

## Crystallographic studies of the $\beta$ -lactamases from *B. cereus*

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**Abstract** - The Gram-positive bacterium *B. cereus* produces both  $\beta$ -lactamase I, a class A serine enzyme, and  $\beta$ -lactamase II, a zinc dependent enzyme of class B. Crystallographic studies of these two enzymes have shown that  $\beta$ -lactamase I is homologous with the penicillin-sensitive D-alanyl-D-alanine carboxypeptidase-transpeptidase of *Streptomyces* R61 while  $\beta$ -lactamase II has a hitherto unknown structure. New methods of studying enzyme activity crystallographically promise to illuminate the mechanisms of action of these enzymes.

### INTRODUCTION

Alexander Fleming discovered both lysozyme and penicillin (ref. 1,2) and it is intriguing and significant that the sites of action of these two discoveries can be identified on a single diagram showing the chemical structure of a bacterial cell wall.

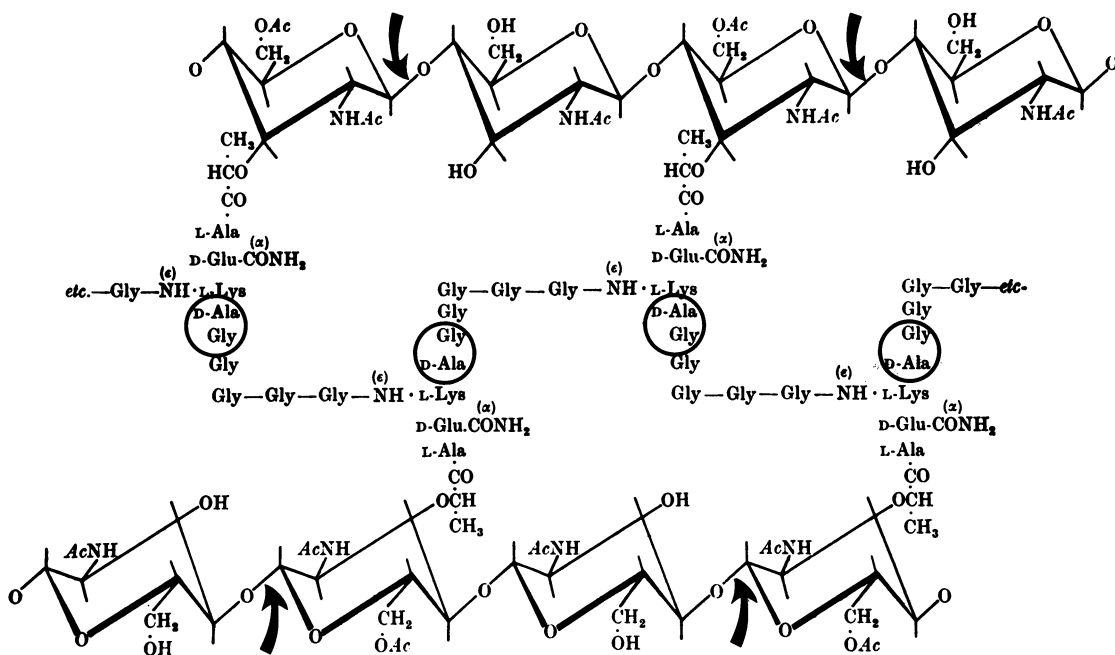


Fig. 1. Chemical structure of cell wall of *B. aureus* (ref. 3) with sites of action of lysozyme and penicillin indicated respectively by arrows and circles (cf. Ivanov, this Symposium).

Lysozyme promotes the hydrolysis of the  $\beta(1-4)$  glycosidic linkage between N-acetyl muramyl and N-acetyl-glucosaminyll sugar residues in the oligosaccharide component of the cell wall. Penicillin inhibits enzymes involved in the final stages of cell-wall synthesis - in particular the transpeptidases that promote the formation of peptide links between the N terminal glycine and C terminal D-Alanyl-D-Alanine residues of oligopeptide chains, with the release of D-Alanine. The activity of penicillin is believed to be based upon its structural and chemical resemblance to the intermediate formed in this reaction (ref. 4).

