the *Escherichia coli* low molecular mass penicillin-binding proteins. However, other than in cases such as these, where single, short amino acid sequences are being considered, graphical techniques are of limited use, primarily because of their requirement for subjective judgements. To overcome these limitations quantitative techniques for the detection of amphiphilic α-helices have been devised. The rose diagram, a two dimensional axial projection based on the helical wheel projections of Schiffer and Edmundson allowed some quantification of hydrophobicity. In these diagrams, the sector of the helical wheel representing a given amino acid includes a graphical representation which is a measure of the hydrophobicity of the given amino acid (Phoenix and Harris, 1995). However, probably the most widely used technique to detect amphiphilic α-helical structure is hydrophobic moment plot analysis, where the hydrophobic moment is an expression of amphiphilicity (Eisenberg *et al.* 1982). These authors considered the hydrophobicities of consecutive residues in a given amino acid sequence as vectors with positive values implying that a residue is hydrophobic. The hydrophobic moment is obtained from a summation of these vectors in two dimensions, perpendicular to the helical long axis and assuming a periodicity ω, which in the case of an idealised α-helix would be 100°, representing 3.6 residues per turn. This is represented by the expression:

$$\mu(\omega) = \{[\sum_{k=1}^{n} H_k \sin(k\omega)]^2 + [\sum_{k=1}^{n} H_k \cos(k\omega)]^2\}^{1/2}$$

where $\mu(\omega)$ is the hydrophobic moment of a sequence of length n residues, H_k is the hydrophobicity of residue k and ω is the periodicity of the residues side chain orientations. The usual method of analysis is to consider a window of eleven residues, representative of three turns of an α-helix, progressing along the amino acid sequence and for each window the hydrophobic moment and average hydrophobicity is calculated. The size of the hydrophobic moment allows the level of amphiphilicity to be estimated. The window possessing the largest value for the hydrophobic moment, together with the mean hydrophobicity at this point is then plotted onto the Eisenberg hydrophobic moment plot diagram (Eisenberg *et al.* 1984a). For a given data point on the plot diagram, the hydrophobicity measures the affinity of the sequence for the membrane interior and the hydrophobic moment measures the structured partitioning of hydrophilic and hydrophobic residues in the sequence and hence provides a measure of its amphiphilicity (figure 1.10). The Eisenberg hydrophobic moment plot diagram has been used to identify sequences likely to form amphiphilic α-helices and additionally to suggest possible membrane locations for the interaction of such helices (Eisenberg *et al.* 1984a). This use of the Eisenberg hydrophobic moment plot is illustrated in section 1.6.3.5 where figure 1.10 shows data points representing the C-terminal regions of PBP4, PBP5 and PBP6. The points of all three PBP s lie in the area defining surface active proteins, thus these C-terminal sequences would be predicted to be active at the interface (Pewsey *et al.* 1996).

However, despite the utility of the hydrophobic moment there are disadvantages to its use. One of the main problems stems from the fact that the hydrophobic moment is a function of hydrophobicity. There are a variety of scales expressing amino acid hydrophobicities and these may be empirical (Chou and Fasman, 1978), determined experimentally (Urry and Luan, 1995; Chakrabartty *et al.* 1994) or derived statistically (Miyazawa and Jerginan, 1985; Rose *et al.* 1985). Variations in the relative values of these hydrophobicities, both within and between scales, can lead to difficulties in interpreting the significance of the hydrophobic moment (Pewsey *et al.* 1996). Additionally, in their original analysis Eisenberg *et al.* (1982) assumed that μ(maximum) would occur when $\omega = 100^{o}$ which is the periodicity of an idealised amphiphilic α-helix. However, in practice μ(maximum) for an amphiphilic α-helical region usually occurs when ω is closer to 97.5^{o} (Cornette *et al.* 1987), although values between 95^{o} and 105^{o} are possible (Auger, 1993). Accordingly, hydrophobic moment plot analysis has been extended to include all periodicities between $\omega = 0^{o}$ to $\omega = 180^{o}$. This analysis allows the detection of periodic hydrophobicity which is characteristic of not only putative amphiphilic α-helical regions but also other regular structures such as β-sheet (Eisenberg *et al.* 1984b). However, hydrophobic moment plot analysis appears to have difficulty in identifying weakly membrane interactive α-helices, particularly those which occur near hydrophobic moment plot boundary regions (Harris and Phoenix, 1997b) and efforts have been made to refine this methodology. Brasseur (1991) has considered the molecular hydrophobic potential of membrane interactive protein and peptide sequences. This technique provides a three dimensional graphical summary of hydrophobicity. A numerical value for hydrophobicity is assigned to the sequence of interest but this method also considers the relative locations and size of the hydrophilic and hydrophobic arcs of the helix (Brasseur, 1991). Pewsey *et al.* (1996) have developed a technique which statistically tests the

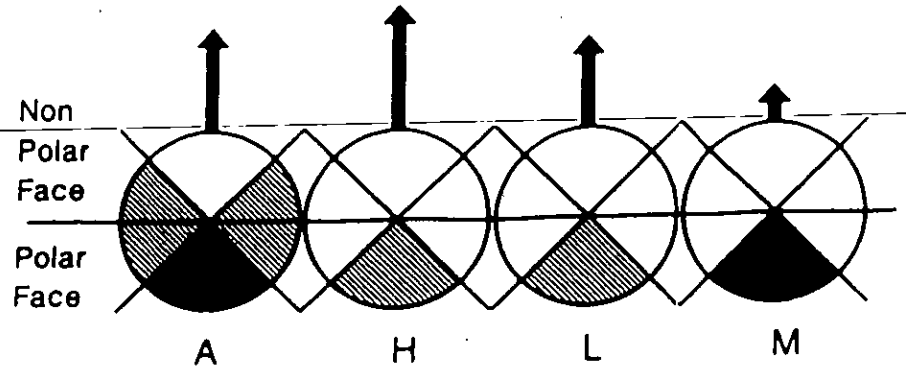
significance of the hydrophobic moment with respect to both the hydrophilic / hydrophobic nature and the spatial organisation of the residues present in the putative helical sequence (Pewsey *et al.* 1996). Other, mathematically more rigorous techniques have been developed for the prediction of amphiphilic α-helical domains. For example, the amphipathic index (AI), which is based on a Fourier transform, compares the hydrophobic moment with a power spectrum, with amphiphilicity being indicated by the intensity of the power spectrum around $\omega = 97.5^{o}$ (Cornette *et al.* 1987). More recently, the development of computer based techniques and algorithms (Roberts *et al.* 1997; Auger, 1993; Cornette *et al.* 1993; Segrest *et al.* 1990) means that the primary sequence of a protein may now be routinely screened for the presence of putative amphiphilic α-helical architecture. Typically, this methodology was employed in structure - function studies on the calmodulin-binding protein caldesmon. This protein was known to possess a phospholipid binding site (Bogatcheva *et al.* 1994) and the use of a computer algorithm showed that a potential amphiphilic α-helix existed within the sequence predicted to form the lipid interactive region (Bogatcheva and Gusev, 1995). Most recently, Roberts *et al.* (1997) have defined a new measure of membrane interactive potential - the depth-weighted insertion hydrophobicity (DWIH). This methodology employs an algorithm which models the putatitve α-helical sequence in three dimensions and determines the potential for membrane insertion, taking into account the exclusion of hydrophilic residues, insertion angles and insertion depth of the helix. Compared with hydrophobic moment plot analysis, this methodology appears to have several advantages, primarily the ability to detect a greater range of amphiphilic geometries and greater discrimination, particularly when attempting to identify weakly membrane interactive α-helices which occur near hydrophobic moment plot boundary regions (Roberts *et al.* 1997). The use of these computer-based methodologies has permitted the structural analyses of many

proteins and peptides which, in turn, has led to the the generation of databases. The existence of such databases has allowed amphiphilic α-helices from diverse sources to be compared and classified.

1.6.3 Classes of amphiphilic α-helices and their lipid interactions

Segrest *et al.* (1990) derived a database from known and putative amphiphilic α-helical regions of a large number of proteins and polypeptides. Comparisons between these helical arrangements revealed correlations between biological function and molecular architecture - principally the distribution of charged residues in the polar faces of the α-helices. These correlations led to the definition of seven classes of α-helix; three (K, C and G) are predominantly involved in protein

Figure 1.6. Schematic representation of classes of amphiphilic α-helces



Segrest et al. (1990) defined four classes of α-helix, A, H, L and M, which are concerned with protein - lipid associations (figure 1.6). Classification is based on a correlation between biological activity and distribution of charged residues in the polar face of these α-helices. Dark areas represent negatively charged regions, shaded areas represent positively charged regions and white areas represent hydrophobic regions. Arrows represent the hydrophobic moments of these classses of α-helix.

- protein interactions and four (A, H, L and M) are primarily concerned with protein - lipid associations (figure 1.6). For the most part, the properties of protein interactive amphiphilic α-helices lie outside the focus of this research and so will not be considered

By virtue of their dual nature amphiphilic α-helices are well designed to interact with lipids and membranes. However, these interactions may proceed by a variety of mechanisms. Lytic peptides possess class L amphiphilic α-helices (Segrest *et al.* 1990) and it has been proposed that these helices facilitate the initial binding of the peptide to the membrane. Orientated with the helical long axis parallel to the bilayer, membrane insertion of these helices then destabilises local lipid packing and leads to cell lysis (Tytler *et al.* 1993). Some polypeptide hormones which have putative amphiphilic α-helical regions self associate in aqueous solution, presumably resulting in helix stabilisation. However, other hormones are conformationally flexible in solution and require the proximity of lipid or a membrane to adopt α-helical conformations (Taylor, 1993). Amphiphilic α-helices from both groups have been analysed and classified as class H helices (Segrest *et al.* 1990). This membrane-induced formation of helical structure results in α-helices which orientate with the helix parallel to the membrane (Taylor and Kaiser, 1986) and has been proposed as an initial step leading to hormone - receptor binding (Taylor, 1993). Amphiphilic α-helices may also interact with the bilayer in transmembrane orientations, with the helical long axis roughly perpendicular to the membrane. These helices can associate within the membrane such that their apolar surfaces interact with phospholipid acyl chains whilst their polar faces can form hydrophilic channels or pores. Such amphiphilic α-helices from a number of proteins were analysed by Segrest *et al.* (1990) and were designated class M α-helices. Nonetheless, there are many other bioactive molecules which possess amphiphilic α-helices able to form such pores and channels but which would fall into one of the other categories defined by Segrest *et al.* (1990). A

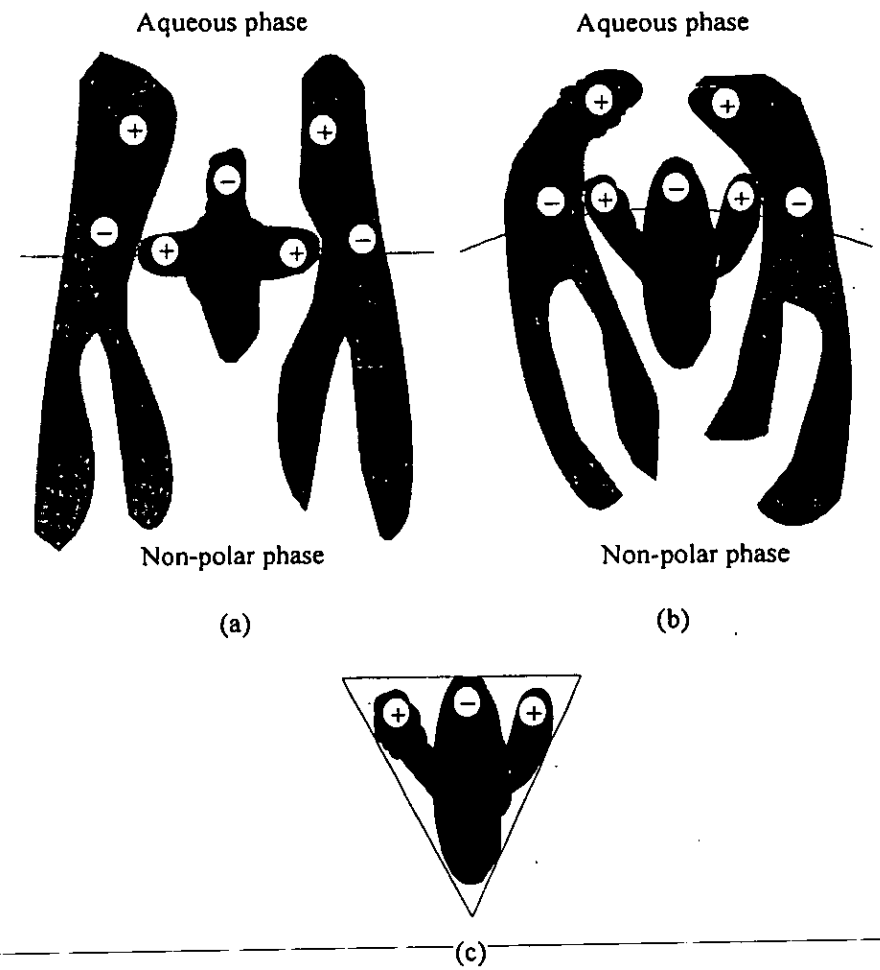
more detailed method of analysing the pore forming amphiphilic α-helices has been proposed and is discussed later (Tomich, 1993). However, historically, amphiphilic α-helices were first described as a unique structure / function motif in studies on the lipid interactions of the apolipoproteins (Segrest *et al.* 1974).

1.6.3.1 Apolipoproteins

One of the main biological activities of the exchangeable human apolipoproteins (Apo) is to associate with lipids to form lipoproteins in the plasma and thereby assist in the transport of these lipids to or from the tissues. There are various classes of lipoproteins and each class is associated with characteristic apolipoproteins and distinctive lipid compositions. However, these classes share common structural features, notably their spherical shapes. These spherical bodies possess hydrophobic cores, comprising lipid and apolar amino acid residues within the apolipoprotein structure, and hydrophilic external surfaces formed from phospholipid headgroups and the apolipoprotein charged and polar residues. The structures of the apolipoproteins possess internal tandem repeats of eleven amino acid residues within their sequences and the majority of these regions display the periodicity associated with amphiphilic α-helices. Such regions have been identified in Apo-AI, Apo-II , Apo-IV, Apo-C, Apo-CII, Apo-CIII and Apo-E (Anantharamaiah *et al.* 1993). In addition, potential α-helical domains have been identified in the predicted apolipoprotein gene product of the recently discovered gene *Apo-C4*, (Allan *et al.* 1995) and in Apo-B100, another class of apolipoprotein (Segrest *et al.* 1994). A computer-based analysis of 28 of these putative α-helical regions showed a unique bunching of arginyl and lysyl residues at the polar / non-polar interface, accompanied by clusters of negatively-charged residues at the centre

of the 180° polar face. Initially, these helices were categorised as class A amphipihilic α-helices (figure 1.6; Segrest *et al.* 1990) and a model had been proposed for their lipid associations. In this model, the positively-charged residues at the polar / non-polar interface and negatively-charged residues in the polar face of an apolipoprotein α-helical domain, interacted with phospholipid headgroups. Concomitant hydrophobic interactions between the non-polar face of the α-helix and the bilayer core were believed to result in the α-helix being half buried in the membrane (figure 1.7a; Segrest *et al.* 1974). The results of many studies on peptide analogues of apolipoprotein amphiphilic α-helical domains have shown that the clustering of positively-charged residues at the polar / non-polar interface and the centrally placed negatively-charged residues in the polar face of these helices is important for lipid association (Anantharamaiah *et al.* 1993; Epand *et al.* 1987). However, more recently it has been suggested that the amphiphilic nature of these positively charged residues themselves, namely those of lysine and arginine, contribute to the lipid affinity of the apolipoproteins. It has been proposed that, when interacting with phospholipids, the hydrophobic alkyl chains of these lysine and arginine side chains are able to snorkel or extend and insert their charged moieties into the aqueous phase (Segrest *et al.* 1992a) which would allow the class A helices much deeper penetration of the membrane (figure 1.7b) than that proposed by the original model (figure 1.7a) of Segrest *et al.* (1974). More recently, a further analysis of class A helices has revealed differences in the clustering patterns of the charged residues in the polar faces of these helices, both within a given apolipoprotein structure and amongst different structural classes of apolipoproteins. Based on these differences, the original class A α-helices were further separated into subclasses A_1, A_2 and A_4 which are proposed to be largely responsible for the lipid association of the apolipoproteins and the newly defined class G* helices and class Y helices (Anantharamaiah *et al.* 1993; Segrest *et al.* 1992b).

Figure 1.7 Models for the lipid association of apolipoprotein class A helices



In the original model (1.7a) the helix lies half buried in the non-polar phase. Positively charged residues at the polar / non-polar interface (—) and negatively charged residues in the polar face of an apolipoprotein α-helical domain interact with phospholipid headgroups. In the "snorkel" model (1.7b) the amphiphilic nature of the positively charged side chains allows these residues to extend out to the aqueous phase. This permits much deeper penetration of the membrane by the helix than that allowed by the original model. The molecular architecture of the class A helices gives rise to a helical side projection which effectively has an inverted cone shape (1.7c). This architecture may contribute to thecurved micellar surface of high density lipoprotein, as depicted (—) in figure 1.7b.

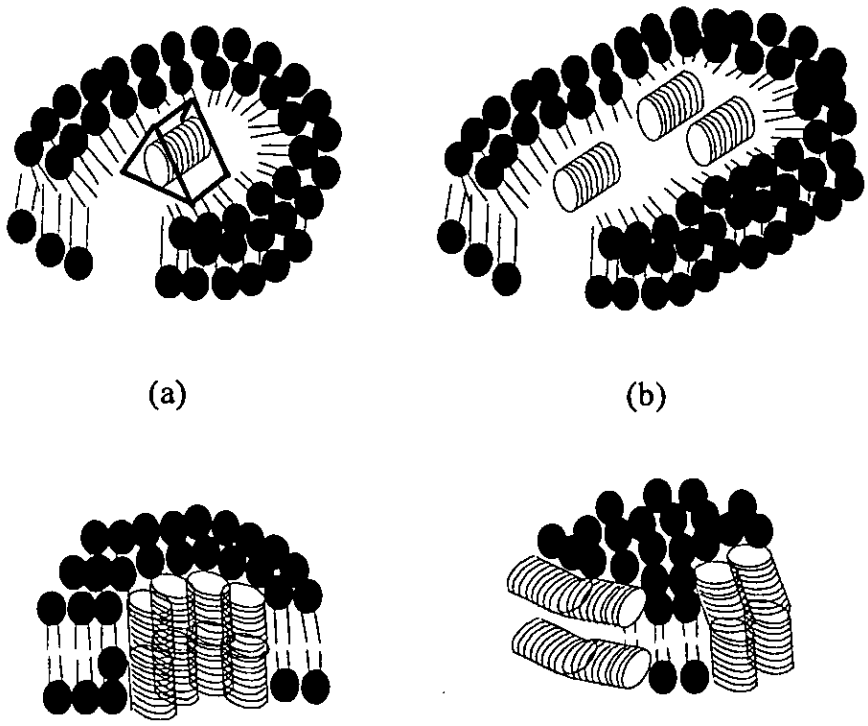
It has been suggested that this variation in structure and / or the number of class A helices present in apolipoprotein molecules may be related to the observed differences in lipid affinity, both between apolipoprotein molecules and between regions of the same molecule (Anantharamaiah *et al.* 1993). Such differences in lipid affinity are shown by results from recent work on Apo-AI which suggested that the strong lipid associating properties of the apolipoprotein are localised in two of the proteins eight amphiphilic α-helical domains, these being located in the C and N terminal regions of the apolipoprotein (Palgunachari *et al.* 1994). Based on the "snorkel" hypothesis it has been proposed that the molecular architecture of the class A helices gives rise to a helical side projection which effectively has a wedge or inverted cone shape (figure 1.7c). This structural arrangement could be important in the membane interactions of the apolipoproteins and may contribute to the curved micellar surface of high density lipoprotein (figure 1.7b; Segrest *et al.* 1992b). Interestingly, in the presence of class A α-helices the lytic action of some class L peptides are inhibited. This led to the idea that the wedge shape of the class A helices are able to compensate for the perturbations in membrane lipid structure caused by the molecular shapes of class L helices and the lytic peptides described in the next section (Tytler *et al.* 1993).

1.6.3.2 Lytic peptides.

Lytic peptides are functional components in the venoms, toxins and secretions of a diverse number of living organisms. The activity of these peptides involve invasion of the membrane and for many lytic peptides this process is facilitated by the presence of amphiphilic α-helical structure which forms most of the molecular architecture. Segrest *et al.* (1990) analysed thirteen such helical domains from cationic peptides, namely magainins, bombolitins, mastoparans and

crabolin, and so defined class L amphiphilic α-helices. These helices are characterised by high hydrophobic moments, narrow polar faces and the clustering of lysine residues at the polar / non-polar interfaces (figure 1.6; Segrest *et al.* 1990). The association of lytic peptides with the membrane can produce a variety of effects such as the stimulation / inhibition of enzymes (Tytler *et al.*, 1994) but their primary interaction is with lipids. A general mechanism describing how this lipid interaction may vary with increasing lytic peptide concentration (figure 1.8) has been suggested by Cornut *et al.* (1993). Membrane interaction is not believed to be receptor mediated and at low concentrations, the peptide penetrates the bilayer in a parallel orientation. Aligned in this manner, the helical hydrophobic face(s) of the peptide interact with the bilayer lipid core, whilst the polar face(s) associate with phospholipid headgroups and together these actions have the nett effect of perturbing the bilayer. This mechanism is generally accepted for the binding of melittin to erythrocytes (Dempsey, 1990). For class L helices and based on the "snorkel" hypothesis, Tytler *et al.* (1993) have suggested that this membrane perturbation results from the molecular architecture of these peptides. Class L helices are proposed to have helical side projections which effectively have an inverted wedge or cone shape. These arrangements are able to mimic phospholipid dynamic molecular shapes which favour non-bilayer phospholipid packing (Tytler *et al.* 1993) and thus peptide insertion into the membrane destabilises local bilayer lipid packing (figure 1.8a). In Figures 1.8b and 1.8c, it can be seen that as the peptide concentration increases the tendency of monomers to self associate within the membrane increases until oligomers are able to engage in channel forming activities. Oligomerisation can then increase until in the presence of excess peptide membrane fragmentation and cell lysis occurs (figure 1.8d). Evidence in support of this general mechanism comes from many recent reports. For example, magnainin 2 and alamethicin interact with phospholipids in parallel

Figure 1.8 A general scheme for the effects of lytic peptides on membrane structure



At low concentrations, monomers of lytic peptide destabilise local bilayer lipid packing by the induction of a wedge effect (1.8a). As peptide concentration rises, the tendency of monomers to self associate within the membrane increases until oligomers are able engage in channel forming activities (1.8b and 1.8c). Oligomerisation can then increase until, in the presence of excess peptide, membrane fragmentation occurs (18d).

orientations at sub-lytic peptide concentrations but insert at higher peptide concentrations (Ludtke *et al.* 1995). Pardaxin aligns with phospholipids in the bilayer and forms a pore which increases in diameter with the progressive addition of monomers (Shai, 1994). For δ-toxin, channel

formation is proposed to involve parallel bundles of amphiphilic α-helices and is a distinct event from cell lysis (Kerr *et al.* 1995). For potent haemolysins such as melittin, at high concentrations, membrane interaction results not only in total lysis but the substantial solubilisation of phospholipids (Katsu *et al.* 1989). Nonetheless, many questions remain to be answered as to the membrane interaction of lytic peptides and even for extensively studied lysins such as melittin and alamethicin, precise details on bilayer orientations and the aggregation states of the peptides are still lacking (Saberwal and Ramakrishnan, 1994). Further, despite many similarities, there are many fundamental structural differences between the architectures of lytic peptides. Mellitin is probably the most potent cationic haemolysin known (Dempsey, 1990), yet Segrest *et al.* (1990) propose that the helical domains of this peptide are more characteristic of the class M helices of transmembrane proteins rather than class L helices. Other lysins are not cationic but may be zwitterionic such as δ-haemolysin or uncharged as is alamethicin (Cornut *et al.* 1993). This indicates that the α-helical domains of lytic peptides are a heterogeneous group and this heterogeneity is not accounted for by the analysis of Segrest *et al.* (1990). However, all cytolytic peptides show channel forming activity and as such constitute a subset of channel forming peptides (CFPs). The CFPs also include synthetic peptides and peptides derived from larger proteins, and form part of a family of channel forming structures which facilitate the selective transport of various ions. Individual CFPs could be placed in many of the categories of membrane interactive amphiphilic helices described by either Segrest *et al.* (1990) or Anantharamaiah *et al.* (1993). It has been suggested that the structures of the CFPs are better described by considering the amphiphilic helix as being composed of four functional regions: the polar face (P), the non-polar face (NP) and two separate regions (A and A') which participate in helix-helix interactions with adjacent peptide molecules. The surfaces defined by these functional regions may also be

described by the angles which they subtend: Θ_P, Θ_{NP}, Θ_A and $\Theta_{A'}$. Analysis of the distribution of amino acid residues forming the regions defined by A and A' revealed that although these two regions possessed nearly identical hydrophobic moments there was a specific localisation of certain amino acid residues, generating an asymmetry between A and A'. The CFP helices always pack in parallel bundles with region A interacting with region A' of the adjacent helix. The apparent asymmetry involved suggested conserved complementary structures between certain residues and may indicate the existence of packing motifs in the assembled structures. Thus the amino acid residues which form the amphiphilic α-helices of CFP s and hence some lytic peptides, may not only serve the function of membrane invasion but of ordered self association and ion channel formation (Tomich, 1993). It appears that such a packing motif is responsible for the ordered assembly of the dimeric transmembrane domain of glycophorin A. Steric considerations (MacKenzie *et al.* 1997) and mutagenesis data (Lemmon *et al.* 1994) have suggested that the presence of a seven residue motif, located within the single transmembrane domain of monomeric glycophorin A, induces dimerisation *via* α-helix - α-helix packing and this packing is the main determinant of stability of the dimer. Another subgroup of the channel forming structures is that comprising large complex multimeric proteins such as the nicotinic acetylcholine receptor. These proteins possess transmembrane amphiphilic α-helices some of which were analysed by Segrest *et al.* (1990) and are included in the class M transmembrane helices described in the next section.

