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β-Lactamases: Molecular Studies

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Introduction

Much contemporary research in molecular biology makes use of β-lactamases ('penicillinases' or 'cephalosporinases', EC 3.5.2.6) and their genes, not as the primary objects of study but because their properties make them useful tools for studying phenomena of more general importance. Some recent examples of this are provided by studies of prokaryotic promoters and the control of transcription (Stuber and Bujard, 1981; Brosius, Cate and Perlmutter, 1982; Jaurin, Grundstom and Normark, 1982; McLaughlin, Chang and Chang, 1982; Olsson, Bergstrom and Normark, 1982; Yamamoto *et al.*, 1982; Kreft, Burger and Goebel, 1983); expression of the genes in unnatural environments (Brammar, Muir and McMorris, 1980; Chan *et al.*, 1981; Crabeel *et al.*, 1981; Gray and Chand, 1981; Imanaka *et al.*, 1981; Neugebauer, Sprengel and Schaller, 1981; Roggenkamp, Kustermann-Kuhn and Hollenberg, 1981; Breunig, Mackedonski and Hollenberg, 1982; Fujii, Imanaka and Aiba, 1982; Kreft, Burger and Goebel, 1983; Sarvas and Palva, 1983; Saunders *et al.*, 1984); secretion (Chan *et al.*, 1981; Gray and Chand, 1981; Neugebauer, Sprengel and Schaller, 1981; Inouye *et al.*, 1982; Koshland and Botstein, 1982; Koshland, Sauer and Botstein, 1982; Palva *et al.*, 1982; Pollitt and Zalkin, 1983; Kadanoga *et al.*, 1984) and the control of translation (Lai *et al.*, 1981; McLaughlin, Murray and Rabinowitz, 1981; Koshland, Sauer and Botstein, 1982; Mezes *et al.*, 1983a).

The gene is a natural component of many of the plasmids from which cloning vectors have been constructed. It makes a convenient selectable genetic marker, and is therefore often retained as part of the construct. In addition, the protein is easily expressed and purified, and many of the techniques for site-directed mutagenesis were first applied to this enzyme Shortle *et al.*, 1980, 1982; Castagnoli, Cesareni and Brenner, 1982; Dalbadie-McFarland *et al.*, 1982; Koshland and Botstein, 1982). For much the same reasons, many fusion proteins have part of the β-lactamase of transposon A, originally derived from an R_{TEM}-like plasmid (So, Gill and Falkow, 1975), as

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their amino-terminal component (Derynck *et al.*, 1980; Talmadge, Stahl and Gilbert, 1980; Chan *et al.*, 1981).

The object of this review is to summarize what is now known about the properties of these enzymes as proteins, in the hope that this will be of interest and value to those who make use of the genes for other reasons, and may also indicate how these studies can assist those whose primary interest is the structure and function of the proteins.

β-Lactamases have fulfilled this role of convenient model system at many stages in the development of molecular biology; the results of this work have been summarized several times in the past. The review by Citri (1971) still contains relevant material. A multi-author book devoted to the enzyme was published in 1979 (Hamilton-Miller and Smith, 1979). The latter is complemented by the Proceedings of a Royal Society Discussion Meeting held in 1978 (*Philosophical Transactions of the Royal Society, Series B* **289**, 165–378 (1980)). To a large extent the present review is an effort to update these accounts by a further five years, but it will necessarily have to concentrate on a few aspects of β-lactamase studies.

β-Lactamases are elaborated by bacteria as a defence mechanism against β-lactam antibiotics; these compounds are completely inactivated by hydrolysis of the β-lactam, and the hydrolysis is catalysed by the enzyme (*Figure 1*).

β-Lactamase production is not the only mechanism by which strains of bacteria may be resistant to the antibiotics, but it is particularly important because it depends on the presence of a single gene. β-Lactamase genes are often carried by plasmids, and so can be transferred between species. They are frequently found as components of transposons, and may transfer between plasmids and from plasmids to bacterial chromosomes. Some of the recent clinical problems arising in these ways are described by Broda (1979).

The increasing incidence of resistance in bacteria has been met by the use of new semi-synthetic β-lactams which are poor substrates for the enzymes concerned. However, as will be described below, the variety of substrate specificities to be found among the β-lactamases is very large, and it is by no means certain that any particular antibiotic will remain clinically useful indefinitely. In addition, β-lactams which are poor substrates for β-lactamases are often less effective as antibiotics. It may be hoped that molecular studies of the β-lactamase enzymes—and particularly of the basis of substrate specificity—will lead to the rational design of more effective antibiotics.

β-Lactamase activity has been detected from non-bacterial sources (e.g. human tissue (Hamilton-Miller, 1982)), but almost nothing is known about the proteins in these cases.

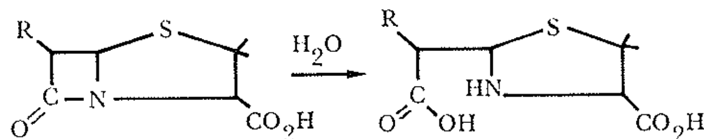

Figure 1. The β-lactam hydrolysis reaction catalysed by β-lactamases.

β-Lactams and the proteins which interact with them

MECHANISM OF ACTION OF β-LACTAM ANTIBIOTICS

The general nature of the molecular mechanism by which penicillins kill bacteria has been known for many years; the molecular details are becoming more clearly understood as a result of the positive identification of the bacterial enzymes involved.

Bacterial cell walls are made of oligosaccharides cross-linked by short polypeptides (Hammond, Lambert and Rycroft, 1984). The structure of the polypeptides differs from one species to another, but in every case the final cross-link is made by transfer of the acyl group of a –D–Ala–D–Ala bond at the *C*-terminus of one oligopeptide to an amino group on another (*Figure 2*). The cell-wall synthesis enzymes which catalyse this reaction are called transpeptidases; other cell-wall enzymes ('carboxypeptidases') hydrolyse the same bond.

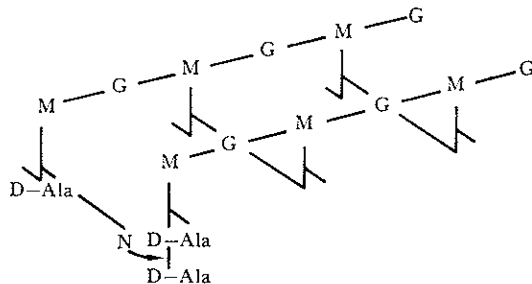

Figure 2. Schematic representation of the structure of the bacterial cell wall. Chains of alternating *N*-acetyl muramic acid (M) and *N*-acetyl glucosamine (G) residues are cross-linked by branched-chain oligopeptides. The sequence of the oligopeptides differs in different species, but the final cross-link is always made by acyl transfer from a *C*-terminal –D–Ala–D–Ala bond to an amino group on a neighbouring chain. Not all peptides form cross-bridges; when they do not, the terminal D–Ala is removed by hydrolysis.

It was proposed by Tipper and Strominger (1965) that the fused β-lactam ring of penicillins and cephalosporins mimics the –D–Ala–D–Ala bond as it binds to these enzymes and then acylates the active site irreversibly so that the enzymes are inactivated (*Figure 3*). The ring strain of the β-lactams makes

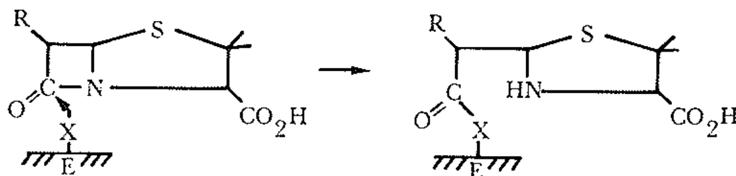

Figure 3. General mode of interaction of β-lactams with their targets.

them good acylating agents (their reactivity to nucleophiles is more like that of an ester than a 'normal' peptide), and of course it is also likely that some at least of the target enzymes become transiently acylated by the normal substrate in the course of their reactions. The irreversibility of the acylation by antibiotics is presumably mainly (or entirely) attributable to the fact that the newly formed amino group cannot leave the active site and cannot, therefore, be replaced by a nucleophile, such as water, to which the acyl group can be transferred to release it from the enzyme. The Strominger hypothesis has been generally confirmed by the studies outlined below, which demonstrate the identity of the penicillin target sites with the penicillin-binding proteins and the enzymes whose normal substrates include the –D–Ala–D–Ala bond.