The mode of action of penicillin was, of course, unknown to Fleming and to the Oxford workers led by Florey who developed a promising observation into a useful antibiotic. Indeed penicillin must be included as an outstanding example among discoveries that have been useful before they have been understood and have presented a challenge to scientific research that has led to an advance in fundamental understanding.

Even as the susceptibility of micro-organisms to the action of penicillin was being studied in Oxford in 1940 (ref. 5), Abraham and Chain observed that bacteria developed resistance to the antibiotic and they attributed this resistance to the production of a bacterial enzyme that promoted its inactivation (ref. 6). They named this enzyme penicillinase: it was the first observation of what is now known to be a heterogeneous group of enzymes that promote the hydrolysis of a wide range of  $\beta$ -lactam antibiotics, the  $\beta$ -lactamases.

### $\beta$ -LACTAMASES

The  $\beta$ -lactamases catalyse the hydrolysis of the four-membered  $\beta$ -lactam ring in penicillins, cephalosporins and related compounds to give products that are antibiotically inactive.

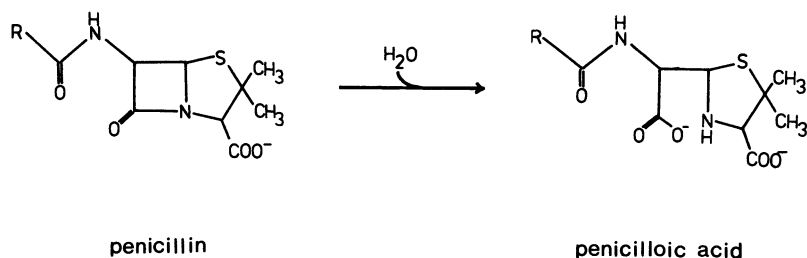


Fig. 2. The hydrolysis of a  $\beta$ -lactam that is catalysed by  $\beta$ -lactamases.

They are produced by a wide range of bacteria, both Gram-positive and Gram-negative, and are expressed both as membrane-bound and as exocellular enzymes. Various methods of classifying them have been proposed (ref. 7) of which the most appropriate here is that based on chemical analysis suggested by Ambler (ref. 8). Three classes have been identified.

Class A enzymes have been characterized from the Gram-positive species *B. cereus*, *B. licheniformis* and *S. aureus* and from the Gram-negative *E. coli*. They have clearly homologous amino-acid sequences and include a conserved serine residue that is involved in the catalytic mechanism.

Class C  $\beta$ -lactamases have been identified in a variety of Gram-negative organisms. They are also serine enzymes but sequence comparisons suggest that they are only very distantly, if at all, related to Class A.

*B. cereus* also produces a zinc-dependent  $\beta$ -lactamase II (ref. 9) that has been assigned, as the only member yet identified, to class B.

Tipper and Strominger (ref. 10) suggested the  $\beta$ -lactamases might have evolved from penicillin target proteins and this idea was reinforced by the discovery that many proteins of both type include active serine residues. Detailed sequence comparisons, however, have failed to find convincing evidence for homology (ref. 11) though there are some similarities of sequence especially on the N-terminal side of the active serine. Sequences are compared in Table 1.

TABLE 1. Amino-acid sequences near the active serine in  $\beta$ -lactamases and penicillin-target proteins.

$\beta$ -lactamases class A	50	60	70	
<i>B. cereus</i> 569/H	DARLGVY AID	TGTNQT- ISY	RPNERFAFAS	TYKAL
<i>B. licheniformis</i>	DAKLGIFALD	TGTNRT-VAY	RPDERFAFAS	TIKAL
<i>S. aureus</i>	NAHIGVYALD	TKSGKE-VKF	NSDKRFAYAS	TSKAI
<i>E. coli</i> RTEM	GARVGYIELD	LNSGKILESF	RPEERFPMMS	TFKVL
$\beta$ -lactamase class C				
<i>E. coli</i> AmpC	QGKPYFTWG	YADIAKKQPV	TQQLFELGS	VSKTF
Penicillin target proteins				
<i>E. coli</i> PBP5	DAE-SYILID	YNSGKVLAEQ	NADVRRDPAS	LTKMM
<i>B. subtilis</i> CPase	NAS-AAIMIE	ASSGKILYSK	NADKRLPIAS	MTKMM
<i>Streptomyces</i> R61 CPase			VGS	VTK

It is well known from crystallographic studies of a variety of proteins that resemblances between the three dimensional structures of homologous proteins persist when the relationship between their amino-acid sequences has effectively vanished (ref. 12). One motive for crystallographic studies of these enzymes, therefore, is to illuminate the evolutionary relationships between them. Others are to characterize the active sites of the enzymes, to define as closely as possible their specificities and the structural constraints upon their catalytic mechanisms, and to observe the structures of enzyme-inhibitor complexes and of intermediates on the reaction pathways.

