Penicillin-binding Proteins and the Future of β -Lactam Antibiotics

The Seventh Fleming Lecture

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INTRODUCTION

The development of β -lactam antibiotics has come a long way since the discovery of penicillin by Fleming in 1929 and of cephalosporins by Abraham and Newton in 1953. Over the last twenty years manipulation of the sidechains of the penicillin and cephalosporin nuclei has produced a wide range of potent antibiotics, and in recent years active β -lactam antibiotics with novel ring structures have been discovered or synthesized.

The β -lactams are likely to remain a most important group of antibiotics for the foreseeable future. The current generation of β -lactams are at present very effective in the treatment of a wide range of bacterial infections but bacteria resistant to their action will inevitably arise and reduce their efficacy. The constant need to develop new β -lactam antibiotics, and to understand and predict the mechanisms of resistance that may arise, provides a major reason for the continuing interest in the mechanism of action of these compounds.

In this article I review briefly the structure and function of penicillin-binding proteins and their role in intrinsic resistance to β -lactam antibiotics.

THE ROLE OF PENICILLIN-BINDING PROTEINS IN THE KILLING ACTION OF β -LACTAM ANTIBIOTICS

The early work on the mechanism of action of penicillin culminated in the discovery that it inhibited the final crosslinking of the peptide sidechains of nascent peptidoglycan and led to the suggestion that it acted as an analogue of acyl-D-alanyl-D-alanine (Tipper & Strominger, 1965). The transpeptidation reaction was soon achieved in crude cell envelope preparations of *Escherichia coli* and was shown to be inhibited by β -lactam antibiotics (Izaki *et al.*, 1966). In addition to the peptidoglycan transpeptidase activity, a second penicillin-sensitive reaction which removed the terminal D-alanine from the pentapeptide sidechains of peptidoglycan precursors was detected and called D-alanine carboxypeptidase (Izaki *et al.*, 1966).

By the mid 1970s it was clear that bacteria possessed multiple D-alanine carboxypeptidases and peptidoglycan transpeptidases, but the precise role of these enzymes in cell growth and the mechanism of action of penicillin was unknown (Blumberg & Strominger, 1974). The demonstration that penicillin bound covalently to the enzymes that it inhibited (see Blumberg & Strominger, 1974) provided a method for the detection of penicillin-sensitive enzymes as penicillin-binding proteins (PBPs), and while working in Art Pardee's laboratory I developed a convenient autoradiographic method for the detection and study of PBPs (Spratt & Pardee, 1975). This PBP assay proved to be extremely useful as it allowed the study of all of the penicillin-sensitive enzymes of bacterial cells regardless of which reactions of peptidoglycan synthesis they catalysed, and opened the way to the identification of the killing targets for β -lactam antibiotics (Spratt, 1975).

Table 1. *Properties of the penicillin-binding proteins of Escherichia coli*

PBP	Mol. wt	Consequence of inactivation of PBP	Enzymic activity of PBP
1A 1B	92000 90000	Rapid cell lysis occurs if both PBP 1A and PBP 1B are inactivated	Both PBP 1A and PBP 1B are transglycosylase/transpeptidases involved in cell elongation
2	66000	Growth as spherical cells	A transpeptidase (and possibly also a transglycosylase) that may initiate peptidoglycan insertion at new growth sites
3	60000	Inhibition of cell division leading to filamentous growth	A transglycosylase/transpeptidase required specifically for formation of the cross wall at cell division
4	49000	No obvious growth defect	A D-alanine carboxypeptidase that may function <i>in vivo</i> as a secondary transpeptidase in the maturation of peptidoglycan
5	42000	No obvious growth defect	A D-alanine carboxypeptidase
6	40000	No obvious growth defect	A D-alanine carboxypeptidase

Escherichia coli contains seven PBPs in the cytoplasmic membrane and each of these corresponds to a distinct penicillin-sensitive enzyme (see below). The role of the PBPs of *E. coli* in growth, morphogenesis, and the killing action of β -lactam antibiotics has been elucidated in two main ways (Spratt, 1980*a*). Firstly, β -lactam antibiotics produce at least three morphological effects on *E. coli* (filamentation, rapid cell lysis, and growth into spherical cells) and the degree to which β -lactams produce these responses has been correlated with their binding to particular PBPs (Spratt & Pardee, 1975; Spratt, 1975). Secondly, the consequences of the inactivation of each of the PBPs has been determined by the isolation of mutants that lack or produce thermolabile forms of these proteins (Spratt, 1975, 1977*b*; Spratt *et al.*, 1977; Suzuki *et al.*, 1978; Spratt, 1980*b*; Broome-Smith & Spratt, 1982).

Inactivation of PBP 3, either with a β -lactam antibiotic, or by transferring a mutant that produces a thermolabile form of PBP 3 to the restrictive temperature, results in the inhibition of cell division and the growth of *E. coli* into long filamentous cells, and eventual cell death (Spratt, 1975, 1977*c*). In a similar way the inactivation of PBP 2 has been shown to result in the growth of *E. coli* as spherical cells and leads to eventual cell lysis, at least in media which afford rapid growth (Spratt, 1975, 1978).

Rapid lysis of *E. coli* occurs when both PBPs 1A and 1B are inhibited (Spratt, 1975, 1977*a*; Tamaki *et al.*, 1977; Suzuki *et al.*, 1978). Mutants that lack detectable activity of PBP 1A or PBP 1B are viable, but a double mutant that lacks PBP 1B and produces a thermolabile PBP 1A is temperature-sensitive and lyses when shifted to 42 °C. PBP 1A has a much higher affinity for β -lactam antibiotics than PBP 1B, and the increased sensitivity of PBP 1B mutants to β -lactams has been suggested to be due to their dependence on PBP 1A activity for cell growth. It appears that the activity of either PBP 1A or PBP 1B is sufficient for cell elongation and the two enzymes may have similar roles in peptidoglycan synthesis. This conclusion is complicated by the report of a temperature-sensitive mutant that lacks only PBP 1B and lyses at the restrictive temperature (Tamaki *et al.*, 1977). It may be that the complete lack of PBP 1B is lethal and that most of the PBP 1B mutants survive because they retain residual activity *in vitro*. Attempts to isolate mutants with deletions of the PBP 1A or PBP 1B gene should clarify the role of these PBPs in the rapid cell lysis produced by β -lactam antibiotics.