The natural history of penicillins is complex and poorly understood; it can be viewed simply as an attack mechanism devised by soil-dwelling fungi against bacteria which compete against them in the same environment. Bacteria have evolved several defence mechanisms, of which the β-lactamase enzymes, which catalyse the hydrolysis of the β-lactam ring, are the simplest and most important. Saz and Lowery (1979) discuss the evidence—principally the observed distribution of antibiotics and enzymes—which is hard to reconcile with this simple account of the enzyme's biological role. In the clinical context, however, it has been shown repeatedly (*see* e.g. Richmond and Sykes, 1973) that the enzymes have the simple function of conferring resistance on the bacteria producing them, by catalysing the hydrolysis of the antibiotic. A slightly more complex situation appears to arise for 'third generation' cephalosporins such as cefotaxime, moxalactam and ceftriaxone. It has been clearly shown (Seeberg, Tolxdorff-Neutzling and Wiedemann, 1983; Seeberg and Wiedemann, 1984) that, in some cases, resistance to these antibiotics can be produced by the presence of a β-lactamase for which, however, the compounds are known to be very poor substrates and may be inhibitors. A potential explanation is that the antibiotics are sequestered or inactivated by stoichiometric binding to the proteins. Experiments on model systems have been described (Then and Angehrn, 1982; Gutmann and Williamson, 1983) which provide some plausibility for this explanation, but Seeberg, Tolxdorff-Neutzling and Weidemann (1983) show that (at least in one special case) it would require the presence of an implausibly large amount of protein. These authors suggest, alternatively, that a new permeability barrier arises in a way somehow determined by the presence of the β-lactamase, and this implies some as yet unknown additional function for the enzyme.

Because β-lactams bind to the active sites of the cell-wall synthesis enzymes, it is tempting to speculate (Tipper and Strominger, 1965) that the β-lactamases might have evolved from them—somehow having found a way to avoid irreversible acylation, perhaps trading on the fact that the β-lactam ring is so much more labile than the peptide bond. The cycle of development of weapon and counter-weapon has been taken at least one stage further: a number of naturally occurring penicillinase inhibitors have been discovered, mostly produced by *Streptomyces* species. The best understood of these, clavulanic acid, is effective because it has found a way of making the acylation of the β-lactamase active site irreversible.

The suggestion that there is some evolutionary relationship between the cell-wall synthesis enzymes and the β-lactamases could not be seriously tested until recently, but there is currently a rapid increase in knowledge of the targets for the β-lactam antibiotics. The subject was recently reviewed by Waxman and Strominger (1983).

Two main routes have been used to identify the targets for β-lactam antibiotics. One method is to incubate membrane preparations or whole cells with a radioactive antibiotic, to solubilize the membrane proteins, to separate them (by electrophoresis) and to identify by autoradiography those to which the antibiotic has become covalently bound. The second approach, largely due to Ghuysen (Ghuysen *et al.*, 1979), arises because in some Actinomycetes, some of the target enzymes, identified by their use of –D–Ala–D–Ala peptides as substrates and by their sensitivity to penicillins, are secreted into the culture medium and can be purified from it and studied as soluble, relatively low-molecular-weight proteins.

PENICILLIN-BINDING PROTEINS

The first of these approaches leads most directly to the identification of the killing target in pathogenic organisms. The technique has been applied to a large range of species. The number of distinct penicillin-binding proteins (PBPs) ranges between three and 10, depending on the species, and on how carefully minor components are sought. There is no consistent nomenclature, or indeed any simple way of relating corresponding proteins from different organisms. The SDS-gel electrophoresis system which is used to resolve the proteins, separates on the basis of chain length, and the proteins in a given organism are generally numbered in molecular-weight order, starting with the heaviest. There is not necessarily any similarity between proteins with the same number in different species.

The proteins differ in their affinity for any one β-lactam, and this specificity pattern differs from one antibiotic to another. These differential sensitivities have been exploited, together with the selection of mutants, to infer the activity of a given protein by observing the morphologically damaging or lethal effects of inactivating each individual PBP.

Most work has been done with *Escherichia coli*, and so far there is little reason to doubt that the nature and properties of the PBPs in this case are typical of those in more clinically important Gram-negative organisms. In general, the Gram-positive organisms appear to have a smaller and simpler array of penicillin target sites. In *E. coli* there are two classes of PBP; the lower-molecular-weight (40 000–49 000) proteins, '4', '5', and '6', are not essential (individually or in pairs) to the organism (at least, not while it is growing in the luxurious conditions of laboratory culture). On isolation these proteins are found to be DD-carboxypeptidases. It is believed that their function is to help establish the precise structure of the cell wall, for example by regulating the degree of cross-linking.

The lethal effects of β-lactam antibiotics are produced by inhibition of one or more of the higher-molecular-weight PBPs. PBP-1A and PBP-1B appear to be mainly responsible for peptidoglycan biosynthesis. PBP-1B can com-

pensate for the absence of PBP-1A, and the inverse may also be true to some extent. Absence or inhibition of PBP-2 (M.W. 66 000) gives rise to spherical cells, and inhibition or deletion of PBP-3 produces filamentation, so that it appears that these enzymes are topologically limited in their activity, and are responsible for the control of cell elongation and septation. Studies of isolated PBPs 1-A, 1-B and 3 have shown that they are all bifunctional enzymes catalysing both transpeptidase and transglycosylase activity. There is indirect evidence (Ishino *et al.*, 1982) that the same is true of PBP-2.

The genes for all of these proteins have been cloned, and the sequences of PBP-3 reported by Nakamura *et al.* (1983), of PBP1-A and 1-B by Broome-Smith *et al.* (1985), and of PBP-5 by Broome-Smith, Edelman and Spratt (1983).

DD-TRANSPEPTIDASES AND CARBOXYPEPTIDASES

The second main approach to the biochemical study of penicillin target enzymes is that due to Ghuysen and co-workers (Ghuysen *et al.*, 1979), who have concentrated on examining soluble proteins which are secreted by Actinomycetes into the medium, and which can be shown to catalyse DD-carboxypeptidase–transpeptidase reactions of –D–Ala–D–Ala peptides, and to be sensitive to penicillins. The three enzymes principally concerned are those secreted by *Actinomadura* R39 (M.W. 53 000) and *Streptomyces* R61 (M.W. 38 000), and the Zn-containing enzyme from *Streptomyces albus* G (M.W. 20 000).

The soluble nature of these proteins has made possible extensive biochemical studies of their behaviour; the drawback to these studies is the doubt that must remain about the relevance that these proteins have to clinically important enzymes. Nguyen-Disteche, Leyh-Bouille and Ghuysen (1982) have recently described the isolation of an efficient transpeptidase from *Streptomyces* K15 which is closely similar to the R61 enzyme, but which is normally membrane-bound and is therefore more likely to represent the physiologically significant enzyme of cell-wall synthesis.

Earlier studies have been reviewed by Ghuysen *et al.* (1979) particularly as they bear on the mechanism of action of the antibiotics. For present purposes the most important of these results was the finding (Frere *et al.*, 1976) that the reaction of radioactive penicillin with the R61 enzyme leads to the acylation of a serine residue in the sequence –Val–Gly–Ser–. After incubation of the enzyme with di-isopropylfluorophosphate, benzylpenicillin no longer reacts with it. A similar result was obtained with R39, the labelled peptide having the sequence Leu–Pro–Ala–Ser–Asn–Glu–Val. Studies on the protein chemistry of the two enzymes (Duez *et al.*, 1981a) seem to show that they share an unusual, uneven distribution of basic residues; digestion with trypsin leads in each case to a number of rather short soluble peptides and to an insoluble core with at least three high-molecular-weight components. This paper also reports the *N*-terminal 28 residues of R61, which does not, however, contain the 'active site' serine; the label is attached to one of the core peptides. In R39 it is a soluble peptide which is labelled.

A high-resolution X-ray crystal structure of the R39 enzyme has been published (Kelly *et al.*, 1982; Bartolone *et al.*, 1985).

The R61 and R39 enzymes are primarily transpeptidases and are very sensitive to penicillin, whereas the *Streptomyces albus* G enzyme is primarily a carboxypeptidase which is resistant to inhibition by penicillin. Molecular studies of this enzyme have proceeded somewhat further; Joris *et al.* (1983) report the complete sequence of 212 amino acids. A 2·5A X-ray crystal structure has also been published for this enzyme (Dideberg *et al.*, 1982).

Studies of the target sites of penicillin have, therefore, led to a number of complete protein sequences and X-ray crystal structures of proteins which resemble the β-lactamases in binding penicillins at their active sites. Whether these proteins have any closer relationship to the β-lactamases will be examined in a later section.

Variety and classification of β-lactamases

The genes for β-lactamases can be transferred easily, even between species, and it might have been expected that the number of distinguishably different enzymes would be rather small: in fact, clinical scientists have recognized a large variety of these proteins.