1.6.3.3 Transmembrane proteins

Integral membrane proteins possess lipophilic α-helical regions which are able to traverse the bilayer. These helical regions may be either, hydrophobic as are those found in the photoreaction centres of *Rhodobacter sphaeroides* (Rees *et al* 1989a; Allen *et al.* 1987) and *Rhodopseudomonas viridis* (Diesenhofer *et al.* 1985), or amphiphilic. These amphiphilic α-helices are generally arranged in the membrane such that a hydrophilic pore or channel is formed. Typical of such an arrangement is that found in bacteriorhodopsin, the proton pump of *Halobacterium halobium*. The structure of this protein has been obtained to near atomic resolution by electron diffraction (Ceska *et al.* 1992) and it possesses seven amphiphilic α-helices which associate to form a membrane pore. Analysis of these seven helices, along with amphiphilic helical sequences from various receptors, transporters and channel forming proteins, defined the class M helices (figure 1.7) which are distinguished by very low charge densities on the polar face, low mean hydrophobic moments but high mean non-polar face hydrophobicity. These parameters reflect the fact that the amphiphilicity of the class M helices is slightly different in nature to that of the other helices described (Segrest *et al.* 1990). The residues which make up the hydrophilic faces of the class M helices are predominantly polar in nature rather than charged and these polarities are comparable to those of the interior residues of soluble proteins. However, highly hydrophobic residues form the exterior residues of the class M helices whereas, of course, hydrophilic residues form the exterior faces of soluble proteins (Rees *et* al. 1989b). With these properties, class M helices differ markedly from the other classes of helices considered by Segrest *et al.* (1990). Most notably, whereas the class M helices form lipid interactive structural components of membrane

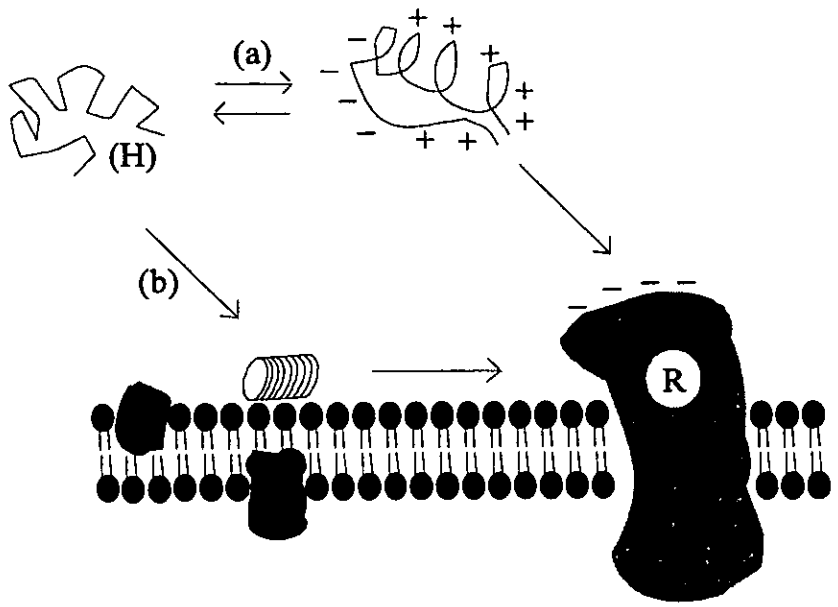
embedded proteins, the class H helices of the next section are inducible conformations in the structures of some polypeptide hormones.

1.6.3.4 Polypeptide Hormones

Amphiphilic α-helices have been postulated as being structural components and having functional roles in a wide variety of polypeptides that function as hormones. These polypeptides fall into two general groups: Those of higher molecular weight where amphiphilic α-helical regions are generally an integral part of the biologically active tertiary structure, and those of lower molecular weight polypeptides, such as β-endorphin, which possess little ordered structure in solution (Taylor, 1993). Despite this, studies on modelled peptides suggested that amphiphilic α-helical structures were important determinants in the biological activity of β-endorphin. It was proposed that an amphiphilic environment, such as a membrane surface, could induce α-helical conformations within the β-endorphin molecule and that similar processes may occur in the structures of other polypeptide hormones (Kaiser and Kezdy, 1983). A similar mechanism has been suggested for the induction of α-helical structure in the lytic peptide melittin which also shows little ordered structure in solution (Dempsey, 1990). In the case of polypeptide hormones, evidence supporting this mechanism is provided by recent data from the CD analyses of various calcitonins which revealed the formation of α-helical secondary structure in membrane mimetic environments (Siligardi *et al.* 1994) and from molecular dynamics simulations which showed that corticotropin releasing factor had the capacity to adopt α-helical conformations in the presence of a phospholipid bilayer surface (Huang and Leow, 1995). A number of amphiphilic α-helical sequences derived from hormone polypeptides were analysed by Segrest *et al.* (1990) and defined

as class H helices (figure 1.7). This class of helices tend to be strongly cationic, possess polar faces which subtend angles of 100^{o} or less and have high hydrophobic moments. These properties are similar to the class L helices of lytic peptides discussed previously and presumably reflect the fact that both classes of helix function at the interface and usually orientate parallel to the bilayer to interact with lipids (Segrest *et al.* 1990). In general, these hormone helices are located in the C-terminal regions of the molecule and serve to enhance the receptor binding of an N-terminal site which constitutes the essential basic unit for specific receptor binding and pharmacological activity (Taylor and Kaiser, 1986). Typically, such structural organisation has been proposed for β-endorphin (Kaiser and Kezdy, 1983) and more recently for the intestinal peptide hormone, motilin (Miller *et al.*, 1995). It has been suggested that one of the ways in which α-helical regions of molecules such as β-endorphin enhance receptor binding is to assist in the facilitated diffusion of the hormone to its receptor. Two models have been proposed for this process. In the first of these mechanisms, the hormone is in solution and the potential for amphiphilic α-helix formation in the molecule, leads to a partially folded structure and the segregation of charged residues. This segregation of charged residues enables long range electrostatic interactions to guide the hormone to its receptor with subsequent binding. Such a mechanism has been proposed for neuropeptide Y (figure 1.9). In the second proposed pathway, membrane catalysed α-helix formationpermits non-specific adsorption of the hormone polypeptide onto the membrane surface and subsequent surface diffusion leads to receptor binding (figure 1.9; Taylor, 1993). Strong support for this latter mechanism comes from studies on the membrane interaction of a N-terminal lipo-peptide derivative of the neuropeptide cholecystokinin. This peptide derivative was predicted to interact with the membrane *via* a

Figure 1.9 Two hypothetical mechanisms for receptor location by polypeptide hormones



In the first mechanism (a) receptor location occurs in solution. The potential of the hormone molecule (H) for amphiphilic α-helical formation favours a partially folded structure. The resulting segregation of charged residues enables long range electrostatic interactions to guide the hormone to its receptor (R), with subsequent binding. In the second mechanism (b), receptor location proceeds via the membrane catalysed induction of α-helical structure in the hormone molecule. Subsequent surface diffusion leads to receptor binding.

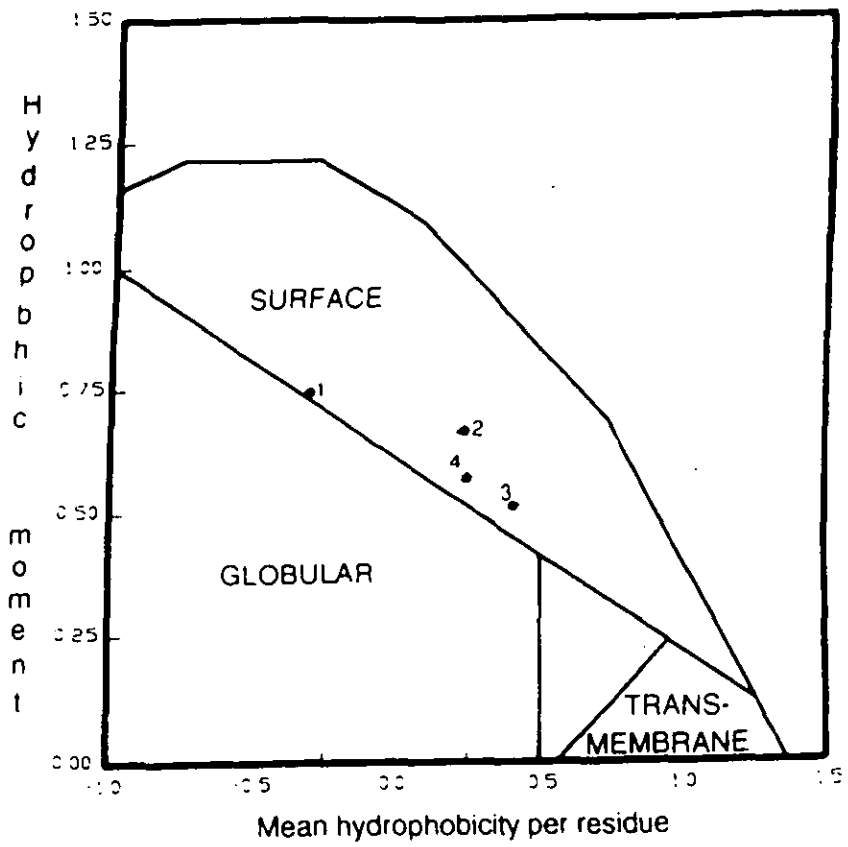
conventional C-terminal amphiphilic α-helical region but in addition the N-terminal lipid moiety was designed to interdigitate with bilayer lipids to form a strong complementary membrane association. Despite these constraints, the strength of binding of the cholecystokinin lipo-peptide derivative to receptors was comparable to that of the parent hormone peptide, albeit at a lower rate. These results implied that lateral diffusion of the lipo-peptide through the bilayer lipid phase had occurred to facilitate receptor binding. In turn, this suggested that adsorption of polypeptide hormones to the cell surface may indeed be a first step in the receptor recognition process (Moroder *et al.* 1995). Thus, it appears that the induction of amphiphilic α-helical

structure at the membrane surface is a feature of the biological activities of at least some polypeptide hormones. It has been suggested that a similar process may be involved in the membrane interactions of *E. coli* low molecular mass penicillin-binding proteins (Phoenix and Harris, 1995; Gittins *et al.* 1993).

1.6.3.5 The *E. coli* low molecular mass penicillin-binding proteins.

Deletion analysis (Pratt *et al.* 1986; Jackson and Pratt, 1987; Van der Linden *et al.* 1992) and Schiffer-Edmundson graphical analysis (Schiffer and Edmundson, 1967) have led to the suggestion that amphiphilic C-terminal architecture (figure 1.5) may feature in the membrane interactions of the low molecular mass PBPs. More recent theoretical work, including hydrophobic moment plot analysis (Phoenix, 1990), statistical analysis (Pewsey *et al.* 1996) and the DWIH analysis of Roberts *et al.* (1997) have also predicted that the C-terminal regions of the low molecular mass PBPs may have the potential for α-helix formation and surface activity. In the approach of Pewsey *et al.* (1996), hydrophobic moment profile analysis suggested that the most likely amphiphilic structure to be formed by the PBP5 and PBP6 C-terminal regions were amphiphilic α-helices. However, in the case of the PBP4 C-terminal region this analysis suggested that amphiphilic α-helical structure or amphiphilic β-sheet structure could be formed by the region with almost equal probabilities. When the C-terminal regions of the low molecular mass PBP s were represented as data points on a hydrophobic moment plot diagram (figure 1.10), the data points were found to lie in the region defining surface active proteins (Pewsey *et al.* 1996) which also included the data point of melittin, a cytolysin known to interact with the

Figure 1.10 Hydrophobic moment plots of the C-terminal regions of PBP4, PBP5, PBP6 and melittin



The data points of the PBP5 and PBP6 C-terminal regions, (2) and (3), cluster around that of melittin (4), a toxic molecule, known to possess amphiphilic α-helical secondary structure and to be surface active (Dempsey, 1990). It was predicted that these PBP C-terminal regions possess high potential for α-helix formation and may have surface activities comparable to that of melittin. In contrast, the data point of the PBP4 C-terminal region (1) is removed from the cluster. It was predicted that if the PBP4 C-terminal region does form an amphiphilic α-helix, then this region would be only weakly membrane interactive.

membrane *via* amphiphilic α-helices (Dempsey, 1990). It has been suggested that if similar structural arrangements facilitated the membrane interaction of PBP4, PBP5 and PBP6 C-terminal α-helices then the hydrophilic faces of these helices would project into the aqueous

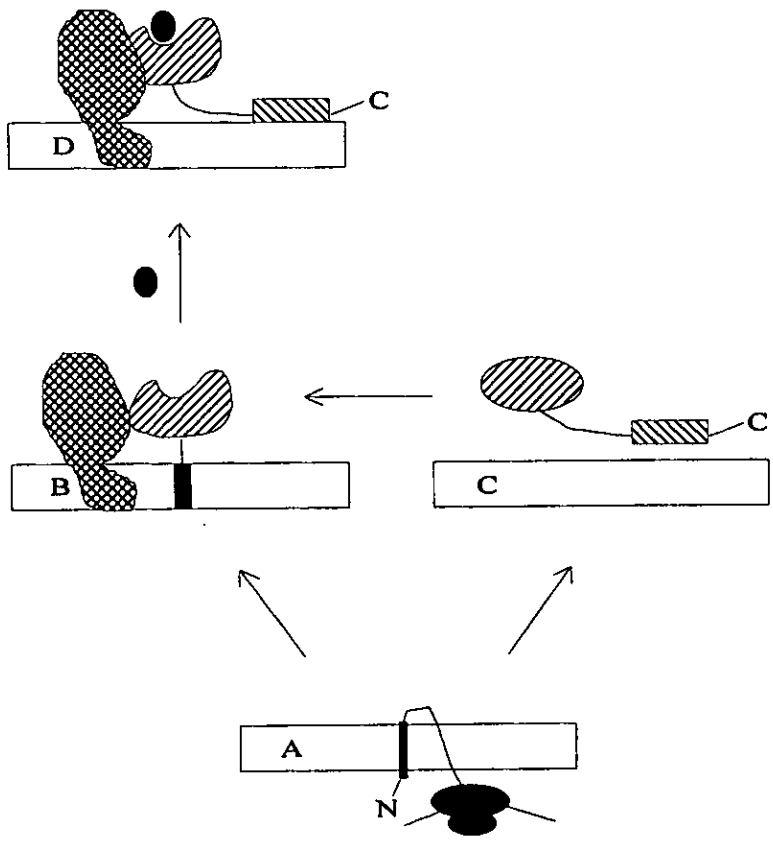
environment whilst the hydrophobic faces would interact with the lipid bilayer core (Phoenix and Harris 1995). However, various analyses have also predicted that the PBP4 C-terminal region has a lower potential for α-helix formation than those of PBP5 and PBP6. In particular, it can be seen from figure 1.5 that in an α-helical conformation, the segregation of hydrophilic and hydrophobic residues in the PBP4 C-terminal region is less well defined than those of the corresponding PBP5 and PBP6 regions (Gittins *et al.* 1993). It can be seen from figure 1.10 that the data points representing the PBP5 and PBP6 C-terminal regions cluster around that of melittin, suggesting that these regions may have amphiphilic α-helix forming potential and surface activity comparable to those of melittin but the data point representing the PBP4 C-terminal region is removed from the main cluster. Although this latter data point is statistically significant with respect to levels of amphiphilicity, based on its location (figure 1.10), Pewsey *et al.* (1996) concluded that even in amphiphilic α-helical conformation, the potential of the PBP4 C-terminal region for membrane interaction was low. This conclusion was supported by the results of a DWIH analysis of the same C-terminal region (Roberts *et al.* 1997). Whether the PBP4 C-terminal region plays a role in the membrane anchoring of PBP4 and if so, whether amphiphilic α-helical architecture is involved are, as yet, unresolved questions but recent studies have shown that the membrane association of PBP4 appears to be fundamentally different to that of the other low molecular mass PBPs (Phoenix and Harris, 1995; Gittins *et al.* 1993). Nonetheless it appears to be generally accepted that PBP5 and PBP6 anchor to the membrane *via* amphiphilic α-helices at their C-termini (Phoenix *et al.* 1994; Phoenix and Pratt, 1990).

1.6.3.5.1 PBP5 and PBP6

Experimental results have suggested that PBP5 and PBP6 have similar anchoring characteristics and in both cases, over-expression results in proteins which are exclusively membrane bound (Phoenix and Harris, 1995; Gittins *et al.* 1993). Experiments have shown that when *E. coli* inner membrane fragments are treated with perturbants, PBP5 and PBP6 are displaced from the membrane. In particular, PBP5 and PBP6 show a strong susceptibility to the chaotropic thiocyanate ion and the denaturant urea but a weak susceptibility to ionic perturbants suggests a major role for hydrophobic forces and a minor role for electrostatic interactions in the membrane anchoring of these proteins (Phoenix *et al.* 1994; Phoenix and Pratt, 1990). These results are consistent with the predicted involvement of amphiphilic architecture in the membrane interactions of PBP5 and PBP6. The susceptibility of PBP5 and PBP6 to perturbants is accompanied by a pH dependence, in particular, at low pH, these proteins are resistant to the action of urea but as pH is increased above neutrality, progressively greater amounts of the protein are washed off the membrane (Phoenix *et al.* 1994; Phoenix and Pratt, 1990). It has been postulated that this pH dependent release from the membrane may result from increasing accessibility of the protein's anchor regions to urea. In turn, increasing accessibility of the PBP5 and PBP6 anchor domains may arise from decreased affinity for a membrane receptor or may be a reflection of pH related changes in the conformations these proteins. In support of the latter idea, several lines of evidence have suggested that the overall conformation of PBP5 can affect the strength of binding of the protein to the membrane. When the anchor domain of PBP5 was fused to a soluble periplasmic β-lactamase the hybrid protein was able to bind to the membrane but was unable to attain the urea inaccessible state (Phoenix and Pratt, 1993). Additionally, it has

been shown, using an active site mutant, that when PBP5 interacts with a β-lactam antibiotic and is therefore in an active conformation, the urea accessible form of the protein predominates (Phoenix and Pratt, 1993). Together, these results suggest that the PBP5 C-terminal anchor region is sufficient for membrane association but that the presence of the ectomembranous domain is also necessary to attain the urea inaccessible state and for efficient membrane interaction. Furthermore, it has been speculated that if the binding of a substrate analogue to PBP5 is instrumental in maintaining the urea accessible state of the protein, then this may indicate that this state corresponds to the enzymatically active form of PBP5 with the urea inaccessible state corresponding to the enzymatically inactive form (Phoenix and Pratt, 1993). It could be that in this active form the PBP5 ectomembranous domain is restrained from engaging in interactions with other membrane components which are necessary for the higher levels of membrane binding associated with the urea inaccessible state. One possibilty is that PBP5 may interact with other membrane bound proteins. In support of this idea it was found that PBP5 would not reconstitute into vesicles when the cytoplasmic face was accessible but would reconstitute into vesicles when the periplasmic face was accessible (Phoenix, personal communication). This suggests that some component of the membrane periplasmic face, possibly proteinaceous, specifically facilitates PBP5 - membrane association (Phoenix and Pratt, unpublished data). Furthermore, cross-linking studies have suggested the possibility of a protein complex involving PBP1a / 1b, PBP3 and PBP5 (Said and Holtje, 1983). Overall, these latter findings have led to the suggestion that PBP5 may participate in a protein complex which includes other PBP's and plays a role in peptidoglycan biosynthesis (Harris and Phoenix, 1997a; Gittins *et al.* 1993). A suggested scheme for the membrane anchoring of PBP5 and by analogy that of PBP6 is shown in figure 1.11.

Figure 1.11 A suggested scheme for the membrane interaction of PBP5 and PBP6



After translocation has been initiated and the signal sequence cleaved ("A"), two courses of action may be available for PBP5: as the C-terminus of the protein crosses the membrane it may adopt an amphilphilic alpha-helical conformation which acts as a stop transfer sequence, preventing complete translocation and anchoring the protein to the membrane ("B"). Alternatively PBP5 may be completely translocated and exist as a short lived periplasmic intermediate ("C"). Charge and pH effects could then stabilise the amphiphilic alpha-helical secondary structure of the protein which then interacts with the membrane ("B"). In either case, the PBP5 C-terminal interaction may be stabilised by other interactions involving the protein's ectomembranous domain and other membrane bound proteins ("B"). This could correspond to the urea inaccessible state and enzymatically inactive form of PBP5. On binding of substrate ("D") PBP5 adopts an enzymatically active form with concomitant accessibility of the proteins C-terminal anchor region to urea (Gittins et al. 1993; Phoenix and Pratt, 1993) and changes in the levels of interaction of the protein's ectomembranous domain. It could be that this enzymatically active form of PBP5 corresponds to the urea accessible form of the protein.

The analysis and classification of the C-terminal regions of PBP5 and PBP6 using the techniques of Segrest *et al.* (1990) has not yet been done. Nonetheless, using a qualitative approach, it can be seen from figure 1.5. that the PBP5 and PBP6 C-terminal helices are cationic, have positively charged residues at or near the polar - non-polar interface and possess wide polar faces of 140^{o} and 180^{o} respectively. With these structural characteristics, the PBP5 and PBP6 C-terminal helices show no particular similarities to any of the classes of helix described by Segrest *et al.* (1990). It may be that further analysis will show that these helical domains belong to a class of helix hitherto undescribed.

1.6.3.5.2 PBP4

Recently, light scattering experiments have shown that PBP4 exhibits aggregation behaviour in the presence of various precipitating agents and additives. However, in the presence of EDTA, PEG 4000 and ammonium sulphate the determined molecular mass was approximately 110 kDa (Fusetti and Dijkstra, 1996) which is consistent with a dimeric form of the protein. Under appropriate conditions, a crystalline form of PBP4 has been precipitated from ammonium sulphate solution (Thunnissen *et al.* 1995). There is some debate as to whether *in vivo*, PBP4 is a soluble protein or a legitimately membrane bound protein and if the latter is true, as to whether the membrane binding of the protein involves amphiphilic C-terminal architecture (Phoenix and Harris, 1995). Examination of the primary sequence of PBP4 did not reveal either a potential C-terminal amphiphilic α-helical domain or other regions likely to be involved in the anchoring of PBP4 (Mottl *et al.* 1991). This may be supported by the observation that only 10% of overproduced PBP4 is recovered with the membrane fraction after osmotic lysis (Korat *et al.*

1991), compared with 100% of PBP5 and PBP6 (Gittins *et al.* 1993). The lack of a strongly amphiphilic C-terminus (figure 1.5), combined with the recovery of the majority of the overproduced protein in the soluble fraction, led to the suggestion that PBP4 is actually a soluble protein and that the PBP4 found associated with the membrane fraction results from non-specific interactions between PBP4 and the membrane (Mottl *et al.* 1991). However, various theoretical analyses have predicted that the PBP4 C-terminal domain may have a weak potential for α-helical formation and membrane interaction (Roberts *et al.* 1997; Pewsey *et al.* 1996). This could be supported by the recent demonstration that PBP4 and other penicillin-binding proteins were able to engage in protein - protein interactions with immobilised transglycosylases. This led to the suggestion that multi-enzyme complexes which could involve PBP4 may function in cell wall synthesis (Ehlert and Holtje, 1996; Holtje, 1996a;. Holtje, 1996b).