Mutants that lack detectable levels of PBP 4, PBP 5, or PBP 6 are viable and do not show significant morphological abnormalities. This implies that the latter PBPs are non-essential at least under laboratory conditions. The isolation of strains with deletions of the PBP 5 and PBP 6 gene has proved that these PBPs are non-essential (Spratt, 1980*b*; Broome-Smith & Spratt, 1982), but only point mutants of PBP 4 have been described and the effect of the total lack of this protein has not been established. Similarly the effect of the combined loss of PBPs 4, 5 and 6 is unknown. The properties of the *E. coli* PBPs are summarized in Table 1.

The study of mutants shows that inactivation of PBP 1A/1B, or PBP 2, or PBP 3, is sufficient

for the death of *E. coli*, and β -lactam antibiotics exist that kill by each of these three routes. The β -lactams can therefore kill by three completely different mechanisms (rapid lysis, production of spherical cells, or filamentation) and effective derivatives are known that kill at their minimal inhibitory concentration (MIC) exclusively by rapid lysis (e.g. cefsulodine), by production of spherical cells (mecillinam), or by filamentation (cephalexin). More typical β -lactams (e.g. ampicillin or cephaloridine), at the concentrations achieved in serum, kill by two or all three of these mechanisms (Spratt, 1980*a*; Curtis *et al.*, 1979).

THE ENZYMIC ACTIVITY OF PENICILLIN-BINDING PROTEINS

All seven PBP genes of *E. coli* have been cloned and strains are available that overproduce each of the PBPs (Spratt, 1980*a*; Spratt *et al.*, 1980; Takeda *et al.*, 1981). These strains have aided the purification of PBPs and enzymic activity has now been attributed to each of the seven PBPs of *E. coli* (Matsuhashi *et al.*, 1982). The PBPs can be conveniently considered as two groups: the high molecular weight essential PBPs that are the killing targets for β -lactam antibiotics, and the lower molecular weight PBPs that appear to play a less important role in peptidoglycan synthesis.

Considerable difficulties have been encountered in the detection of the enzymic activity of purified high molecular weight PBPs. In several cases these PBPs have been enzymically active as demonstrated by their ability to bind penicillin but have not shown any activity with various peptidoglycan substrates. The most progress has been made with PBP 1B of *E. coli*. The properties of PBP 1B suggested that it was the major peptidoglycan transpeptidase involved in the incorporation of nascent peptidoglycan into the pre-existing cell wall (Spratt, 1975; Spratt *et al.*, 1977), and this was confirmed by the demonstration that the synthesis of crosslinked peptidoglycan in cell envelopes prepared from mutants lacking PBP 1B activity was very low compared to that in cell envelopes from the parent bacteria (Tamaki *et al.*, 1977; Suzuki *et al.*, 1978). Two groups have recently demonstrated that purified PBP 1B catalyses the formation of crosslinked peptidoglycan from lipid-linked disaccharide pentapeptide precursor (Nakagawa *et al.*, 1979; Suzuki *et al.*, 1980). PBP 1B therefore catalyses both the polymerization of the disaccharide units into glycan chains and also the crosslinking of their pentapeptide sidechains, i.e. the enzyme is a bifunctional peptidoglycan transglycosylase/transpeptidase. In the presence of β -lactam antibiotics PBP 1B synthesizes uncrosslinked peptidoglycan, indicating that the transpeptidation, but not the transglycosylation, is penicillin-sensitive. The demonstration that PBP 1B catalyses both the synthesis and crosslinking of peptidoglycan is clearly consistent with the finding that both of these activities are very low in cell envelopes prepared from PBP 1B mutants.

The presence of transglycosylase and transpeptidase activities on the same polypeptide has not yet been rigorously proved but this idea has recently received strong support from studies with *Bacillus megaterium* where a major peptidoglycan transglycosylase has been purified and subsequently shown to be a PBP (Taku *et al.*, 1982). As the synthesis and crosslinking of peptidoglycan needs strict topological control it makes sense to combine the transglycosylase and transpeptidase activities in a single enzyme.

As cell envelopes from PBP 1B mutants show very low levels of peptidoglycan synthesis it appears that the other PBPs contribute little to this activity under these conditions. When purified, however, both PBP 1A and PBP 3 have been reported to be bifunctional transglycosylase/transpeptidases but the conditions required to obtain activity with these PBPs apparently differ from those required for PBP 1B activity (Ishino *et al.*, 1980; Ishino & Matsuhashi, 1981). Recently we have obtained preliminary evidence that PBP 2 is a peptidoglycan transpeptidase although it is not yet known if it also acts as a transglycosylase (Ishino *et al.*, 1982). With each of the above PBPs the crosslinking activity is inhibited by β -lactam antibiotics at concentrations that correspond well with those expected from their penicillin-binding activity in cell envelopes. For example, the crosslinking activity of purified PBP 1B is inhibited by low concentrations of benzylpenicillin, which binds with high affinity to PBP 1B, but is only inhibited by very high concentrations of cephalexin, which has low affinity for this

PBP (Matsushashi *et al.*, 1982). Similarly, the crosslinking activity attributed to PBP 2 is inhibited by low concentrations of mecillinam, which is known to bind exclusively to PBP 2 (Ishino *et al.*, 1982).