### $\beta$ -LACTAMASE II FROM *B. CEREUS*

The amino-acid sequence of  $\beta$ -lactamase II (Table 2) has been determined from the DNA sequence and from direct analysis of the protein (ref. 7).

TABLE 2. Amino acid sequence of  $\beta$ -lactamase II. The sequence is numbered from the observed N-terminal residue of the secreted enzyme. Zinc ligands are marked by \* and the glutamic acid residue implicated in the activity by +.

-30	MKKNTLLKVG	LCVGLLGTIQ	FVSTISSVQA	<u>1</u>	SQKVEKTVIK	NETGTISISQ
21	LNKNVWHTE	LGSFN <sup>+</sup> GEAVP	SNGLVLNTSK	GLVLVDSSWD	DKLTKELIEM	
71	VEKFKQRVT	DVIITH <sup>**</sup> AHAD	RIGGIKTLKE	RGIKAHSTAL	TAELAKNGY	
121	EEPLGDLQTV	TNLKFGNMKV	ETFYPGKGHT	EDNIVVWLPQ	YNILVGG <sup>*</sup> CLV	
171	KSTSADLGN	VADAYVNEWS	TSIENVLKRY	RNINAVVPGH <sup>*</sup>	GEVGDKGLLL	
221	HTL <sup>227</sup> DLLK					

The essential zinc is liganded by three histidine residues 86, 88 and 210 (ref. 13) and cysteine 168 may also be involved (ref. 14).

Crystallographic studies were long frustrated by the difficulty of preparing stable and reproducible crystals but this problem was solved in 1985 when Professor Clive Little, a visitor from the University of Tromsø to Stephen Waley's laboratory in Oxford, showed that good crystals were obtained when the zinc was replaced by cadmium. These crystals had cell dimensions  $a = 54.4$ ,  $b = 63.8$ ,  $c = 70.9$  Å and  $\beta = 93.6^\circ$  with space group C2. When cross linked with glutaraldehyde they proved stable to treatment by various heavy-atom reagents and tolerated removal of the cadmium by treatment with EDTA. Thanks to the efforts of Drs. B.J. Sutton, A. Cordero-Borboa and P.J. Artymiuk, crystallographic analysis by the method of multiple isomorphous replacement proceeded smoothly and has provided interpretable electron density maps at 6, 4.5 and 3.5 Å resolution. At 6 Å resolution the boundary of the molecule was clear; at 4.5 the cadmium atom was the strongest feature and the course of the polypeptide could be traced; and at 3.5 Å resolution many of the side chains could be identified. Figure 3 shows a part of the map at 3.5 Å resolution in which the density corresponding to cadmium and two of the histidine ligands can be clearly seen.

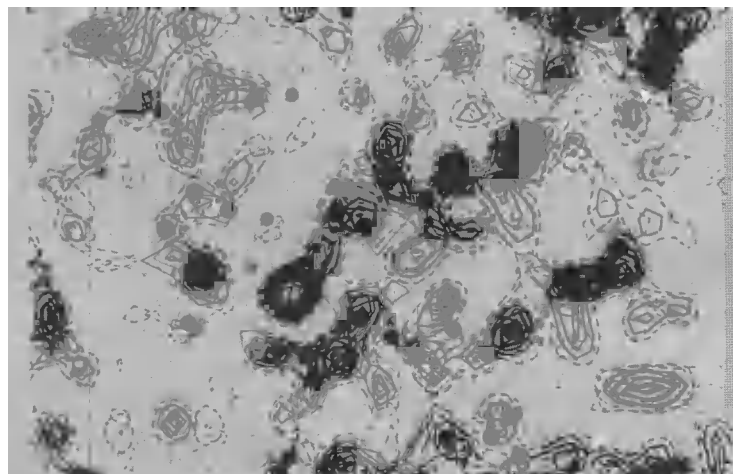


Fig. 3. Part of the electron density in  $\beta$ -lactamase II at 3.5 Å resolution. The strong central feature corresponds to the cadmium atom. Markers indicate the positions of Ca atoms.