1.7 CONCLUSION

Nature has decreed that cellular processes occur in an aqueous environment. As a necessary consequence and for biological function, the cell also has a requirement for hydrophobic and amphiphilic agents - particularly the lipid components of membrane barriers. To interact with the amphiphilic environments and at the phase boundaries created by the presence of lipids within the cell, proteins and peptides have also evolved the ability to assume amphiphilic character. Of the various mechanisms used by proteins and peptides to achieve this, the most commonly employed is the adoption of amphiphilic α-helical secondary structure. Major examples of the lipid interactions of this molecular architecture include those of apolipoproteins, lytic peptides,

transmembrane proteins and polypeptide hormones. It appears that amphiphilic α-helical architecture at the C-termini of the *E. coli* low molecular mass penicillin-binding proteins may feature in the membrane interactions of these proteins. To help establish if these C-terminal sequences have the capacity for α-helix formation and membrane association, the conformational properties and lipid interactions of the peptides, P4, P5 and P6 which are homologues of the PBP4, PBP5 and PBP6 C-terminal regions have been investigated. The membrane binding requirements of the parent proteins PBP4 and PBP5 have been studied and preliminary experiments have been conducted with a view to localising low molecular mass PBPs to the *E. coli* inner membrane. In chapter 2, CD analysis is utilised to determine if P4, P5 and P6 have the capacity for α-helix formation in the presence of various lipids and detergents and in chapter 3, haemolytic analysis is used to investigate the potential of these peptides for membrane interaction. In chapter 4, P5 and P6 are further examined for the presence of α-helical structure by the use of CD and pressure - area isotherm analysis on monolayers formed from these peptides. Monolayers derived from the inner membranes of various strains *E. coli* and pure lipid monolayers are then used to determine the membrane interactive potential of P5 and P6 and the specific lipid requirements for such interactions. In chapter 5, the PBP5 requirement for anionic lipids is investigated. Using the *E. coli* mutant strain, HDL11 (Kusters *et al.* 1991), in which the membrane levels of negatively charged lipids can be controlled, the action of perturbants on PBP5 associated with membrane extracts which possess either wild type levels or depleted levels of anionic lipids were observed. In chapter 6, using chemiluminesence, PBP4 is localised in the wild type *E. coli*, MRE600 (Cammack and Wade, 1965). Chemiluminesence and biochemical wash procedures are used to investigate the lipid requirements and forces involved in the binding of PBP4 to membranes of the PBP4 overproducing *E. coli* strain, HB101/pBK4 (Korat *et al.*

1991) and the mutant strain HDL11. The potential of the PBP4 C-terminal region for membrane association is further studied by observing the interactions of P4 with lipid monolayers, in particular, those derived from the wild type *E. coli*, SD12 (De Vrije *et al.* 1988) and those from the mutant strain HDL11.

CHAPTER 2.

α-HELICAL CONFORMATION IN THE C-TERMINAL ANCHORING DOMAINS OF *ESCHERICHIA COLI* PENICILLIN-BINDING PROTEINS 4, 5 AND 6.

HARRIS, F., SILIGARDI, G. AND PHOENIX, D. A. (1997) BIOCHIMICA BIOPHYSICA ET ACTA. **1329**, 278-284.

2.1 ABSTRACT

The conformational behaviour of synthetic peptides corresponding to the C-terminal anchoring domains of PBP4, PBP5 and PBP6 were studied as a function of solvent, pH, sodium dodecyl sulphate micelles and phospholipid (DOPC, DOPG) vesicles using circular dichroism spectroscopy. Although the CD data showed that in 2, 2, 2-trifluoroethanol or sodium dodecylsulphate, all three peptides have the capacity to form an α-helical conformation, in the presence of phospholipid vesicles only those peptides corresponding to the PBP5 and PBP6 C-termini were observed to do so. A pH dependent loss of α-helical conformation in the peptide corresponding to the PBP5 C-terminus was found to correlate with the susceptibility of PBP5 to membrane extraction.

2.2 INTRODUCTION

For the proteins PBP5 and PBP6, much indirect biochemical evidence (section 1.5.2; section 1.6.3.5.1) and theoretical analyses (section 1.5.2; section 1.6.3.5; figure 1.5) have supported the idea that these proteins are anchored to the membrane *via* C-terminal amphiphilic α-helices. This is now the generally accepted model for the membrane anchoring of these proteins (section 1.5.2, section 1.6.3.5.1).

In contrast, various theoretical analyses have predicted that the PBP4 C-terminal region has the potential to form only a weakly amphiphilic α-helix (section 1.5.2, section 1.6.3.5). Furthermore, Pewsey *et al.* (1996) has predicted that this region would form α-helix or β-sheet structure with almost equal levels of amphiphilicity and if the region was to adopt an α-helical conformation, then the helix would be only weakly surface active compared to PBP5 and PBP6. Taken with the fact that the anchoring mechanism of PBP4 appears to be fundamentally different to that of the other low molecular mass PBP s (chapter 6), it is uncertain as to whether the PBP4 anchoring mechanism does involve the C-terminal region of the protein.

As yet, the ability of the C-terminal regions of PBP4, PBP5 and PBP6 to form α-helices is untested. In the present work, circular dichroism (CD) is used to determine if the homologues of these C-terminal regions, P4, P5 and P6 (table 2.1), are able to form an α-helical conformation, as a function of pH, solvent and membrane mimetic environments.

Table 2.1 The primary structures of P4, P5 and P6

PROTEIN	C-TERMINAL RESIDUES
PBP4	++ + - + + - - RRIPLVRFESRLYKDIYQNN-COO
PBP5	+ - + ++ - GNFFGKIIDYIKLMFHHWFG-COO
PBP6	+ - + + - GGFFGRVWDFVMMKFHQWFGSWFS-COO

The peptides P4, P5 and P6 possess sequence identity with the C-terminal domains of PBP4 (Mottl et al. *1991) PBP5 and PBP6 (Broome-Smith* et al. *1988) respectively.*

2.3 MATERIALS AND METHODS

Materials

Synthetic peptides P4, P5 and P6, possessing primary structures which correspond to the C-terminal domains of PBP4, PBP5 and PBP6 (table 2.1) respectively were purchased from the Department of Biochemistry, University of Liverpool. The peptides were synthesised by solid state synthesis, purified by HPLC and showed a purity of 99% or greater. Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG) were purchased from Sigma. All solvents were of spectroscopic grade.

Preparation of phospholipid vesicles

Small unilamellar vesicles (SUVs) of DOPC and DOPG (phospholipid content 4.5 mM) were prepared according to Keller *et al.* (1992). The lipid / chloroform solutions were dried with

nitrogen gas and hydrated with aqueous buffer (pH 7) composed of 50 mM NaCl, 5 mM piperazine-N, N'-bis (2-ethane-sulphonic acid) (PIPES) and 1 mM ethylenediaminetetraacetic acid (EDTA). The resulting cloudy suspensions were ultra-sonicated at 4°C with a Soniprep 150 sonicator (amplitude 10 microns) until clear suspensions resulted (30 cycles of 30 seconds) which were then centrifuged (15 min, 3000 g, 4°C).

Preparation of peptide solutions

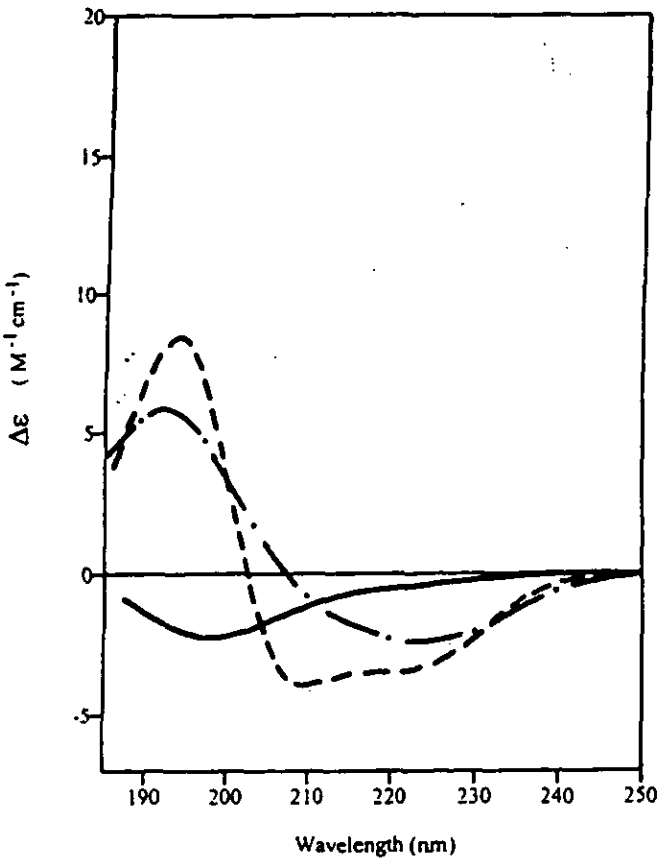
Peptide solutions (0.1 mM) were prepared in either water, 5% (v/v) 2, 2, 2-trifluoroethanol (TFE) or 25 mM sodium dodecylsulphate (SDS). The peptides were also solubilised in suspensions of SUVs (DOPC or DOPG) to give a molar ratio of peptide to lipid of 1:50 (Keller *et al.* 1992).

Determination of CD spectra

CD spectra were recorded using a nitrogen flushed JASCO J720 Spectropolarimeter, employing a 4 s time constant, a 10 nm min^{-1} scan speed, a spectral bandwidth of 1 nm and a 0.02 cm cell pathlength. Spectra obtained from peptides in the presence of DOPC and DOPG vesicles were reported as changes in absorbance, ($\Delta A = A_L - A_R$), since problems were encountered with the determination of peptide concentration. In all other cases, spectra were reported in terms of $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ (M^{-1} cm^{-1}). The estimation of secondary structure from CD data was analysed with GRAMS / 386 using a Principle Component Regression (PCR) method (Drake, unpublished data).

2.4 RESULTS AND DISCUSSION

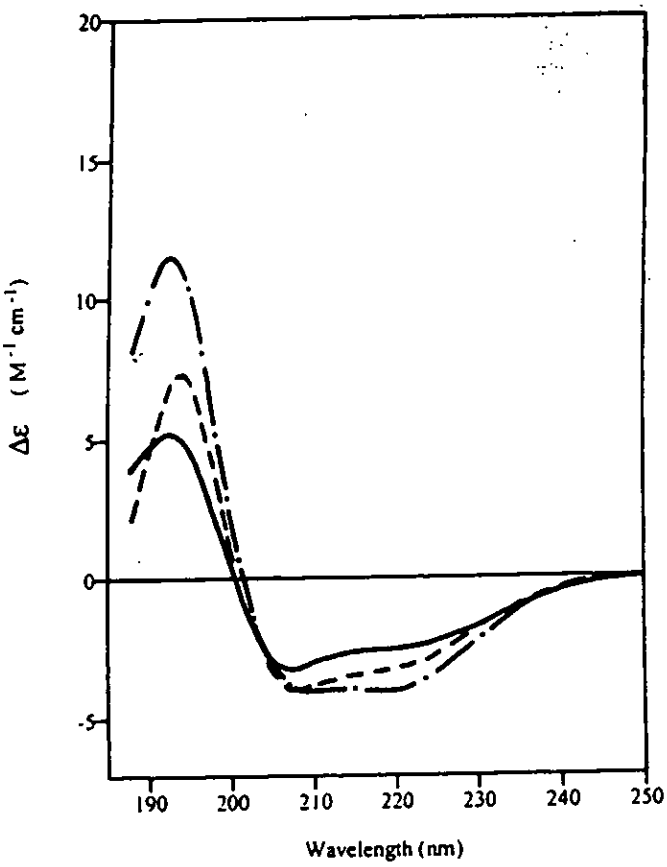
Figure 2.1 The CD spectra of P4, P5 and P6 in aqueous solution



CD was used to determine the structures of P4 (solid line), P5 (dashed line) and P6 (dash-dotted line) in aqueous solution, at their intrinsic pH. For 0.1 mM P5 this pH was measured as pH 4.1 and the peptide readily adopted an α-helical conformation which constituted 29% of the P5 structure. By contrast, P6 was sparingly soluble, possessed an intrinsic pH of 4.3 and exhibited a β-strand structure typical of aggregation. For 0.1 mM P4 the intrinsic pH was determined as pH 4.4 and the peptide demonstrated an irregular structure.

Figure 2.1 indicates that in aqueous solution P4 has an irregular structure and that P6, which has very low water solubility, possesses a spectrum characteristic of β-strand aggregation. P5 shows an estimated 29% of α-helical conformation in its structure.

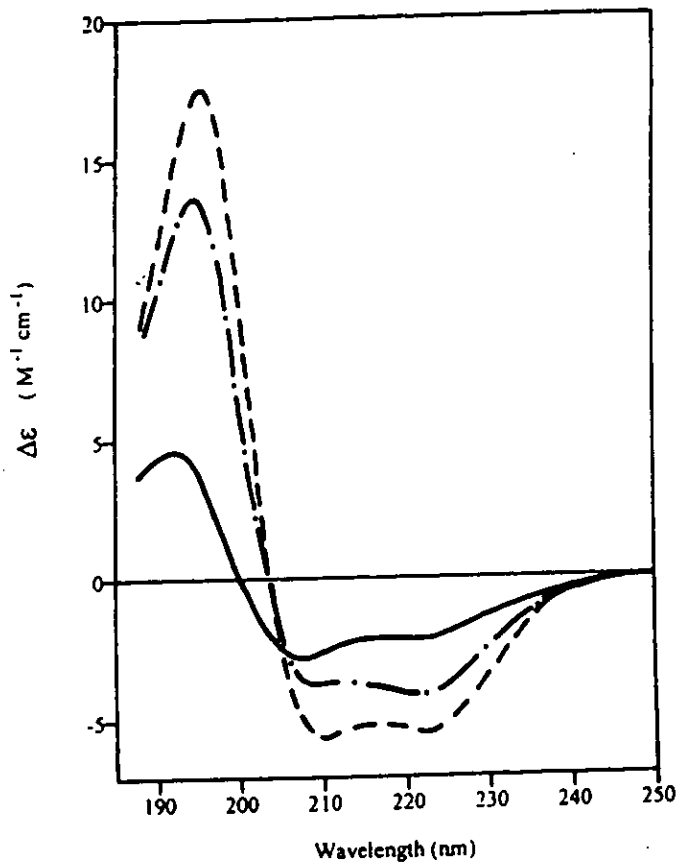
Figure 2.2 The CD spectra of P4, P5 and P6 in the presence of SDS



CD was used to determine the structures of P4 (solid line), P5 (dashed line) and P6 (dash-dotted line) in 5% (v/v) TFE. At a concentration of 0.1 mM, the intrinsic pH of these peptide solutions were determined as pH 5.7, pH 4.1 and pH 4.9 and with estimated α-helix contribution to the peptide structures of 25%, 28% and 32% respectively.

Figure 2.3 shows that in a supramicellular concentration of SDS P5 shows a remarkably high α-helical content of 62% compared to 40% and 20% α-helical content for P6 and P4 respectively. In contrast, it can be seen from figure 2.2 that in the presence of TFE the structure of P6 shows 32% α-helical content whereas those of P5 and P4 show 28% and 25% respectively.

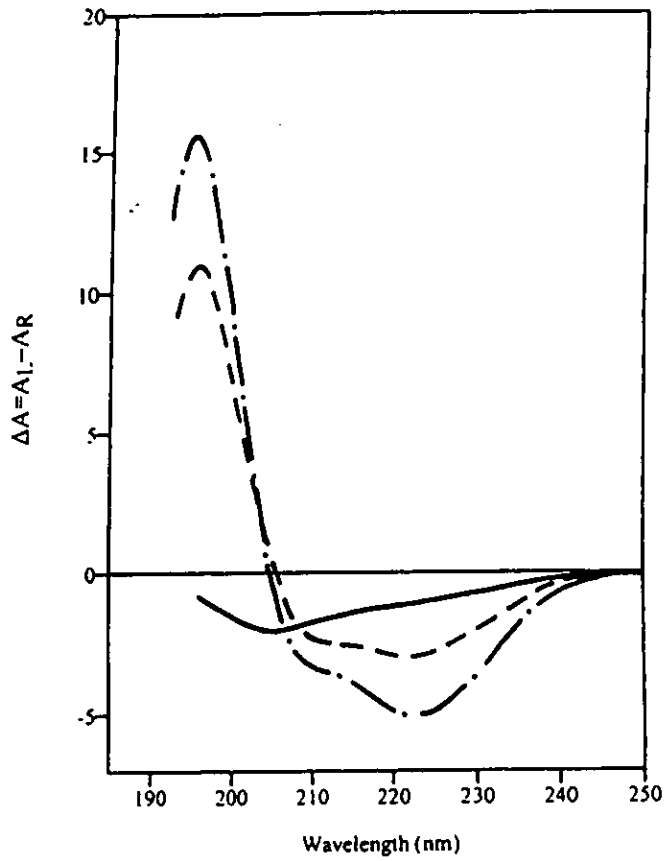
Figure 2.3 The CD spectra of P4, P5 and P6 in aqueous TFE.



CD was used to determine the structures of P4 (solid line), P5 (dashed line) and P6 (dash-dotted line) in the presence of 25 mM SDS. All of the peptides were soluble and were able to adopt α-helical conformation. For each peptide at a concentration of 0.1 mM, the estimated α-helical contribution to their respective structures was 20%, 62% and 40%.

These results show that these peptides possess the ability to adopt α-helical conformations depending upon their environment. This implies that the C-termini of PBP4, PBP5 and PBP6 may have similar abilities to form amphiphilic α-helices and supports the hypothesis that these C-terminal regions may contribute to the membrane anchoring of these proteins (section 1.5.2; section 1.6.3.5).

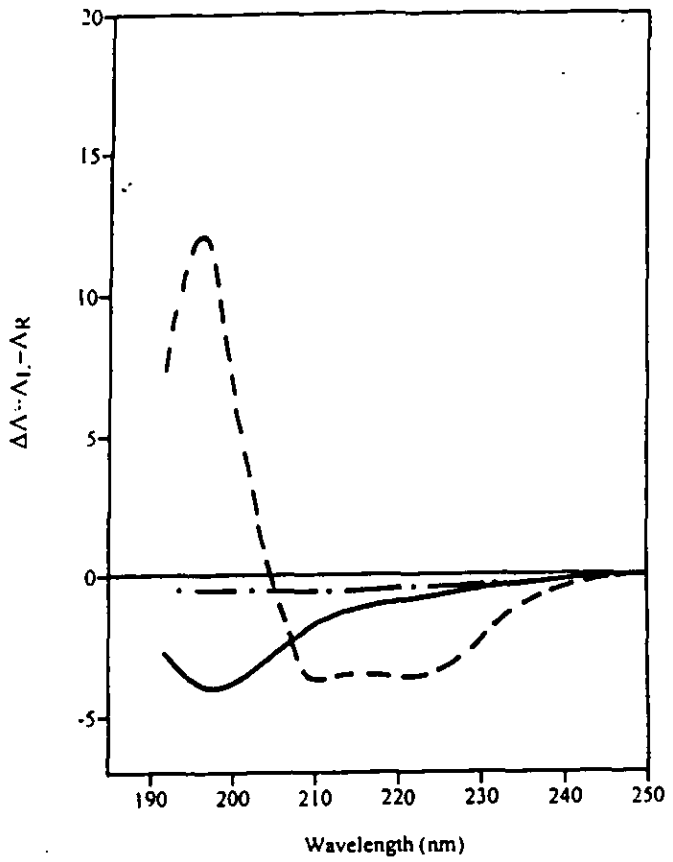
Figure 2.4 The CD spectra of P4, P5 and P6 in the presence of DOPG vesicles



CD was used to determine the structures P4 (solid line), P5 (dashed line) and P6 (dash-dotted line) in the presence of SUV's of DOPG (phospholipid content 4.5 mM). P5 and P6 exhibited spectra indicating the presence of α-helical conformation whereas that of P4 denoted a largely irregular structure.

In vesicles of DOPG (figure 2.4) and DOPC (figure 2.5) P5 exhibited spectra which were characteristic of α-helical conformation. P6 showed a predominantly soluble α-helical conformation in the presence of DOPG vescicles (figure 2.4) but an irregular structure with DOPC vescicles (figure 2.5).

Figure 2.5 The CD spectra of P4, P5 and P6 in the presence of DOPC vesicles



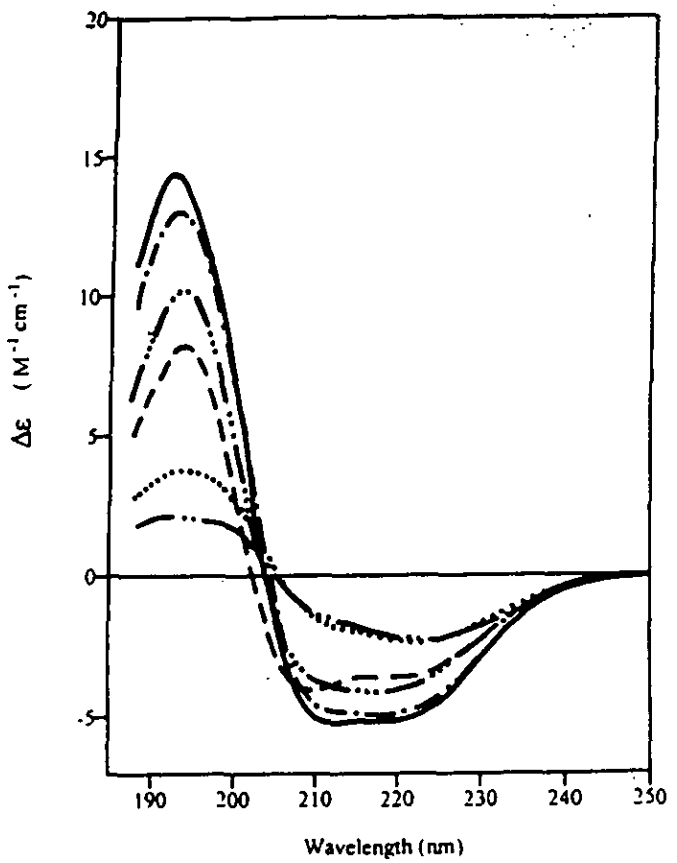
CD was used to determine the structures of P4 (solid line), (P5 dashed line) and P6 (dash-dotted line) in the presence of SUVs of DOPC (phospholipid content 4.5 mM). P6 was not soluble under these conditions. P5 displayed a spectrum characteristic of α-helical conformation whereas that of P4 indicated a largely irregular structure.

In the presence of both DOPG and DOPC vesicles, P4 adopted a largely irregular structure (figure 2.4 and figure 2.5). Phospholipid vesicles are able to mimic membrane environments more appropriately than either TFE or SDS micelles. The fact that under membrane mimetic conditions P4 did not adopt an α-helical conformation may be interpreted to support the theoretical prediction that the PBP4 C-terminal region may not form an α-helix or would be only weakly membrane interactive (Pewsey *et al.* 1996). In contrast, P5 not only readily adopted an α-

helical conformation in the presence of membrane mimetic vesicles but showed a strong tendency to do so in all cases examined (figures 2.1 - 2.6). This further supports the generally accepted idea that PBP5 associates with the membrane *via* an amphiphilic C-terminal α-helix (section 1.6.3.5.1; section 1.5.2) and that this membrane association has no requirement for the presence of anionic phospholipids (Harris *et al.* 1995b). PBP6 possesses similar anchoring characteristics to PBP5 (Phoenix *et al.* 1994) and therefore, it might have been expected that the peptide P6 would have an α-helix forming capacity comparable to that of P5 which indeed was observed in TFE, a supramicellular concentration of SDS and DOPG vesicles (figures 2.2, 2.3 and 2.4). However, unlike P5, in the presence of DOPC vesicles, P6 showed no α-helical content (figure 2.5). This suggests that under membrane or membrane mimetic conditions P6 may require the presence of phosphatidylglycerol (PG) or anionic phospholipids to stabilise α-helix formation. This supports the idea that a C-terminal amphiphilic α-helix is necessary for PBP6 - membrane interaction but also suggests that, in contrast to PBP5 (Harris *et al.* 1995b), the presence of PG or other anionic phospholipids may be required for efficient PBP6 - membrane anchoring. A higher level of hydrophobicity is associated with the structure of P6 than P5 (section 1.6.4.5; Pewsey *et al.* 1996). This hydrophobicity is probably responsible for the low water solubility of P6 and the aggregation of the peptide in DOPC vesicles.