At present the activity of purified high molecular weight PBPs is feeble [e.g. < 1 mol product (mol enzyme) $^{-1}$ min $^{-1}$]. Presumably this is because the conditions *in vitro* are very different from those *in vivo* where the enzymes may be part of a multienzyme complex within the cytoplasmic membrane. In addition, the substrate used for the *in vitro* assays of the purified high molecular weight PBPs may be rather different from the normal substrates used *in vivo*.

PBP 1A, PBP 1B, PBP 3, and perhaps PBP 2, therefore appear to be bifunctional peptidoglycan transglycosylase/transpeptidases and these PBPs exhibit little or no ability to act as D-alanine carboxypeptidases (Matsushashi *et al.*, 1982). Presumably each of these enzymes has a specific function in the synthesis and insertion of peptidoglycan into the cell wall during the cell cycle. Thus PBP 1B (and PBP 1A?) may be used for peptidoglycan synthesis for cell elongation, PBP 3 for the synthesis of the septum at cell division, and PBP 2 for the initiation of synthesis at new sites of cell wall growth.

The low molecular weight PBPs 4, 5 and 6 of *E. coli* have all been purified on the basis of their ability to catalyse the D-alanine carboxypeptidase reaction, although this does not imply that they necessarily do this *in vivo*, as under the appropriate conditions these PBPs will also catalyse a model transpeptidase reaction, and PBP 4 will act as an endopeptidase hydrolysing the peptide crosslinks in peptidoglycan (Tamura *et al.*, 1976; Spratt & Strominger, 1976; Amanuma & Strominger, 1980; Spratt, 1980a).

In vitro, and probably also *in vivo*, PBPs 5 and 6 catalyse the D-alanine carboxypeptidase reaction. This reaction has been suggested to control the extent of peptidoglycan crosslinking by removing the terminal D-alanine residues from the pentapeptide sidechains of nascent peptidoglycan and thereby preventing them from acting as peptide donors in transpeptidation (Izaki *et al.*, 1966). However, there is no evidence that these (or any) D-alanine carboxypeptidases regulate the level of crosslinking and some experiments argue against the idea. For example, increasing or decreasing the level of PBP 5 produces effects on the level of pentapeptide sidechains in nascent peptidoglycan that are consistent with this PBP acting as a D-alanine carboxypeptidase *in vivo*, but produces no significant effect on the extent of crosslinking in the mature peptidoglycan (De Pedro *et al.*, 1980; Markiewicz *et al.*, 1982). The role of D-alanine carboxypeptidases in bacterial cell wall synthesis therefore remains unclear.

Strains that have deletions of either the PBP 5 or the PBP 6 gene have been constructed recently and this has established that the complete absence of PBP 5 or PBP 6 has no marked effect on cell morphology (Spratt, 1980b; Broome-Smith & Spratt, 1982). It remains possible that these PBPs have very similar functions, and that the loss of one of them is compensated for by the presence of the other. The construction of strains that completely lack both PBP 5 and PBP 6 will establish whether the loss of the two most abundant PBPs of *E. coli* is tolerated.

De Pedro and Schwarz (1981) have suggested that peptidoglycan precursors are polymerized and inserted into the pre-existing cell wall by the high molecular weight transglycosylase/transpeptidases, and that further secondary transpeptidation then occurs to produce the final level of crosslinking characteristic of the mature cell wall. The secondary crosslinking is proposed to be the function of PBP 4, as mutants that lack the activity of this enzyme are unable to carry out the second phase of crosslinking, and have a low level of crosslinking in their peptidoglycan. Apparently secondary transpeptidation is not vital for the survival of the cell under laboratory conditions, or for the strength of the peptidoglycan, as the mutants that lack detectable levels of PBP 4 show no obvious morphological defects. The available PBP 4 mutants may still retain some activity *in vivo* and a rigorous demonstration that this enzyme is non-essential requires the construction of a strain with a deletion of the PBP 4 gene. The lowest molecular weight PBP of *Staphylococcus aureus* (PBP 4) also appears to be a secondary transpeptidase as its inactivation, by mutation or with β -lactam antibiotics, results in a substantial reduction in peptidoglycan crosslinking (Wyke *et al.*, 1981). This organism also tolerates the loss of the secondary transpeptidase and the resulting reduction in the extent of peptidoglycan crosslinking.

The low molecular weight PBPs of *E. coli* and probably of other bacteria (e.g. PBP 5 of

B. subtilis, PBP 4 of *S. aureus* and PBP 3 of *Neisseria gonorrhoeae*) are therefore separated by several criteria from the high molecular weight PBPs. The latter PBPs are responsible for the synthesis and initial crosslinking of glycan chains to pre-existing peptidoglycan, are essential for the normal growth of the bacteria, and are the killing targets for β -lactam antibiotics. The low molecular weight PBPs are apparently dispensable (at least under laboratory conditions) and function in the maturation of peptidoglycan.

THE STRUCTURE OF PENICILLIN-BINDING PROTEINS

The determination of the structure of PBPs is of interest for several reasons. One of the main reasons is to understand the molecular details of the interaction of penicillin with those PBPs that are the killing targets for β -lactam antibiotics. As this knowledge will come from X-ray crystallographic studies, it is important to know how similar PBPs are to each other, and therefore whether the solution of the structure of one PBP will allow a rapid solution to the structure of other PBPs. At first sight PBPs seem to form a heterogeneous collection of proteins. Some are D-alanine carboxypeptidases whereas others are peptidoglycan transglycosylase/transpeptidases. There are large differences in molecular weight between PBPs and probably in cell location, e.g. some may be integral membrane proteins, whereas others may be peripheral membrane proteins, or exocellular. Only the determination of the amino acid sequence of PBPs can provide a rapid answer to the relationships between these proteins.