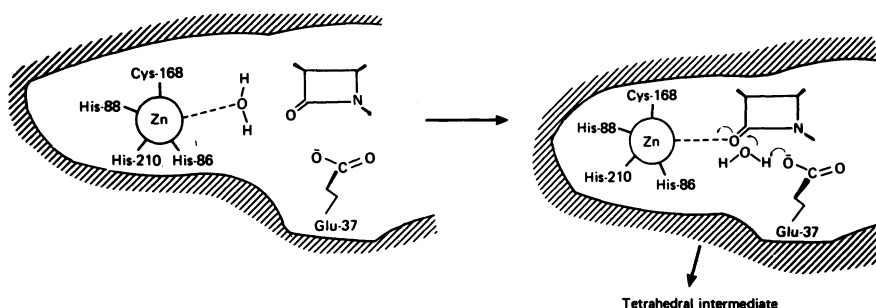


Fig. 4. Hypothetical outline mechanism for  $\beta$ -lactamase II (ref. 15)

Detailed studies of the activity and inactivation of  $\beta$ -lactamase II are not yet well developed but Waley and his colleagues have recently (ref. 15) shown that reaction with a water-soluble carbodi-imide and a nucleophile inactivated the enzyme and labelled glutamic acid residue 37. They concluded that this residue is catalytically essential and proposed the outline mechanism indicated in Figure 5 where it is suggested that a water molecule bound to the catalytic  $Zn^{2+}$  attacks the  $\beta$ -lactam carbonyl group, with glutamate 37 acting as a general base deprotonating the water molecule. Subsequently the proton may be donated to the nitrogen atom of the  $\beta$ -lactam ring.

Preliminary interpretation of the latest electron-density map provides some support for this hypothesis. Figure 5 shows the arrangement of the polypeptide chain in the vicinity of  $Cd^{2+}$ , as seen at 3.5 Å resolution. Glutamate 37 appears to lie in a shallow depression at some distance from the metal as suggested by Waley and his colleagues.

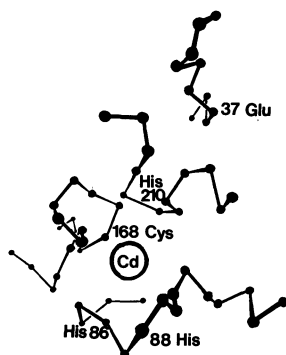


Fig. 5. Arrangement of protein chain in the vicinity of the metal site in  $\beta$ -lactamase II as determined from the electron-density map at 3.5 Å resolution.

Further details must await full interpretation of the electron-density map and refinement of the structure. At that stage it will also be possible to study the association of enzyme and substrates in the crystal, by methods discussed below. Happily preliminary experiments on the hydrolysis of nitrocefin, a chromophoric substrate that changes colour from yellow to red on hydrolysis, have shown already that the enzyme is active in the crystals.

The overall arrangement of the polypeptide chain in  $\beta$ -lactamase II is not yet fully defined but it appears to include two short  $\alpha$ -helices together with some  $\beta$ -pleated sheet. The initial impression is that the proportion of regular secondary structure is low. There is no marked similarity to any other known structure and, in particular, no clear resemblance to the zinc-containing D-alanyl-D-alanine cleaving carboxypeptidase from *Streptomyces albus*, the structure of which is known (ref. 16). This is not entirely surprising since the carboxypeptidase is known not to be penicillin sensitive. The zinc of carbonic anhydrase also has three histidine ligands, two of which are next nearest neighbours in the protein chain, but any other resemblance to  $\beta$ -lactamase II seems slight. At present, therefore, the three dimensional structure of  $\beta$ -lactamase II appears to be providing no indication of its evolutionary origin.

