REVIEWS

The Molecular Basis of β-Lactamase Catalysis and Inhibition

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Abstract: The β-lactamases catalyze the hydrolysis of the lactam bond in β-lactams, thus rendering the β-lactam ineffective as an antibiotic. The increasing spread of resistance to β-lactam antibiotics is largely due to this class of enzyme. Mechanistically these enzymes appear to be related to the transpeptidases and carboxypeptidases involved in the synthesis of the bacterial cell wall. Interest in the basic mechanism of action of the β-lactamases has been spurred by the potential for mechanism-based drug design. The past seven years have seen a significant increase in our knowledge of the catalysis and inhibition of the β-lactamases. The presence of an essential, conserved, serine residue which participates in the formation of a covalent acyl-enzyme intermediate in catalysis, inhibition and inactivation by β-lactams has been established. Unfortunately, few additional details regarding the catalytic mechanism are well established. A generalized reaction pathway can be formulated for most β-lactam inhibitors (reversible or irreversible). This scheme involves partitioning of the initially-formed acyl-enzyme by three pathways: 1) hydrolysis leading to turnover, 2) transient inhibition probably involving formation of an imine or enamine acyl-enzyme, or possibly involving a substantial conformational change in some cases, and 3) imine formation followed by additional covalent modification of the enzyme leading to irreversible inactivation. The flux through each of these pathways varies with the nature of the "substrate" and the particular β-lactamase.

Throughout the history of β-lactam antibiotics[3], the β-lactamases have played an important role, both as a source of resistance, and as an incentive for the development of new β-lactams. Since the first reports in 1940 of enzymes which rendered penicillin ineffective as an antibiotic (1), there has been a continuing effort to produce antibiotics which would not be affected by the β-lactamases of resistant strains of pathogenic microorganisms. This has been a major impetus in the development of the newer cephalosporins in particular. Although the β-lactamases are still believed to account for the majority of instances of bacterial resistance, the role of membrane permeability, as well as other penicillin-sensitive enzymes is becoming more recognized (2).

Any discourse on the β-lactamases should also recognize the intimate relation with the enzymes involved in cell wall biosynthesis. Twenty years ago Tipper and Strominger (3) proposed that the β-lactam antibiotics were structural analogs of the D-Ala-D-Ala terminus of the peptidoglycan cross-link, and that their antibiotic function derived from inhibition of enzymes involved in cross-linking the bacterial cell wall. The main features of this theory have continued to stand the test of time.

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[3] For chemical structures of the β-lactams, see Pharm. Res., issue 1, 1985 (78).

Until recently it was assumed that the penicillin antibiotic targets in cell wall biosynthesis (transpeptidases and carboxypeptidases) and the β-lactamases were distinct moieties; however, recent work strongly suggests some common origins and similarities in function. For example, current hypotheses involve the formation of a penicilloyl-enzyme intermediate in both cases, as well as some sequence homology (4–6). The difference between the transpeptidases and carboxypeptidases of the cell wall synthetic machinery and the β-lactamases arises from the stability of this acyl-enzyme in the former case, and its lability in the latter (6). Both sequence homology and cross-specificity have been observed.

β-Lactamases are found throughout the microbial world, including virtually all the bacteria, and most actinomycetes (7). The majority of β-lactamases produced by gram-positive organisms are inducible and exocellular, although membrane-bound examples have been reported (8, 9). β-Lactamases produced by gram-negative organisms may be inducible or constitutive, and are found in the periplasm. The β-lactamases may be either chromosomally or plasmid coded. β-Lactamase genes are highly mobile, accounting for their facile spreading among bacteria.

A major reason for interest in the details of the catalytic reaction of β-lactamases stems from the potential for mechanism-based drug design. A detailed understanding of the mechanisms of action of the cell wall transpeptidases, the target enzymes for β-lactam antibiotics, and the β-lactamases, which render such antibiotics ineffective, and of cell wall permeability, should allow the rational design and development of drugs which would be effective against β-lactam-resistant organisms.

Of necessity this brief review is selective in its coverage of the subject, and the reader is referred to other recent reviews for further information (6, 7, 10–15). The emphasis of this article will be on the *molecular* basis of catalytic (and inhibitory) function of β-lactamases. Only a very limited number of the total known β-lactamases have been studied in any detail at this level.