The number and type of distinguishable varieties have been profoundly affected by the therapeutic history of the antibiotics. The first penicillins were principally effective against Gram-positive organisms, such as *Staphylococcus aureus*. Because these organisms lack permeability barriers to the penetration of antibiotics, β-lactamases are particularly important in determining resistance. The clinical use of penicillin led to the very widespread occurrence of penicillinase-producing staphylococci, and by the early 1960s there was a serious risk that the penicillins might become therapeutically useless against these infections. At this stage most penicillinase studies concentrated on the enzymes from Gram-positive pathogens. The invention of semi-synthetic penicillins relieved this problem, and also led to drugs which were effective against Gram-negative organisms. Clinical interest in the β-lactamases then shifted to the vast variety of enzymes to be found in Gram-negative organisms. A similar process has occurred with the introduction of each new class of β-lactam antibiotic, clinical attention naturally focusing on the enzymes active against the most recently introduced drugs.

About ten years ago, Matthew (Matthew *et al.*, 1975; Matthew and Harris, 1976; Matthew and Hedges, 1976) developed a technique which allows β-lactamases to be identified and partly characterized quickly and using small quantities of material. This technique uses isoelectric focusing to concentrate and separate the proteins, and the chromogenic substrate 'Nitrocefin' to stain the β-lactamase bands specifically. The method has demonstrated the presence of the enzyme in many strains from which it was thought to be absent, and the number of distinguishable β-lactamases must probably now be numbered in hundreds.

Efforts to classify these proteins have, naturally, been generally based on properties which are easy to measure on small quantities of unpurified

material. These properties include substrate specificity ('substrate profile'), molecular weight (estimated by gel filtration), isoelectric point, and susceptibility to various classes of inhibitor. Several authors have proposed formal classifications (e.g. Sawai, Kanno and Tsukamoto, 1982) but the most extensive and systematic scheme is that proposed by Richmond and Sykes (1973) and expounded at length by Sykes and Matthew (1976). In a recent note, Sykes (1982) argues for the continued value of this scheme.

RICHMOND AND SYKES CLASSIFICATION

The Richmond and Sykes classification applies primarily to the enzymes from Gram-negative sources, and contains five types of enzyme. Classes II and V are primarily penicillinases rather than cephalosporinases (although rather confusingly, Richmond and Sykes also state that the Class V enzymes have a broad profile similar to Classes III and IV). The main difference between Classes II and V is that enzymes of the latter type are not inhibited by cloxacillin. Class I enzymes are chromosomally mediated cephalosporinases. Classes III and IV of this scheme both contain enzymes of broad specificity, and the distinction between them appears, again, to be made mainly on the basis that Class III but not Class IV enzymes are inhibited by cloxacillin. The addition to the classification scheme made by Sykes and Matthew (1976) was to point out that the enzymes in Classes III and V are plasmid-borne and the rest are chromosomal.

This classification scheme was very useful in introducing order into the large variety of enzymes which have been discovered in clinical laboratories. However, as more of the enzymes have been characterized in greater detail, it has become clear that the scheme has both theoretical and practical limitations as a basis for molecular studies.

Firstly, it strictly refers only to the enzymes from Gram-negative organisms. The best-known Gram-positive enzymes (from *Staphylococcus aureus, Bacillus cereus* and *Bacillus licheniformis*) must in any reasonable classification be placed with the Richmond and Sykes Class III enzymes. However, in the bacilli the enzymes are chromosomal, not plasmid-borne. Other clinically significant Gram-positive enzymes are those from mycobacteria (Kasik, 1979) and from clostridia (Magot, 1981). Some enzymes from *Streptomyces* have been extensively studied at the molecular level (Kelly *et al.*, 1981; Duez *et al.*, 1981b, 1982; Frere *et al.*, 1982a,b).

Secondly, many of the measurements on which the scheme depends to identify similarities and differences amongst the enzymes are unreliable or of doubtful fundamental significance. The shortcomings of gel filtration as a method for the estimate of molecular weight are well known (Andrews, 1970): they are well illustrated in the case of β-lactamases by the isolation of two enzymes; estimates of the molecular weights of these enzymes, made in this way, were seriously erroneous in opposite directions (Duez *et al.*, 1981b, 1982). Inducibility is often regarded as characteristic of a class of enzyme, but Bergstrom *et al.* (1983) have described a case in which homologous β-lactamase genes are inducible in one of the species, and non-inducible in the other.

The substrate profile is taken as fundamentally significant in the Richmond and Sykes scheme, but there is ample evidence that the substrate specificity of β-lactamases can be radically altered by a small number of amino-acid substitutions. Pollock (1968) described two biotypes of the *Bacillus licheniformis* enzyme of significantly different substrate specificity which differ only at about half-a-dozen positions in the sequence (R. P. Ambler, personal communication). Hall and Knowles (1976) described the isolation of point mutants of a penicillinase which had acquired cephalosporinase activity. Conversely, Baldwin *et al.* (1980) induced and isolated mutants which shifted a *Bacillus cereus* cephalosporinase into a penicillinase.

Finally, as the Richmond and Sykes scheme makes use of the presence or absence of inhibition by cloxacillin to characterize the enzymes, it should be pointed out that the molecular basis of this inhibition is not known (and may well not be the same for all enzymes). There are several distinct ways in which a protein could avoid being inhibited by this compound.

A justification for continued use of this classification scheme rests on its 'naturalness'—if the enzymes within a class are basically similar, then the points made in the last few paragraphs do not destroy the value of the scheme. The trouble is that one cannot establish this similarity for any particular enzyme with any certainty, without carrying out a far more extensive study than merely characterizing unpurified material in these few simple ways. The main purpose in classifying enzymes (at least for molecular studies) is to gain some confidence that studies made on one species at least have some application to another, and to allow a general view of function and mechanism to be formed by synthesis of studies on different proteins. In practice, this means that one wants to put into the same category only those enzymes which are related to each other by divergent evolution from a common ancestor. This cannot be established with certainty before the amino-acid sequences of the proteins concerned have been determined, and its negative can rarely or never be established unequivocally. Consequently, a classification based on these fundamental principles must always be incomplete or tentative (or both).

AMBLER CLASSIFICATION

The process of creating such a fundamental and sequence-based classification was started by Ambler (1980). At that time sequences were known for the enzyme from three Gram-positive sources and one Gram-negative plasmid. As the sequences were clearly homologous, they were all put into Class A. Mechanistic studies (to be described in a later section) have confirmed the expected similarity of function and mechanism.

Class A

All four of these sequences had been determined in the protein form: a DNA sequence almost identical to the *E. coli* plasmid (TEM-2) protein had been determined in the plasmid pBR322 (TEM-1; Sutcliffe, 1978). Other genes for this group of β-lactamases have been cloned (*S. aureus*, Chang and Cohen,

1974, Timmis, Cabello and Cohen, 1975; *B. licheniformis*, Brammar, Muir and McMorris, 1980, Gray and Chand, 1981, Imanaka *et al.*, 1981; *B. cereus*, Mezes *et al.*, 1983b, Sloma and Gross, 1983, W. Wang, P. S. F. Mezes, Y. Q. Yang and J. O. Lampen, unpublished work), and sequences from these genes have been reported (*see below*).

These gene sequences established the sequences of the leader peptides which are responsible for the transfer of the protein across the cell membrane. The membrane-bound and secreted forms of the enzyme in *B. licheniformis* are products of the same gene (Chang *et al.*, 1982). Maturation of the bound form occurs with cleavage at a cysteine residue in the leader sequence, and attachment of glyceryl palmitate to provide the hydrophobic handle which anchors the protein in the membrane. In the secreted protein a further 16 or so residues are removed. Similar mechanisms have been demonstrated (Nielsen and Lampen, 1982) for the Class A enzyme in *S. aureus*, and in a Class C (*see below*) enzyme in the same species.

Related studies (Connolly and Waley, 1983; Nielsen and Lampen, 1983) revealed a remarkable situation in *B. cereus*. The major secreted Class A enzyme in this case is not found in a cell-bound form and the gene sequence shows that there is no appropriate Cys in the leader sequence. The cell-bound ('γ' or 'III') enzyme is attached by the modified-Cys mechanism, and immunological and biochemical studies suggest strongly that it is also a Class A enzyme.

Class B

Ambler (1980) also proposed the creation of a Class B, to contain one enzyme. This protein is also secreted by *B. cereus* but is more active against cephalosporins than the Class A enzyme in the same organism, and is known to contain an essential Zn ion. On this basis, and with some peptide sequences already determined, Ambler concluded that the enzyme must be the product of a non-homologous gene, and could therefore be placed (alone) in Class B. The sequence and structure of this protein is discussed further below; no other enzymes can yet be added to this Class.