In aqueous solution the conformation of P5 was found to be pH dependent (figure 2.6) whereas both P6 and P4 showed no pH dependence under the conditions tested. At pH 4.1 P5 shows a CD spectrum rich in α-helix conformation. With increasing pH, the α-helix content of P5 rises until at pH 6.2 a maximum is observed. Thereafter the α-helical contribution falls until at pH 8.3 and pH 10.3 peptide aggregation / precipitation accompanied by a large loss of α-helical structure is

Figure 2.6 The CD spectra of aqueous P5 as a function of pH



CD was used to determine the structure of 0.1 mM P5 at pH 4.1 (dashed line), pH 6.2 (solid line), pH 7 (dash-dot line), pH 8.3 (dash-dot-dot line), pH 10.3 (dotted line) and pH 11.6 (dash-dot-dot-dot line). At pH 4.1, the intrinsic pH of the peptide, α-helical structure was indicated. At pH 6.2 a maximum in the α-helix content occured which then decreased with increasing pH until at pH 8.3 and pH 10.3 the major contribution to P5 structure was from β-strand conformations. At pH 11.6 the α-helical contribution toP5 structure predominated.

observed. At pH 11.6 the peptide regains solubility and the predominant conformation is α-helical. This model would be consistent with the proposal of Phoenix and Pratt (1990) who suggested that upon translocation a pH-related stabilisation of the PBP5 C-terminal region leads to α-helix formation (figure 1.11). These latter findings correlate well with the pH dependent loss

of α-helical content in P5 and together these data suggest that the strength of PBP5 - membrane binding is related to the α-helical content of the proteins C-terminal region, with high α-helicity corresponding to strong PBP5 - membrane interaction.

In conclusion, for the first time, it has been shown directly that the amino acid residues comprising the C-terminal domains of PBP4, PBP5 and PBP6 have the capacity to adopt α-helical conformations. Despite this, in the presence of phospholipid vesicles, those residues corresponding to the PBP4 C-terminal domain formed no detectable α-helix. This is consistent with other data suggesting that this domain would only be weakly surface active and may not have a role in the PBP4 anchoring mechanism. In contrast, a peptide corresponding to the C-terminal domain of PBP5 demonstrated a strong tendency towards α-helical formation in the presence of phospholipid vesicles. Taken with the correlation between the pH dependent loss of α-helix in this peptide and the pH dependent susceptibility of PBP5 to membrane extraction (Phoenix and Pratt, 1990), this strongly supports the hypothesis that an amphiphilic α-helical domain at the C-terminus of the protein is necessary for membrane interaction. A peptide corresponding to the C-terminal domain of PBP6 exhibited a strong tendency towards α-helical formation in the presence of vesicles formed from DOPG but not those formed from DOPC. This supports the view that an amphiphilic α-helical region at the PBP6 C-terminus is necessary for membrane interaction but also suggests that PG or other anionic phospholipids may be involved in the anchoring mechanism of the protein.

CHAPTER 3.

AN INVESTIGATION INTO THE MEMBRANE INTERACTIONS OF C-TERMINAL HOMOLOGUES *OF ESCHERICHIA COLI* PENICILLIN-BINDING PROTEINS 4, 5 AND 6.

HARRIS, F. AND PHOENIX, D. A. (1997) BIOCHEMIE, 79, 171-174.

3.1 ABSTRACT

Theoretical analysis has predicted that C-terminal helical regions of PBP4, PBP5 and PBP6 may be membrane interactive. This hypothesis has been tested by assaying PBP4, PBP5 and PBP6 C-terminal homologues, P4, P5 and P6, for haemolytic activity. Our results show that the PBP5 and PBP6 C-terminal homologues readily lyse sheep erythrocytes in a pH dependent manner with LD_{50} values of 3.5×10^{-6} M and 6.8×10^{-7} M respectively at pH 7. These results appear to support the present model for the membrane anchoring of PBP5 and PBP6. The PBP4 C-terminal homologue shows no evidence of haemolytic activity which could imply a different means of membrane association for PBP4.

3.2 INTRODUCTION

Theoretical analyses have predicted that the membrane interactive properties of the PBP4 C-terminal region would differ from those of the other low molecular mass PBP's. Hydrophobic moment plots have predicted that the PBP5 and PBP6 C-terminal sequences, will be strongly surface active (section 1.6.2; section 1.6.3.5; figure 1.10). However, the same analyses predicted that the PBP4 C-terminal region had only a low potential for membrane interaction (section 1.6.2; section 1.6.3.5; figure 1.10).

In chapter 2, PBP - membrane interaction was invetigated by the use of CD spectroscopy to study the conformational behaviour of synthetic peptides, P4, P5 and P6 which share sequence homology with the C-terminal regions of PBP4, PBP5 and PBP6 (table 2.1). This analysis revealed that in the presence of phospholipid vesicles, both P5 and P6 showed a capacity for α-helix formation whereas under corresponding conditions, no helical structure could be detected in P4. To date, it is still a matter of debate as to whether PBP4 employs a similar anchoring mechanism to PBP5 and PBP6 (section 1.6.3.5.1; section 1.6.3.5.2; Phoenix and Harris, 1995).

The PBP5 and PBP6 C-terminal anchors appear to possess properties which are similar to those of many haemolytic peptides, examples being melittin (Dempsey, 1990; Cornut, *et al.* 1993), cardiotoxins and thionins (section 1.6.3.2; Chien *et al.* 1994; Vernon and Rogers, 1992). This suggests that the peptide analogues, P5 and P6, corresponding to these regions, may have the capacity for haemolysis. If the theoretical analyses are correct (section 1.6.2; section 1.6.3.5; Roberts *et al.* 1997; Pewsey *et al.* 1996). P4 would have a very low capacity for haemolytic action. An investigation into the interaction of these peptides with erythrocyte membranes may provide insight into the association of PBP4, PBP5 and PBP6 with the

membrane and provide evidence for the accuracy of the theoretical studies performed on these systems (Pewsey *et al.* 1996). Accordingly, we have studied the action of P4, P5 and P6 on sheep red blood cells and compared it to that of melittin.

3.3 MATERIALS AND METHODS

The peptides P4, P5 and P6, which correspond to the C-terminal domains of PBP4, PBP5 and PBP6 (table 2.1) respectively were purchased from the Department of Biochemistry, University of Liverpool, UK. These peptides were manufactured by solid state synthesis, purified by HPLC and were of 99% purity or greater. P6 stock solutions were solubilised in 50 % (v/v) 2, 2, 2-trifluoroethanol (TFE; Sigma). TFE had no detectable lytic effects on red blood cells at the levels used in the assay. Fresh red blood cells were isolated from sheep's blood using Histopaque (Sigma) according to makers instructions. Packed red blood cells were washed three times in Tris buffered sucrose (TBS; 0.25 M sucrose, 10 mM Tris) at either pH 6, pH 7 or pH 8 and then resuspended in fresh TBS, at pH corresponding to that of the TBS wash. The initial blood cell concentration (approximately 0.05 % v/v) was adjusted such that incubation with 0.1% (v/v) Triton X-100 for 1 hour produced a supernatant with A_{416} = 0.6 and this was taken as 100% haemolysis. Aliquots (1 ml) of the red blood cell suspensions were incubated with varying concentrations of the peptides P4, P5, P6 or melittin (Sigma), at room temperature with gentle shaking. After 1 hour the suspensions were centrifuged at low speed (300 g, 15 min, 25°C) and the A_{416} of the supernatants determined. Basal lysis due to the incubation medium was less than 3% in all cases.

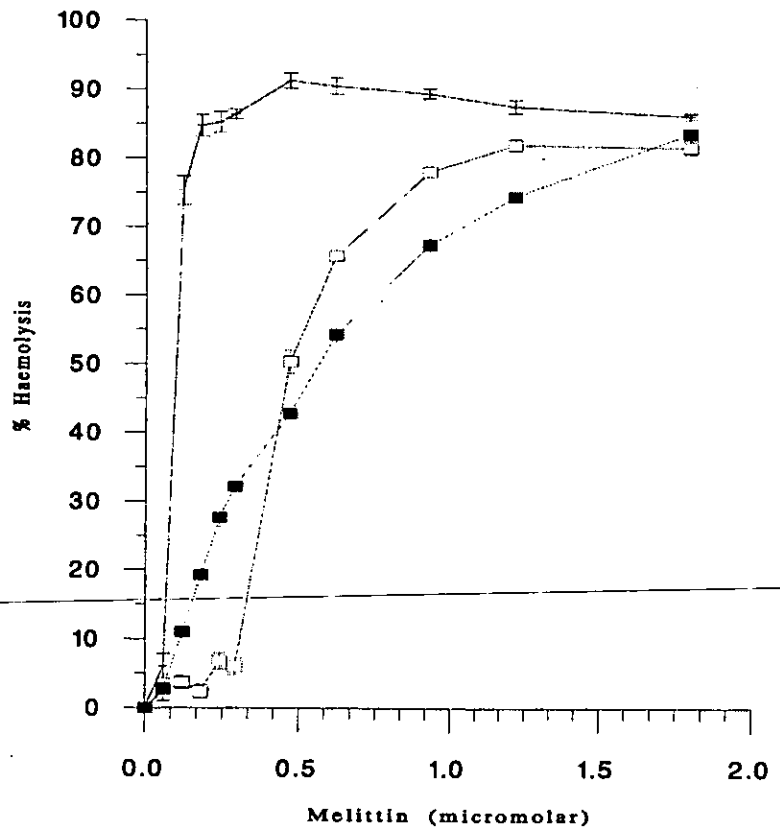
3.4 RESULTS AND DISCUSSION

Haemolytic peptides are able to penetrate the erythrocyte membrane outer leaflet and induce changes in permeability. However, despite many studies, the molecular basis for the haemolytic mechanism(s) of these peptides is still unclear (Cornut *et al.* 1993). One of the most potent haemolysins and probably the best characterised is melittin (Dempsey, 1990). Hydrophobic moment plot analysis has predicted that P5 and P6 would possess membrane interactive potentials comparable to that of melittin (section 1.6.3.5; figure 1.10.; Roberts *et al.* 1997; Pewsey *et al.* 1996) and as such melittin was selected as a control for this series of experiments. It can be seen from figure 3.1 that at low pH, the haemolytic activity of melittin varies with peptide concentration in a sigmoidal manner, implying that a cooperative effect is involved in the process. At neutral and particularly alkaline pH, levels of haemolysis are greatly enhanced and hyperbolic kinetics are observed. A Hanes-Woolf analysis (Chaplin and Bucke, 1990) of the melittin haemolytic curve, at pH 7 (figure 3.1), yielded a value of 6.7×10^{-7} M for the LD_{50} of the toxin which is in good agreement with published data (Cornut *et al.* 1993). The haemolytic capacities of the peptides P4, P5 and P6 were compared to this value.

The PBP4 C-terminal homologue, P4, was examined for haemolytic activity. No such activity was detected across a P4 concentration range of 8×10^{-3} M to 8×10^{-8} M all at pH 6, pH 7 or pH 8 and this could mean that P4 does not interact with eryrthocyte membranes. Alternatively, it may be that P4 interacts only weakly with erythrocyte membranes which would agree with the theoretical prediction that in an α-helical conformation P4 would be weakly membrane interactive (section 1.6.3.5; figure 1.10; Roberts *et al.* 1997; Pewsey *et al.* 1996). However, in the presence of either DOPG or DOPC vesicles, CD analysis could detect no α-helical structure in P4 (chapter 2). Overall, these results imply that P4 does not possesses a significant level of α-helicity and either does not interact with

the membrane or that the level of interaction does not facilitate haemolysis. This could agree with the theory that the PBP4 C-terminal domain may not play a major role in the protein's anchoring mechanism (chapter 6; Phoenix and Harris, 1995).

Figure 3.1 The haemolytic action of melittin



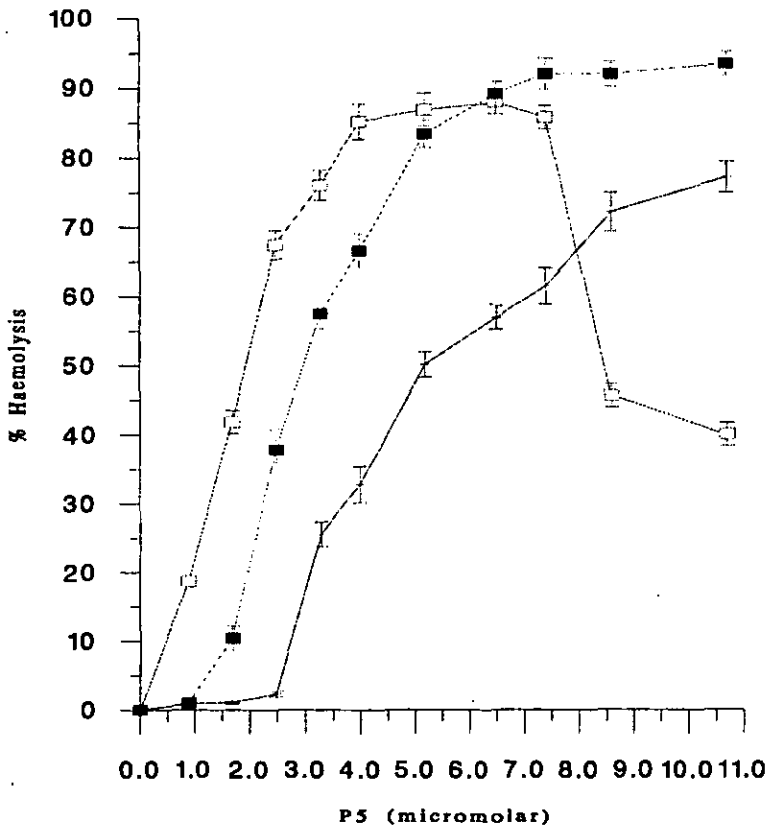
Varying concentrations of melittin were incubated with suspensions of sheep erythrocytes in Tris buffered sucrose, at pH 6 (□), pH 7 (■) or pH 8 (+) and % lysis determined, all as described. (Error bars are the standard deviation for n = 3).

The PBP5 and PBP6 C-terminal homologues, P5 and P6, were also examined for haemolytic activity. It can be seen from figures 3.2 and 3.3 that in contrast to P4, levels of haemolysis by

P5 and P6 are considerable. Nevertheless, unlike the control peptide, melittin, the haemolytic activities of P5 and P6 are enhanced by acid conditions. This makes comparison of the peptides difficult as different haemolytic mechanisms appear to be involved. However, at pH 7, the LD_{50} of P6 is 6.8×10^{-7} M (figure 3.3) which implies that it has a haemolytic activity comparable to that of melittin (figure 3.1). The LD_{50} of P5 at pH 7 is 3.5×10^{-6} M (figure 3.2) which is approximately 5% of that for melittin (figure 3.1), indicating that P5 has a lower haemolytic activity than melittin. Nonetheless, the haemolytic activity of P5 is comparable to that of other surface active toxins, including mastoparans (Argolias and Pisano, 1983), pardaxins (Lazarovici *et al.* 1986) and magainins (Bevins and Zasloff, 1990) which interact with the membrane *via* amphiphilic, α-helical regions (Cornut *et al.* 1993). These data appear to support the predictions that the PBP5 and PBP6 C-terminal regions are able to interact with membranes at a level comparable to those of melittin and other natural toxins (Pewsey *et al.* 1996). This would support the theory that these C-terminal regions have the ability to anchor PBP5 and PBP6 to the membrane (Phoenix and Harris, 1995; Gittins *et al.* 1993). A CD analysis of aqueous P5 showed that the peptide possessed α-helical structure, the levels of which were maximal at pH 6.2 (chapter 2). Maximal values for the haemolytic action of P5 (figure 3.2) were also observed at 6.0 and these findings suggest that there may be a relationship between the ability of P5 to penetrate the membrane and the levels of α-helical structure present in the P5 molecule.

It can be seen from figures 3.2 and 3.3 that at acid pH, haemolytic action varies with the concentrations of P5 and P6 in a hyperbolic manner but at higher pH the relationship becomes sigmoidal. This implies that under alkaline and neutral conditions cooperative effects are involved in the haemolytic action of these peptides.

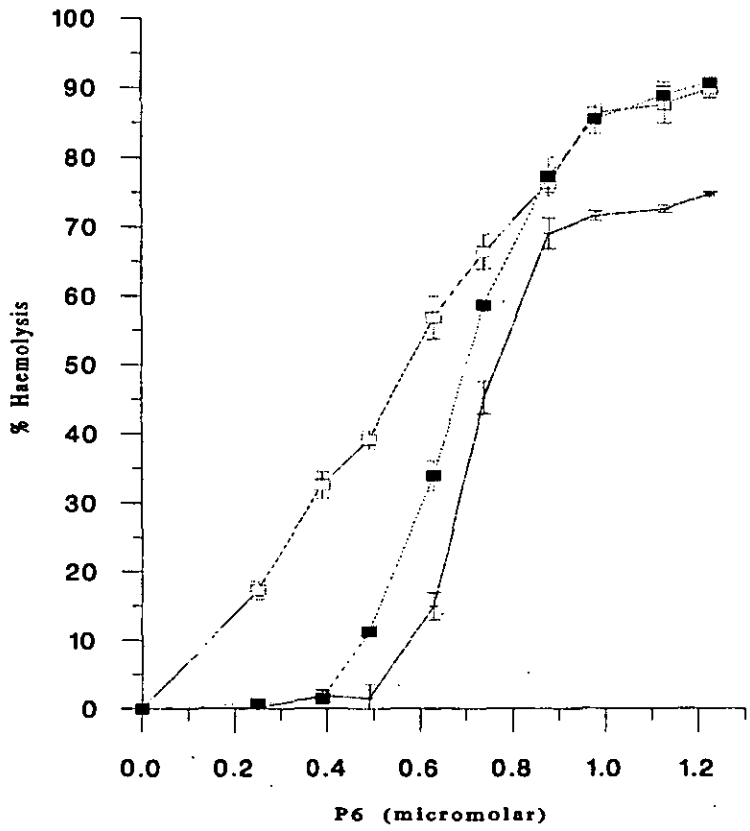
Figure 3.2 The haemolytic action of P5



Varying concentrations of P5 were incubated with suspensions of sheep erythrocytes in Tris buffered sucrose at pH 6 (□), pH 7 (■) or pH 8 (+) and % lysis determined, all as described. (Error bars are the standard deviation for n = 3).

This could occur if helicity and therefore haemolysis was induced *via* the self association of peptide molecules either within the membrane or in the aqueous environment (Cornut *et al.* 1993). The large drop in haemolytic activity of P5, observed at pH 6 and at concentrations above 6 μM, could be due to aggregation of the peptide.

Figure 3.3 The haemolytic action of P6



Varying concentrations of P6 were incubated with suspensions of sheep erythrocytes in Tris buffered sucrose at pH 6 (□), pH 7 (■) or pH 8 (+) and % lysis determined, all as described. (Error bars are the standard deviation for n = 3).

If acidic pH does indeed activate the membrane insertion of P5 by the induction of high levels of α-helicity in the molecule, then this may support the model suggested for the membrane interaction of newly translocated PBP5 (Phoenix and Pratt, 1993) and by analogy PBP6. This model proposed that upon translocation, one option for PBP5 or PBP6 may be to exist as a short lived periplasmic intermediate. The protein could then experience a decrease in pH due to the membrane proton gradient and membrane proximity effects. Low pH and charge effects at the membrane surface could then stabilise amphiphilic α-helical secondary structure in the

protein's C-terminal anchoring region with subsequent interaction of the protein with the membrane (section 1.6.5.3.1; figure 1.11).

In conclusion we have shown that P4 appears to have no haemolytic activity whereas P5 and P6 are highly haemolytic. This confirms the theoretical predictions made for these peptides and reinforces the idea that the membrane anchoring of PBP4 may involve a different mechanism to PBP5 and PBP6. We have suggested that for P5 and P6, haemolytic activity is related to the degree of α-helicity in the peptide structures and to the ability of these peptides to penetrate the membrane.

CHAPTER 4.

AN INVESTIGATION INTO THE LIPID INTERACTIONS OF PEPTIDES CORRESPONDING TO THE C-TERMINAL ANCHORING DOMAINS OF *ESCHERICHIA COLI* PENICILLIN-BINDING PROTEINS 5 AND 6

HARRIS, F., DEMEL, R.A., PHOENIX, D. A. AND DE KRUIJFF, B. (1997)

BIOCHIM. BIOPHYS. ACTA SUBMITTED

4.1 ABSTRACT

Pressure - area isotherm analysis of monolayers formed from peptide homologues of the PBP5 and PBP6 C-terminal regions, P5 and P6, gave molecular areas of *circa* 200 $Å^2$ for the peptides and this implied the presence of α-helical structure, which was confirmed by CD analysis. P5 and P6 were able to form stable monolayers and showed surface activities of 33.7 mN m^{-1} and 22.3 mN m^{-1} respectively at pH 7. The surface pressure changes induced by P5 and P6 with monolayers of: DOPC, DPPC, DOPG, those derived from membrane lipids of a wild type *E. coli* strain SD12 or those from a mutant strain *E. coli* HDL11 which were depleted in anionic lipids, appeared to proceed *via* predominantly hydrophobic forces with only minor requirements for anionic lipid. Interactions were generally high (in the range 10.0 -15.0 mN m^{-1}) and enhanced by low pH. Overall these results support the view that C-terminal amphiphilic α-helices are involved in the membrane anchoring of PBP5 and PBP6. Furthermore, it is speculated that the membrane interaction of P5 may involve cationic residues in the hydrophilic face of the P5 α-helix and that for membrane interaction of P6, α-helix stabilisation by low pH may be necessary.

4.2 INTRODUCTION

Theoretical analyses have predicted that the PBP5 and PBP6 C-terminal regions have the potential to form strongly amphiphilic α-helical conformations and have membrane interactive potentials comparable to that of the haemolysin melittin (section 1.5.2; section 1.6.3.5.1; figure 1.10). Haemolytic analysis has supported these predictions (chapter 3). Melittin is known to interact with the membrane *via* amphiphilic α-helices (Dempsey, 1990) and this led to the suggestion that the PBP5 and PBP6 C-terminal regions may utilise similar mechanisms and lie at the membrane interface with their hydrophobic arcs interacting with the bilayer core (section 1.6.3.5). This model appears to be supported by the strong susceptibility shown by PBP5 and PBP6 to the perturbant action of the chaotropic thiocyanate ion (section 1.6.3.5.1).

Overall, these data strongly, but indirectly, support the hypothesis that the membrane associations of PBP5 and PBP6 involve C-terminal amphiphilic α-helices. In an effort to directly determine if the PBP5 and PBP6 C-terminal sequences have the capacity for α-helix formation at an amphiphilic interface, monolayers formed from synthetic homologues of these regions, the peptides, P5 and P6 (table 2.1), were subjected to CD and pressure - area isotherm analysis. To try and identify lipid requirements of the PBP5 and PBP6 C-terminal regions for membrane association, the interaction of P5 and P6 with monolayers formed from dioleoylphosphatidylglycerol (DOPG), dioleoylphosphatidylcholine (DOPC), and dipalmitoylphosphatidylcholine (DPPC) were studied. Additionally, the interactions of P5 and P6 with monolayers formed from lipid extracts of membranes derived from the wild type *E. coli* SD12 (De Vrije *et al.* 1988), and the *E. coli* mutant strain HDL11 (Kusters *et al.* 1991) were studied. In this latter strain, the *pgsA* gene, which encodes phosphatidylglycerolphosphate

synthetase, an enzyme involved in PG and DPG synthesis, has been placed under the control of a *lac* promoter (Kusters *et al.* 1991) and *pgsA* expression can be induced by addition of the *lac* inducer isopropylthiogalactoside (IPTG). Thus *pgsA* expression, and that of the major membrane anionic phospholipids, can be controlled by the presence of IPTG, allowing lipid to be extracted from membranes which are depleted in anionic phospholipid. In the absence of IPTG, phosphatidylgylcerolphosphate synthetase is still produced at a low, basal level and this is sufficient to permit the viability of this strain given the additional presence of a lipoprotein gene, *lpp2*, deletion (Asai *et al.* 1989).