Catalytic Mechanism

At the present time it seems reasonable to conclude that there are only a small number of distinct classes (structural and functional) of these enzymes. Consequently information relating to a particular enzyme may be general to all members of that class. Several classifications have been proposed for β-lactamases, based mainly on substrate specificity patterns [reviewed by Sykes (16)] we will use that of Ambler (17). Three

structural classes (A, B, and C) are recognized; however, it is likely that there are additional, as yet uncharacterized, classes. Classes A and C have an active-site serine, and molecular weights (MW) of about 30,000 and 39,000 daltons respectively. Class B enzymes have an active-site Zn^{2+}, and MW of around 22,000 daltons. Since the bulk of our knowledge concerns the class A enzymes, we will focus on these. Interestingly, class A enzymes are found in both gram-positive and gram-negative bacteria. Membrane-associated forms are also known (8, 9). A discussion of different β-lactamases, based on their origin, may be found in a recent review (7).

Specificity

Individual β-lactamases vary considerably in their specificity toward penicillins, cephalosporins and the newer classes of β-lactam antibiotic [such as cephems, penems, cephamycins, carbapenems, monocyclic β-lactams (18)]. It would appear that any β-lactam is a potential substrate for a β-lactamase. β-Lactamases have not been found to hydrolyze acyclic peptides, involving D-Ala-D-Ala. However, acyclic depsipeptides, analogous in structure to the terminal D-Ala-D-Ala moiety of the peptidoglycan cross-link, are substrates (19).

Active-Site Residues

The limited knowledge we have of the essential catalytic groups comes predominantly from chemical and inhibitor modification studies, with some input from mutagenesis, kinetics, sequence homology and crystallography (of an evolutionarily related DD-carboxypeptidase). Most of the pertinent studies have been carried out on class A enzymes. Early chemical modification studies, reviewed in detail in ref. (20), implicated almost every type of amino acid with a reactive side-chain. These investigations dramatically illustrate the limitations of this approach, and must, in general, be considered suspect. For example, recent investigations (21, 22) have shown that the previously reported essential tyrosine is an experimental artifact. Some potential catalytic residues may be eliminated on the basis of sequence data. For example, there are no conserved histidine residues, β-lactamase I from *B. cereus* lacks cysteine, and the enzyme from *Staph. aureus* PC-1 lacks tryptophan.

In fact, the only residue for which there is strong evidence of its functional involvement is serine-70 [in the numbering system of Ambler, (17)]. This residue is implicated by covalent modification by inhibitors, by site-specific mutagenesis, by conservation of sequence homology and by analogy to the related D-Ala-D-Ala carboxypeptidase crystallographic structure. The first indication of the involvement of this Ser came from independent investigations by Pratt and Waley on the inactivation of the enzyme from *B. cereus* by 6-β-bromopenicillanic acid (23–26).

6-β-Bromopenicillanic acid is a very potent inhibitor for class A β-lactamases. The inactivated enzyme has a unique chromophore which has been shown to be due to the dihydrothiazine moiety (25, 26). It has been postulated that this compound is formed by rearrangement from an initially-formed acyl-enzyme in which the bromine is intramolecularly displaced by the thiazolidine ring sulfur (24–27) (see below). Enzymatic digestion, following inactivation with radioactive-labeled 6-β-bromopenicillanic acid, led to identification of Ser-70 as the site of attachment (24, 25). Subsequent investigations with a number of other β-lactam inhibitors have also demonstrated covalent attachment to this serine residue (28–30).

The probable involvement of an essential serine in the class C enzymes has been demonstrated in a similar manner using radioactive cloxacillin, a poor substrate[4] of the β-lactamase from *Pseudomonas aeruginosa* (31).

Site-specific mutagenesis experiments have been undertaken on the pBR322 RTEM enzyme from *E. coli*. Replacement of the active-site serine by threonine (32) leads to the absence of catalytic activity in the mutant. The replacement of the active-site Ser by Cys leads to reduced catalytic activity (33, 34), sensitivity to thiol reagents (previously absent), and a different substrate specificity to that of the wild-type (34). These observations are consistent with the essentiality of Ser-70 in catalysis.