Class C

Cloning of the gene for a chromosomal, inducible 'cephalosporinase' from *E. coli* (Jaurin and Grundstrom, 1981) revealed a protein sequence that was sufficiently unlike the Class A enzymes for Class C to be suggested to receive it. Use of the cloned DNA as a probe has revealed that a wide range of Gram-negative organisms (including *Pseudomonas, Shigella, Klebsiella, Serratia* (Bergstrom, Olsson and Normark, 1982) and *Citrobacter* (Bergstrom *et al.*, 1983)) contain homologous genes in a similar-sized restriction fragment. It has been carefully established that a purified enzyme from *Pseudomonas aeruginosa* belongs to the same class (Knott-Hunziker *et al.*, 1982a), and so does the *Enterobacter cloacae* P99 enzyme (Charlier *et al.*, 1983). The general properties of at least three other recently purified enzymes make it seem likely that they belong to the same class. These are the enzymes from *Proteus*

morganii (Toda, Inoue and Mitsuhashi, 1981), *Proteus rettgeri* (Matsuura *et al.*, 1980) and *Citrobacter freundii* (Tajima *et al.*, 1980).

Possible future classes

No other classes have been proposed yet, but the study of several other enzymes is approaching the point at which it will be possible to say whether they belong to one of the first three classes. One such protein is the OXA-2 enzyme called R46. The 'OXA' enzymes are R-factor (plasmid) coded enzymes which hydrolyse oxacillin. Dale and Smith (1974) examined the enzymes on a number of different plasmids, and proposed a division into two main groups—the lower-molecular-weight OXA-1 and higher-molecular-weight OXA-2 enzymes. The OXA-3 enzymes, of which R55 is an example, are similar to OXA-2 proteins, but distinguishable in physical properties. Both OXA-2 and OXA-3 enzymes were shown to be dimers (Dale and Smith, 1976), and the best current estimate is that the R46 enzyme is composed of two identical subunits of molecular weight 32 000 (Holland and Dale, 1984). These enzymes are also remarkable in binding to Cibacron Blue, other anthraquinone dyes, and Blue Sepharose. It has been claimed that binding of these materials indicates the presence of a 'nucleotide binding domain' related in structure to that found, for example, in alcohol dehydrogenase. However, there is no evidence for nucleotide binding in the β-lactamases. The OXA-2 enzyme has been cloned, and the determination of its DNA sequence is nearing completion (J. W. Dale, personal communication).

The enzymes from two actinomycete strains have been purified and extensively studied enzymologically by Ghuysen's group (Kelly *et al.*, 1981; Duez *et al.*, 1981b, 1982; Frere *et al.*, 1982a,b). That from *Streptomyces albus* G is a penicillinase with a molecular weight of about 30 000. The enzyme from *Actinomadura* R39 is highly unusual, both in having a very low molecular weight for a catalytically effective subunit of 15 000, and in containing, as isolated, substantial quantities of bound DNA—which can, however, be removed without affecting the enzyme activity. It seems unlikely that these properties can be accommodated within an existing class, but it is not safe to reach this conclusion until at least some sequence information is available.

The same comment applies even more strongly to other enzymes whose purification has been reported recently, and the reported properties of which at least suggest that they may not belong to established classes. These include an enzyme from *Proteus vulgaris* (Matsubara *et al.*, 1981) which has a cephalosporinase activity, but an apparent molecular weight substantially less than that of the Class C enzymes. Conversely, an enzyme isolated from *Salmonella wien* (Ravagnan *et al.*, 1981) has a TEM-like substrate profile, but a molecular weight of 44 000. A group of enzymes from Gram-negative sources has been recognized by their ability to hydrolyse carbenicillin, and one of these has been isolated from *Pseudomonas aeruginosa* (Labia *et al.*, 1981). Finally, a penicillinase has been isolated from *Pseudomonas maltophila* (Saino *et al.*, 1982; Bicknell *et al.*, 1985) which has an essential Zn atom; however, unlike the Class B enzyme, this is primarily a penicillinase and is a

tetramer of subunit molecular weight 31 600. Bicknell *et al.* (1985) report the sequence of the *N*-terminal 32 residues of this protein; there is no recognizable similarity to the corresponding sequence of any other β-lactamase.

In summary, these studies of apparently new types of enzyme suggest that another three or four classes will be required to accommodate them, but at least a complete amino-acid sequence should be determined before any new class is named.

Structure of β-lactamases and related proteins

There is no substitute in a study of protein mechanism for a high-resolution X-ray crystal structure. Several β-lactamases have been under investigation by this technique for several years. As these enzymes are in general so much more accessible than the penicillin target enzymes, it has also been hoped that studies of the former might lead to sufficient structural understanding of the target proteins for structural principles to be employed in the more rational design of synthetic antibiotics. In fact, completion of high-resolution structures has proved remarkably difficult for all the enzymes of this group, and greater progress has been made with proteins which are closer analogues of the normal killing targets.

X-RAY CRYSTALLOGRAPHY OF β-LACTAMASES

Preliminary crystallographic data have been published for four enzymes—three in Class A (TEM, Knox *et al.*, 1976; *Bacillus cereus* I enzyme, Aschaffenburg *et al.*, 1978; *Bacillus licheniformis*, Dideberg *et al.*, 1985), and one in Class C (*Enterobacter cloacae* P99, Charlier *et al.*, 1983). Low-resolution structures of the TEM enzyme (DeLucia *et al.*, 1980) and of the *Staphylococcus* enzyme (Moult *et al.*, 1985) have been described. At this level of resolution there is little of biochemical interest which can be said about the structures, but both molecules are apparently roughly spherical; the *S. aureus* enzyme is dimeric in the crystal, but there is no evidence that dimerization is significant at biochemically significant protein concentrations. It has been claimed (Amicosante, Crifo and Strom, 1982) that kinetically significant association phenomena occur at low protein concentrations with the Class A enzyme from *Bacillus cereus*.

X-RAY CRYSTALLOGRAPHY OF RELATED PROTEINS

Two high-resolution structures have been described for proteins which interact with β-lactams—the carboxypeptidase–transpeptidase from *Streptomyces* R61 (Kelly *et al.*, 1982; Bartolone *et al.*, 1985) and the Zn-containing carboxypeptidase from *Streptomyces albus* G (Dideberg *et al.*, 1982). The first of these is a globular protein and contains about 40% secondary structure. The secondary structure is described as of the $\alpha + \beta$ type, but the two outer strands of the β-sheet are anti-parallel, and two of the helices appear to be present in $\alpha\beta\alpha$ units. The substrate-binding site has been tentatively iden-

tified, and some active site contacts postulated. The acylatable serine is satisfactorily close to the β-lactam ring. The lysine residue in the conserved Ser–X–Y–Lys sequence does not have an obvious role, although it can be brought close to the C3′ substituent in cephalosporins. An ion-pair with the free carboxylic acid of penicillins and cephalosporins seems to be ruled out.

Secondary-structure predictions have been made for the Class A β-lactamases (DeLucia *et al.*, 1980). These suggest that the enzymes contain a large number of reverse turns (at conserved sites) and that the helical and extended regions in the chain, if present, should all be short. To the extent that it is possible to judge from published ribbon diagrams, the transpeptidase–carboxypeptidase has a significantly different structure from that predicted for the β-lactamases. This is particularly true in the *C*-terminal part of the molecule. The R61 enzyme is 30% larger, and if the two are related in structure, there must be one or more large relative deletions in the β-lactamases.

The second high-resolution structure of a penicillin-interacting protein is that of the Zn^{2+}-containing carboxypeptidase from *Streptomyces albus* G (Dideberg *et al.*, 1982). There is no reason to expect that this structure is related to that of any of the β-lactamases, so it will not be discussed in detail here. The protein contains two distinct domains. The *N*-terminal (80 residue) region contains three helices and is of unknown function. The *C*-terminal domain contains the active site; its secondary structure consists of one long and two short helices, and a five-strand mixed β-sheet.

Primary structures of β-lactamases

CLASS A

Figure 4 shows the amino-acid sequences of the five Class A enzymes whose sequences are completely known. All these sequences are derived from primarily determined DNA sequences, but there is complete agreement with the protein sequences in all cases in which the latter has been completely determined. (However, note that the TEM sequence given is that in the plasmid pBR322; the sequence of the protein determined by Ambler and Scott (1978) has lysine instead of glutamine at position 62. Most studies of the TEM enzyme at the protein level have probably used material with lysine at this position; site-directed mutagenesis experiments, however, have all used the glutamine enzyme.)