### $\beta$ -LACTAMASE I FROM *B. CEREBUS*

Crystallographic studies of the class A  $\beta$ -lactamases have been in progress for many years with singularly little result until this year. Crystallographic data have been published on the enzymes from the four bacterial species listed in Table 1 (ref. 7) and preliminary descriptions of the homologous enzymes from *B. cereus* 569/H (ref. 17) and *B. licheniformis* (ref. 18) have now been published.

Crystals of  $\beta$ -lactamase I from *B. cereus* are very small thin plates (ref. 19). The analysis of their structure has depended upon the availability of synchrotron radiation at the Daresbury Laboratory of the SERC, UK and at Le laboratoire pour l'utilisation du Rayonnement

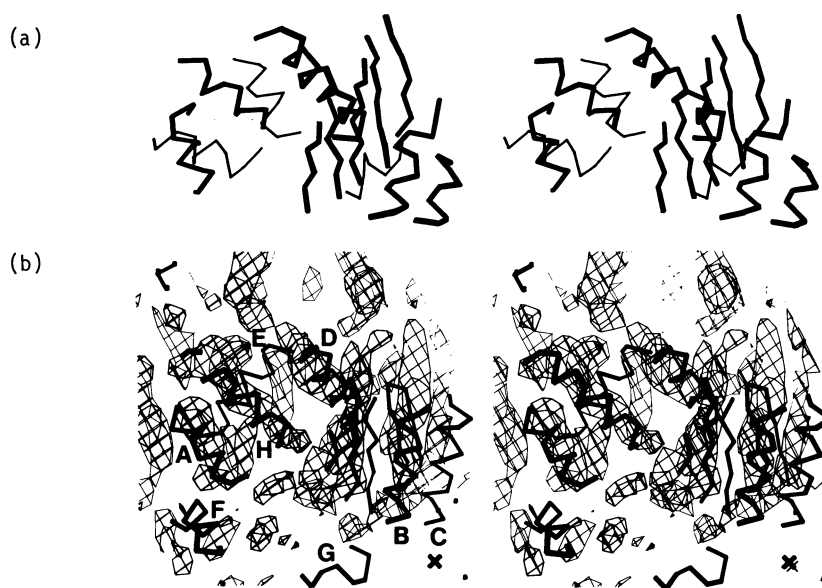


Fig. 6. (a) the secondary structure elements of  $\beta$ -lactamase I. Those in faint line do not correspond to features in the R61 CPase. (b) a stereo view of part of the electron-density map of  $\beta$ -lactamase I at 6 Å resolution with secondary structure elements of the R61 CPase superimposed. There is no density in the map which corresponds to helices E, F, G of the R61 CPase but helix H is present.

Electromagnetique, CNRS, France, a splendid example of the way in which crystallography and chemistry are becoming "big" sciences dependent upon the provision of major facilities and international cooperation.

The crystals have unit cell dimensions  $a = 143.0$ ,  $b = 35.8$ ,  $c = 57.2$  Å and  $\beta = 97.8^\circ$  in space group C2 and their structure has been analysed at 6 and at 2.5 Å resolution by the method of multiple isomorphous replacement. At 6 Å resolution the boundary of the molecule was clear and rod-like features within it suggested the presence of some eight  $\alpha$ -helices (Figure 6). The map at 2.5 Å resolution, when improved by Wang's method of solvent-density modification (ref. 19), revealed the greater part of the structure of the molecule, and provided the basis for the iterative process of map interpretation, restrained least-squares refinement, phase calculation and map recalculation that is now being used to refine the description of the molecule.

The initial interpretation of the modified electron density map showed immediately that the arrangement of secondary-structure elements in  $\beta$ -lactamase I closely resembles that in the penicillin sensitive D-Ala-D-Ala carboxypeptidase from *Streptomyces R-61* (R-61 CPase) which has been described by Kelly *et al.* (ref. 20).

In  $\beta$ -lactamase I, there is a five stranded  $\beta$ -pleated sheet with three helices on one side and five on the other (Figure 6a). Five of these helices have counterparts in the R61 CPase (Figure 6b) which also has three helices not present in  $\beta$ -lactamase I. These three helices (E, F and G in the current description of the structure of R61 enzyme) are close to the carboxyl end of the molecule so that this result is in accord with the prediction of Kelly *et al.* (ref. 20) that  $\beta$ -lactamases of class A would lack a part of the carboxy-terminal domain of the R61-CPase. Curiously, however, the carboxy-terminal helix H of the R61 enzyme is present in  $\beta$ -lactamase I.