Although protein crystallographers have been working on the structure of β-lactamase for almost 15 years, progress has been slow, and no high resolution structures are yet available. At present X-ray analysis is underway on the following class A enzymes: *E. coli* TEM (35), *B. cereus* (36), *S. aureus* (37), *B. licheniformis* (38), as well as that from *E. cloacae* P99 (39), a class C β-lactamase. Recent crystallographic investigations of the R61 DD-carboxypeptidase from *Streptomyces,* which is assumed to be evolutionarily related to the β-lactamases (5, 40, 41), have located the penicillin-binding site in a 2.8 Å resolution map (42). The serine homologous to Ser-70 is located in this area. The β-lactam binding site is at the *N*-terminus of two helices, which may provide the positive electrostatic field to bind the carboxylate. Chemical modification implicates a lysine or arginine, 12 residues to the *N*-terminal side of the active-site Ser, which spatially is located in the vicinity of the β-lactam binding site. The relatively low resolution, and lack of a complete amino acid sequence, complicate detailed interpretation of the structure.

The presence of a conserved Lys three residues to the carboxyl side of the active-site serine in all the sequenced β-lactamases and penicillin-binding proteins is good evidence for an essential lysine. This residue is also observed to be in the vicinity of the penicillin-binding site in the crystallographic study (42). Modification of most of the amino groups in the β-lactamase from *Staph. aureus* by methyl acetimidate leads to 99 % inactivation (20). The presence of cephaloridine prevents such inactivation. Further support for an essential lysine in catalysis comes from the observation that the heat of ionization for the alkaline limb of the bell-shaped pH-dependence of k_{cat} is ~10 kcal mol^{-1}, with a pK of 8.5 at 25°C (S. J. Cartwright & A. L. Fink, unpublished results). Recent studies showing the irreversible inhibition of β-lactamase by phenylpropynal (43) and phenylglyoxal (44) are consistent with the presence of a lysine or arginine in the active-site vicinity.

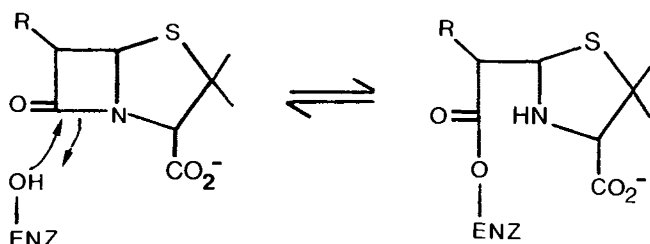
Catalytic Pathway

Until quite recently there was insufficient data available to postulate a catalytic pathway for β-lactam hydrolysis by β-lactamases. Formally the reaction is analogous to peptide hydrolysis, and one might therefore anticipate that β-lactamases may have evolved, by analogy to the proteases, to utilize nucleophilic, electrophilic or general acid-general base catalysis involving serine, thiol, carboxyl or metal ion residues. So far

[4] The value for k_{cat} is very low (2.5×10^{-2} s^{-1}), and K_m is estimated as 17 nM. Thus on the basis of k_{cat}/K_m cloxacillin is a good substrate. However, based on rates of hydrolysis it acts as an inhibitor because of the very slow breakdown of the acyl-enzyme. This lack of discrimination at the kinetics level between substrate and inhibitor is common to many β-lactams usually considered inhibitors.

only examples of serine (class A & C) and zinc ion (class B) have been found, although some β-lactamases are known to be sensitive to thiol reagents (7). Since virtually nothing is known about the details of the mechanism of the class B enzymes, our discussion will be limited to the class A and C enzymes.

The results from several investigations in the past five years are consistent with a covalent, acyl-enzyme (penicilloyl-enzyme) intermediate (Scheme 1).



Scheme 1

The first evidence for a covalent intermediate in catalysis involved the poor substrate cefoxitin and the *E. coli* RTEM-2 enzyme (45). This substrate possesses an α-methoxy group on C7 which results in its very slow hydrolysis ($k_{cat}/K_m = 6\ M^{-1}\ s^{-1}$, compared to $1 \times 10^8\ M^{-1}\ s^{-1}$ for benzyl penicillin), providing the potential to observe directly the build-up and decay of the putative acyl-enzyme intermediate. The nature of the covalent linkage was established by gel filtration following denaturation of the intermediate (using radioisotope-labeled cefoxitin) and Fourier transform infrared measurements (45).