The alignment is based on that proposed by Ambler (1980) but the completion of the *Bacillus cereus* sequences has required a few changes. No attempt has been made to align the 'leader' sequences *N*-terminal to the mature proteins, because they do not all fulfil the same function. The TEM enzyme is transported into the periplasmic space of Gram-negative organisms. All of the Gram-positive enzymes are secreted into the medium, but in some cases (*described above*) a proportion of the gene product remains attached to the cell surface. The cysteine residue which provides the anchorage point is that at position 33 of the *B. licheniformis* sequence in this alignment.

```
                    1                                                 50
B.cereus 5/B        MIVLKNKKML KIGMCVGILG LSITSLVTFT GGALQVEAKE KTGQVKHKNQ
B.cereus 569/H       MILKNKRML KIGICVGILG LSITSLEAFT GESLQVEAKE KTGQVKHKNQ
B.licheniformis           MKLW FSTLKLKKAA AVLLFSCVAL AGCANNQTNA SQPAEKNEKT
S.aureus                                           M KKLIFLIVIA LVLSACNSNS
TEM                                            MSIQH FRVALIPFFA AFCLPVFAHP
Identities          ---------- ---------- ---------- ---------- ----------

                    51                                               100
B.cereus 5/B        ATHKEFSQLE KKFDARLGVY AIDTGTNQT. IAYRPNERFA FASTYKALAA
B.cereus 569/H      ATHKEFSQLE KKFDARLGVY AIDTGTNQT. ISYRPNERFA FASTYKALAA
B.licheniformis     EMKDDFAKLE EQFDAKLGIF ALDTGTNRT. VAYRPDERFA FASTIKALTV
S.aureus            SHAKELNDLE KKYNAHIGVY ALDTKSGKE. VKFNSDKRFA YASTSKAINS
TEM                 ETLVKVKDAE DQLGARVGYI ELDLNSGKIL ESFRPEERFP MMSTFKVLLC
Identities          ---------e ----a--g-- --d------- -------rf- --st-k----

                    101                                              150
B.cereus 5/B        GVLLQQNSTK KLDEV..ITY TKEDLVDYSP VTEKHVDTGM TLGEIAEAAV
B.cereus 569/H      GVLLQQNSID SLNEV..ITY TKEDLVDYSP VTEKHVDTGM KLGEIAEAAV
B.licheniformis     GVLLQQKSIE DLNQR..ITY TRDDLVNYNP ITEKHVDTGM TLKELADASL
S.aureus            AILLEQVPYN KLNKKVHI.. NKDDIVAYSP ILEKYVGKDI TLKALIEASM
TEM                 GAVLSRVDAG QEQLGRRIHY SQNDLVEYSP VTEKHLTDGM TVRELCSAAI
Identities          ---l------ --------i- ---d-v-y-p --ek------ --------a-

                    151                                              200
B.cereus 5/B        RYSDNTAGNI LFHKIGGPKG YEKALRKMGD RVTMSDRFET ELNEAIPGDI
B.cereus 569/H      RSSDNTAGNI LFNKIGGPKG YEKALRHMGD RITMSNRFET ELNEAIPGDI
B.licheniformis     RYSDNAAQNL ILKQIGGPES LKKELRKIGD EVTNPERFEP ELNEVNPGET
S.aureus            TYSDNTANNK IIKEIGGIKK VKQRLKELGD KVTNPVRYEI ELNYYSPKSK
TEM                 TMSDNTAANL LLTTIGGPKE LTAFLHNMGD HVTRLDRWEP ELNEAIPNDE
Identities          --sdn-a-n- ----igg--- ----l---gd --t---r-e- eln---p---

                    201                                              250
B.cereus 5/B        RDTSTAKAIA RNLKDFTVGN ALPHQKRNIL TEWMKGNATG DKLIRAGVPT
B.cereus 569/H      RDTSTAKAIA TNLKAFTVGN ALPAEKRKIL TEWMKGNATG DKLIRAGIPT
B.licheniformis     QDTSTARALV TSLRAFALED KLPSEKRELL IDWMKRNTTG DALIRAGVPD
S.aureus            KDTSTPAAFG KTLNKLIANG KLSKENKKFL LDLMLNNKSG DTLIKDGVPK
TEM                 RDTTMPAAMA TTLRKLLTGE LLTLASRQQL IDWMEADKVA GPLLRSALPA
Identities          -dt----a-- --l------- -l-------l ---m------ --l-----p-

                    251                                              300
B.cereus 5/B        DWVDADKSGA G.SYGTRNDI AIVWP.PNRS PIII.AILSS KDEKEATYDN
B.cereus 569/H      DWVVGDKSGA G.SYGTRNDI AVVWP.PNSA PII..VLISS KDEKEAIYND
B.licheniformis     GWEVADKTGA A.SYGTRNDI AIIWP.PKGD PVVLAVL.SS RDKKDAKYDD
S.aureus            DYKVADKSGQ AITYASRNDV AFVYPKGQSE PIVLVIF.TN KDNKSDKPND
TEM                 GWFIADKSGA G.ERGSRGII AALGP.DGKP SRIVVIY.TT GSQATMDERN
Identities          -----dk-g- ------r--- a---p----- ---------- ----------

                    301        319
B.cereus 5/B        QLIKEAAEVV IDAI.....
B.cereus 569/H      QLIAEATKVI VKGS.....
B.licheniformis     KLIAEATKVV MKALNMNGK
S.aureus            KLISETAKSV MKEF.....
TEM                 RQIAEIGASL IKHW.....
Identities          --i-e----- ---------
```

Because it is necessary to postulate deletions in all the sequences to give good sequence similarity over the full lengths of the proteins, the alignment given here is not unique. X-ray crystal structures, when they become available, will allow the extent and position of deletions to be determined with greater confidence.

About 20% of the sequences are identical in all five proteins, and a further 10% are identical in the Gram-positive sequences alone. The extent of these identities, and their distribution through the whole chain, leaves no doubt that all these enzymes are derived by divergent evolution from a common ancestor. Evidence outlined in the last section of this review has established that the conserved serine residue at position 93 is a principal component of the active site, playing an identical part in the mechanism of all these enzymes.

The similarity of the TEM enzyme to the others is weakest in the last 50 residues of the sequence. If this is not coincidental, it might be connected with the different environment in which the Gram-negative enzyme is found, or with its substrate specificity.

CLASS B

Figure 5 shows the sequence of the *B. cereus* II (Class B) enzyme. This sequence also is derived from the determined DNA sequence, but once again the protein sequence is in complete agreement with it (R. P. Ambler, personal communication). The enzyme activity in this case depends on an active-site Zn ion, and Baldwin, Waley and Abraham (1979) showed that the essential Zn is liganded by three histidine residues and a cysteine residue. The only cysteine is that at position 198 in the sequence, and the data given by Baldwin, Waley and Abraham (1979) are sufficient to identify the liganding histidine residues as those at positions 116, 118 and 240. The only other penicillin-interacting protein with which this sequence might be compared is the Zn-containing carboxypeptidase from *Streptomyces albus* G, the complete sequence of which is given by Joris *et al.* (1983). These authors could detect no similarity between the protein sequences. Three histidine ligands have been identified in this case as well, but there is no detectable similarity in the histidine environments in the two proteins (except that in each protein, two of the histidine residues are separated by a single residue in the sequence).

Figure 4. Sequences of Class A β-lactamases. Protein sequences derived by translation from the gene sequences (*B. cereus* 5/B, W. Wang, P.S.F. Mezes, Y.Q. Yang and J.O. Lampen, unpublished work; *B. cereus* 569/H, Sloma and Gross, 1983; *B. licheniformis*, Neugebauer, Sprengel and Schaller, 1981; TEM, Sutcliffe, 1978), or by a combination of DNA and protein sequencing (*S. aureus*, Ambler, 1980, McLaughlin, Murray and Rabinowitz, 1981). In each case where the protein sequence is complete, there is complete agreement with gene-derived sequences, except as described in the text (Ambler, 1980). The observed *N*-terminal residues of secreted forms of the proteins are underlined. The one-letter notation used in Figures 4, 5 and 6 is that recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (*Biochemical Journal* (1969) **113**, 1–4), except that . instead of – is used to indicate a deletion. The single-letter code is as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

```
1                                                   50
 MKKNTLLKVG LCVGLLGTIQ FVSTISSVQA SQKVEKTVIK NETGTISISQ

51                                                 100
 LNKNVWVHTE LGSFNGEAVP SNGLVLNTSK GLVLVDSSWD DKLTKELIEM

101                                                150
 VEKKFQKRVT DVIITHAHAD RIGGIKTLKE RGIKAHSTAL TAELAKKNGY

151                                                200
 EEPLGDLQTV TNLKFGNMKV ETFYPGKGHT EDNIVVWLPQ YNILVGGCLV

201                                                250
 KSTSAKDLGN VADAYVNEWS TSIENVLKRY RNINAVVPGH GEVGDKGLLL

251   257
 HTLDLLK
```

Figure 5. Sequence of Class B β-lactamase, the 'Type II' enzyme from *B. cereus* 569/H. Protein sequence derived by translation from the gene sequence (J.O. Lampen, personal communication); the protein determined sequence is in agreement (R.P. Ambler, personal communication). The observed *N*-terminal residue of the secreted form of the enzyme is underlined.