Another interest in the structure of PBPs relates to their similarity with β -lactamases. The suggestion that β -lactamases may have evolved from penicillin-sensitive enzymes involved in bacterial cell wall synthesis has often been made (e.g. Tipper & Strominger, 1965). Certainly several similarities exist between the two classes of enzymes. Of particular relevance is the fact that several β -lactamases and PBPs have been shown to use an acyl-enzyme mechanism in which the penicilloyl moiety, and also substrate in the case of PBPs, is attached (transiently in β -lactamases and rather stably in PBPs) to an active site serine residue (Frère *et al.*, 1976; Knott-Hunziker *et al.*, 1979; Waxman & Strominger, 1980). With some PBPs (e.g. PBP 4 of *S. aureus* and PBPs 5 and 6 of *E. coli*) the penicilloyl-PBP complex breaks down at an appreciable rate to regenerate the active PBP and produce penicilloic acid; these PBPs therefore catalyse a very weak β -lactamase reaction (Spratt, 1980*a*).

The binding of penicillin to a serine residue has so far only been shown for the low molecular weight PBPs that are believed to act *in vivo* as D-alanine carboxypeptidases, and for these enzymes there is strong evidence that the same serine residue is acylated by substrate in the normal enzyme mechanism, in accord with the substrate analogue model for the mechanism of action of penicillin proposed by Tipper & Strominger (1965). The involvement of an active site serine, and the validity of the substrate analogue hypothesis, is still not proven for the high molecular weight PBPs which are the killing targets for penicillin.

If PBPs and β -lactamases have a common evolutionary history, this should emerge from a comparison of their amino acid sequences. At present a small amount of NH₂-terminal protein sequence is available for PBPs 5 and 6 of *E. coli* and for PBP 5 of *B. subtilis* and *B. stearothermophilus* (Waxman & Strominger, 1980; Waxman *et al.*, 1982) and we have recently obtained the complete sequence of *E. coli* PBP 5 from the nucleotide sequence of the *dacA* gene (J. K. Broome-Smith, A. Edelman & B. G. Spratt, unpublished). Figure 1 shows the alignment of the NH₂-terminal regions of these D-alanine carboxypeptidases to illustrate their sequence similarities. All four sequences can be aligned without the introduction of gaps and, although the data are still fragmentary, there is little doubt that these low molecular weight PBPs form a homologous group. For example, PBP 5 of *E. coli* and PBP 5 of *B. subtilis* have identical amino acids in 25 out of the 65 amino acids for which sequence data are available for both proteins (39% identity).

Serine-36 has been shown to be the residue acylated by both penicillin and substrate in PBP 5 of *B. subtilis* and *B. stearothermophilus* (Waxman & Strominger, 1980) and the extent of homology in this region allows the assignment of serine-44 as the corresponding active site serine in PBP 5 of *E. coli*.

Waxman & Strominger (1980) have pointed out sequence similarities between the NH₂-terminal regions of low molecular weight PBPs and class A β -lactamases (the latter are a homologous group of 'serine enzymes' and include the TEM, *S. aureus*, and *B. licheniformis* enzymes and the type I enzyme from *B. cereus*; Ambler, 1980). An alignment of the NH₂-terminal sequence of the TEM-1 β -lactamase with the available sequences of low molecular weight PBPs is shown in Fig. 1. Within the NH₂-terminal 65 amino acids there is 23% identity between TEM-1 β -lactamase and PBP 5 of both *E. coli* and *B. subtilis*. At several positions identical, or structurally similar, amino acids are present in all of the low molecular weight PBPs and class A β -lactamases that have been examined (Fig. 1). The secondary structure of this region of PBPs and β -lactamases has also been predicted to be similar (Moews *et al.*, 1981). Maximum similarity between the D-alanine carboxypeptidases and class A β -lactamases occurs immediately to the NH₂-terminal side of the active site serine and the similarity decreases rapidly on the COOH-terminal side of this residue. Comparison of the complete amino acid sequence of PBP 5 of *E. coli* with the available sequences of class A β -lactamases shows that the sequence similarities are restricted to the NH₂-terminal region and no further significant similarity is found outside the region shown in Fig. 1 (J. K. Broome-Smith, A. Edelman & B. G. Spratt, unpublished).

Although the sequence similarities between the NH₂-terminal regions of the D-alanine carboxypeptidases and the class A β -lactamases are fairly convincing, the two classes of proteins are clearly not closely related. Presumably, if these proteins have evolved from a common ancestor, then the divergence in their cellular functions (and cellular location) has been paralleled by extensive divergence in their primary sequences, and the existing similarity around the active site serine residue may reflect the conservation of a region involved in the recognition of penicillin. The situation is complicated by the fact that other classes of β -lactamases and PBPs exist. For example, the sequence of the chromosomal β -lactamase of *E. coli* (a class C β -lactamase) shows little similarity to either the class A β -lactamases (Jaurin & Grundstrom, 1981) or to the available sequences of PBPs.

Rather little is known about the organization of PBPs within the cytoplasmic membrane. In *B. stearothermophilus* (and *B. subtilis*) the major D-alanine carboxypeptidase (PBP 5) appears to be an ectoprotein inserted into the cytoplasmic membrane by a COOH-terminal hydrophobic membrane anchoring peptide (Waxman & Strominger, 1979). Removal of the COOH-terminal region with trypsin results in an enzymically active water-soluble PBP. The anchoring of this enzyme by a COOH-terminal peptide presumably allows the bulk of the protein to extend away from the membrane towards its peptidoglycan substrate. This may be particularly important for D-alanine carboxypeptidases and secondary transpeptidases that have to act on the remaining pentapeptide sidechains of peptidoglycan that have already been linked to the pre-existing cell wall and are some distance from the cytoplasmic membrane. PBP 5 of *E. coli* may be inserted in a different way to PBP 5 of *Bacillus* as there is no strongly hydrophobic COOH-terminal region (J. K. Broome-Smith, A. Edelman & B. G. Spratt, unpublished) and enzymically active fragments produced by trypsin treatment remain water insoluble (W. Keck, personal communication). The high molecular weight PBPs also presumably have substantial amounts of their polypeptide exposed on the outer surface of the cytoplasmic membrane where they catalyse the synthesis and crosslinking of peptidoglycan.