This discovery, which is confirmed by the parallel studies of the class A  $\beta$ -lactamase from *B. licheniformis* (ref. 18), that a class A  $\beta$ -lactamase and a penicillin-sensitive D-Ala-D-Ala carboxypeptidase-transpeptidase have an extensive region of common tertiary structure suggests very strongly that the two groups of enzymes have evolved by divergence from a common ancestor. As we have seen, this idea was mooted earlier but, apart from the fact that the R61 GPase and  $\beta$ -lactamases I are both serine enzymes, the available sequence data appear to show no strong relationship between them. The resemblance between their tertiary structures may therefore provide a further example of the persistence of similarities between three dimensional structures when the similarity between primary structures has disappeared (ref. 12). More detailed comparison must await the availability of refined structures but it is interesting to note the further speculation of Kelly *et al.* (ref. 20) that the carboxyl terminal domain of the R61 CPase may be concerned with binding of the enzyme to the cell wall peptidoglycan (Figure 1). It would be remarkable if this part of the R61 CPase resembled lysozyme.

Crystallographic studies of the activity of  $\beta$ -lactamase I are still at an early stage but experiments with nitrocefin have indicated that the enzyme is active in the crystals and experiments with the inactivator 6- $\beta$ -bromo-penicillanic acid (ref. 21) have located the active site of the enzyme and point the way to more detailed studies.

The exciting feature of inactivators such as 6- $\beta$ -bromo-penicillanic acid is that their interaction with the class A  $\beta$ -lactamases is complex, involving not only specific binding to the enzyme and the formation of an acyl intermediate at the active serine but also additional chemical reactions that may be catalysed by the enzyme (Fig. 7). 6- $\beta$ -Bromo-penicillanic acid undergoes reactions which lead to the formation of a product, possibly a dihydrothiazine (ref. 22), which is resistant to deacylation.

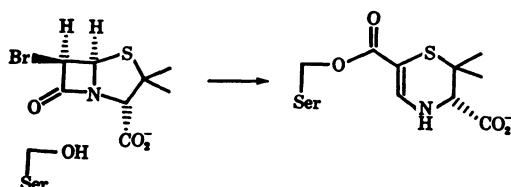


Fig. 7. Interaction of 6- $\beta$ -bromo-penicillanic acid with  $\beta$ -lactamase I with proposed dehydrothiazine product, resistant to deacylation (ref. 22).

A crystallographic study of this interaction shows clearly an increase in electron density corresponding to the bound product. Its location is shown in Figure 8 in relation to the latest model of the polypeptide chain. This result shows that the active site of  $\beta$ -lactamase I lies in a pocket that has helix A (Figure 6) on one side and helix H at its base.



Fig. 8. Course of the polypeptide chain in  $\beta$ -lactamase I with location of active site indicated by van der Waals envelope of inactivator derived from 6- $\beta$ -bromo-penicillanic acid.

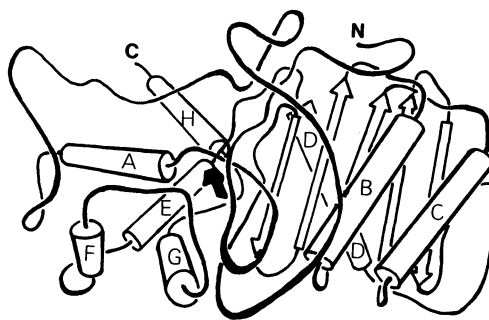


Fig. 9. Course of the polypeptide chain in R61 CPase, showing location of  $\beta$ -lactam binding site (from ref. 20).

Comparison with Figure 9 again shows the clear relationship in overall structure and active-site location between  $\beta$ -lactamase I and the R61 carboxypeptidase-transpeptidase.

#### PROSPECTS FOR CRYSTALLOGRAPHIC STUDIES OF $\beta$ -LACTAMASE ACTIVITY

The difficulty with crystallographic studies of enzyme activity is that the X-ray observations required to synthesize the image of a structure take a long time while catalysed reactions take a relatively short time. Clearly there are two approaches to closing the gap: the reactions can be slowed down and the measurements speeded up. Both of these approaches are being followed with increasing success and a brief description of two different studies will serve to show what can be done.