The existence of a covalent intermediate in the reaction of β-lactamase I (from *B. cereus*) with a very good penicillin substrate was demonstrated by the use of low pH or subzero temperature to trap the intermediate formed in the reaction with dansyl-penicillin ($k_{cat}/K_m = 1 \times 10^7\ M^{-1}$ (46). A good dansyl-labeled cephalosporin substrate ($k_{cat}/K_m = 3.3 \times 10^5\ M^{-1}\ s^{-1}$) was used to demonstrate the existence of a covalent acyl-enzyme intermediate using the enzyme from *Staph. aureus* PC1 (47, 48). Surprisingly, a detailed kinetic analysis of the reaction revealed that the rate of the reverse acylation step (reclosure of the β-lactam ring with expulsion of the enzyme) was approximately one third that of the corresponding forward (acylation) rate constant (48). This suggests that in the acyl-enzyme the cephalosporinoyl moiety is maintained in a very similar conformation to that in the substrate, and that the acyl-bond may be strained. Recent studies (49, 50) have shown that in the β-lactamase-catalyzed hydrolysis of cephalosporins with good 3'-leaving groups the departure of the 3'-leaving group is not necessarily concurrent with β-lactam cleavage. Thus two types of acyl-enzyme may be formed, one containing the 3'-leaving group, the other not. The latter has a greatly reduced rate of hydrolysis. Detailed kinetic studies on the effect of pH and solvent kinetic isotope effects on β-lactamase I catalysis have been reported (51).

Kinetic data consistent with the intermediacy of an acyl-enzyme have been obtained in reactions of the class C enzymes from *P. aeruginosa* and *E. coli* K12 (the *ampC* chromosomal enzyme) with cloxacillin (31). This work pointed out two significant mechanistic differences between the class A and C enzymes. The former do not show transferase activity toward simple added nucleophiles such as hydroxylamine or methanol. However, not only do the class C enzymes exhibit transferase activity toward alcohols, they also catalyze the hydrolysis of the resulting esters, such as α-methyl benzyl-penicilloate ester (31).

Several investigators have remarked on the apparent conformational mobility of the class A β-lactamases (20, 30, 52, 53). This appears to be of more importance in the reactions with some inhibitors, such as cloxacillin and methicillin, than with substrates, since conformationally-constrained β-lactamase (by cross-linking or antibodies) retains full activity toward good substrates such as benzyl penicillin (20, 54, 55). The conformational mobility of the β-lactamases has been known for a long time, and the term "conformational response" was coined to describe the reversible inhibition brought about by certain substrates (52).

Ring strain in the β-lactam ring of substrates results in their reactivity typically being comparable to that of a methyl ester (12). Thus the required rapid formation of the acyl-enzyme necessitates facilitation of the serine hydroxide attack. There are three probable mechanisms for this: activation of the serine, analogous to the serine proteases; electrophilic catalysis to polarize the lactam carbonyl; or enzyme-induced conformational change in the substrate to increase the ring strain.

The first mechanism would involve base-catalyzed activation of the serine hydroxyl; the most likely candidate for the base is carboxylate. This would be expected to result in a hyper-reactive serine residue. Because such hyperactivity has not been observed one can conclude that either this mechanism does not occur, or that the activation of the serine occurs only after a conformational change induced by substrate binding, or that the putative hyper-reactive serine is shielded from reaction with probes. Electrophilic catalysis could involve acid catalysis in which a proton is donated to the carbonyl oxygen to facilitate the nucleophilic attack by the serine hydroxyl. Electrophilic catalysis by the Zn^{+2} is a likely mechanism for the class B enzymes. Significant ground state distortion of the β-lactam ring is also a good possibility, given the conformational flexibility of the class A enzymes. In this case intrinsic binding energy would be used to raise the ground state energy of the substrate toward that of the transition state.

Inhibitory Mechanisms

Since the most common source of bacterial resistance to β-lactam antibiotics is the presence of β-lactamases, there has been much interest in the design of β-lactamase inhibitors. At present two directions have been explored in depth. The first is synthesis of β-lactams that are both resistant to the action of β-lactamases and effective and wide-spectrum antibiotics. These are exemplified by the third generation cephalosporins [for example moxalactam, cefuroxime (56)]. The second is synthesis of compounds that effectively inactivate the β-lactamases, but have poor antibiotic properties and must be used in conjunction with a good antibiotic. The first example of such a combination drug is Augmentin, a mixture of clavulanic acid and amoxycillin, which was recently approved for clinical use in the United States (57).