In eukaryotes, cytoplasmic membrane proteins that have the bulk of their polypeptides on the exterior of the cytoplasmic membrane are synthesized, by a similar mechanism to secreted proteins, as preproteins with NH₂-terminal hydrophobic signal peptides. At least some PBPs might therefore be expected to be synthesized as preproteins and this has been shown for PBPs 5 and 6 of *E. coli* (Pratt *et al.*, 1981). In the case of PBP 5 the signal peptide is 29 amino acids long and has all of the features characteristic of the signal peptides of *E. coli* periplasmic and outer membrane proteins (J. K. Broome-Smith, A. Edelman & B. G. Spratt, unpublished). PBP 5 of *B. subtilis* and PBP 5 of *B. stearothermophilus* are also probably made as preproteins since the NH₂-terminal sequence of the mature proteins shows no hydrophobic signal-like region that could direct their transfer across the cytoplasmic membrane.

The structures of the high molecular weight PBPs that are the killing targets for β -lactam

(a) <i>E. coli</i> PBP 6	NH ₂ -AEQTVEAPSVDSASWFLMDYXGKV----
	* ** * ** * ** *
<i>E. coli</i> PBP 5	NH ₂ -DDLNIKTMPGVQIDAESYILIDYNSGKVLAEQNADVRRDPASLT
	* ** * ** * ** *
<i>B. subtilis</i> PBP 5	NH ₂ -ASDPIDINASAAIMIEASSGKILYSKNADKRLPIASMTKMMTEYLLLEAIDQGVKWDQTYTTPD----
	* ** * ** * ** *
<i>B. stearothermophilus</i> PBP 5	NH ₂ -ESAPLDIRADAAIILVDAQTGKILYEKNIDTVLGIASMTKM----
	* ** * ** * ** *
(b) <i>E. coli</i> PBP 5	NH ₂ -DDLNIKTMPGVQIDAESYILIDYNSGKVLAEQNADVRRDPASLT
	* ** * ** * ** *
TEM β -lactamase	NH ₂ -HPETLVKVKDAEDQLGARVGYIELDLNSGKILLESFRPEERFPMSTFKVLLCGAVLSRVDAGQEQLGRRIHYS----
	* ** * ** * ** *
<i>B. subtilis</i> PBP 5	NH ₂ -ASDPIDINASAAIMIEASSGKILYSKNADKRLPIASMTKMMTEYLLLEAIDQGVKWDQTYTTPD----
	* ** * ** * ** *

Fig. 1. (a) Alignment of the available NH₂-terminal regions of D-alanine carboxypeptidases. The sequences of PBP 5 of *B. subtilis* and *B. stearothermophilus* and PBP 6 of *E. coli* are from Waxman & Strominger (1980) and Waxman *et al.* (1982), and the sequence of PBP 5 of *E. coli* is from unpublished data of J. K. Broome-Smith, A. Edelman & B. G. Spratt. The alignment of the *Bacillus* and *E. coli* sequences differs considerably from that given by Waxman *et al.* (1982). Identical residues are marked with an asterisk and the position of the active site serine with ●. The one-letter notation of amino acids is used.

(b) Alignment of the NH₂-terminal regions of PBPs 5 of *E. coli* and *B. subtilis* with that of TEM β -lactamase (Ambler, 1980). Identical residues and the position of the active site serine are marked as above.

Fig. 2. A possible alignment of a bifunctional peptidoglycan transglycosylase/transpeptidase with a D-alanine carboxypeptidase. The solid blocks represent a region of similarity between the sequences of PBP 3 (a transglycosylase/transpeptidase; *M_r* 60000) and PBP 5 (a D-alanine carboxypeptidase; *M_r* 42000) of *E. coli*. The sequence of this region and that of the corresponding region of *B. stearothermophilus* are shown at the bottom of the figure. Identical amino acids are marked with an asterisk. The one-letter notation of amino acids is used.

antibiotics are of particular interest. Recently the nucleotide sequence of the PBP 3 gene of *E. coli* has been reported (Maruyama *et al.*, 1982). Comparison of the amino acid sequence of this bifunctional transglycosylase/transpeptidase with that of PBP 5 shows little similarity except in one small region (Fig. 2). Interestingly, the region in PBP 5 that shows similarity with PBP 3 is the same region that shows maximum similarity in the comparisons of D-alanine carboxypeptidases and β -lactamases and may be part of the region that recognizes penicillin. Whereas this sequence occurs close to the NH₂-terminus of both D-alanine carboxypeptidases and β -lactamases, it occurs towards the middle of PBP 3. This raises the possibility that the larger bifunctional peptidoglycan transglycosylase/transpeptidases may have an NH₂-terminal domain catalysing the transglycosylase reaction and a COOH-terminal domain carrying the penicillin-sensitive transpeptidase activity as shown in Fig. 2. Sequencing of further PBP genes should rapidly provide the additional data that are required to clarify the relationship between D-alanine carboxypeptidases, peptidoglycan transglycosylase/transpeptidases, and β -lactamases.

Eventually a detailed knowledge of the interaction of penicillin with penicillin-sensitive enzymes and with β -lactamases will come from a knowledge of their three-dimensional structure. Recently the structure of an exocellular D-alanine carboxypeptidase from *Streptomyces* R61 has been determined to a resolution of 2.8 Å and the binding of β -lactam antibiotics to the enzyme has been visualized (Kelly *et al.*, 1982). Although the *Streptomyces* R61 enzyme has been shown to be a 'serine enzyme', the amino acid sequence is not yet available, and its similarity to other PBPs, and its *in vivo* function, are unknown (Ghuysen *et al.*, 1980).