First, it is very well known that enzyme catalysed reactions can be slowed down very significantly by relatively modest reduction in temperature. Douzou and his colleagues (ref. 23) have pioneered the use of this approach and have described cryosolvents, especially methanol-water mixtures, that can be used to study reactions in enzyme crystals. Some ten years ago, Petsko and his colleagues (ref. 24) used this method to observe the acyl-enzyme intermediate in the hydrolysis of *N*-carbobenzoxy-L-alanyl-*p*-nitrophenol ester that is catalysed by porcine pancreatic elastase. The acyl-enzyme intermediate was stabilized at  $-55^{\circ}\text{C}$  in a mixture of 70% methanol - 30% water and deacylation was observed to take place when the temperature was raised.

This experiment, which has yet to be extended to high resolution, indicated clearly the potential of low-temperature methods in crystallographic studies of enzyme activity. It is also amusing to note its new-found relevance to studies of  $\beta$ -lactam sensitive enzymes. Modified cephalosporins have been shown recently (ref. 25) to inhibit human leukocyte elastase and crystallographic studies (ref. 26) have shown the detailed structure of porcine pancreatic elastase inactivated by a cephalosporin sulphone.

The second approach to observing intermediates in enzyme-catalysed reactions is to speed up the measurements and this is being facilitated by the use of synchrotron radiation. Current methods of data collection permit the recording of diffraction data in less than one hour from crystals of large proteins and this has been exploited by Louise Johnson and her colleagues in Oxford (ref. 27) in a study of the phosphorylation of heptenitol to form heptulose-2-phosphate that is catalysed by rabbit muscle phosphorylase. By judicious selection of reaction conditions it was possible to observe the build up of heptulose-2-phosphate as the reaction progressed.

The prospects for this kind of experiment are very much improved by the latest developments in rapid data collection by means of synchrotron radiation. The current method employs highly monochromatic X-rays that are isolated from the continuous spectrum by the use of a crystal monochromator. Recording the complete diffraction pattern characteristically requires some 50 photographs taken with the crystal in a variety of orientations and with exposure times of many minutes. Use of polychromatic X-rays, with wavelengths ranging from 0.5 to 2.5 Å, however, permits the recording of a large proportion of the diffraction pattern on a single photograph. Such Laue photographs of phosphorylase crystals (ref. 28) record some 70% of the diffraction data, quite enough for a clear image of the structure at 2 Å resolution, in exposure times of 250 mseconds.

Clearly, the use of this Laue method of recording diffraction data combined with modestly low temperatures opens the way to direct crystallographic observation of a wide range of enzyme complexes with substrates, intermediates and products. In studies of  $\beta$ -lactamase complexes with inactivators such as penicillanic acid sulphone (ref. 29), for example, (Figure 10) it might well be possible to observe the initial complex, the initial acyl-enzyme intermediate, the contribution of the more stable acyl enzyme produced by tautomerization to the  $\beta$ -amino acrylate ester, and the relatively slow development of permanent inactivation.

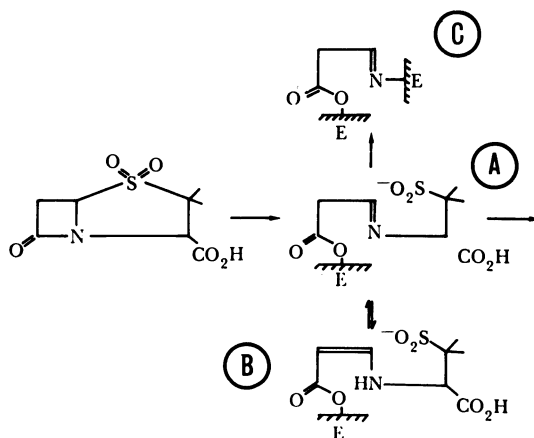


Fig. 10. Inactivation of  $\beta$ -lactamase by penicillanic acid sulphone (ref. 29), with acyl enzyme complex leading to hydrolysis products (A); tautomerization to a stable acyl enzyme (B); and irreversible inactivation through reaction with second functional group on the enzyme (C).

### Acknowledgements

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