Types of Inhibitors

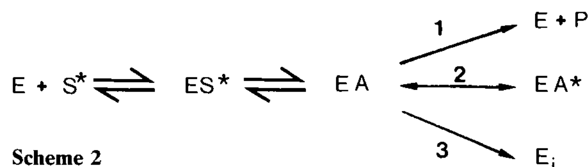
Since the late seventies, several naturally-occurring and semi-synthetic β-lactam derivatives have been reported which are good inhibitors of the β-lactamases. In fact, until the discovery of clavulanic acid in 1976 (58), no good inhibitors of these enzymes were known. Several different categories of β-lactam

inhibitors and suicide substrates of the β-lactamases are now recognized. These include weak competitive inhibitors [such as penicilloic acids (59)]; reversible inhibitors, which are really suicide substrates [such as methicillin, cloxacillin (30)]; tight-binding substrates with very low turnover rates, which therefore can appear to be inhibitors by tying up the enzyme for long time periods [such as cephamycins, and several third-generation cephalosporins, such as moxalactam, cefotaxime, cefotetan (60)]; and irreversible inactivators, also suicide substrates, such as clavulanic acids (61), penicillin sulfones (62, 63), 6-β-halopenicillins (64), 6-methylenepenicillanic acid derivatives (65). This last class has been subjected to detailed investigations into the mechanistic basis of their inhibition. The impetus for such studies has in part been to provide data for mechanism-based drug design.

In addition a number of non-β-lactam inhibitors, other than chemical modification reagents, have been reported. These include derivatives of boronic acid (59, 66), and the macrolides izumenolide (67) and dotriacolide (68).

Inhibition Pathways

A general scheme for the mechanism of inactivation of β-lactamase by β-lactams that cause irreversible inhibition, such as penem sulfones, 6-β-halopenems, carbapenems and the clavulanates can be formulated based mainly on the work of Knowles and coworkers (61–63, 69). The common feature of this Scheme 2 is the formation of an acyl-enzyme intermediate (EA) which can then react by different pathways leading to different types of products.