4.3 MATERIALS AND METHODS

The peptides P5 and P6 (table 2.1) were supplied by the Department of Biochemistry, University of Liverpool, England. The peptides were synthesised by solid state synthesis, purified by HPLC and showed a purity of 99% or greater. Buffers and solutions for all monolayer experiments were prepared from milli Q water. Phospholipids of *E. coli* were extracted by Bligh and Dyer extraction (Bligh and Dyer, 1959) of cells in the late log phase and purified by column chromatography with Polygosil (63 - 100 μm, Macherey-Nagel) as stationary phase and chloroform / methanol (1:1, v/v) as eluant, after first eluting neutral lipids and other contaminants with 100% (v/v) chloroform. DOPG and DOPC and DPPC were supplied by Avanti. Monolayer surface tension was monitored by the (platinum) Wilhelmy plate method (Demel, 1994) using a Cahn C202 microbalance. Monolayers were formed by spreading 0.1 mM stock solutions of either P5 or P6 in water / 2, 2, 2-trifluoroethanol (5:2, v/v), pure phospholipids (10 mM) or total phospholipid extracts of *E. coli* strains, in chloroform / methanol (8:2, v/v), until the desired

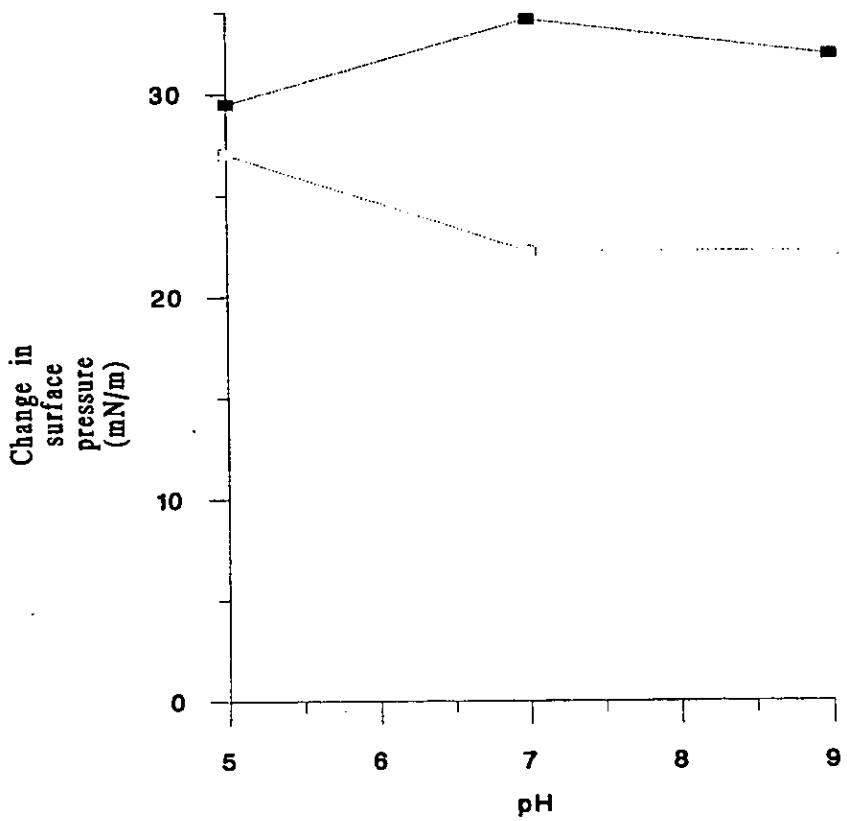
initial surface pressure was achieved. Peptide pressure - area isotherm determinations and the CD analysis of peptide monolayers at constant pressure were performed using a 5 × 15 cm Teflon trough containing 60 ml of buffer subphase (10 mM acetate buffer at pH 5 and 10 mM Tris buffer at pH 7 and pH 9). The trough was equipped with a moveable barrier, the position of which could be adjusted by an ABU11 autoburette, controlled by a TTT2 titrator (both from Radiometer, Copenhagen) which was connected to the Cahn C202 microbalance. For pressure - area isotherm determinations, starting with a 5 × 15 cm area, P5 or P6 stock solutions were spread to form monolayers. The monolayer area was then decreased by the moveable barrier at a rate of 3 cm min^{-1} until the monolayers had reached the point of over-compression. For the CD analysis of peptide monolayers at constant surface pressure, starting with a 5 × 11 cm area, P5 or P6 stock solutions were spread to form monolayers which could be maintained at surface pressures of 20 mN m^{-1} and 30 mN m^{-1} respectively. Samples of monolayer were transferred to glass solid supports using the *x* type Langmuir-Blodgett film technique (Demel, 1994) and subjected to CD analysis. CD spectra were recorded using a nitrogen flushed JASCO J600 spectropolarimeter, employing a 0.25 s time constant, a 50 nm min^{-1} scan speed, a spectral bandwidth of 1 nm and a 0.02 cm cell pathlength. The estimation of secondary structure from CD data was analysed with GRAMS / 386 using a Principle Component Regression (PCR) method. Constant area measurements were performed in a Teflon trough with a volume of 5 ml and a surface area of 8.04 cm^2. Stock P5 or P6 solutions were added to the subphase *via* a reservoir extending into the subphase. The subphase was continuously stirred by a magnetic bar.

4.4 RESULTS

Theoretical analyses have predicted that the amino acid sequences comprising the PBP5 and PBP6 C-terminal regions have high potential to adopt amphiphilic α-helical conformations (section 1.5.2; section 1.6.3.5; figure 1.5; Roberts *et al.* 1997; Pewsey *et al.* 1996; Phoenix, 1993). These same analyses have also predicted that in such α-helical conformations these sequences would possess high levels of surface activity. It can be seen from figure 4.1 that the determined equilibrium surface pressures of the PBP5 and PBP6 C-terminal homologues, P5 and P6, were 33.7 mN m^{-1} and 22.3 mN m^{-1} at pH 7 respectively and such values are typical of peptides possessing high levels of surface activity (Demel, 1994). These observations appear to support the predicted surface activity of the PBP5 and PBP6 C-terminal regions.

A surface pressure of 30 mN m^{-1} may be taken to be typical of a biological membrane (Rojo *et al.* 1991). At this surface pressure, P6 formed stable monolayers but in the case of P5, although the peptide showed the ability to form monolayers, these monolayers showed a slow but significant decrease in surface pressure with time. Monolayers of P5 were found to be stable at 20 mN m^{-1} and were therefore examined at this surface pressure. The gradual decrease in stability of a P5 monolayer at 30 mN m^{-1} could reflect a loss of amphilphilic structure in the peptide, possibly resulting from molecular associations in the monolayer environment. Nonetheless, the fact that these peptides were able to form stable monolayers shows that both P5 and P6 are able to partition at an amphiphilic interface and suggests that under these conditions, the peptides themselves may possess amphiphilic characteristics. The presence of α-helical structure in a monolayer environment can often be inferred from a determination of molecular area. Using monolayers formed from P5 or P6, pressure - area isotherms have been derived for these peptides.

Figure 4.1 The variation of P5 and P6 surface activity with pH



The surface activity of P5 (■) and P6 (□) were determined at various pH, as the change in surface pressure caused by the presence of peptide monolayers in equilibrium on a buffer subphase at 73 mN m^{-1}, all as described.

In deriving these isotherms, monolayers are under continuous compression but at the data points representing monolayer surface pressures of 20 mN m^{-1} (data not shown) and 30 mN m^{-1} (table 4.1), the determined molecular areas for P5 and P6 were all *circa* 200 $Å^2$. Such values (Batenburg *et al.* 1988) are in accordance with P5 and P6 being orientated approximately normal

Table 4.1 The determined molecular areas of P5 and P6 at a surface pressure of 30 mN m^{-1} and at various pH values

pH	Determined molecular area of P5 at a surface pressure of 30 mN m^{-1} ($Å^2$)	Determined molecular area of P6 at a surface pressure of 30 mN m^{-1} ($Å^2$)
5	205	198
7	213	203
9	199	203

Shown above are determined molecular areas of P5 and P6 at 30 mN m^{-1} and at various pH values, all as described. These values were derived from pressure - area isotherm analysis of monolayers formed from the pure peptides.

to the plane of the interface and possessing α-helical secondary structure. A CD analysis of P5 and P6 monolayers, at surface pressures of 20 mN m^{-1} and 30 mN m^{-1} respectively, revealed α-helical contributions to the structures of both peptides in every case examined (table 4.2). Overall, these data support the predictions that the PBP5 and PBP6 C-terminal sequences have the potential for amphiphilic α-helix formation (section 1.5.2; section 1.6.3.5; Roberts *et al.* 1997; Pewsey *et al.* 1996; Phoenix and Harris 1995).

The membrane binding of PBP5 and PBP6 shows a pH-dependent susceptibility to perturbants. Membrane binding is enhanced under acidic conditions but as pH progresses to alkaline conditions increasingly greater amounts of the proteins are displaced from the membrane by perturbants (section 1.6.3.5.1; Phoenix *et al.* 1994; Phoenix and Pratt, 1990). In the CD analysis (table 4.2), no apparent correlation between peptide α-helical content and pH could be

Table 4.2 The percentage α-helical contribution to the structures of P5 and P6

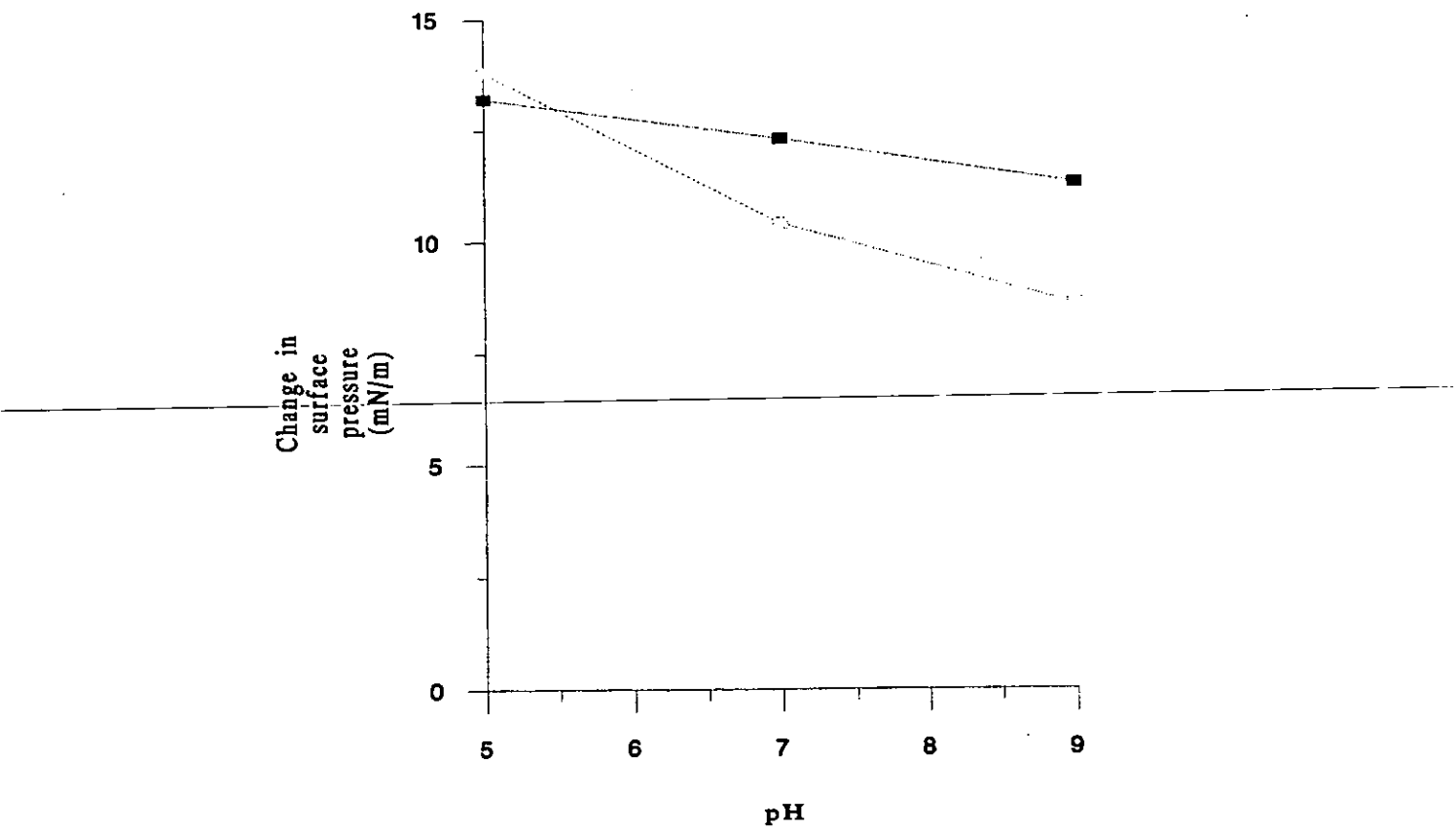
	Percentage α-helical contribution to peptide structure	
pH	**P5 at a monolayer surface pressure of 20 mN m^{-1}**	**P6 at a monolayer surface pressure of 30 mN m^{-1}**
5	64	60
6	36	25
7	55	34
9	64	16

Monolayers of P5 and P6, at surface pressures of 20 mN m^{-1} and 30 mN m^{-1} respectively, were subjected to CD analysis and the percentage α-helical content of the peptides at various pH were determined, all as described.

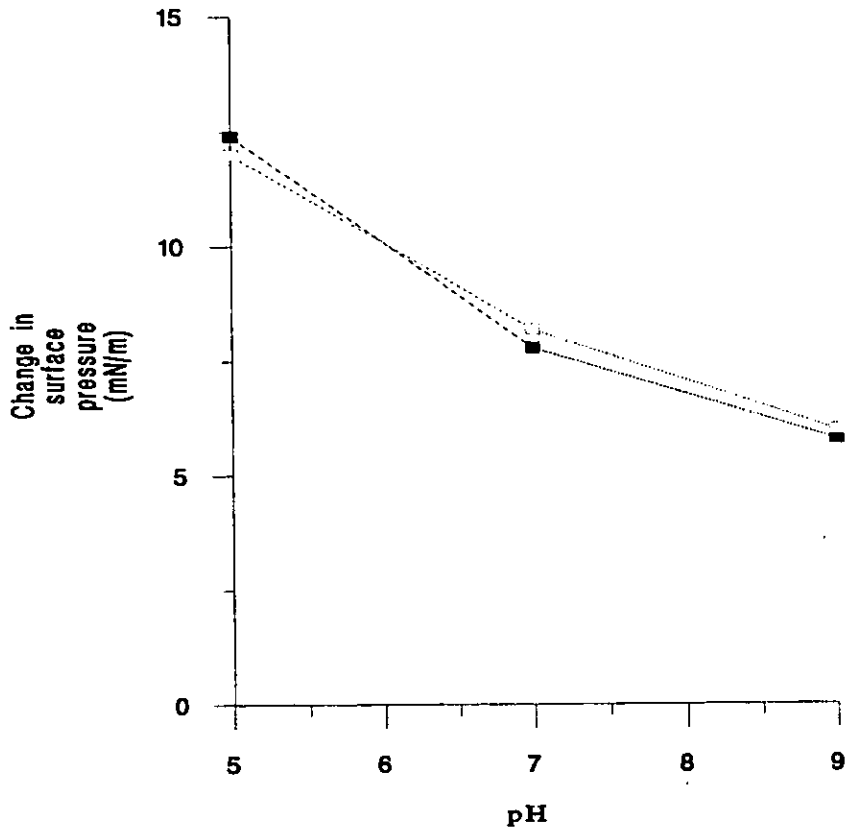
detected. Nonetheless. it can be seen from figures 4.2. and 4.3. that both P5 and P6 show high levels of interaction with lipid monolayers which are generally enhanced under acid conditions but decrease with increasing pH in a manner that correlates to that of the parent protein's susceptibility to perturbants. This correlation could be interpreted to support a role for the PBP5 and PBP6 C-terminal regions in the membrane anchoring of the parent proteins. The overall membrane anchoring of PBP5 and PBP6 have been shown to involve predominantly hydrophobic forces (section 1.6.3.5.1; Phoenix *et al.* 1994; Phoenix and Pratt, 1990). To determine if the C-terminal regions of these proteins have the potential for membrane interaction and the nature of those forces involved, the interactions of P5 and P6 with monolayers formed from the total phospholipid extracts of various *E. coli* strains and pure lipid monolayers (figure 4.2; figure 4.3)

were investigated. Both peptides were found to interact with these lipid monolayers across a range of initial pressures (data not shown) but at an initial surface pressure of 30 mN m^{-1}, mimetic of a biological membrane, was chosen for further study. Both peptides were found to interact with monolayers derived from the inner membranes of a wild type *E. coli* SD12 (figure 4.2). In these monolayers the normal membrane phospholipid composition was conserved and both peptides were found to induce large surface pressure changes, in the range 9.2 - 13.2 mN m^{-1} for P5 (figure

Figure 4.2 The interaction of P5 and P6 with lipid monolayers derived from *E. coli* membranes, all at an initial surface pressure of 30 mN m^{-1} and at various pH values



(a)



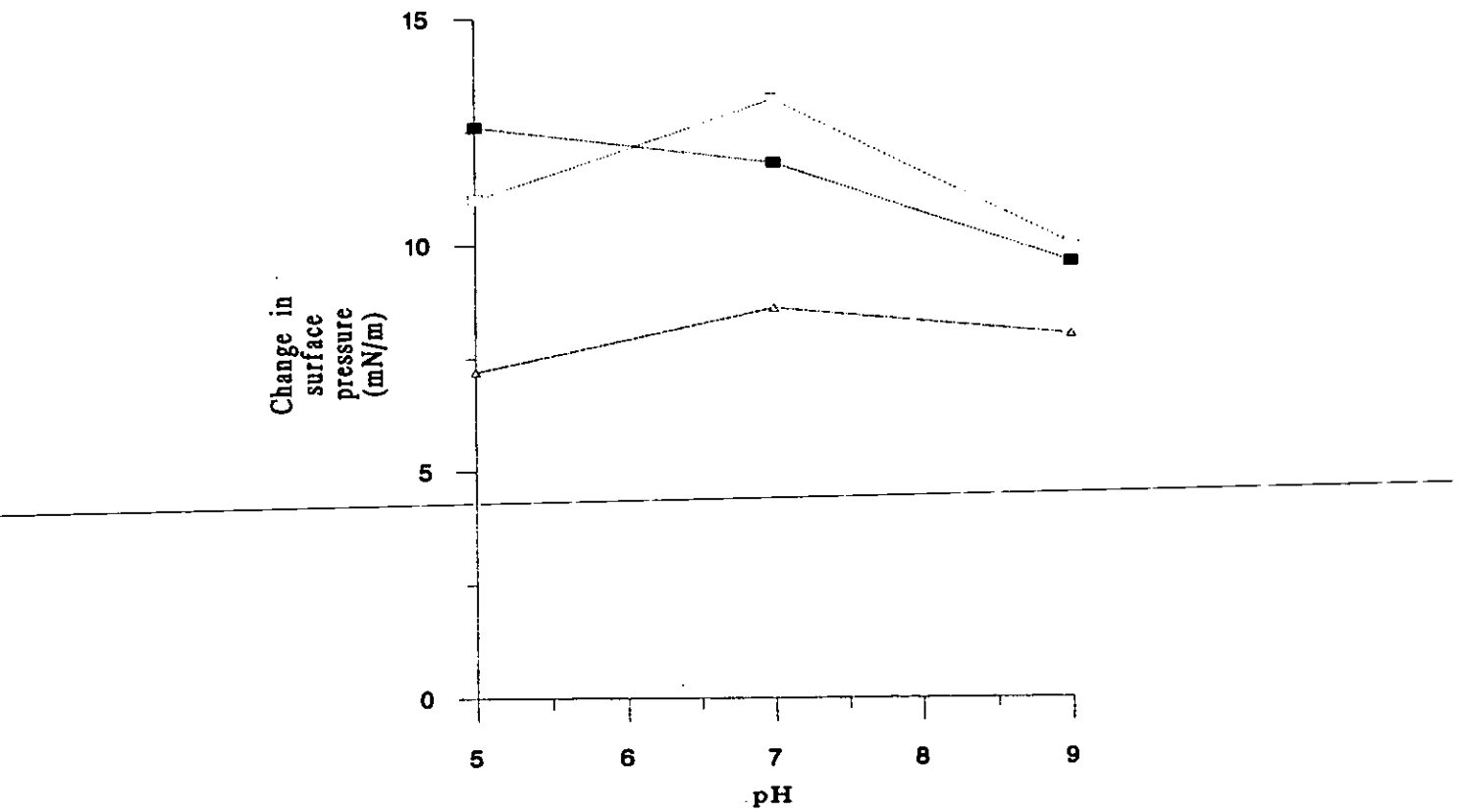
(b)

Figures 4.2a and 4.2b show the changes in surface pressure induced by the interaction of P5 and P6 respectively, with monolayers formed from total phospholipid extracts of the membranes of strains of E. coli. The strains used were the wild type E. coli, SD12 (■) and the mutant E. coli, HDL11, in which membranes were depleted in anionic phospholipids (□). All were performed at an initial surface pressure of 30 mN m^{-1} and at various pH values.

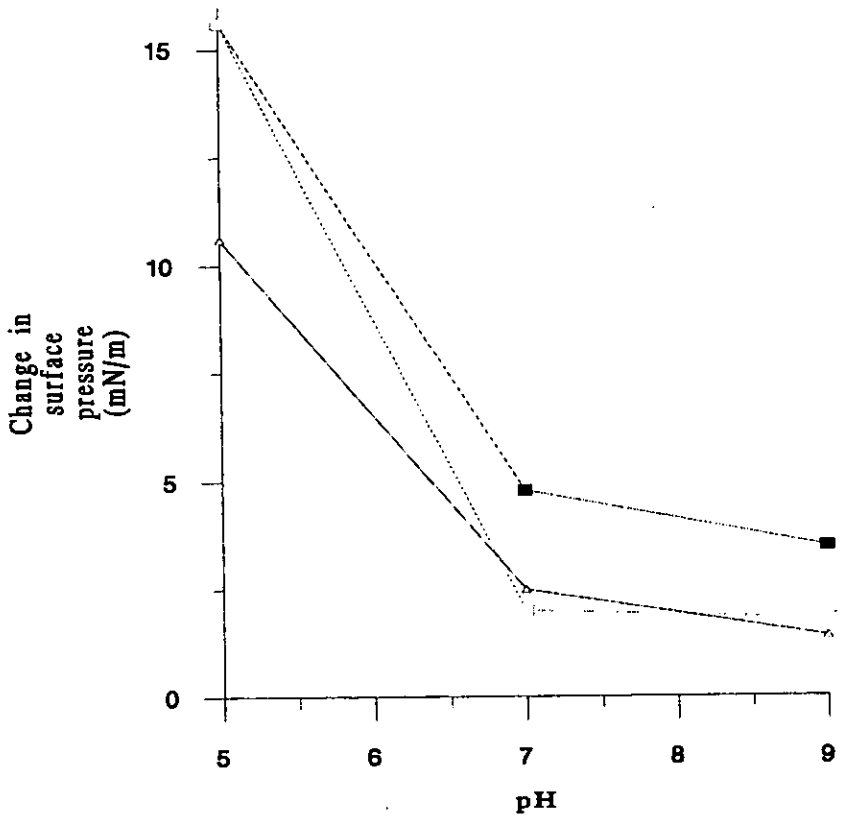
4.2a) and 6.0 - 12.6 mN m^{-1} for P6 (figure 4.2b). Pressure changes associated with peptides which predominantly interact with the phospholipid headgroup region, (e.g. poly-lysine), are typically of the order of 3.0 mN m^{-1} (Rojo *et al.* 1991) and thus the levels of interaction shown by P5 and P6 suggest penetration of the monolayer acyl chain region and the involvement of

hydrophobic forces in the lipid interaction of these peptides. In the case of P5, this suggestion could be supported by the large change in surface pressure induced by the peptide with monolayers formed from DOPC and DPPC (figure 4.3a). The ability of a peptide to show high levels of interaction with monolayers formed from zwitterionic lipids, in particular those formed

Figure 4.3 The interaction of P5 and P6 with pure lipid monolayers all at an initial surface pressure of 30 mN m^{-1} and at various pH values



(a)



(b)

Figures 4.3a and 4.3b show the changes in surface pressure induced by the interaction of P5 and P6 respectively with monolayers formed from DOPG (■), DOPC (□), DPPC (Δ) respectively, all at an initial surface pressure of 30 mN m^{-1} and at various pH values.

from DPPC with its densely packed acyl chain region is often indicative of the involvement of hydrophobic forces (Demel, 1994). For DOPC these levels of interaction are in the range 10.0 - 12.2 mN m^{-1} and for DPPC in the range 7.2 - 8.6 mN m^{-1} (figure 4.3a). These levels are generally comparable to those induced in the corresponding P5 interactions with SD12 monolayers (figure 4.2a). However, in contrast to these latter interactions, the levels of

interaction of P5 with monolayers formed from DOPC and DPPC are decreased at low pH rather than enhanced. These results show that P5 has generally high levels of interaction with monolayers formed from zwitterionic lipids and suggest that hydrophobic forces may play a major role in P5 - lipid monolayer interactions but that at low pH there may be other factors affecting the efficiency of interaction with wild type SD12 monolayers. In the case of P6, at low pH, the peptide induced large changes in surface pressure of 15.6 mN m^{-1} and 10.8 mN m^{-1} with DOPC and DPPC monolayers respectively (figure 4.2b) and these levels are comparable to those induced in the corresponding P6 - SD12 monolayer interactions (figure 4.2b). However, at higher pH these levels of interaction are greatly reduced, being in the range 1.4 - 2.6 mN m^{-1} in both cases (figure 4.3b).