The available data suggest that the D-alanine carboxypeptidases and the essential peptidoglycan transglycosylase/transpeptidases will show little sequence similarity although, of course, some similarity may be apparent in their three-dimensional structures. Also the multiple transglycosylase/transpeptidases must differ considerably in structure as they vary markedly in size (e.g. in *E. coli* from M_r 92000 for PBP 1A to M_r 60000 for PBP 3). Thus, although the elegant crystallographic studies of the *Streptomyces* R61 enzyme have shown the feasibility of using X-ray studies to observe the interaction of β -lactam antibiotics with PBPs at high resolution, the likely differences between the structure of this enzyme and the essential transglycosylase/transpeptidases indicate the need for similar studies on the latter enzymes. Until recently the low abundance of these enzymes and their membrane location has made such studies impractical but the availability of the high molecular weight PBPs of *E. coli* in substantial amounts through gene cloning, and progress in the crystallization of membrane proteins, may make the determination of the three-dimensional structure of these killing targets feasible in the not too distant future.

X-ray crystallographic studies of several β -lactamases are in progress and a medium resolution structure of the TEM β -lactamase has been reported (DeLucia *et al.*, 1980). Solution of the three-dimensional structure of the essential PBPs and of β -lactamases should allow the rational design of novel β -lactam antibiotics, or other inhibitors, that inactivate essential PBPs without being recognized by β -lactamases, or which inactivate both groups of enzymes at low concentration.

THE ROLE OF PENICILLIN-BINDING PROTEINS IN INTRINSIC RESISTANCE TO β -LACTAM ANTIBIOTICS

In the majority of bacteria by far the most common and effective mechanism of resistance to β -lactam antibiotics is the production of a β -lactamase that destroys the antibiotic. In some cases, however, resistance is not due to production of β -lactamase and has been called intrinsic resistance.

In Gram-negative bacteria intrinsic resistance is often due to poor penetration of the antibiotic through the outer membrane to its targets in the cytoplasmic membrane. This mechanism of resistance has been shown in clinical isolates of *Neisseria gonorrhoeae* that have low level resistance to benzylpenicillin (although PBP changes are also involved; see below). Similarly, in the laboratory, and also clinically, *E. coli* can obtain resistance to β -lactam antibiotics by mutations that result in decreased penetration through the outer membrane due to loss of the *ompF* porin. Apart from the intrinsic resistance of atypical isolates of a normally

susceptible species there are examples where all isolates of a bacterial species show intrinsic resistance. For example, *Pseudomonas aeruginosa* is highly resistant to most β -lactam antibiotics. The inducible chromosomal β -lactamase of this organism is a contributory factor in the resistance, but it is not the only factor, as the organism is also resistant to β -lactams that are not hydrolysed by the β -lactamase, and mutants that produce only very low levels of the β -lactamase are still resistant to β -lactam antibiotics. Recent studies have shown that the intrinsic resistance of *P. aeruginosa* is due to the extremely poor penetration of most β -lactams through the outer membrane rather than to an insensitivity of the PBPs for antibiotic. Mutants that allow unhindered penetration of β -lactams through the outer membrane show greatly increased sensitivity to β -lactam antibiotics, reflecting the high affinity of the PBPs for the antibiotics (Zimmermann, 1980).

In Gram-positive bacteria β -lactam antibiotics have only to penetrate the thick peptidoglycan layer to reach the PBPs and, as this layer does not appear to be a significant barrier to molecules of the size of β -lactam antibiotics, intrinsic resistance due to altered penetration is unknown in these organisms.

The second major cause of intrinsic resistance to β -lactam antibiotics is the production of PBPs that have decreased affinity for antibiotic. This method of resistance was first shown clearly in laboratory mutants of *E. coli* that had increased resistance to mecillinam due to the decreased affinity of PBP 2 for antibiotic (Spratt, 1978). Mutants of this type are easy to obtain as mecillinam kills *E. coli* exclusively by inactivation of PBP 2. As expected these mutants show cross-resistance to other β -lactams that kill by inactivation of PBP 2 but show no cross-resistance to β -lactams that kill by binding to PBPs 1A/1B or PBP 3. Resistance by alteration of PBP 3 can also be obtained in the laboratory by selecting for low level resistance to β -lactam antibiotics that preferentially bind to PBP 3 and therefore kill by inducing filamentation (Spratt, 1979).

I have suggested that high level resistance to β -lactam antibiotics through alteration of the affinity of PBPs would be unlikely to occur with most β -lactams (Spratt, 1978). Firstly, typical β -lactam antibiotics at concentrations above their MIC kill by inactivating more than one essential PBP and high level resistance would therefore require a decrease in the affinity of each of these essential PBPs. Secondly, penicillin is proposed to be a structural analogue of the acyl-D-alanyl-D-alanine part of the substrate of PBPs. Mutants that drastically reduce the affinity of a PBP for penicillin might in many cases also greatly reduce its affinity for substrate and prevent it from performing its essential function in peptidoglycan synthesis. In practice neither of these theoretical reasons has precluded the appearance of intrinsic resistance to β -lactam antibiotics through PBP changes. It now appears that although the presence of multiple killing targets may greatly reduce the rate of appearance of intrinsic resistance through target changes, it does not prevent it (see below). Also, altered PBPs with very considerable decreases in their affinity for β -lactam antibiotics have been found in clinical isolates, and it may be that although β -lactams and substrate bind to the same site on PBPs the amino acid residues involved in the binding of antibiotic and substrate differ, so that amino acid substitutions can occur which reduce binding of the antibiotic but do not hinder binding of the substrate.