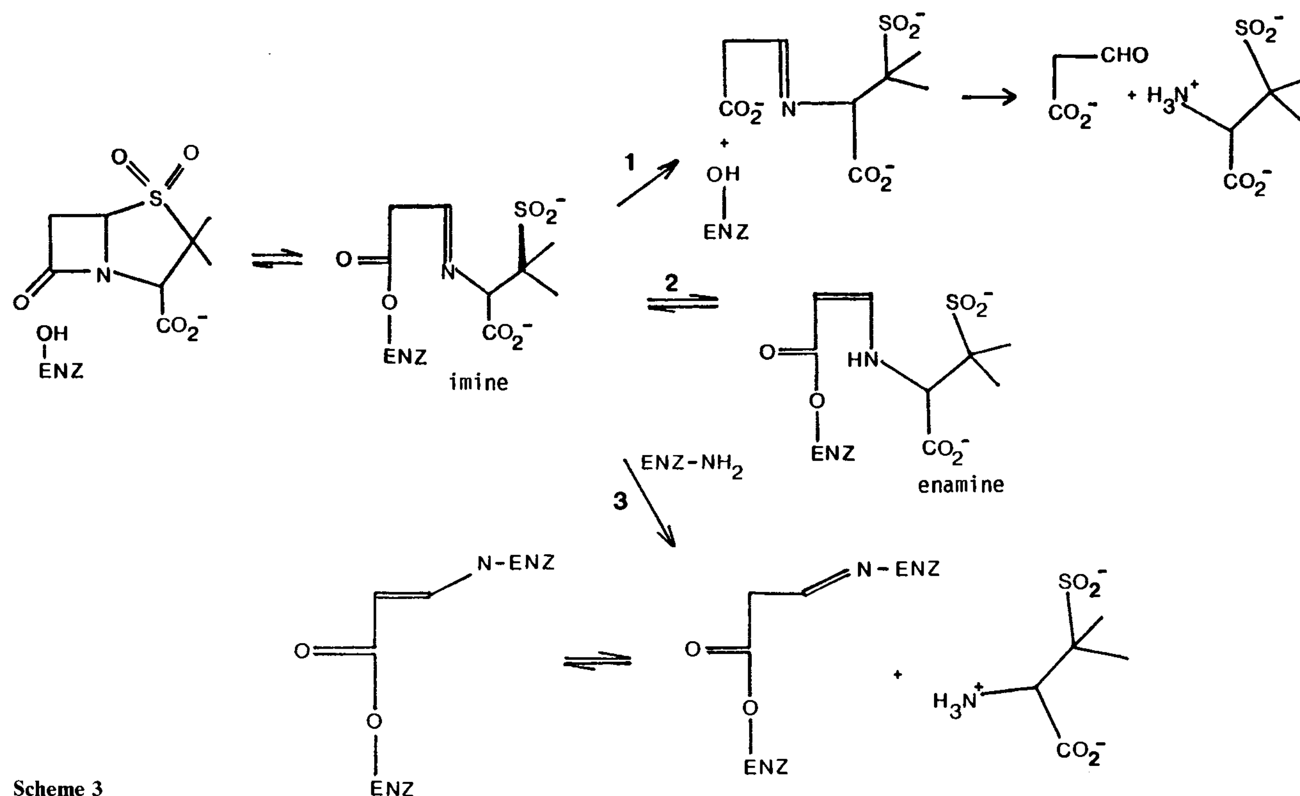
Scheme 2

Typical products are: (1) the corresponding penicilloic acid (P) resulting from hydrolysis of the β-lactam (the normal turnover reaction), (2) a transiently inhibited enzyme species (EA*), and (3) an irreversibly inactivated enzyme product (E_i). The exact details and relative rates of these multiple pathways are determined by the particular combination of inhibitor structure and enzyme.

Currently the best understood case is that of penicillanic acid sulfone (also known as Sulbactam) (63, 69, 70). Its mechanism of inhibition probably typifies that for most inhibitors in which the five-membered ring can readily open by cleavage of the C5-heteroatom bond. Formation of the acyl-enzyme is accompanied by scission of the C5-S bond, due to the sulfinate being a good leaving group, and formation of an imine (Scheme 3).

Hydrolysis of this form of the acyl-enzyme leads to regeneration of the active enzyme and formation of the unstable product which undergoes non-enzymic hydrolysis to malonyl semialdehyde and penicillamine sulfinate. The imine form of the acyl-enzyme can undergo tautomerization to the more stable enamine (β-aminoacrylate), in which conjugation with the carbonyl of the acyl-bond results in stabilization of the acyl-linkage and consequent resistance to hydrolysis. Since the tautomerization equilibrium greatly favors the enamine form, most of the acyl-enzyme (90%) initially ends up in this transiently inhibited form. The enamine form of the acyl-enzyme is characterized by a distinct chromophore at 290 nm.

It is postulated that the irreversible inactivation of the enzyme occurs by transimination of the imine acyl-enzyme by an enzyme group, presumably lysine. Alternatively, a Michael addition on the enamine form could occur. The end result is the same, namely the attachment of part of the inhibitor molecule to a reactive side chain in the active-site region to form a stable linkage. Recent results confirm the expulsion of the penicillamine sulfinate moiety (69).