CLASS C

Figure 6 gives the sequence of the original Class C β-lactamase. The sequence has been derived from the DNA sequence, and has not been confirmed in the protein. Evidence outlined in the last section of this review has shown that the serine residue at position 80 is essential to the activity. Jaurin and Grundstrom (1981) could detect no overall sequence similarity with the Class A enzymes. Knott-Hunziker *et al.* (1982a) compared the neighbourhoods of the active-site serines (both of which are fairly close to the *N*-termini of their respective sequences), and pointed out that the only identities with the Class A sequences are provided by a phenylalanine residue four positions earlier, and a lysine residue three positions later in the sequence than the acylatable serine. If this represents a genuine homology between the sequences, it is at least clear that the two classes of protein are separated by a very great evolutionary distance.

SEQUENCE SIMILARITIES BETWEEN β-LACTAMASES AND RELATED ENZYMES

It was suggested originally by Tipper and Strominger (1965) that the β-lactamases might have evolved from penicillin target proteins, and this idea was reinforced as it was discovered that many proteins in both classes contained an active-site serine residue. The strongest claim of evidence for this homology was put forward by Waxman and Strominger (1980), who found significant similarity between the *N*-terminal sections of Class A β-lactamases and the corresponding regions (including the active-site serines) of two DD-carboxypeptidases from *Bacillus subtilis* and *Bacillus stearothermophilus*. The similarity was most marked in a section of about 15 residues to

```
1                                                 50
MFKTTLCALL ITASCSTFAA PQQINDIVHR TITPLIEQQK IPGMAVAVIY

51                                               100
QGKPYYFTWG YADIAKKQPV TQQTLFELGS VSKTFTGVLG GDAIARGEIK

101                                              150
LSDPTTKYWP ELTAKQWNGI TLLHLATYTA GGLPLQVPDE VKSSSDLLRF

151                                              200
YQNWQPAWAP GTQRLYANSS IGLFGALAVK PSGLSFEQAM QTRVFQPLKL

201                                              250
NHTWINVPPA EEKNYAWGYR EGKAVHVSPG ALDAEAYGVK STIEDMARWV

251                                              300
QSNLKPLDIN EKTLQQGIQL AQSRYWQTGD MYQGLGWEML DWPVNPDSII

301                                              350
NGSDNKIALA ARPVKAITPP TPAVRASWVH KTGATGGFGS YVAFIPEKEL

351                       377
GIVMLANKNY PNPARVDAAW QILNALQ
```

Figure 6. Sequence of Class C β-lactamase, the *ampC* gene from *E. coli* strain K12. Protein sequence derived by translation from the gene sequence (Jaurin and Grundstrom, 1981).

the *N*-terminal side of the active-site serine. Most recently the similarity between the two classes of protein has been re-examined by Broome-Smith *et al*. (1985) in the light of the larger range of PBP sequences which are now available. These authors failed to find any large-scale unequivocal similarity between any individual PBP and any β-lactamase. However, they did find a region of significant similarity among all the proteins examined, about a dozen residues in length, centred about 19 residues on the *N*-terminal side of the active-site serine. The serine itself is always found in the sequence –Ser–Xaa–Xaa–Lys– (except in the *Actinomadura* R39 carboxypeptidase (Duez *et al*., 1981c)).

A practical consequence of this comparison is that such a low degree of similarity certainly means that the determination of the structures and mechanisms of all these classes of enzyme must continue to be treated as separate problems. X-ray crystal structures, when available, should help to show what evolutionary relationships exist among them.

Mechanism of action of β-lactamases

OVERALL CHEMISTRY

The nucleus of penicillins and cephalosporins has a very high ratio of hetero- to carbon atoms. These compounds therefore have a variety of possible decomposition routes, and it was the resulting complexity of decomposition

products which obstructed the chemical determination of structure (Clarke, Johnson and Robinson, 1949). Very little of this chemistry is relevant to the reactions catalysed by β-lactamases with good substrates (more complex chemistry is found with active-site-directed inhibitors, *see below*). For penicillins, the reaction is complete with the simple ring-opening hydrolysis of the β-lactam bond.

The release of the antibiotic molecule from the active sites of penicillin target enzymes occurs in some cases at a measurable rate (Ghuysen *et al.*, 1979) and in these cases also it is generally the penicilloate which is released. For the *Actinomadura* R39 and the *Streptomyces* R61 enzyme, a competing pathway in which the antibiotic fragments before deacylation is also observed.

The interaction of cephalosporins with β-lactamases is a little more complex, because of the presence in many of these compounds of a good leaving group at position 3′ of the thiazoline ring. It was shown by Hamilton-Miller, Newton and Abraham (1970) that the first isolatable product on enzymic hydrolysis with these compounds is the imino compound formed by loss of the 3′ leaving group from the initially formed cephalosporoate (*Figure 7*). O'Callaghan *et al.* (1972a) showed that for several cephalosporins and for three enzymes from Classes A and C, loss of the leaving group appeared to be a simple and rapid consequence of the β-lactam ring opening. Cephalosporins lacking a leaving group, such as the chromogenic compound 'Nitrocefin' (O'Callaghan *et al.*, 1972b) may be just as rapidly hydrolysed, and the stable cephalosporoate is the isolated product. Most recently, Faraci and Pratt (1984) have found two cases in which the loss of the leaving group is clearly a slow non-enzyme-catalysed step. There is no reason to believe that for the Class A and Class C enzymes there is catalysis of anything other than the β-lactam hydrolysis. However, in a note in the same paper, Pratt claims that, in Class B, the loss of the 3′ group is catalysed.

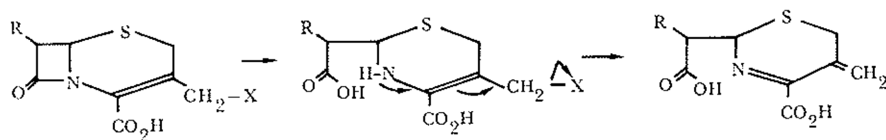

Figure 7. Path of breakdown of cephalosporins on hydrolysis by β-lactamases.

SUBSTRATE SPECIFICITY

Most studies of the specificities of the enzyme have concentrated on the effects of the substituents at the 6 position in penicillins and the 7 and 3′ positions in cephalosporins. Broad generalizations can sometimes be made from kinetic data of this kind. For example, penicillins with side-chains having considerable steric bulk just beyond the carbonyl group (such as methicillin, but also compounds with strongly branched aliphatic side-chains

(Gourevitch, Pursiano and Lein, 1962; Novick, 1962; Brandl, 1971)) are poorly hydrolysed by *S. aureus* penicillinase. Presumably this indicates steric constraint in the corresponding region of the substrate-binding site. Confirmation of conclusions such as this, and any detailed understanding of the molecular basis for substrate specificity, will have to await the completion of high-resolution X-ray crystal structures.

There is very little to say about the kinetic consequences of changing the structure of the nucleus of potential substrates. The difficulties of organic synthesis of this class of compound have presumably deterred extensive study of this kind, and it is hard to base firm conclusions on much of the early work because of the use of poor assays and poorly characterized enzymes. More recently, Pratt has published two careful studies of the reactions of substrates which lack the normal nuclear structure of β-lactam antibiotics. Pratt, Surh and Shaskus (1983) showed that anhydropenicillin V was a poor substrate for two Class A enzymes, and that the catalysed reaction involved only the usual β-lactam ring-opening reaction. More interestingly, Pratt and Govandhan (1984) have also shown that acyclic depsipeptides are substrates for enzymes in Classes A, B and C, and that for A and C the acyl group can be transferred to a suitable amino acceptor. This implies in turn that acyclic peptides themselves must be substrates, a fact which had been asserted by Saz, Lowery and Jackson (1961) on the basis of experiments which have not since been repeated.

THE NON-ENZYME-CATALYSED REACTION

The hydrolysed bond in β-lactams is a peptide link, but sterically and electronically it is different in structure from acyclic peptides, and it is not certain that the enzymes are mechanistically analogous to other peptidases. The ring strain, and the geometry which prevents development of the normal partially double-bonded character of the peptide bond, enhance the reactivity of the carbonyl group to nucleophiles; the rates of hydrolysis by OH^- are comparable to those of esters rather than amides (Indelicato *et al.*, 1974). The purpose of this hyperreactivity is presumably to improve the reactivity towards the active site of the target enzymes. Penicillins are effective acylating agents for proteins: Han *et al.* (1981) showed that penicillin G will inactivate glucose-6-phosphate dehydrogenase by acylation of an active-site lysine. The acylation of serum proteins which is associated with the antigenic properties of the penicillins is usually attributed to penicillanic acid generated in a rearrangement reaction, but in Han's case there is no evidence that it is not the β-lactam ring itself which is the effective acylating agent. A slightly different view of the significance of the structure of the β-lactam ring was taken by Lee (1971) who suggested that it is designed to resemble the transition state for the attack of nucleophiles on the –D–Ala–D–Ala bond. Strictly speaking, this would be most appropriate if the β-lactam antibiotics were acting as competitive rather than as covalent inhibitors.