To determine if anionic phospholipid may feature in the putative membrane interactions of the PBP5 and PBP6 C-terminal regions, the interactions of P5 and P6 with monolayers derived from the inner membranes of an *E. coli* mutant, HDL11 were studied. In this *E. coli* mutant, the inner membranes have reduced anionic lipid content (Kusters *et al.* 1991). It can be seen from figure 4.2a that the levels of surface pressure change induced by P5 interaction with HDL11 monolayers are in the range 8.6 - 13.8 mN m^{-1} and when these are compared to those of the corresponding interactions with SD12 monolayers (figure 4.2a), although the level of interaction is reduced above neutral pH, this reduction is maximal at approximately 25%. In the case of P6, it can be seen from figures 4.2b. that there appears to be no significant differences between the levels of surface pressure change induced by the peptide with HDL11 monolayers and those of the interactions with the corresponding SD12 monolayers. Overall, these observations suggest that anionic lipids and electrostatic forces do not play a major role in the interactions of P5 and P6 with SD12 monolayers and in the case of P5, could support the findings of Harris *et al.* (1995b)

who concluded that there was no requirement for anionic lipids in the membrane anchoring of PBP5. It can be seen from figures 4.3a. and 4.3b. that both P5 and P6 interact with monolayers formed from DOPG. The levels of surface pressure change for these interactions are in the range 9.6 - 12.6 mN m^{-1} for P5 (figure 4.2b) and 3.2 - 15.6 mN m^{-1} for P6 (figure 4.3b). When these levels of interaction are compared to those of the corresponding interactions of the peptides with SD12 and HDL11 monolayers (figure 4.2), they are are comparable for P5 but are enhanced at low pH and greatly reduced at higher pH for P6. Nonetheless, the maximal surface pressure changes induced in DOPG monolayers by P5 (12.6 mN m^{-1}; figure 4.3a) and P6 (15.6 mNm^{-1}; figure 4.3b) were not significantly affected by the presence of 500 mM NaCl (data not shown). Overall, these results strongly support the suggestion that P5 and P6 do not have a major requirement for anionic phospholipids and that electrostatic forces may play only a minor role in the interaction of the peptides with SD12 monolayers. In the case of P6, it seems likely that α-helix stabilisation by low pH is a major factor in the high levels of interaction observed between the peptide and DOPG monolayers under acid conditions. The greatly reduced levels of interaction observed at higher pH (figure 4.3b), may indicate other requirements for the efficient interaction of P6 with SD12 monolayers under these conditions. Alternatively, it could be that aggregation of P6 occurs and effectively lowers the concentration of the peptide.

4.5 DISCUSSION

The results show that the peptides P5 and P6 are surface active (figure 4.1) and able to interact with pure lipid monolayers (figure 4.3) and those mimetic of naturally occurring membranes (figure 4.2). Molecular area determinations (table 4.1) and CD data (table 4.2) have implied that

P5 and P6 have the ability to adopt α-helical secondary structure. Overall, these results support the view that that membrane interactive α-helical architecture at the PBP5 and PBP6 C-termini may contribute to the membrane anchoring of these proteins.

It can be seen from table 4.2 that the relationship between peptide α-helical content when in a monolayer environment and pH is complex with no apparent pattern. However, the interactions of both P5 and P6 with lipid monolayers were generally enhanced under acid conditions but decreased with increasing pH (figures 4.2 and 4.3). Mimicking this variation with pH, it has been shown for the peptide P5 that under acid conditions, the peptide possesses a high level of α-helicity which decreases with increasing pH (chapter 2; Siligardi *et al.* 1997). The correlation amongst these pH trends suggests that the levels of monolayer interaction shown by P5 and P6 may be related to levels of α-helicity in the peptide, with high levels of interaction corresponding to high levels of α-helicity. If this is the case then this could support the suggestion that the strength of membrane binding of PBP5 and PBP6 is related to the levels of α-helicity in the C-terminal regions of these proteins, with high levels of membrane binding corresponding to high levels of α-helicity (Phoenix and Harris, 1995).

P5 showed generally high levels of interaction with zwitterionic lipids (figure 4.3a), which in the case of DOPC was comparable to those with corresponding monolayers formed from membrane lipids of the wild type *E. coli* SD12 and the mutant *E. coli* strain HDL11 (figure 4.2a). Taken with the fact that P5 showed either no or a minor requirement for anionic lipids, these results suggest a major role for hydrophobic forces and a minor role for electrostatic forces in the interactions of this peptide with lipid monolayers. However, in contrast to the general pH trend shown by the interactions of P5 with lipid monolayers, the levels of interaction of the peptide with zwitterionic lipid monolayers were decreased at low pH rather than enhanced (figure 4.3a). This

The primary sequence of the PBP5 C-terminal region (table 2.1) contains four basic amino acid residues (two histidines and two lysines) which would occur on the hydrophilic face of its putative α-helical anchor (section 1.5.2; figure 1.5). The presence of the histidine residues would provide a cationic region within the PBP5 anchor which could vary in magnitude in a pH dependent manner which mimics that of the anchor region's susceptibility to perturbants (chapter 4). This suggests that this positively charged region may contribute to an electrostatic interaction in PBP5 membrane binding. Since the PBP5 anchor possesses molecular architecture of a similar nature to other surface active agents, it seems reasonable to postulate that this electrostatic interaction may involve anionic phospholipids, by analogy with cationic regions of these surface active molecules.

The main anionic phospholipids found in *E. coli* cells are diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG), which constitute approximately 5% and 20% respectively of the total cellular phospholipid (Gennis, 1989). If an electrostatic interaction involving anionic phospholipids does contribute to the PBP5 anchoring process then removing PG and DPG from the membrane should destabilise membrane binding. Under these conditions increased amounts of PBP5 may be expected to be displaced from the inner membrane by pertubants.

To investigate this possibility, the *E. coli* strain HDL11/pLG364 was used in washing experiments. In this strain, the expression of PG and DPG synthesis genes, has been placed under the control of a *lac* promoter and can be induced by addition of the *lac* inducer IPTG. Thus the level of anionic lipids in the membrane can be controlled (section 4.2; Kusters *et al.* 1991). The plasmid pLG364 carries the *dacA* 11191 PBP5 allele, and overproduces this protein (Broome-Smith and Spratt, 1984).

latter result may not have been expected when it is considered that, at low pH, P5 possesses high levels of α-helicity (chapter 2; Siligardi *et al.* 1997) and that electrostatic interactions appear to play only a minor role in P5 - monolayer interactions. However, examination of the primary structure of P5 (table 2.1) shows that in an α-helical conformation, two lysine residues (pK_a 11.0) and two histidine residues (pK_a 6.5) would occur in the hydrophilic face of the helix (figure 1.5). At low pH, these histidine residues would contribute to a cationic region which could decrease penetration into zwitterionic monolayers. At higher pH, these histidine residues and the cationic region would experience a decreased positive charge and this could facilitate "snorkelling" into the DOPC and DPPC monolayers by the hydrophobic alkyl chains of the lysine residues in the cationic region (section 1.6.3.1; Segrest *et al.* 1992a) leading to deeper penetration and higher levels of interaction with zwitterionic monolayers by P5. With lipid monolayers derived from SD12 or HDL11 membranes and those formed from DOPG, the presence of anionic lipid headgroups could stabilise the positive histidine residues in the P5 helical polar face and thus decrease the effect of protonation observed at low pH with zwitterionic lipid monolayers.

In the case of P6, at low pH the peptide showed high levels of interaction with zwitterionic lipids (figure 4.3b) which were either similar or enhanced when compared to those with corresponding monolayers formed from the membranes of the wild type *E. coli* SD12 and the mutant *E. coli* strain HDL11 (figure 4.2b). Examination of the primary sequence of P6 (table 2.1) shows that in an α-helical conformation there would also be a histidine residue in the hydrophilic face of the P6 helix (figure 1.5). However, this histidine residue is situated next to a negatively-charged aspartic acid residue and since there is no option for the snorkelling effect in the absence of a positive lysine residue, the histidine would not have such a great effect on P6 - monolayer interactions. P6 has been shown to be strongly hydrophobic and was found to form an α-helix much less readily

than P5 (chapter 2; Siligardi *et al.* 1997). It seems likely that low pH is necessary to stabilise α-helix formation in P6 and is a major requirement to facilitate the high levels of hydrophobic interaction shown by the peptide with DOPC, DPPC and other lipid monolayers under acid conditions. However, at higher pH, the levels of interaction of P6 with zwitterionic lipids are greatly reduced (figure 4.3b) when compared to the corresponding interactions of the peptide with lipid monolayers derived from the membranes of the wild type *E. coli*, SD12 and the mutant *E. coli* strain, HDL11 (figure 4.2b). This may be because in the monolayers derived from SD12 and HDL11, the presence of negatively-charged lipids is able to cause a local drop in pH near the membrane interface which could stabilise helix formation in P6. Furthermore, the nature and packing order of the lipids forming the monolayer could affect penetration by P6.

In summary, these results appear to show that P5 and P6 - lipid monolayer interactions are likely to involve α-helical secondary structure and predominantly hydrophobic forces with a minor electrostatic contribution. It has been suggested that the levels of interaction of P5 and P6 with lipid monolayers are related to the levels of α-helicity in the peptide structure, with high levels of interaction corresponding to high levels of α-helicity. These levels of α-helicity appear to be enhanced by low pH. In the case of P6, α-helix stabilisation by low pH seems to be a strong requirement for efficient monolayer interaction and it is speculated that the ability of P6 to penetrate monolayers may be related to the nature and packing order of lipids forming the monolayer. In the case of P5, it is speculated that levels of monolayer penentration may be related to the presence of residues which occur in the polar face of the helical peptide, namely the level of charge carried by histidine residues and the "snorkelling" abilities of lysine residues. Overall these results support the view that C-terminal amphiphilic α-helices are involved in the membrane anchoring of PBP5 and PBP6.

CHAPTER 5

DEPLETION OF ANIONIC PHOSPHOLIPIDS HAS NO OBSERVABLE EFFECT ON THE MEMBRANE ANCHORING OF *ESCHERICHIA COLI* PENICILLIN-BINDING PROTEIN 5.

HARRIS, F., CHATFIELD, L. and PHOENIX, D. A. (1995) FEMS LETT. **129**, 215-220.

5.1 ABSTRACT

The results of washing experiments have suggested a minor electrostatic contribution to the *Escherichia coli* PBP5 anchoring mechanism which may involve the cationic region of the C-terminal α-helix. Similarities between this anchor domain and some surface active agents, such as melittin, suggest that the cationic region of the PBP5 anchor may require the presence of anionic phospholipids for membrane interaction *in vivo*. Washing experiments performed on membranes of HDL11, an *E. coli* mutant in which the occurrence of the major anionic phospholipids is under *lac* control, found no such requirement. The results are discussed in relation to the hypothesis that the cationic region may interact with other sources of negative charge, possibly arising from a protein complex involving PBPs.

5.2 INTRODUCTION

Theoretical analyses have predicted that the *E. coli* PBP5 and PBP6 C-terminal regions may have surface activities comparable to that of the cytotoxin, melittin (section 1.6.2, section 1.6.3.5; figure 1.10) and haemolytic analysis has supported this prediction (chapter 3). Melittin is known to interact with the membrane *via* amphiphilic α-helices (section 1.6.3.2; Dempsey, 1990) and this led to the suggestion that the PBP5 and PBP6 C-terminal region may utilise a similar mechanism and lie at the membrane interface with their hydrophobic arcs interacting with the bilayer core (section 1.6.3.5). This model appears to be supported by the strong susceptibility shown by PBP5 and PBP6 to the perturbant action of the chaotropic thiocyanate ion and to that of urea (section 1.6.3.5.1). However it was also found that this perturbant susceptibility was dependent on pH. At low pH, PBP5 extraction was resistant to perturbants but as pH was increased above neutrality progressively greater amounts of PBP5 were displaced from the inner membrane (section 1.6.3.5.1). This observation suggested that the PBP5 anchoring mechanism may involve an electrostatic contribution.

Electrostatic interactions are also a feature of the membrane interactions of some surface active proteins and polypeptides. For example, melittin from bee venom (Dempsey, 1990) and the bacterial toxin, colicin A (Van der Goot *et al.* 1993) require the presence of anionic phospholipids for membrane association. Interaction between the negatively charged phospholipid headgroups and cationic regions within the structure of the protein or polypeptide acts to stabilise membrane association. The cationic regions involved in these interactions are frequently located on the hydrophilic face of the amphiphilic α-helices found in such molecules. For example, melittin possesses positively charged N-terminal and C-terminal regions, both of which are situated on the hydrophilic face of the polypeptide's single α-helix (Dempsey, 1990).

5.3 MATERIALS AND METHODS

Bacterial strains and growth conditions

The *E. coli* strains used were: SP1048 (*his, tsx, supF, srl*::Tn10,*ΔdacC1,ΔdacA*::Km^r) (Phoenix and Pratt, 1990) and HDL11 (*pgsA*::*kan*, *ϕ*(*lac*OP-*pgsA*$^+$)1, *lacZ'*, *lacY*::Tn9, *lpp2*, *zdg*::Tn10) (Kusters *et al.* 1991). SP1048 and HDL11 were each transformed with the multicopy plasmid pLG364 (Hanahan, 1986) which carries the *dacA* 11191 mutation of PBP5 on a *Bam*H1-*Eco*R1 fragment in pBR322 (Broome-Smith and Spratt, 1984). All strains were grown in nutrient broth (Lab M, No 2) supplemented with ampicillin (25 μg ml^{-1}) at 37°C with aeration. When required, HDL11 strains were grown in the presence of 50 μM IPTG to induce high levels of *pgsA* gene expression.

Determination of phospholipid content of bacterial strains

Cultures (1 litre) were grown in the presence of 370 GBq of [^{14}C]-sodium acetate (specific activity; 1.36 kBq μmol^{-1}, Sigma) to an A_{660} of 0·6. Lipids were extracted from cells (Bolton and Harwood, 1977) and separated by thin layer chromatography (Silica gel, type G, Sigma) using chloroform / methanol / acetic acid / water (170:30:20:7, v:v:v:v) as solvent. The lipids were visualised by iodine vapour and identified using appropriate standards. Incorporation of [^{14}C]-acetate into lipids was quantified using a Beckman LS5801 scintillation counter.

Preparation of envelope fractions

Cultures (1 litre) were grown to an A_{660} of 0·6. Envelope fractions were prepared by osmotic lysis (Osborne *et al.* 1972). The DNA released from cells was sheared mechanically using 23G and 25G syringes and unlysed cells were removed by low speed centrifugation (1100 g,

15 min, 4°C). Samples (8 ml) of the supernatant were centrifuged (100,000 g, 60 min, 4°C) and the resulting pellets were stored at -20°C.

The effect of perturbants on the anchoring of PBP5 in envelope fractions

Envelope pellets were resuspended in 1 ml of 4 M urea, 2 M sodium thiocyanate or 2 M sodium chloride at pH 6, 7 or 8 and left on ice for one hour at 0°C. Solutions were prepared in 10 mM phosphate buffer. Envelope pellets were also resuspended in 10 mM phosphate buffer alone at pH 6, 7 or 8 (Phoenix and Pratt, 1990). The volume of the resuspended pellets was made up to 8 ml with the appropriate perturbant at the appropriate pH and centrifuged (100,000 g, 60 min, 4°C). The pellet was resuspended in a minimum volume of resuspension buffer (13 parts of wash buffer, (KH_2PO_4, 3 g l^{-1}; $Na_2HPO_4.2H_2O$, 8.9 g l^{-1}; NaCl 4.0 g l^{-1}; $MgSO_4.7H_2O$, 0.1 g l^{-1}): 5 parts of saturated Tris base: 2 parts of phenylmethylsulphonylfluoride (PMSF) in dimethyl sulphoxide (6 mg ml^{-1}). Trichloroacetic acid was added to the supernatant to give a final concentration of 10% (w/v), the mixture was left on ice for 1 hour, centrifuged (3000 g, 15 min, 4°C) and the pellet resuspended in a minimum volume of resuspension buffer (10 µl - 25 µl). Protein in the resuspended pellets was analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (12% resolving gel; Laemelli, 1970). PBP5 was visualised by Western blotting (Towbin *et al.* 1979) with the exception that 3,3'-diaminobenzidene (Sigma) was used as substrate and quantified by densitometry using a Shimadazu CS-9000 dual wavelength flying spot scanner at 500 nm.

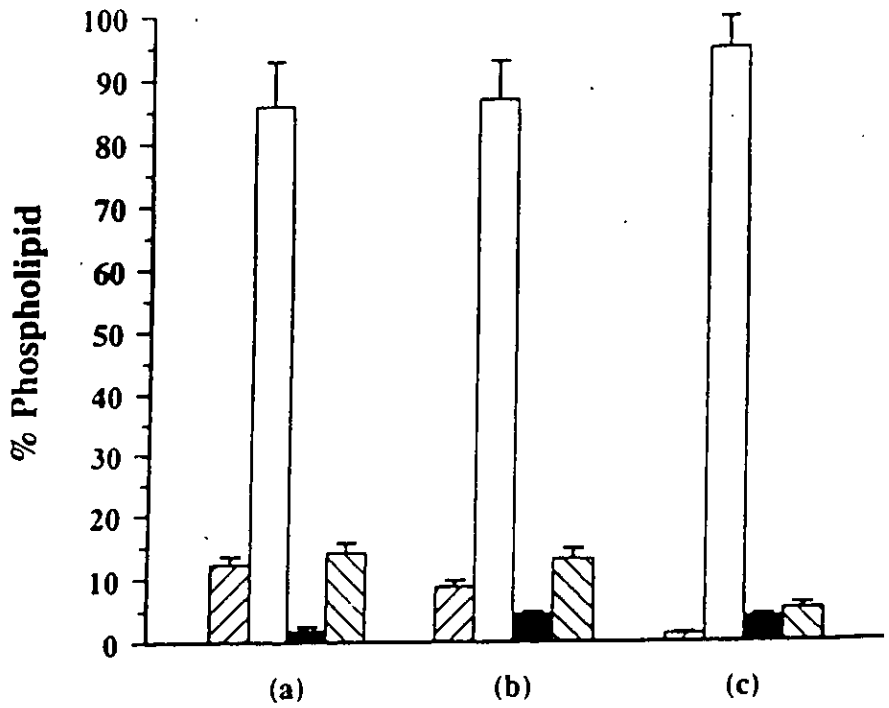
5.4 RESULTS AND DISCUSSION

When HDL11/pLG364 was grown in the presence of IPTG the overall level of anionic phospholipids was comparable to the control strain SP1048/pLG364 (figure 5.1). However,

when the organism was grown in the absence of IPTG this level was decreased by approximately 60%. The predominant anionic phospholipid in the membrane is PG, and levels of this phospholipid were found to be decreased by 85% in uninduced cells of HDL11/pLG364, which agrees well with the data of Van der Goot *et al.* (1993). These latter authors showed that the surface active bacterial toxin, colicin A, has an *in vivo* requirement for the presence of anionic phospholipids if it is to interact with the membrane. Using HDL11, they demonstrated that the toxin was fully active against this strain in the presence of induced levels of anionic phospholipids, but toxicity was reduced by a factor of three in cells with reduced levels of these phospholipids (Van der Goot *et al.* 1993).

A reduction of approximately 60% in the overall level of anionic phospholipid had no significant effect on the amounts of PBP5 displaced from membranes of HDL11 by perturbant action. The levels of PBP5 displaced from membrane fragments of SP1048/pLG364 and HDL11/pLG364 grown in the presence of IPTG are comparable to those displaced from HDL11/pLG364 grown in the absence of IPTG (figure 5.2). This could indicate that the presence of anionic phospholipids is not a general requirement for PBP5 - membrane anchoring, although the possibility exists that the magnitude of their interaction is too small to be detected by this experimental system. It can also be seen from figure 5.2 that despite the overall decrease in anionic lipids in membranes of HDL11/pLG364 grown in the absence of IPTG, the level of DPG in these membranes is comparable to that of HDL11/pLG364 grown in the presence of IPTG. Thus it is possible that DPG plays a role in the membrane anchoring mechanism of PBP5. If anionic phospholipids do not play a role in PBP5 - membrane anchoring, these results do not exclude phospholipids, charge interaction or the cationic region of the PBP5 anchor from contributing to the anchoring process. The trends shown by the results of these washing experiments are in agreement with the findings of previous

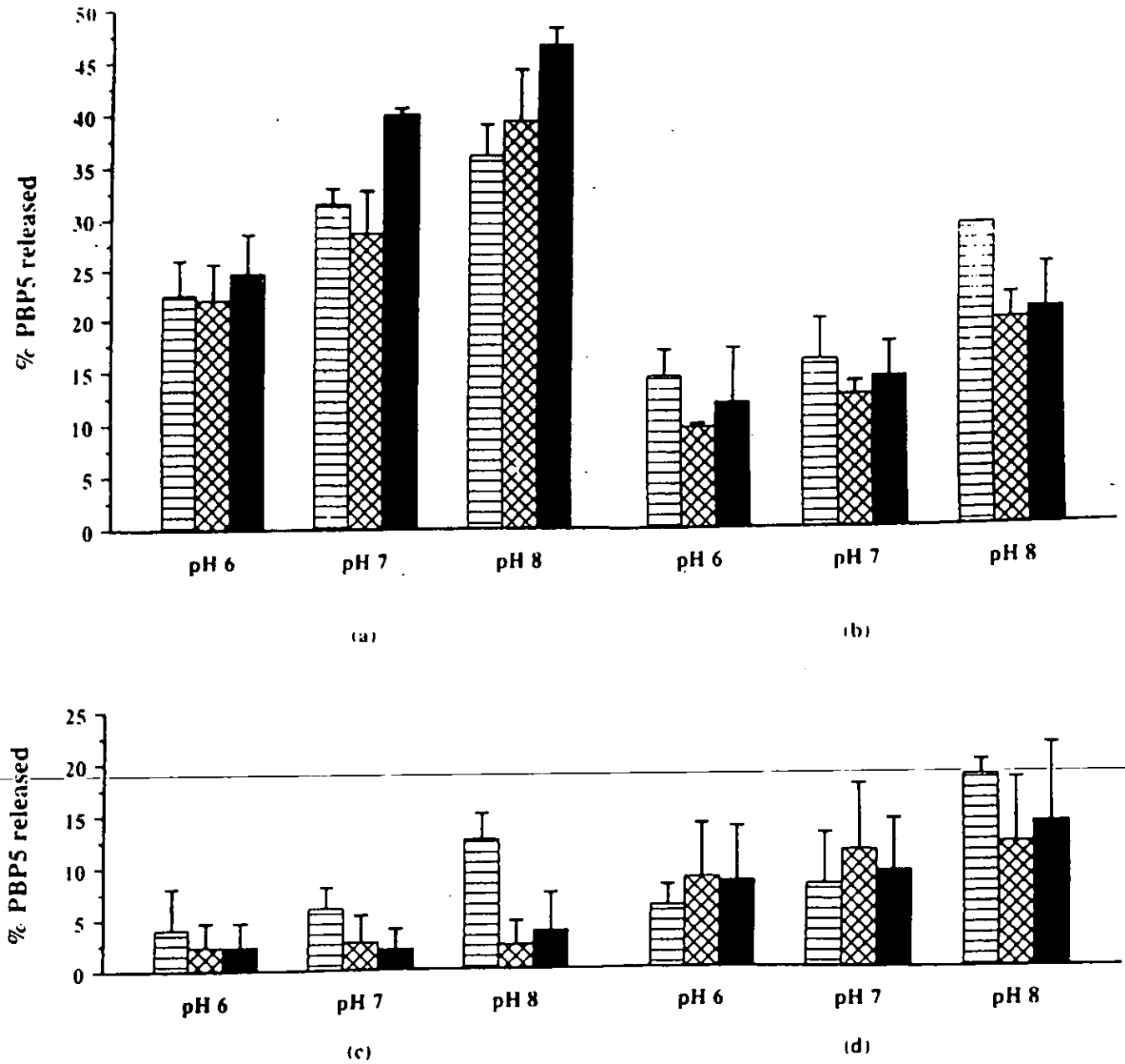
Figure 5.1 Phospholipid content of bacterial strains examined



The relative percentages of the major phospholipids in the membranes of the E. coli strains (a) SP1048/pLG364, (b) HDL11/pLG364 grown in the presence of 50 μM IPTG and (c) HDL11/pLG364 grown in the absence of IPTG were determined by radio-labelling with [^{14}C]-acetate and quantification via scintillation counting, all as previously described. Phosphatidylethanolamine (▭) was the major component with contributions from the anionic phospholipids phosphatidylglycerol (▨) and diphosphatidylglycerol (■). The total anionic phospholipid content is also shown (▧). Error bars denote the standard deviation for n = 3.

studies (Phoenix and Pratt, 1990) and indicate that the PBP5 anchor region becomes progressively more susceptible to chaotropic agents with increasing pH, but maintains its resistance to extraction by ionic perturbants. The overall pH dependance of perturbant action reiterates the possibility of an electrostatic contribution to membrane binding and, if anionic phospholipids are not involved in the binding process, this could mean that the anchor's positively charged region may interact with negative charge from other sources.