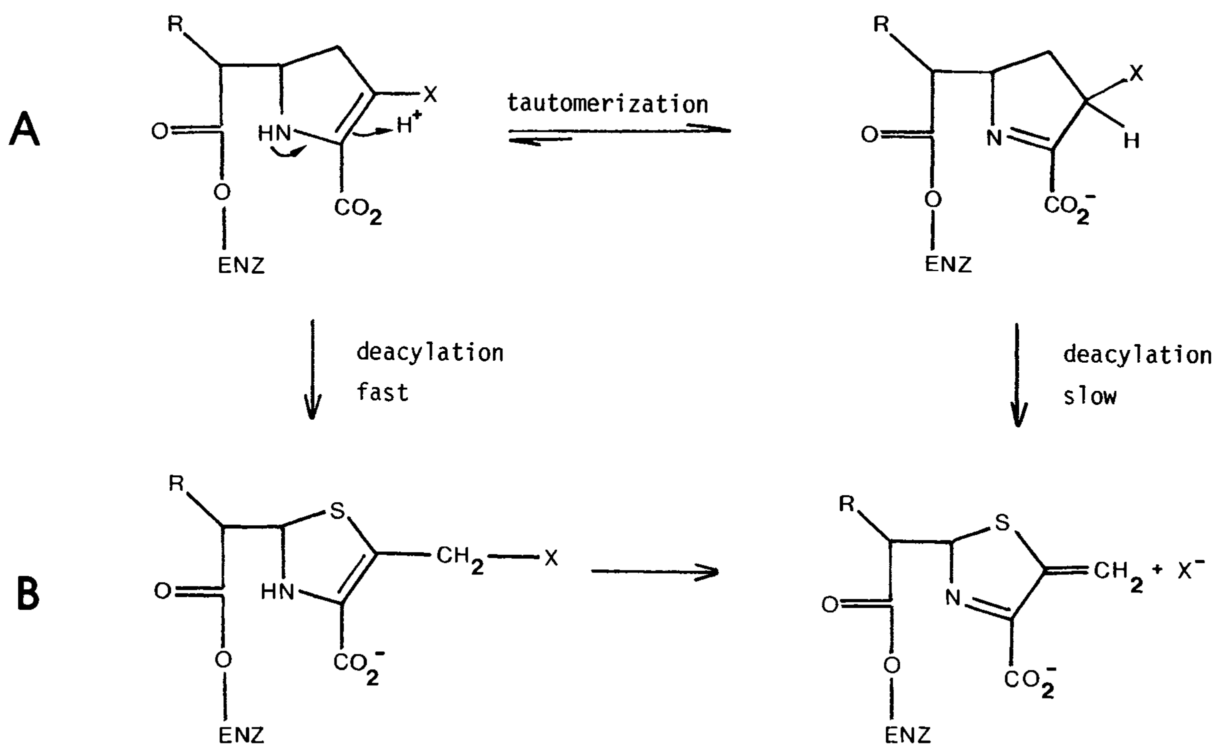
Scheme 3

Additional support for the generality of the postulated mechanism is found in the reported results of inhibition by quinacillin sulfone (62) and 6-β-(trifluoromethanesulfonyl)-amidopenicillanic acid sulfone (71). The inhibitors were covalently linked to the enzyme by Ser-70 in the inactivated species.

In the case of carbapenems such as the thienamycins and cephems with good leaving groups at C3', cleavage of the 5- or 6-membered ring cannot occur. However, the acyl-enzyme initially formed (Scheme 4) can tautomerize from an enamine to an imine (conjugated with the exo-cyclic carboxylate).

lanic acid derivatives (69, 73) have been prepared and found to be inhibitors. A detailed study of 6-(methoxymethylene) penicillanic acid revealed an inhibitory mechanism comparable to that of penicillanic acid sulfone, with the acyl-enzyme partitioning between hydrolysis, transient inhibition and irreversible inactivation (69). The results with 6-(acetylmethylene)penicillanic acid are also consistent with such a pathway (73).

The earliest type of inhibitor of the β-lactamases was the class exemplified by methicillin and cloxacillin, and designated



Scheme 4

For reasons as yet unknown, the imine form of the acyl-enzyme is hydrolyzed much less readily than the enamine form, resulting in transient inhibition of the enzyme. This has been observed both with carbapenems such as the olivanic acids (72) (Scheme 4A) and with cephems such as PADAC (49, 50) (Scheme 4B).

For the 6-β-halopenicillins, such as 6β-bromopenicillanic acid, the enzyme is irreversibly inactivated by formation of a dihydrothiazine (25, 26). The postulated mechanism involves formation of the corresponding acyl-enzyme, which hydrolyzes very slowly. The net result is a relatively long-lived acyl-enzyme. Cleavage of the S1-C5 bond leads to formation of the imine form of the acyl-enzyme and permits the resulting thiolate to displace the bromine. Tautomerization then leads to the more stable enamine form, which is the dihydrothiazine. In this case the vinylogous ester must be very stable in order to lead to the apparent irreversibility of inhibition.