The fact that the β-lactam ring has an enhanced reactivity to nucleophiles does not itself give any clue to the mechanism of the catalysed hydrolysis. A

number of low-molecular-weight catalysts of β-lactam hydrolysis have been found which could have analogues in the active sites of enzymes. Martin, Morris and Page (1976) demonstrated base catalysis of the aminolysis of benzylpenicillin, and showed that intramolecular catalysis occurred with ethylenediamine. Kinetic measurements were consistent with a mechanism in which a positively charged tetrahedral intermediate is formed at equilibrium, and the role of the base is to accelerate the breakdown of the intermediate by deprotonating it. By analogy with the serine proteases, this is much the sort of mechanism one would expect for a serine-dependent β-lactamase, and an analogous mechanism may be assumed for the cycloheptaamylose-catalysed hydrolysis observed by Tutt and Schwartz (1971).

Recently Schwartz (1982) has reported that hydrolysis and aminolysis of benzylpenicillin can be catalysed in a ternary complex of penicillin with Zn^{2+} and tris(hydroxymethyl)aminomethane. Apparently, a penicilloyl ester is an intermediate in the reaction and this then partitions between water and the amino group. It is postulated that, in the catalytic complex, the metal ion is co-ordinated to the carbonyl and the nitrogen atom of the β-lactam bond. It is at least possible that this reaction is analogous to the mechanism of the Class B β-lactamase.

ACTIVE-SITE-DIRECTED INHIBITORS

Until X-ray crystal structures are available, the most effective method of identifying active-site residues is by chemical modification. This approach has been attempted for some time, and early work of this kind is described in previous reviews. No conclusive results were obtained until there was a burst of activity about 1978, largely stimulated by the discovery of the naturally occurring active-site-directed inactivator, clavulanic acid (Howarth, Brown and King, 1976; Reading and Cole, 1977).

Clavulanic acid is a metabolite of *Streptomyces clavuligerus* which was discovered by a group at Beecham Research Laboratories Limited as a result of a large-scale programme of screening for naturally occurring β-lactamase inhibitors. The compound is effectively synergistic with penicillins against a range of pathogens whose resistance to the antibiotics is due to a β-lactamase, and a combination of clavulanate and amoxycillin is now sold under the name 'Augmentin'. Incubation of the compound with Class A β-lactamases gives rise to an intense UV absorption at about 280 nm. This observation led two groups (Cartwright and Coulson, 1980; Fisher *et al.*, 1980a) to propose that inhibition or inactivation of the enzymes was due to the formation of an α,β-unsaturated acyl enzyme (*Figure 8*). It was fortunate that this structure should have such a characteristic spectrum; other plausible arrangements of the atoms and multiple bond capacity of clavulanate are much weaker chromophores. This postulated mechanism led to the design of compounds capable of elimination across the C6–C5 bond as potential active-site-directed inhibitors. For the TEM enzyme, quinacillin sulphone was shown to inactivate by labelling the serine residue in the sequence Phe–Pro–Met–Met–Ser–Thr–Phe–Lys (Fisher *et al.*, 1981) and, for the *S. aureus* enzyme, chloropeni-

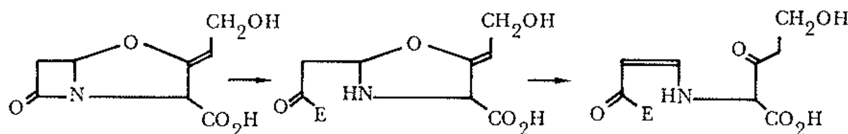

Figure 8. Proposed path for the reaction of clavulanic acid with the β-lactamase from *Staphylococcus aureus*.

cillanic acid sulphone was shown to label the homologous serine in the sequence Ala–Ser–Thr–Ser–Lys (Cartwright and Coulson, 1980; A. F. W. Coulson, unpublished work).

At about the same time, two other groups (also independently of each other) found that β-bromopenicillanate was an effective inactivator of the *B. cereus* I enzyme, and that this compound labels a serine residue in a homologous sequence in this enzyme also (Knott-Hunziker *et al.*, 1979; Cohen and Pratt, 1980). The chemical mechanism of inactivation is different in this case, but the final acyl enzyme also owes its stability to α,β-unsaturation (Orlek *et al.*, 1980). Unsaturated esters of this type are more stable to nucleophiles than their saturated counterparts; in addition, there may of course be steric reasons why deacylation of the enzyme is not catalysed in these cases.

The behaviour of these compounds strongly suggests not only that the homologous serine residue is part of the active site, but also that this residue becomes transiently acylated in the course of the hydrolysis of normal substrates. In most of these inhibitor/enzyme interactions, a catalysed hydrolysis of the inactivator competes with inhibition. Presumably, the acyl-serine intermediate may hydrolyse, or undergo the rearrangement reactions which lead to the various slowly hydrolysed unsaturated compounds.

There is some more direct evidence for the acyl-serine mechanism with Class A enzymes. Fisher *et al.* (1980b) showed that the poor substrate cefoxitin is hydrolysed by the TEM enzyme with a covalent intermediate having the spectral properties of an ester, and Anderson and Pratt (1981, 1983) showed that the pre-steady-state kinetics for a good substrate (a dansyl cephalosporin) and the *S. aureus* enzyme were consistent with the intermediacy of an acyl enzyme which could be trapped by lowering the pH. Similar results were obtained by Cartwright and Fink (1982) with the *B. cereus* I enzyme and dansyl penicillin. The expected labelled peptide was isolated after peptic digestion.

A number of other active-site-directed inactivators of Class A enzymes are known (and at least one—penicillanic acid sulphone (Retsema *et al.*, 1981)—is being evaluated for potential clinical use, by Pfizer Limited). The reactions of some of these compounds are more complex than has been implied so far, and at least in some cases it is not clear that the possibility of α,β-elimination is the key structural feature in producing an inhibitor. For the interaction of penicillanic acid sulphone with the TEM enzyme, Knowles (Brenner and

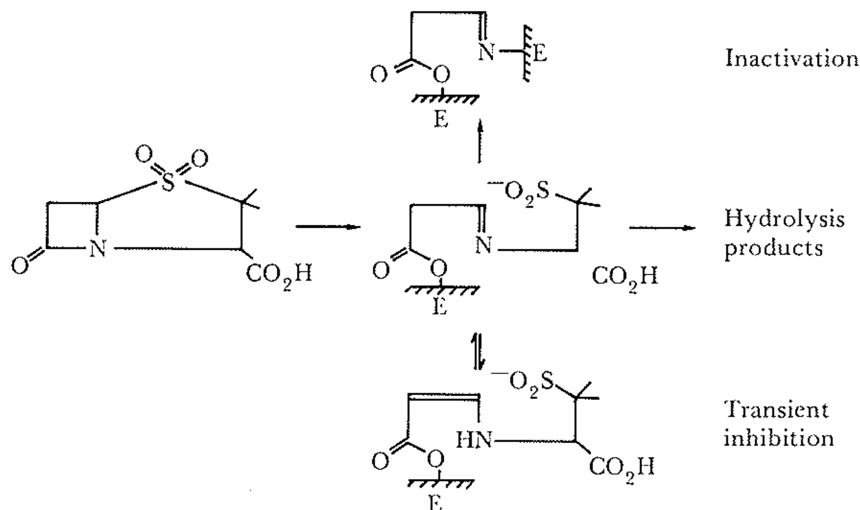

Figure 9. Simplified version of the scheme proposed by Brenner and Knowles (1981) for the interaction of penicillanic acid sulphone with the TEM β-lactamase.

Knowles, 1981; Kemal and Knowles, 1981) argues that the elimination reaction of the acyl enzyme leads to the enamine (*Figure 9*). This may hydrolyse (leading to substrate turnover), or tautomerize to the more stable β-aminoacrylate. However, this structure is responsible for only a transient inhibition, because it may either hydrolyse or tautomerize back to the enamine. In this case it is suggested that permanent inactivation is produced by a transimination reaction of the enamine to block an active-site lysine residue. A similar scheme was proposed for the interaction of the TEM-2 enzyme with clavulanate (and with desoxyclavulanate) except that here there are three distinguishable irreversibly inactivated products (Charnas and Knowles, 1981), the structure of which has not yet been determined. These schemes imply the presence of active-site residues in addition to the acylatable serine, but these residues have not yet been identified and their roles, if any, in the normal enzyme mechanism are unknown.

Other β-lactamase inhibitors have been described; in some cases the mechanisms of action have either been shown or can be presumed to be similar to those already described. Heckler and Day (1983) have now shown that diazotized 6-aminopenicillanic acid acylates the active-site serine of the *B. cereus* I enzyme with the formation of a dihydrothiazine, as does β-bromopenicillanate. It seems likely that the reaction of iodopenicillanate (Moore and Brammer, 1981) is also similar. 6-β-(Trifluoromethanesulphonyl)amidopenicillanic acid sulphone is expected to have a ready C5–C6 elimination reaction, and it is an effective inactivator of the *B. cereus* enzyme (Clarke *et al.*, 1983). Keith *et al.* (1983) have described the synthesis of a large number of compounds analogous to penicillins and derivatives, in which the 2

and 3 positions of the thiazolidine ring are bridged by a methylene group. These compounds have poor antibiotic properties, but the analogues of β-lactamase substrates are themselves readily hydrolysed by Class A enzymes. The analogues of β-bromopenicillanate, penicillanic acid sulphone and α-chloropenicillanic acid sulphone are effective β-lactamase inactivators, presumably employing the same mechanisms as the corresponding penicillin derivatives.