One of the most interesting areas of research on PBPs in recent years has been the demonstration of PBP alterations in clinical isolates that show intrinsic resistance to β -lactam antibiotics. *Staphylococcus aureus* provides a good example. In the 1940s the vast majority of isolates of *S. aureus* were extremely sensitive to penicillin. The few isolates that were resistant produced β -lactamase and soon after the introduction of penicillin these strains became prevalent. The problem caused by β -lactamase-producing *S. aureus* was solved by the introduction of methicillin, which was resistant to hydrolysis by the plasmid-mediated β -lactamase of *S. aureus*. After the introduction of methicillin for the treatment of *S. aureus* infections there appeared reports of isolates that were highly resistant to methicillin. Although in many cases these resistant strains produced the *S. aureus* β -lactamase the curing of the β -lactamase plasmid did not reduce the level of resistance to methicillin. These strains are therefore intrinsically resistant to methicillin and are a cause for concern as they are cross-resistant to other β -lactam antibiotics. Isolates have appeared recently that are also resistant to

additional groups of antibiotics, providing very few options for effective antibiotic therapy.

Several reports of PBP changes in methicillin-resistant strains of *S. aureus* have appeared (e.g. Brown & Reynolds, 1980; Hayes *et al.*, 1981). The most striking of these is the study of strain MR1 (Hayes *et al.*, 1981). This strain is resistant to $>1000 \mu\text{g}$ methicillin ml^{-1} and is cross-resistant to other β -lactams. PBPs 1, 2 and 4 of strain MR1 appear identical to those in sensitive strains but the affinity of PBP 3 for all β -lactams that have been examined is extremely low compared to that in a sensitive strain. Apparently in this methicillin-resistant strain a very high level of resistance to β -lactam antibiotics has been achieved by the production of an altered PBP 3 with extremely low affinity for β -lactams. This implies that, at least in this strain, PBP 3 is the only essential PBP, as only this PBP will be enzymically active when strain MR1 is growing in the presence of high levels of methicillin.

Staphylococcus aureus MR1 is in some ways an atypical methicillin-resistant strain since it fails to show the heterogeneity of expression of resistance that is characteristic of most isolates. However, the decreased affinity of PBP 3 has also been found in more typical methicillin-resistant isolates (Brown & Reynolds, 1980).

How do methicillin-resistant strains of *S. aureus* arise? Laboratory studies on the development of resistance to benzylpenicillin in *S. aureus* have shown that high level resistance cannot be achieved in a single step but can only be obtained by repeated selection for small increments in resistance (Demerec, 1945). Presumably the multiple mutational events that are required to obtain high levels of resistance to β -lactam antibiotics involve a series of amino acid substitutions around the active site region of PBP 3 that result in increasing loss of affinity of the enzyme for β -lactam antibiotics, although additional mutations that allow the organism to survive with only one enzymically active PBP may also be involved. Genetic studies of methicillin resistance have shown that it can be transferred to a sensitive strain by transformation, indicating that if multiple mutations have occurred in the development of resistance, then all of the mutations are closely linked, and these studies are consistent with the view that methicillin resistance is due to multiple mutations in the PBP 3 gene resulting in a PBP 3 with multiple amino acid substitutions that affect its affinity for β -lactam antibiotics.

Neisseria gonorrhoeae provides another good example of intrinsic resistance due to alteration of the affinity of PBPs for β -lactam antibiotics (Dougherty *et al.*, 1980; Barbour, 1981). When penicillin was introduced all isolates of *N. gonorrhoeae* were extremely sensitive to benzylpenicillin ($\text{MIC} < 0.01 \mu\text{g ml}^{-1}$). Sensitivity is due to the high affinity of the PBPs for antibiotic, good penetration of antibiotic across the outer membrane, and lack of β -lactamase activity. Leaving aside the highly resistant β -lactamase-producing strains that have appeared recently, there are clinical isolates that show low level resistance to benzylpenicillin (e.g. $\text{MIC } 0.5\text{--}8 \mu\text{g ml}^{-1}$). Over the years the level of resistance of these strains to benzylpenicillin has increased. At present they can still be treated successfully with β -lactam antibiotics but if the level continues to increase this may no longer be possible.

Several unlinked mutations have been identified in intrinsically resistant strains of *N. gonorrhoeae*. Some of these involve alteration of the permeability of the outer membrane whereas others involve the affinity of PBPs. Cytoplasmic membranes of *N. gonorrhoeae* contain three PBPs (Dougherty *et al.*, 1980; Barbour, 1981). Those isolates that show the highest levels of intrinsic resistance possess a normal PBP 3 but produce altered forms of PBP 1 and PBP 2 that have decreased affinity for benzylpenicillin compared to their counterparts in sensitive strains (Dougherty *et al.*, 1980). Genetic studies have allowed a dissection of the mutational events that have led to the development of intrinsic resistance (Dougherty *et al.*, 1980). Incubation of the sensitive strain FA19 ($\text{MIC } 0.007 \mu\text{g ml}^{-1}$) with DNA from the resistant strain CDC77 ($\text{MIC } 2 \mu\text{g ml}^{-1}$) produced transformants that were resistant to low levels of benzylpenicillin ($\text{MIC } 0.06 \mu\text{g ml}^{-1}$). The low level of resistance of these transformants was accompanied by the appearance of the altered PBP 2 with decreased affinity for antibiotic. The low level resistant strain was then further transformed with DNA from strain CDC77 to produce a strain with a higher level of resistance ($0.12 \mu\text{g ml}^{-1}$) and the latter strain was transformed again to produce a strain with an MIC of $0.5 \mu\text{g ml}^{-1}$. Neither of the latter strains had any further PBP alterations

and their increased resistance was probably due to the acquisition from strain CDC77 of mutations that affect outer membrane permeability. Transformation to higher levels of resistance with DNA from strain CDC77 has not so far been achieved but would presumably involve the appearance of an altered PBP 1 with decreased affinity for benzylpenicillin (Dougherty *et al.*, 1980).