Current views of the mechanism of inhibition by suicide-substrate inhibitors of the β-lactamases indicate that the formation of a delocalized electron system involving the acyl-enzyme linkage is important. There have been some recent reports on the design of potential inhibitors based on this mechanistic consideration. For example, methylenepenicil-

Type A substrates by Citri (74). Inhibition by compounds of this class has been called substrate-induced deactivation, and it appears to usually involve only reversible inhibition. These inhibitors have low values of k_{cat}, typically lower than 6-aminopenicillanic acid. Low values of k_{cat} presumably reflect a long-lived acyl-enzyme intermediate. The formation of a covalent link between the enzyme and the substrate has been demonstrated (30), although the nature of the residue involved has not yet been ascertained. A reasonable mechanistic hypothesis is that the covalent species is an acyl-enzyme of some type. Presumably the acylenzyme initially formed partitions between a hydrolytic pathway and a transient inhibition pathway.

Several reports are consistent with the suggestion that the transient inhibition results from a substantial conformational change in the enzyme at the acyl-enzyme stage. β-Lactamase, transiently inhibited by β-lactams such as cloxacillin or methicillin, becomes more sensitive to the effects of iodine (75), ammonium sulfate (20) and guanidinium chloride (30) than the native enzyme. Hydrogen exchange of the polypeptide amide hydrogens is significantly altered in the presence of methicillin or cloxacillin. Reaction with antibodies or chemical cross-linking of the native enzyme can prevent the substrate

deactivation phenomenon (20, 55). Some of these observations can be interpreted to indicate a substantial change in the mobility or dynamics of the protein, rather than a conformational change. More direct evidence for the involvement of a conformational change comes from preliminary results on the stabilized, isolated inactivated complex formed from cloxacillin and β-lactamase I from *B. cereus* (L. Ellerby and A. L. Fink, unpublished results). In this investigation the circular dichroism spectrum in the far uv region, which reflects the secondary structure, is very different for the inactivated species compared to the native enzyme. This type of inhibition may be a variant of the general scheme of inhibition (Scheme 2), in which the rate of inactivation by pathway 3 is negligible.

It should be noted that there are significant differences in the interaction of a given inhibitor or inactivator with different β-lactamases. Consequently, the rates associated with different steps (1–3) in the general mechanism for inhibition will differ sufficiently so that a compound that is a good inhibitor for one enzyme may be a poor substrate for another (76).

Conclusions

During the last few years significant progress has been made toward understanding the molecular basis of the mechanisms of catalysis and inhibition of the β-lactamases, and to a lesser extent of the related cell wall transpeptidases and carboxypeptidases. The presence of an essential serine residue and its role in the formation of an acyl-enzyme intermediate is now well established. The phenomenological basis of reversible and irreversible inhibition is becoming much clearer, although the underlying physical-chemical basis for the events is still unknown. Although we now have enough understanding to make some rational, mechanism-based, choices in designing β-lactamase inhibitors, it is apparent that much more work is required at the molecular level. One point of interest concerns the relationship between the *in vitro* effects of an inhibitor or inactivator, and its *in vivo* action. A recent study (77) has shown that the effects of clavulanate on growing cultures of *E. coli* are kinetically consistent with expectations based on kinectic properties of the isolated clavulanate-β-lactamase system.

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Drug Release from Suppositories[1]

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Abstract: Many studies have been carried out over the past decades on suppositories and their drug release properties. The present state of knowledge is reviewed with the conclusion that our current understanding of the *in vivo* performance of suppositories is deficient. It is therefore not possible to rely on *in vitro* data to predict *in vivo* performance. Suggestions are presented for future studies that are required to enhance our knowledge of suppositiories as drug delivery systems.

Suppositories have been in use as drug dosage forms for a very long time and have been the subject of research ever since. Especially in the past decade basic research has been carried out resulting in an impressive increase in knowledge regarding this system and its application. Since *in vivo* performance still seems rather unpredictable, e.g. with regard to spreading behavior in combination with first pass metabolism, the question remains whether there is still a role for suppositories in modern drug therapy. This is essential with the appearance of sophisticated controlled release systems with many attractive features. The answer to such a question will also have to take into account the cost-benefit ratio and the expected progress in resolving the most crucial remaining uncertainties for the system in question.

In this paper the present status of (suspension) suppositories is discussed, with special emphasis on gaps in our current knowledge. An attempt is made to analyze future research directions and the progress to be expected from this research.

General Aspects

In 1973 Bevernage and Polderman (1) published an overview of the factors involved in the release of drugs from suppositories. This overview is reproduced in Table I.

As a follow up Polderman and his coworkers have published a widely known schematic description of the vicissitudes of a

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[1] On the occasion of the 70th birthday of Prof. J. Polderman.

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