Figure 5.2 The relative amounts of PBP5 displaced from membrane fragments of strains of *E. coli* by the action of chaotropic and ionic pertubants



Membrane fragments prepared from SP1048/pLG364 (▤), HDL11/pLG364 grown in the presence of 50μM IPTG (▩) and HDL11/pLG364 grown in the absence of IPTG (■) were treated with (a) 4M urea, (b) 2M sodium thiocyanate, or (c) 2M sodium chloride all in 10mM phosphate buffer at pH 6, 7 or 8. Fragments were also treated with (d) 10mM phosphate buffer at pH 6, 7 or 8 as a control. PBP5 was visualised by Western blotting and quantified by densitometry, all as previously described. Error bars denote the standard deviation for n = 3.

One possibility is that the cationic region may interact with negatively charged regions in the ectomembranous domain of PBP5. Such an interaction could serve to stabilise membrane binding and may be associated with changes in the conformation of the protein. It has been shown that the overall conformation of PBP5 can affect the strength of binding and, *in vivo*, anchoring may be related to the enzymatic activity of the protein (Phoenix and Pratt, 1993). PBP5 may undergo conformational changes upon membrane interaction since it has been found that purified PBP5 shows resistance to proteolytic action when reconstituted into vesicles but that it is readily degraded by proteases in the unreconstituted form (Phoenix and Pratt, unpublished data).

Another possibility is that the positively charged region of the PBP5 anchor may interact with other proteins, either a receptor protein or as part of a protein complex. Phoenix and Pratt (unpublished data) found that PBP5 would not reconstitute into vesicles derived from prokaryotic membranes when the cytoplasmic face was accessible but would reconstitute into such vesicles when the periplasmic face was accessible. These findings could be consistent with a model for PBP5 anchoring which does not require anionic phospholipids but which requires instead some other component of the periplasmic face of the inner membrane - possibly a protein. There is also, of course, the possibility that PBP5 has a lipid requirement for membrane interaction which is related to the asymmetry of lipid content shown between the cytoplasmic and periplasmic faces of the inner membrane (section 1.6.3.5.1; figure 1.11).

In conclusion, it is suggested that PBP5 in the native state has a minor electrostatic component which contributes to membrane binding, and which may involve the cationic region of the PBP5 anchor domain. The anchor region appears to have no general requirement for anionic phospholipids, but may interact with DPG or other negatively charged regions, either in PBP5 itself or those of other membrane components. Interestingly, evidence from cross-linking

studies has suggested that PBP5, PBP3 and either PBP1a or PBP1b have the capacity to form a protein complex (Said and Holtje, 1983) and at present this possibility is being investigated further.

CHAPTER 6.

MEMBRANE BINDING OF *ESCHERICHIA COLI* PENICILLIN-BINDING PROTEIN 4 IS PREDOMINANTLY ELECTROSTATIC IN NATURE AND OCCURS AT A SPECIFIC BINDING SITE

HARRIS, F., DEMEL, R.A., PHOENIX, D. A. AND DE KRUIJFF, B. (1997) BIOCHIM.. BIOPHYS. ACTA SUBMITTED

6.1 ABSTRACT

Using chemiluminesence and biochemical wash procedures it is shown that in the wild type *E. coli* strain MRE600, PBP4 is membrane bound *via* predominantly electrostatic interactions which it is suggested occurs at a specific binding site. Studies on interactions of a PBP4 C-terminal homologue, P4, with monolayers of DOPC, DPPC, DOPG and those formed from membrane lipids of a wild type *E. coli* strain SD12, showed that these interactions proceeded *via* predominantly electrostatic forces with a minor hydrophobic contribution. A molecular area of 180 $Å^2$, derived from radiolabeled P4 - DOPG monolayer interactions, implied α-helical structure in the peptide. These results suggest that the PBP4 C-terminal region may feature in the protein - anchoring mechanism. The interactions of P4 with monolayers formed from membrane lipids of a mutant *E. coli* strain HDL11, in which levels of anionic phospholipids can be controlled, showed a requirement for anionic lipids whereas PBP4 appeared to show no such requirement in its binding to naturally occurring HDL11 membranes. These latter results suggest that anionic lipids may not play a major role in PBP4 - membrane interaction or that the C-terminal requirement may be masked by other interactions, as might occur at a specific binding site.

6.2 INTRODUCTION

Various data on PBP4 and its C-terminal domain have been interpreted by some analyses to suggest that it is a soluble protein (Mottl *et al.* 1991) and by others to support the idea that the protein is membrane bound (Phoenix, 1993). Theoretical analyses have indicated that if the PBP4 C-terminal region interacts with the membrane, then the protein would be weakly bound (section 1.5.2; section 1.6.3.5, section 1.6.3.5.2; Roberts *et al.* 1997; Pewsey *et al.* 1996). Indeed, Mottl and co-workers (1991) were unable to detect either a potential C-terminal amphiphilic α-helical domain or other regions likely to be involved in the anchoring of PBP4 (Mottl *et al.* 1991). However, more recently the use of affinity chromatography and immobilised enzymes has led to the proposal that PBP4 may be involved in a multi-enzyme complex which could include several high molecular mass PBP s (section 1.6.3.5.2; Holtje, 1996a)

In an effort to resolve these conflicting views, and to establish whether *E. coli* PBP4 normally binds to the inner membrane *in vivo*, the interaction of the protein with membranes of the overproducing strain HB101/pBK4 in which PBP4 is over-expressed *via* temperature-induced runaway replication of the plasmid (Korat *et al.*1991), a wild type strain MRE600 (Cammack and Wade,1965) and a mutant strain HDL11 in which the level of anionic lipids can be controlled (section 4.2; Kusters *et al.* 1991) have been investigated. Cells were fractionated, PBP4 localised and the susceptibility of membrane associated PBP4 to perturbants determined. To help determine any role that the C-terminal region of PBP4 may play in the membrane association of the protein, the interactions of a PBP4 C-terminal homologue, P4 (table 2.1), with monolayers formed from: dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylcholine (DPPC), and

lipids extracted from the membranes of a wild type *E. coli* strain SD12 (De Vrije *et al.* 1988) and those extracted from membranes of the mutant strain HDL11, have been investigated. [^{14}C]-P4 was used to determine the molecular area, and hence the possible conformation, of the peptide in the presence of a DOPG monolayer.

6.3 MATERIALS AND METHODS

Bacterial strains

The *E. coli* strains used were: MRE600 (Korat *et al.*1991), SD12 (De Vrije *et al.* 1988), HB101 (*hsdS20* (r_B^-, m_B^-), *recA13, ara-14, proA2, lacY1, galK2, rpsL20,* (Sm^r), *xyl-5, mtl-1, supE44*; Korat *et al.* 1991) and HDL11 (*pgsA::kan, (lac*OP-*pgsA*$^+$)1, *lacZ*, *lacY::*Tn9, *lpp2, zdg::*Tn10; Kusters *et al.*, 1991). HB101 was transformed with the plasmid pBK4, which carries *dacB* on a 1.9 Kb *Sma*I-*Eco*R1 DNA fragment (Bolivar and Backman.1989).

Growth conditions

MRE600 was grown in nutrient broth (Lab M, No 2). Overnight cultures were used to inoculate 500 ml aliquots of pre-warmed, aerated medium (1:200 dilutions). These were then grown in an orbital shaker (37°C, 180 r.p.m.) to an OD_{660} of 0.6.

HB101/pBK4 was grown in Luria-Bertani medium I (sodium chloride 10 g l^{-1}, tryptone (Lab M) 10 g l^{-1} and yeast extract (Lab M) 5 g l^{-1}, pH 7.5), which was supplemented with kanamycin (final concentration 100 μg ml^{-1}). Overnight cultures were used to inoculate 125 ml aliquots of pre-warmed, aerated media (1:200 dilutions). These were then grown (30°C, 150 r.p.m.) to an OD_{660} of 0.4. To over-express PBP4, the resulting cultures were then added to 375 ml aliquots of pre-warmed, aerated medium at 42°C. These were then grown in an orbital shaker (42°C, 180 r.p.m.) for 1 hour (final OD_{660} approximately 0.35), to amplify the pBK4 plasmid.

HDL11 was grown in Luria-Bertani : medium II (sodium chloride 5 g l^{-1}, tryptone (Difco) 10 g l^{-1} and yeast extract (Difco) 5 g l^{-1}, pH 7.5), which was supplemented with kanamycin (final concentration 50 μg ml^{-1}) and IPTG (60 μmolar) as appropriate. Overnight cultures were used to inoculate 500 ml aliquots of pre-warmed, aerated medium (1:200 dilutions). These were then grown in an orbital shaker (37°C, 150 r.p.m.) to an OD_{660} of 0.6.

Determination of phospholipid content of bacterial strains

Cultures (1 litre) were grown in the presence of 370 GBq of [^{14}C]-sodium acetate (specific activity; 1.36 kBq μmol^{-1}, Sigma) to an A_{660} of 0·6. Lipids were extracted from cells (Bolton and Harwood, 1977) and separated by thin layer chromatography (Silica gel, type G, Sigma) using chloroform / methanol / acetic acid / water (170:30:20:7; v/v/v/v) as solvent. The lipids were visualised by iodine vapour and identified using appropriate standards. Incorporation of [^{14}C]-acetate into lipids was quantified using a Beckman LS5801 scintillation counter.

Preparation of membrane fragments

Bacterial cultures (1 litre) were grown as described. Membrane fractions were prepared by osmotic lysis (Osborne *et al.* 1972). DNA released from cells was sheared mechanically using 23G and 25G syringes and unlysed cells were removed by low speed centrifugation (1100 *g*, 15 min, 4°C). Samples (8 ml) of the supernatant were centrifuged (100,000 *g*, 60 min, 4°C) to produce membrane pellets.

Localisation of PBP4

Membrane pellets were prepared as described above and the supernatant resulting from ultracentrifugation was also retained. Pellets were resuspended in a minimum (10-25 μl) of resuspension buffer [13 parts of wash buffer, (KH_2PO_4, 3 g l^{-1}; $Na_2HPO_4.2H_2O$, 8.9 g l^{-1}; NaCl

4.0, g l^{-1}; $MgSO_4.7H_2O$, 0.1 g l^{-1}): 5 parts saturated Tris base: 2 parts of phenylmethylsulphonylfluoride in dimethyl sulphoxide (6 mg ml^{-1})]. Trichloroacetic acid was added to the retained supernatant to give a final concentration of 10% (w/v) and the mixture was left on ice at 0°C for 1 hour. After centrifugation (3000 *g*, 15 min, 4°C), the resulting pellet was resuspended in a minimum volume of resuspension buffer (10-25 µl). Protein in the resuspended pellets was resolved by sodium dodecylsulphate polyacrylamide gel electrophoresis (12% w/v resolving gel; Laemelli, 1970). PBP4 was visualised by Western blotting (Towbin *et al.* 1979), except that instead of using 3, 3'-diaminobenzidine as substrate, either chemiluminesence (DuPont; Kaufmann *et al.* 1987; Thorpe *et al.* 1985) or amplified chemiluminesence (Bio-Rad; Wilson and Nakane, 1978) was employed. Visualised protein was quantified by densitometry using a Shimadazu CS-9000 dual wavelength flying spot scanner at 500 nm.

The effect of perturbants on the membrane association of PBP4

Membrane pellets were produced as described above and were resuspended in 1 ml aliquots of 10 mM phosphate buffer, at either pH 6, 7 or 8. The resuspension buffer contained 4 M urea or 2 M sodium chloride or 2 M sodium thiocyanate as required. The resuspended pellets were placed on ice for one hour and then the volume of each was made up to 8 ml with the appropriate perturbant at the appropriate pH and centrifuged (100,000 *g*, 60 min, 4°C). The resultant pellets and supernatants were treated as described for those used in the localisation of PBP4.

Monolayer experiments

Buffers and solutions for all monolayer experiments were prepared from milli Q water. Phospholipids of *E. coli* were extracted by the Bligh and Dyer extraction (Bligh and Dyer,

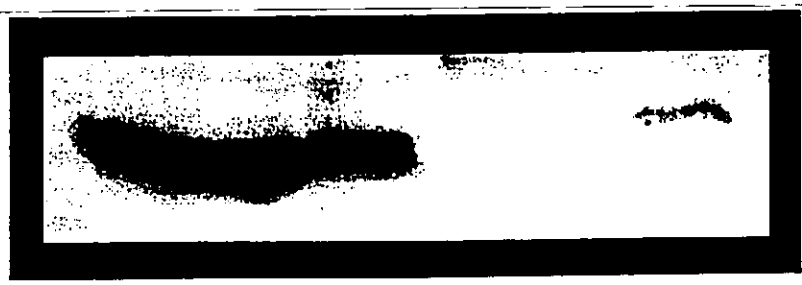
1959) of cells in the late log phase and purified by column chromatography with Polygosil (63 - 100 μm, Macherey-Nagel) as stationary phase and chloroform / methanol (1:1, v/v) as eluant, after a first elution of neutral lipids and other contaminants with 100% chloroform. DOPG and DOPC were supplied by Avanti. P4 was supplied by the Department of Biochemistry, University of Liverpool, England. The peptide was synthesised by solid state synthesis, purified by HPLC and showed a purity of 99% or greater. For surface pressure-area measurments, P4 was radiolabelled by reductive methylation with [^{14}C]-formaldehyde (specific activity 1.49 kBq μmol^{-1}) and sodium cyanoborohydride (Aldrich). By this procedure the net charge of the peptide is not altered (Dottavio-Martin and Ravel, 1978). Sodium cyanoborohydride, P4 and [^{14}C]-formaldehyde (molar ratio 125:2:1) were incubated in 10 mM phosphate buffer (pH 7) for 90 min at 25°C. P4 was separated from the reaction mixture by gel exclusion chromatography on a 20 cm × 0.5 cm Sephadex G25 column, eluted with 10 mM phosphate buffer (pH 7). The radioactivity of 5 μl samples of the 500 μl fractions was determined using a Beckman LS5801 scintillation counter. Fractions eluted between 7 and 9.5 ml were well resolved from the [^{14}C]-formaldehyde. Fractions were pooled and assayed for P4 concentration and [^{14}C] activity. The determined specific activity of labeled P4 was 2.77 kBq μmol^{-1}, implying that on average 93% of P4 molecules had obtained two methylated residues. Monolayer surface tension was monitored by the (platinum) Wilhelmy plate method using a Cahn C202 microbalance (Demel, 1994). Lipid monolayers were formed by spreading either pure phospholipids (10 mM) or total phospholipid extracts of *E. coli* strains, from solutions in chloroform / methanol (8:2, v/v) until the desired initial surface pressure was achieved. The molecular area of [^{14}C]-P4 was determined in the presence of phospholipid monolayers at constant area, using a Teflon dish with a volume of 20 ml and surface area of 29.6 cm^2. Appropriate amounts of [^{14}C]-P4 solution (400 μmolar in phosphate buffer, pH 7) were added to the subphase. The subphase was continuously stirred by a

magnetic bar. The amount of radiolabel at the interface was determined by following the surface radioactivity with a gas flow detector (Demel, 1974). The subphase was refreshed by injecting and ejecting the buffer solution at opposite sides of the dish at a flow rate of 10 ml min^{-1}. The monolayer was collected by sucking into a counting vial (Rietsch *et al.* 1977). P4 - lipid interactions were investigated at constant area using a Teflon dish with a volume of 5 ml and a surface area of 8.04 cm^2.

6.4 RESULTS

When over-expressed, the bulk of PBP4 was recovered in the soluble fraction with only 5% of the total PBP4 being recovered with the membrane fraction (figure 6.1). However, when PBP4 was localised in a wild type strain of *E. coli*, no soluble form of PBP4 could be detected

Figure 6.1 The localisation of PBP4 in *E. coli*



A B C D

Cells were lysed, membrane fractions isolated and visualised by chemiluminesence as described in the methods. "A" and "B" represent PBP4 present in the soluble and membrane fractions of the over-expressing E. coli strain HB101/pBK4 whilst "C" and "D" represent the soluble and membrane fractions of the wild type strain MRE600. Approximately 95% of the total PBP4 produced in the over-expressor was recovered in a soluble form whereas no PBP4 was detected in the soluble fraction of MRE600. The PBP4 recovered in the membrane fraction of MRE600 was of the order of 1% of the total PBP4 recovered in HB101/pBK4. This is in reasonable agreement with the findings of Korat et al. (1991) who estimated 160 fold over-expression of PBP4 in HB101/pBK4 (Mottl et al. 1991).

and the protein was recovered exclusively with the membrane fraction (figure 6.1). This would be explained if the soluble form of PBP4 was an artefact resulting from overproduction and PBP4 is in fact a membrane bound protein. This explanation could be further supported by the observation that membrane bound PBP4 was found to resist total displacement by up to 8M urea (figure 6.2). Such resistance would not be expected from a soluble protein which has been proposed to only loosely associate with the bilayer.

Figure 6.2. The relationship between urea concentration and its ability to displace PBP4 from the membrane.

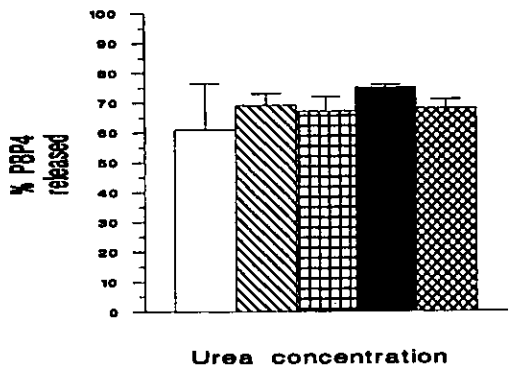
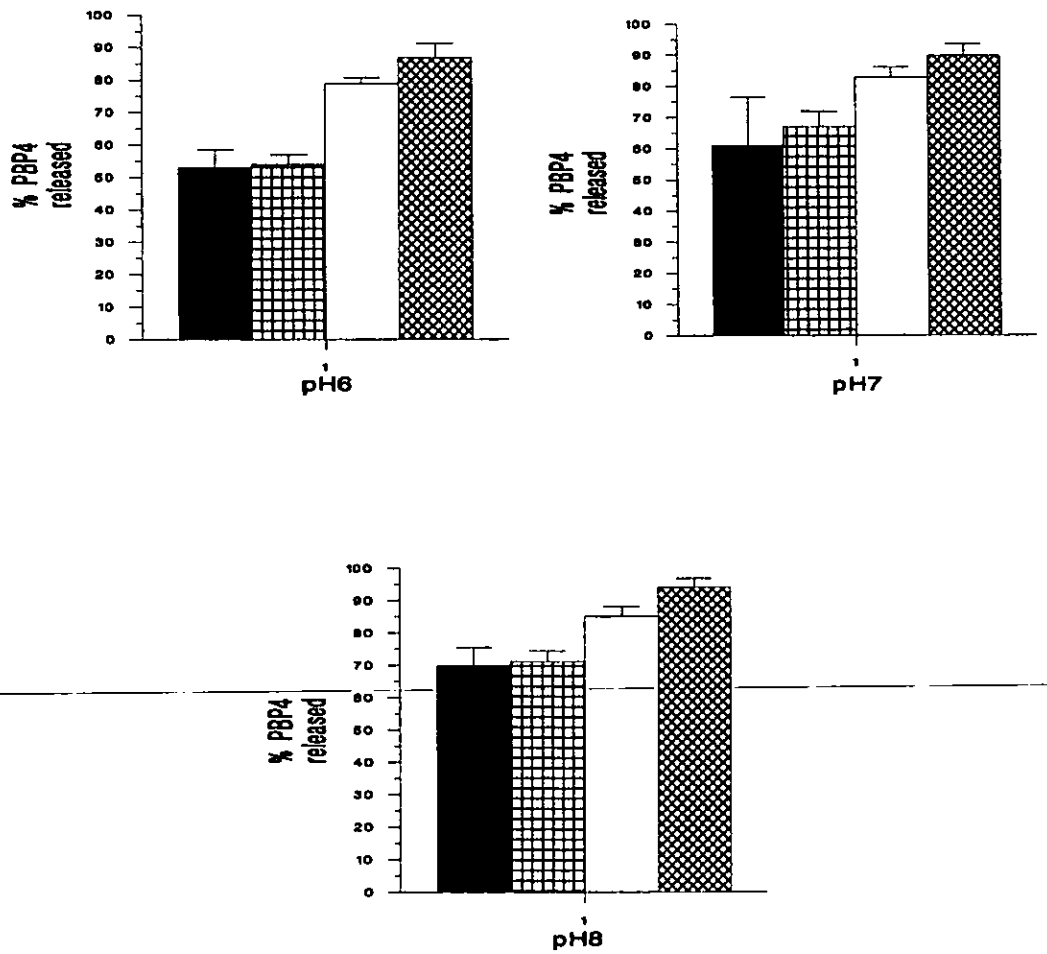


Figure 6.2 shows the percentages of membrane bound PBP4 displaced by the action of urea. Membrane fragments of HB101/pBK4 were treated with 10 mM phosphate buffer (pH 7) ( ) or 2 M urea ( ) or 4 M urea ( ) or 6 M urea ( ) or 8 M urea ( ). The urea solutions were made up in 10 mM phosphate buffer (pH 7).

Furthermore, it was found that the membrane binding of PBP4 showed no greater susceptibility to the chaotropic thiocyanate ion than to sodium chloride and that the perturbant action of phosphate-buffered urea was comparable to that of the corresponding phosphate buffer alone. These findings indicate that PBP4 is susceptible to ionic perturbants, particularly sodium chloride, which displaces over 85% of the protein from the membrane (figure 6.3). Overall, these results suggest that the association of PBP4 with the membrane is

predominantly electrostatic in nature but that there may also be a minor hydrophobic contribution.

Figure 6.3 The pH dependent release of PBP4 from the membrane by perturbants



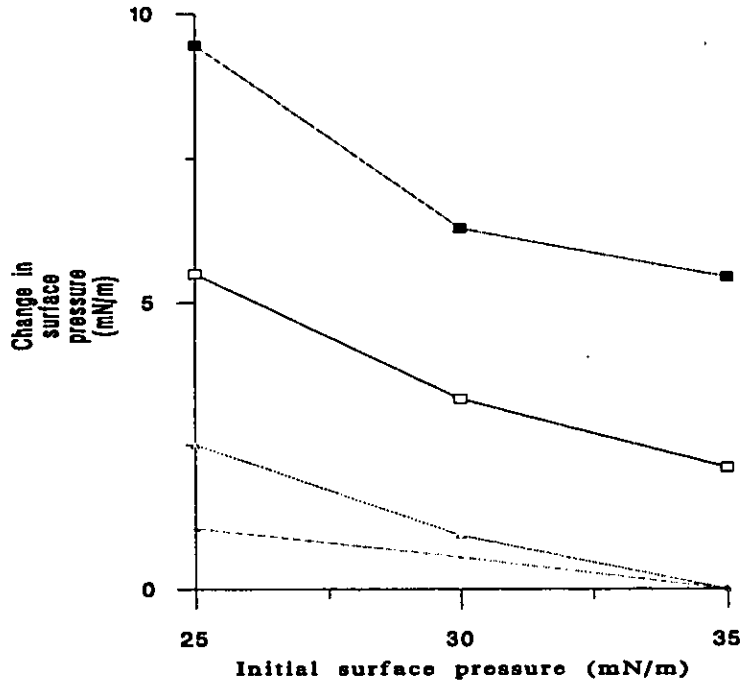
Membrane fragments of HB101/pBK4 were treated with 10 mM phosphate buffer (■) at pH 6, 7 or 8 and either 4 M urea (▦), 2 M sodium thiocyanate (□), or 2 M sodium chloride (▩), all in 10 mM phosphate buffer at pH 6, 7 or 8. PBP4 was visualised by Western blotting and quantified by densitometry, all as described in the methods. Error bars denote the standard error of the mean for n = 3.