There are two other groups of β-lactamase inhibitors for which molecular studies have led to detailed suggestions concerning mechanism. In these cases also (olivanic acid and thienamycins, Easton and Knowles, 1982; 6-acetylmethylene penicillanic acid, Arisawa and Adam, 1983) a stabilized acyl enzyme is central to the proposed mechanism. The use of these compounds has not yet led to further information about the normal mechanism of the enzymes, and their chemistry will not be further discussed.

SITE-DIRECTED MUTAGENESIS

Two applications of site-directed mutagenesis have produced TEM enzymes with alteration of the active-site serine. Dalbadie-McFarland *et al.* (1982) reversed the active-site dipeptide –Ser–Thr– to –Thr–Ser–, and the result had no measured activity. They also briefly report the generation of the –Thr–Thr– and –Ser–Ser– mutants. The former is inactive and the latter has a low activity. Sigal, Hawood and Arentzen (1982) reported the generation of a Ser → Cys mutant; the resulting protein displayed a very low β-lactamase activity which could be inhibited by *p*-chloromercuribenzoate. These results can be taken as providing further evidence for the essential involvement of serine in the mechanism of the enzyme. However, it should be pointed out that studies of two defined point mutants of the *S. aureus* enzyme (obtained by selection) have now shown that the loss of activity is mainly or entirely due to structural disruption, rather than to modification of an active-site residue (Craig *et al.*, 1985; A. F. W. Coulson, unpublished work). Strictly speaking, the same explanation could be invoked to explain a loss of activity in any mutant, however generated.

STEADY-STATE KINETICS

Hardy and Kirsch (1984a,b) and Hardy, Nishida and Kirsch (1984) have recently reported a series of careful studies of the steady-state kinetics of the *B. cereus* I enzyme with good penicillin and cephalosporin substrates. They conclude that measurements of kinetic solvent isotope effects are most simply accommodated by a two-step mechanism, such as the formation and breakdown of an acyl enzyme.

MECHANISM OF CLASS A β-LACTAMASES

The upshot of these studies is that there is every reason to believe that the hydrolysis of β-lactams by Class A enzymes involves an intermediate in which

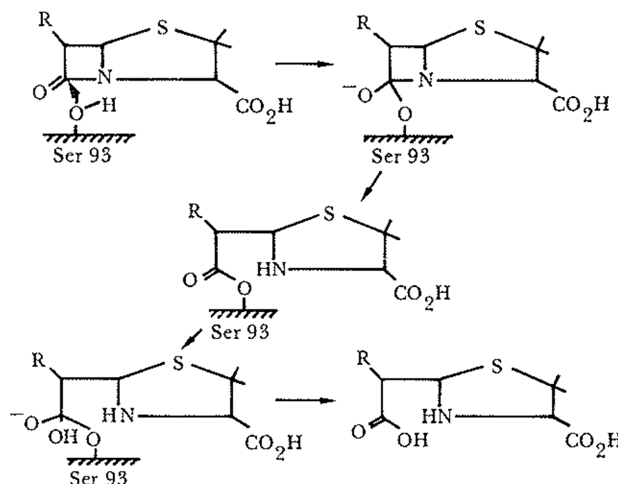

Figure 10. Suggested mechanism for the hydrolysis of penicillin by Class A β-lactamases. Attack by the hydroxyl of serine 93 on the β-lactam carbonyl group leads to the formation of a tetrahedral intermediate. An acyl enzyme is formed by breakdown of the tetrahedral intermediate. A similar two-step reaction with water as the incoming nucleophile releases the hydrolysed product. There is no direct evidence for the participation of tetrahedral intermediates, and the ionization states of these species are unknown.

Ser93 is acylated (*Figure 10*). However, this is really only the start of a full description of the mechanism. By analogy with other serine-dependent hydrolases, one would expect to find an apparatus for transferring protons from the entering nucleophiles and to the leaving groups in the formation and breakdown of the tetrahedral intermediates. Alternatively, or in addition, there might be charged residues to stabilize partial charges in the transition states for the reactions.

Because there is no conserved histidine in the Class A enzymes, it seems unlikely that there is a His. . . .Asp couple like the charge relay system in the serine proteases. Some of the inhibitor studies cited above have suggested the presence of lysine in the active site to act as a transimination acceptor or to take part in addition reactions. Specific inactivation of the *B. cereus* enzyme by phenylpropynal (Schenkein and Pratt, 1980) is thought to implicate lysines or arginine in the active site. Phenylglyoxal also inactivates the *B. cereus* enzyme (Borders *et al.*, 1982), and this also implicates arginine. Finally, observation by Hardy, Nishida and Kirsch (1984) of a shift of 2 pH units in the pK_a of the substrate side-chain carboxyl in carbenicillin was proposed by these authors as due to the presence of a carboxyl residue in the active site. Carboxyl modification has been observed to lead to a loss of activity in Class A enzymes (Waley, 1975). It may be observed from *Figure 4* that the number of acidic and basic residues conserved in all known Class A sequences is quite small, and these presumably include the catalytically active residues.

MECHANISM OF CLASS C β-LACTAMASES

The mechanism of the Class C enzymes also appears to involve the transient acylation of an active-site serine. Much of the most straightforward evidence for this is from Waley's group, and is summarized by Knott-Hunziker *et al.* (1982b). Cloxacillin behaves as a tightly bound, slowly hydrolysed substrate for the *E. coli* ampC protein and for a *Pseudomonas aeruginosa* enzyme—the 'Sabath and Abraham' enzyme. Quenching of mixtures of this substrate with either enzyme leads to the incorporation of the penicilloyl group stably enough for its site of attachment to be identified as the serine numbered 80 in the sequence in *Figure 6*. This implies that the serine residue is part of the active site. Fairly direct evidence that the acyl enzyme is a normal intermediate is provided by the observation that these enzymes, in the presence of methanol or ethanol, catalyse the formation of the penicilloyl esters in addition to the normal hydrolysis. For the good substrate, penicillin G, there is no effect on the overall rate of reaction. These results can be most simply explained by proposing that formation of an acyl enzyme is the rate-determining step in the reaction, and that this intermediate is partitioned among the available nucleophiles. For cloxacillin, the turnover rate is sharply accelerated by the presence of methanol. Presumably the breakdown of the acyl enzyme is rate-limiting; this conclusion is also implied by the fact that the active site can be labelled by cloxacillin.

COMPARISON OF MECHANISMS

Neither of these types of evidence is available for the Class A enzymes, and this fact by itself implies that there are significant mechanistic differences between the two classes. Substrates which have both low K_m and low V_{max} are not commonly found for Class A enzymes. Inhibitory substrates such as quinacillin and cloxacillin have a complex and poorly understood mechanism. 'Competitive substrates' are fairly common for the Class C enzymes. Bush, Freudenberger and Sykes (1982) have described azthreonam, a monobactam, which has this property, and Calverley and Begtrup (1983) have described *N*-(functionalized alkyl) derivatives of 6-aminopenicillanic acid with the same behaviour, both with respect to the *Enterobacter cloacae* P99 enzyme.

Reaction of the acyl enzyme with nucleophiles other than water has often been sought for the Class A enzymes, but found only in the special case described by Pratt and Govandhan (1984). Presumably, either the formation of the acyl enzyme is always the rate-determining step, or there are steric or electronic reasons which prevent other nucleophiles replacing water in the reaction.

The behaviour towards inactivators also differentiates the two classes of enzyme. It is hard to make a confident generalization, because publications in this area do not always clearly classify the enzymes used in terms of either the Richmond and Sykes or the Ambler scheme. However, it does seem to be generally agreed (*see* e.g. Reading and Farmer, 1981; Okonogi *et al.*, 1982) that compounds such as clavulanate and sulbactam are poor inactivators of

the Class C enzymes, while thienamycins may be effective against both types of enzyme.

For the enzymes of Classes A and C, X-ray crystallography and perhaps other techniques may be expected to reveal in the near future the components (other than serine) of the catalytic apparatus.

The importance of the β-lactamases for mechanistic enzymology is that the different classes of enzyme represent different solutions to the single specific problem of opening the β-lactam ring (while avoiding irreversible acylation by the same structure). The range, narrow or wide, of distinct solutions found, should indicate the range of possibilities open to catalysts which are to be constructed out of protein.

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