PBPs 1 and 2 of *N. gonorrhoeae* appear to be killing targets and the affinity of PBP 2 for β -lactam antibiotics is greater than that of PBP 1 (Barbour, 1981). Resistance to benzylpenicillin must therefore first occur by a decrease in the affinity of PBP 2. This produces a strain that has only a low level of resistance since killing still occurs by inactivation of PBP 1. Higher levels of resistance can then be gained by a decrease in the affinity of PBP 1 as well by mutations that decrease outer membrane permeability. It seems likely that further mutations that alternately decrease the affinities of PBPs 1 and 2 will occur and result in the gradual increase in the MIC of these intrinsically resistant strains to the extent that they will no longer be susceptible to treatment with β -lactam antibiotics.

The third clear example of PBP alterations in intrinsically resistant strains is found in *Streptococcus pneumoniae* (Zigheboim & Tomasz, 1980). β -Lactamases are unknown in this species and most clinical isolates are highly sensitive to benzylpenicillin (MIC $<0.01 \mu\text{g ml}^{-1}$), but a number of strains have appeared that show considerable levels of intrinsic resistance (MIC $0.5\text{--}12 \mu\text{g ml}^{-1}$). Analysis of the PBPs of resistant isolates has shown a bewildering array of alterations compared to normal isolates. As in *N. gonorrhoeae* the availability of a transformation system has allowed a genetic dissection of the mutational events that underlie the development of resistance in these clinical isolates (Zigheboim & Tomasz, 1980).

The conclusions to be drawn from the above studies are disturbing. Firstly, very large changes in the affinity of a PBP for β -lactam antibiotics can occur in clinical isolates. In methicillin-resistant *S. aureus* the affinity of PBP 3 for β -lactams is decreased about 1000-fold compared to its affinity in a normal strain.

Secondly, the studies with *N. gonorrhoeae* show that although β -lactams kill most bacteria by inhibiting the activity of more than one essential PBP, this does not prevent the emergence of moderately high levels of resistance.

Thirdly, all of the examples where resistance due to alterations of PBPs has been shown convincingly are in bacteria where β -lactamases are unknown (e.g. *Strep. pneumoniae*), or have only recently appeared (e.g. *N. gonorrhoeae*), or in bacteria where β -lactamases occur but are of little significance as β -lactamase resistant antibiotics have been in use for many years (e.g. *Staphylococcus aureus*).

In the absence of β -lactamases bacteria have little option but to find other ways of protecting themselves from penicillin. In the case of Gram-negative bacteria resistance can be achieved by outer membrane alterations and by PBP changes, and Gram-positive bacteria appear to be limited to the latter mechanism, although the development of tolerance to β -lactam antibiotics is a further option that has been discussed elsewhere (Tomasz, 1980). PBP changes are therefore likely to be a common cause of resistance to β -lactam antibiotics in organisms that are not known to produce β -lactamases.

The widespread occurrence of β -lactamases among Gram-negative bacilli, and their ease of lateral transfer by conjugation, provides these bacteria with an effective mechanism of obtaining resistance to β -lactam antibiotics. In recent years one of the major achievements of the pharmaceutical industry has been the production of potent β -lactam antibiotics that have a high degree of resistance to hydrolysis by β -lactamases. It seems likely that in the hospital environment these β -lactamase stable antibiotics will be very heavily used. If the effectiveness of β -lactamases in protecting *E. coli* or *P. aeruginosa* from β -lactam antibiotics is severely limited by the widespread use of β -lactamase stable antibiotics, we may see the emergence of intrinsic resistance as an important resistance mechanism amongst these organisms. Some reports suggest that this is already happening. For example, clinical isolates of *E. coli* that are resistant to β -lactamase stable derivatives have been shown to have decreased penetration of the antibiotic through the outer membrane and alterations of the affinity of PBPs have been implicated as the cause of resistance in *P. aeruginosa* strains isolated from patients undergoing

long courses of treatment with high levels of β -lactamase stable antibiotics (Godfrey *et al.*, 1981). Patients undergoing very extended periods of therapy with β -lactamase stable antibiotics provide almost perfect conditions for the isolation of highly resistant mutants with multiple alterations of their PBPs and it seems almost inevitable that such strains will arise. If these strains do emerge amongst the Gram-negative bacilli it is likely that they will be cross-resistant to all, or most, β -lactam antibiotics and their treatment will depend on the availability of another group of antibiotics to which they will respond. In *E. coli* most of the new β -lactamase stable antibiotics show a considerably higher affinity for PBP 3 than for the other killing targets and therefore low level intrinsic resistance to these compounds could arise by mutations in the PBP 3 gene that result in the production of a mutant PBP 3 with decreased affinity for β -lactam antibiotics.

A further prospect is that resistance due to the altered affinity of a PBP could become transferable within a species. In the enterobacteria the flanking of a chromosomal gene by insertion sequences provides a documented mechanism by which the gene could become transposed to a plasmid. Whether the transfer of a plasmid carrying a mutant PBP gene to a sensitive strain would result in resistance depends on PBP alterations being dominant and it is unknown if this is the case.

CONCLUDING REMARKS

Over the last few years our knowledge of the role of PBPs in bacterial growth and in the killing mechanism of β -lactam antibiotics has expanded rapidly, and we now have a good understanding of the various factors that determine the effectiveness of these antibiotics. Studies on the structure of PBPs and β -lactamases are progressing, and the prospects of a rational approach to the design of β -lactam antibiotics appear to be good. The role of PBP alterations in the intrinsic resistance of some clinical isolates to β -lactams is well established and, although this type of resistance may increase with the introduction of β -lactamase stable antibiotics, the recognition of the problem, and the means to monitor it, should provide strategies to prevent its emergence as a significant threat to the β -lactams.

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