It has been suggested that the PBP4 C-terminal region may participate in the association of the protein with the membrane (Phoenix and Harris, 1995; Gittins *et al.* 1993). Theoretical analysis has also predicted that the amino acid sequence comprising the PBP4 C-terminal domain has some potential to adopt an α-helical conformation and that in this conformation, the sequence would have a low potential for surface activity and membrane interaction (Pewsey *et al.* 1996; Phoenix, 1993). To test these predictions, the surface properties and lipid monolayer interactions of the PBP4 C-terminal homologue, P4, were investigated.

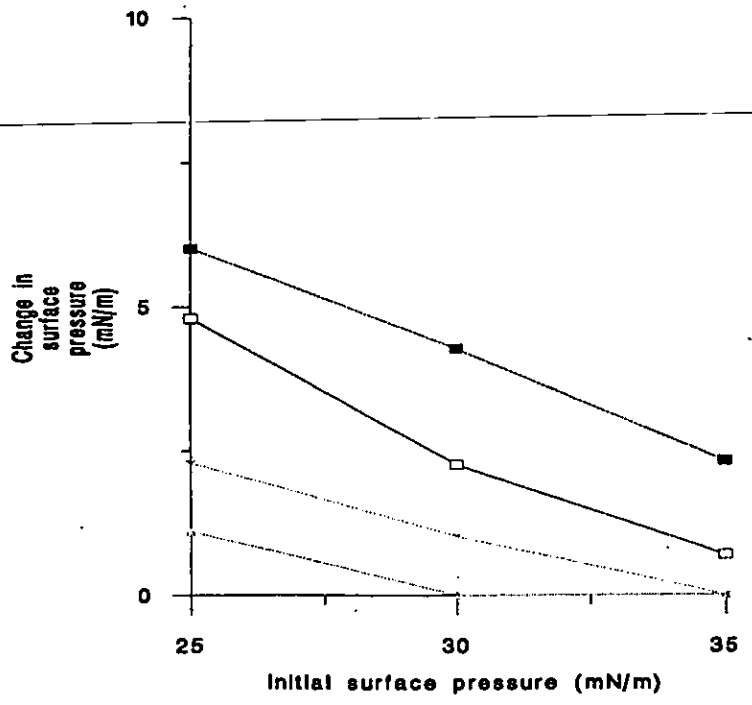
P4 was found to interact with a variety of lipid monolayers over a range of initial surface pressures (figure 6.4). In all cases examined, as initial monolayer surface pressure was increased, the level of surface pressure change induced by the interaction of P4 with the monolayer decreased. This result is probably explained by the fact that increases in monolayer surface pressure generally lead to increased monolayer packing density. Thus, increases in the initial surface pressure of the monolayers depicted in figure 6.4 probably led to a reduced ability of P4 to penetrate the monolayer, in turn leading to reduced levels of surface pressure change upon P4 - monolayer interaction. An initial surface pressure of 30 mN m^{-1} is considered representative of that of a biological membrane (Rojo *et al.* 1991) and further lipid interactions of P4 were investigated at this initial pressure over a range of pH (figure 6.5). It can be seen from figure 6.5 that these interactions of P4 appear to show a slight pH dependency. It can also be seen from figure 6.3 that PBP4 shows a resistance to phosphate washes which appears to show a pH dependency similar to that of the P4 - monolayer interaction shown in figure 6.5, with acid conditions inhibiting protein release (figure 6.3) and causing increased monolayer penetration (figure 6.5).

In the absence of lipid and at pH 7, P4 displayed an equilibrium surface pressure of 13.5 mN m^{-1} at saturation (data not shown) and values of this level are typical of peptides possessing

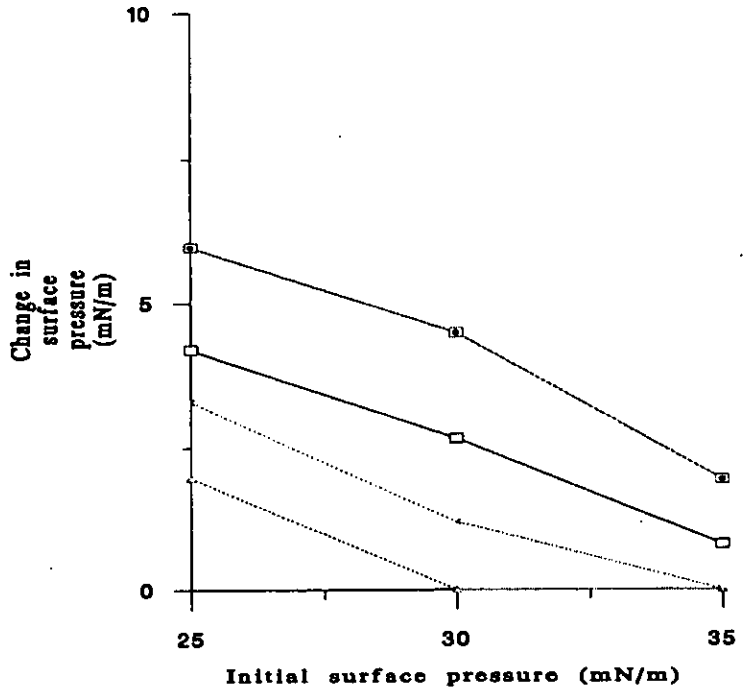
Figure 6.4 The interaction of P4 with lipid monolayers at various initial surface pressures and at various pH values



(a)



(b)



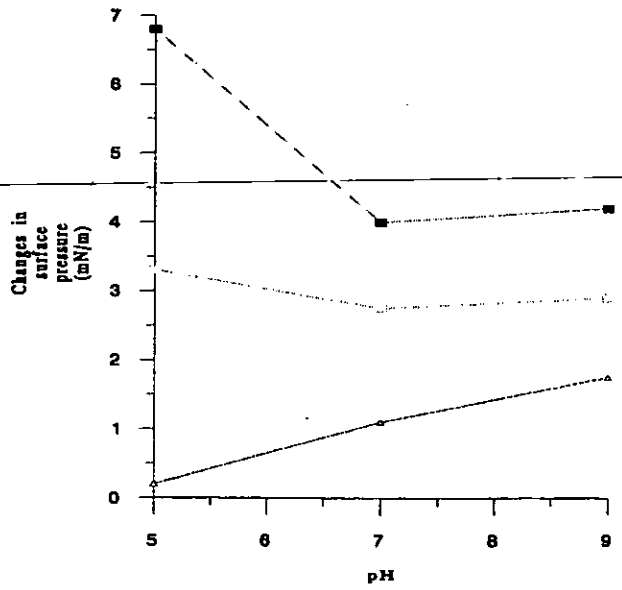
(c)

At various initial surface pressures, figures 6.4a, 6.4b and 6.4c show the surface pressure changes induced by the interaction of P4 with lipid monolayers at pH 5, pH 7 and pH 9 respectively. These lipid monolayers were formed from DOPG (■), DOPG in the presence of 500 mM NaCl (Δ), total phospholipid extracts from membranes of the wild type E. coli, SD12 (□) and total phospholipid extracts from membranes of the mutant E. coli, HDL11, which were depleted in anionic phospholipids (×).

low surface activity (Demel, 1994). P4 was found to interact with monolayers derived from the membranes of a wild type *E. coli* SD12 (figure 6.4 and figure 6.5). In these monolayers the normal membrane phospholipid composition was conserved and at a biological membrane pressure, their interaction with P4 induced pressure changes of the order of 3 mN m^{-1} (figure 6.5). These levels of interaction are those usually associated with peptides such as poly-lysine and prothrombin which mainly interact with the phospholipid headgroup region (Rojo *et al.* 1991). Overall these results appear to confirm theoretical predictions made for the amino acid sequence of P4. These results also suggest that the postulated electrostatic contribution to this involvement could include association with the phospholipid headgroup region. It can be

seen from figure 6.4 that P4 shows high levels of interaction with monolayers formed from the anionic lipid DOPG but that in the presence of 500 mM NaCl, these levels are either greatly reduced or abolished. In particular at biological membrane pressure and in the presence of this high salt concentration, P4 induced no detectable pressure change in DOPG monolayers at higher pH, with only a change of 0.9 mN m^{-1} at pH 5 (data not shown). Furthermore, at all initial surface pressures examined, it was found that in the presence of monolayers formed from the zwitterionic lipid DOPC, P4 induced no detectable pressure change at lower pH and pressure changes of the order of only 1.5 mN m^{-1} were observed at pH

Figure 6.5 The interaction of P4 with phospholipid monolayers at an initial surface pressure of 30 mN m^{-1} and at various pH values



At an initial surface pressure of 30 mN m^{-1} and varying with pH, figure 5 represents the changes in surface pressure induced by the interaction of P4 with monolayers formed from DOPG (■), total phospholipid extracts from membranes of the wild type E. coli, SD12 (□) and total phospholipid extracts from membranes of the mutant E. coli, HDL11, which were depleted in anionic phospholipids (Δ).

9 (data not shown). This suggests that P4 has either little or no affinity for zwitterionic lipid and overall, these observations strongly support the idea that electrostatic interactions are a major factor in the association of P4 with phospholipids. The strong affinity shown by P4 for DOPG monolayers (figures 6.4 and 6.5) suggests that the peptide may have a preference for anionic phospholipids. Accordingly, the interactions of P4 with lipid monolayers, derived from membranes of an *E. coli* mutant, HDL11, were investigated. These membranes were depleted in anionic lipids. It can be seen from figures 6.4 and 6.5 that although P4 shows some interaction with these monolayers, the levels of interaction are reduced when compared to the corresponding interactions with similar SD12 monolayers, particularly at low pH (figure 6.5). This reduction in the levels of interaction strongly suggests that P4 has a requirement for the presence of negatively-charged phospholipids to facilitate efficient membrane interaction. To determine if this apparent requirement for anionic lipid featured in the overall anchoring mechanism of PBP4, the protein was localised in cells of the *E. coli* mutant HDL11 which expressed either wild type levels or depleted levels of anionic phospholipids. In both cases, PBP4 was recovered exclusively in the membrane fraction (data not shown). Together, these results suggest that anionic phospholipids may not play a major role in PBP4 - membrane interaction or that their role is masked by other interactions.

It has been suggested that if the PBP4 C-terminal region does participate in the membrane binding of the protein, then this could involve the adoption of α-helical secondary structure in this C-terminal region (Phoenix and Harris, 1995; Gittins *et al.* 1993). In an effort to obtain direct evidence to support this suggestion, the interaction of radiolabelled P4 with DOPG monolayers were investigated. In a monolayer environment, the presence of α-helical structure in an interacting protein or peptide can often be inferred from a determination of molecular area. For P4 molecular areas of 330 $Å^2$ and 180 $Å^2$ were determined in monolayers

at a surface pressures of 20 mN m^{-1} and 30 mN m^{-1} respectively (table 6.1) and in both cases these values are consistent with the presence of α-helical structure in P4. A molecular area of

Table 6.1 shows the determined molecular areas of radiolabelled P4 in the presence of DOPG monolayers at various surface pressures and at pH 7

Surface pressure of DOPG monolayer (mN m^{-1})	Change in surface pressure of DOPG monolayer induced by radiolabeled P4 (mN m^{-1})	Determined molecular area of radiolabeled P4 ($Å^2$)
20	4.6	330
30	3.9	180

330 $Å^2$ implies that the α-helical region of P4 interacting with the monolayer may be orientated in a plane which favours that of the monolayer and associates with the phospholipid headgroup region. However, a molecular area of 180 $Å^2$ which was determined at a surface pressure mimetic of a bacterial membrane, is consistent with some level of insertion of an α-helical region of P4 into the monolayer hydrophobic region and which is orientated in a plane which is normal to the monolayer (Batenburg *et al.* 1988). It may be that at this higher surface pressure, the greater packing density of the monolayer acyl chain region enables hydrophobic interactions to occur between this region and the membrane interactive segment of P4, leading to penetration of the monolayer hydrophobic region by the peptide. This interpretation may be supported by the fact that at pH 7 and 30 mN m^{-1} although P4 showed no affinity for the zwitterionic lipid DOPC, the peptide was able to induce a pressure change of 1.7 mN m^{-1} in monolayers formed from the zwitterionic lipid DPPC (data not shown). In comparison to monolayers of DOPC, DPPC monolayers possess high molecular packing

densities (Demel, 1974) and the ability of P4 to interact with these latter monolayers may indicate a hydrophobic interaction between the peptide and the monolayer acyl chain region.

6.5 DISCUSSION

It has been suggested previously that PBP4 is a soluble protein (section 1.6.3.5.2; Mottl *et al.* 1991), however, it is shown in the present work that PBP4 is in fact a membrane bound protein. The observation that only 5% of the protein is membrane bound in over-expressing systems but 100% membrane bound in wild type systems could then be explained if PBP4 - membrane association occurred at a specific binding site which has become saturated in the overproducer. Studies carried out on the monolayer interactions of the PBP4 C-terminal homologue, P4, have suggested that the proteins C-terminal region may indeed participate in PBP4-membrane anchoring. The results of these monolayer studies suggest that the PBP4 C-terminal interaction would be predominantly electrostatic in nature (figure 6.4 and figure 6.5), as is the overall interaction of PBP4 with membranes (figures 6.3). However, the monolayer data show a requirement for anionic lipids for P4 - lipid interaction (figure 6.4 and figure 6.5). Molecular area determinations derived from studies on the monolayer interactions of radiolabelled P4 have implied that it is able to form α-helical structure and the penetration of DPPC monolayers (data not shown) would imply that this sequence can penetrate the bilayer core. This implies that despite its apparently predominantly electrostatic nature, there may also be a hydrophobic contribution to the PBP4 C-terminal interaction. Theoretical analysis has shown that the PBP4 C-terminal region has the potential to form a weakly amphiphilic α-helix (section 1.6.3.5; figure 1.5). Approximately one third of the amino acid residues forming this helix are hydrophobic in nature and therefore would have the potential to interact with the bilayer core and make a contribution to PBP4 membrane anchoring. However, the remaining

hydrophilic amino acid residues which form the major polar face of this helix would give this face a strong overall positive charge, mainly due to the presence of the strongly basic residues lysine and arginine (table 2.1; Phoenix and Harris, 1995; Gittins *et al.* 1993). The presence of such a charged region(s) could enable the PBP4 C-terminus to engage in electrostatic interactions with anionic phospholipid headgroups in the membrane and this could account for the strongly electrostatic nature of the putative PBP4 C-terminal contribution to PBP4-membrane interaction.

No requirement for anionic lipid was detected for PBP4 - membrane interaction in HDL11 (data not shown) but this apparent inconsistency would be resolved if more than one interaction was involved in the the binding of PBP4 to the membrane, as would occur at a specific binding site. This speculation may be supported by the recent demonstation that PBP4 is able to engage in protein - protein interactions with immobilised transglycosylases (section 1.4; Holtje, 1996a; Holtje, 1996b). This demonstration has led to the proposal that PBP4 may form part of a protein complex which functions in the synthesis of the *E. coli* sacculus. It is interesting to note that, although the low molecular weight PBPs show some sequence homology, when the structure of PBP4 is compared to those of PBP5 and PBP6, an additional inserted domain of 188 amino acid residues is found between the SXXK and the SXN active site motifs (Mottl *et al.* 1991). The sequence homology could strengthen the argument for comparable C-terminal membrane interactions although the extra domain is predicted to lie on the periphery of the structure of PBP4 (Mottl *et al.* 1992) and as such may form a potential site for PBP4 - membrane interaction.

CHAPTER 7.

CONCLUDING DISCUSSION

7.1 CONCLUDING DISCUSSION

The *E. coli* low molecular mass PBP4, PBP5 and PBP6 are associated with the periplasmic face of the inner membrane. It is generally accepted that PBP5 and PBP6 are membrane bound and a body of indirect experimental evidence and theoretical predictions suggest that these membrane associations involve amphiphilic α-helical structure in the PBP5 and PBP6 C-terminal regions (section 1.5.2; section 1.6.3.5; section 1.6.3.5.1). It has been predicted that the PBP5 and PBP6 C-terminal regions have high potential for α-helix formation (section 1.5.2; section 1.6.3.5; Roberts *et al.* 1997; Pewsey *et al.* 1996) and using the peptides P5 and P6, homologues of the PBP5 and PBP6 C-terminal regions, it has been shown in this work that the amino acid sequences forming these C-terminal regions indeed possess the capacity to adopt α-helical structure. CD analysis has detected the presence of α-helical architecture in both P5 and P6 (chapter 2; chapter 4) and results of these CD analyses are reinforced by molecular area determinations which have implied the presence of α-helical architecture in the peptides at an air - water interface (chapter 4). Theoretical analyses have also predicted that the PBP5 and PBP6 C-terminal regions have high potential for surface activity and membrane interaction. In this work, these predictions appear to have been confirmed, for it is shown that P5 and P6 are highly surface active (chapter 4), are strongly haemolytic (chapter 3) and show generally high levels of interaction with lipid monolayers (chapter 4). Biochemical washing experiments have shown that the membrane binding of PBP5 and PBP6 involve predominantly hydrophobic forces with a minor electrostatic contribution, and show a pH dependent susceptibility to perturbants which is enhanced under acidic conditions (section 1.6.3.5.1; Phoenix *et al.* 1994; Phoenix and Pratt, 1990). In chapter 4 it was shown that P5 and P6 - lipid monolayer interactions involve predominantly hydrophobic

forces with a minor electrostatic contribution. Furthermore, the levels of interactions shown by P5 and P6 with these latter lipid monolayers (chapter 4) and with erythrocyte membranes (chapter 2) generally correlate with the pH dependency shown by the parent proteins to perturbants (section 1.6.3.5.1; Phoenix *et al.* 1994; Phoenix and Pratt, 1990). In chapter 2, it was shcwn that α-helix formation in P5 also shows a pH dependency which correlates with that shown by PBP5 to perturbant action and it was suggested that the levels of interaction of P5 and P6 with lipid monolayers are related to the levels of α-helicity in the peptide structure, with high levels of interaction corresponding to high levels of α-helicity. If this is the case then it could support the suggestion that the strength of membrane binding of PBP5 and PBP6 may be related to the levels of α-helicity in the C-terminal regions of these proteins (section 1.6.3.5.1; Phoenix *et al.* 1994; Phoenix and Pratt, 1993; Phoenix and Pratt, 1990).

In chapter 5, the hypothesis that interactions between histidine residues in the helical polar face of the PBP5 C-terminal region and anionic lipids may stabilise PBP5 - membrane binding was investigated. The results showed that anionic lipids do not appear to be required for efficient PBP5 - membrane anchoring and in chapter 4 it was shown that anionic lipid plays only a minor role in P5 - monolayer interactions. Nonetheless, it was speculated that changes in the levels of charge carried by the histidine residues could affect the ability of lysine residues, also present in the P5 helical polar face, to extend or "snorkel" (Segrest *et al.* 1992) into the monolayer and thus affect levels of monolayer penetration by the peptide.

In chapter 2, at neutral pH, α-helical structure was detected in P6 in the presence of vesicles formed from DOPG but not those formed from DOPC and it was suggested that anionic lipids may be a requirement for the membrane anchoring of the parent protein PBP6. However, in chapter 4 anionic phospholipids were not found to be a requirement for the interactions of P6

with lipid monolayers and at neutral and high pH the peptide showed only low affinity for DOPC monolayers. This low affinity, taken with the fact that P6 is strongly hydrophobic and forms aggregates in solution suggested that in the CD analysis of chapter 2, the levels of interaction of P6 with DOPC vesicles may have been too low to be detected by the system. The generally high levels of interaction shown by P6 with lipid monolayers under acidic conditions (chapter 4) suggesed that α-helix stabilisation by low pH may be required for efficient monolayer interaction of the peptide. It was also speculated that the ability of the peptide to penetrate monolayers may be related to the nature and packing order of lipids forming the monolayer. Overall these results strongly support the view that C-terminal amphiphilic α-helices are involved in the membrane anchoring of PBP5 and PBP6.

In the case of PBP4, it is a matter of debate as to whether this is a soluble or membrane bound protein (section 1.6.3.5.2; Phoenix and Harris, 1995; Gittins *et al.* 1993). In this work, it was shown that in a wild type *E. coli*, PBP4 is exclusively membrane bound and it is suggested that PBP4 occupies a specific binding site with the soluble form of the protein being an artefact of over-expression (chapter 6). It has also been a matter of debate as to whether C-terminal α-helical structure features in the membrane binding of PBP4. In contrast to PBP5 and PBP6, theoretical analysis has predicted that the PBP4 C-terminal region may have a low potential for surface activity and membrane interaction (section 1.5.2; section 1.6.3.5; section 1.6.3.5.2; Roberts *et al.* 1997; Pewsey *et al.* 1996). These predictions appear to be confirmed since, P4, a homologue of the PBP4 C-terminal region, was shown to possess low surface activity (chapter 6), gave no evidence of haemolytic ability (chapter 3) and the levels of interaction of P4 with lipid monolayers (chapter 6) were generally low compared to those of P5 and P6 (chapter 4). However, although these same theoretical analyses have predicted that P4 has a only low potential for

amphiphilic α-helix formation, CD analysis showed that in the presence of SDS and TFE, P4 has the capacity for α-helix formation (chapter 2) and molecular areas determined from the interactions of P4 with DOPG monolayers implied the presence of α-helical structure in the peptide (chapter 6). In chapter 2, P4 appeared to show no evidence of α-helix formation in the presence of either DOPG or DOPC vesicles and this was interpreted to suggest that the PBP4 C-terminal region may either not form an α-helix or may interact with the membrane very weakly (chapter 2). However, in the case of DOPG vesicles, the apparent lack of interaction by P4 could be explained by the fact that the buffer used in the CD analysis of the peptide (chapter 2) contained NaCl which is later shown (chapter 6) to reduce the interaction of P4 with DOPG monolayers to very low levels. Furthermore, in the case of DOPC vesicles, monolayer studies (chapter 6) showed that although, in general, P4 interacts with lipid monolayers, the peptide showed no evidence of interaction with DOPC monolyers and had very low affinity for DPPC monolayers. Overall, these results suggest that amphiphilic C-terminal α-helical architecture could feature in PBP4 - membrane anchoring.

Biochemical wash experiments had not been previously performed on PBP4 but in chapter 6 the results of these experiments demonstrated that PBP4 - membrane binding proceeds *via* predominantly electrostatic forces with a minor hydrophobic contribution. The results of these experiments also showed that PBP4 - membrane binding demonstrates a pH dependent susceptibility to perturbants, which is enhanced by acid conditions. The interactions of P4 with lipid monolayers mirror these characteristics but whereas the membrane binding of PBP4 appeared to have no requirement for anionic lipid, the monolayer interactions of P4 do show such a requirement (chapter 6). This suggests that PBP4 - membrane binding may involve interactions other than that suggested at the protein's C-terminus. Overall, the results of this research have

suggested a model for PBP4 - membrane anchoring which involves electrostatic interactions between a cationic region on the polar face of the protein's putative amphiphilic C-terminal α-helical region and anionic lipid headgroups, supported by other interactions which could involve the PBP4 ectomembranous domain and / or other membrane based factors.

In summary, it appears that PBP4 has similarities to PBP5 and PBP6, in that it is a membrane bound protein, the binding of which could involve C-terminal amphiphilic α-helix formation. However, in contrast to PBP5 and PBP6, the membrane binding of PBP4 proceeds *via* primarily electrostatic forces as opposed to hydrophobic forces, and appears to occur at a specific binding site. Work in progress and work planned to investigate the membrane interactions of the low molecular mass PBPs include: a C-terminal deletion analysis of PBP4 - membrane binding, the use of 2D NMR to detect α-helical structure in the lipid interactions of P4, P5 and P6, the use of fluorescent probes to investigate the lipid interactions of P4, P5, P6 and their parent proteins, cross-linking studies and electron microscopy in conjunction with immunogold labeling to establish the membrane proximity of *E. coli* PBPs, which in turn, could implicate their involvement in a protein complex.

CHAPTER 8.

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8.1 REFERENCES

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CHAPTER 9.

PUBLICATIONS LIST

9.1. PUBLICATIONS LIST

9.1.1 Refereed Publications

Harris, F., Chatfield, L. and Phoenix, D. A. (1995) β-lactams and the future of antibiotics. Biologist **42**, 62-64.

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9.1.3 Conference proceedings

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