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Active sites of β-lactamases from *Bacillus cereus*

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There are two extracellular β-lactamases produced by *Bacillus cereus* 569. One of these enzymes, β-lactamase I, is inactivated by 6-β-bromopenicillanic acid: the site of reaction is serine-44. This is a conserved amino acid residue in the other β-lactamases whose structures have been determined, and it becomes a good candidate for an active-site group in these enzymes. The inactivation may involve a rearrangement leading to a dihydrothiazine. The other extracellular enzyme produced by *B. cereus*, β-lactamase II, is exceptional in requiring metal ions for activity. The Zn II and Co II enzymes (the former is more active) have been studied by nuclear magnetic resonance, and by absorption spectroscopy. The groups that bind the metal ion required for activity are three histidine residues and the enzyme's sole thiol group.

Introduction

Central among the interests of those concerned with molecular or catalytic aspects of enzymes is the study of the active site, where these aspects fuse. Although β-lactamases have been known for nearly 40 years, it is only now that structurally explicit suggestions can be put forward for components of the active sites of any of these enzymes. One of the first β-lactamases (recognized as a penicillin-destroying enzyme) to be investigated was from *Bacillus cereus*, and since then it has been much studied (table 1).

Bacillus cereus 569 produces two extracellular β-lactamases, now known as β-lactamase I and β-lactamase II (Kuwabara & Abraham 1967). The production of two distinct β-lactamases is uncommon, but *B. cereus* 569 is by no means unique; for example, some clinical isolates of *Yersinia enterolitica* produce two β-lactamases (Cornelis & Abraham 1975), as do some resistant strains of *Escherichia coli* and *Providencia stuartii* (Letarte *et al.* 1978). Although β-lactamase I and β-lactamase II can be discussed together from some points of view, work on their active sites is more profitably described separately; there is so far no known common feature in the active sites of the two β-lactamases.

β-Lactamase I

Group modification of β-lactamase I has led to several, somewhat tentative, conclusions. Histidine residues seem unlikely to play any part in the enzyme's action (Ferencz *et al.* 1971), and indeed there are no conserved histidine residues in the amino sequences reported by Ambler (1979). Carboxyl groups, on the other hand, may play a part (Patil & Day 1973; Waley 1975; Durkin *et al.* 1977). This earlier work, which has been reviewed by Abraham & Waley (1979), is overshadowed by the recent finding of some effective inactivators.

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TABLE 1. LANDMARKS IN THE STUDY OF THE ACTIVE SITES OF β-LACTAMASE I AND β-LACTAMASE II

	β-*Lactamase I*		β-*Lactamase II*
1945	Benedict *et al.* Penicillinase from *Bacillus cereus* NRRL B-569		
1956	Kogut *et al.* Purification of penicillinase from *B. cereus*	1956	Newton & Abraham 'Cephalosporinase' in *B. cereus*
1966		1966	Sabath & Abraham Zinc as cofactor
1968		1968	Sabath & Finland Thiol-group binding of zinc
1974	Davies *et al.* Purification of β-lactamase I and β-lactamase II	1974	Davies & Abraham Co II can replace Zn II
1975	Thatcher Partial amino acid sequence of β-lactamase I		

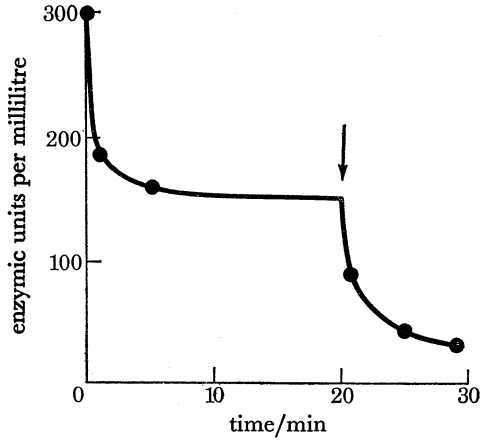
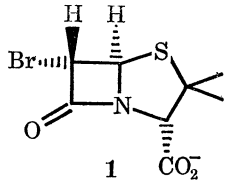


FIGURE 1. Incomplete inactivation of β-lactamase I at pH 7 by a preparation of 6-α-bromopenicillanic ac Portions (100 nmol) of reagent were added to 0.5 ml of buffer, 0.1 M NaH_2PO_4–0.1 M-K_2HPO_4, contain 24 μg (about 0.8 nmol) of enzyme at 20 °C; 5 μl portions were taken for assay. The arrow marks the time at which fresh reagent was added.

Inactivation with 6-bromopenicillanic acid

Our first experiments with preparations of 6-α-bromopenicillanic acid (**1**) gave puzzling results. The enzyme was rapidly inactivated at pH 7, but inactivation was incomplete. Addition of fresh reagent brought about further inactivation (figure 1) yet analysis failed to detect any



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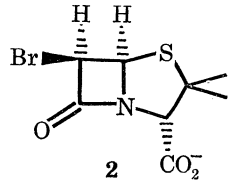

2

decomposition of the reagent. Possibly, then, the 6-α-bromopenicillanic acid was not itself inactivating (nor was it a substrate); instead perhaps the preparation contained a small amount of a powerful inactivator. At a higher pH (9.2), the reagent did inactivate the enzyme completely. Yet here too there were unexpected features. The rate of inactivation did not depend on the concentration of enzyme, only on the concentration of reagent. The inactivator was continually being generated from the reagent at pH 9.2; in fact, this is the rate-determining step. We surmised that the process of generating inactivator might be epimerization: the true inactivator would then be the β-epimer (**2**). Indeed, a solution of the 6-α-bromo compound (**1**)

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at pH 9.2 gradually produced a new compound with properties consistent with those expected for the 6-β epimer (**2**) (Knott-Hunziker *et al.* 1979*a*). Now we can understand the previously puzzling kinetics of inactivation. At this time we learnt that Pratt & Loosemore (1978) had independently established the two important points: **1** could be epimerized to give a mixture containing approximately 10 % **2** (Loosemore & Pratt 1978), and **2** was the effective inactivator of β-lactamase I. We have obtained a mixture containing approximately 54 % **2** by reduction of 6,6-dibromopenicillanic acid with tributyltin hydride, but pure **2** has yet to be prepared.

The β-epimer (**2**) reacted stoichiometrically with β-lactamase I (Pratt & Loosemore 1978), with a rate constant of the order of 10^6 M^{-1} min^{-1} (Knott-Hunziker *et al.* 1979*a*), a value not much less than that of k_{cat}/K_m for 6-aminopenicillanic acid (10^7 M^{-1} min^{-1}; Waley 1974). The rate of inactivation, moreover, was decreased when substrate (benzylpenicillin) was present. These experiments encouraged the hope that 6-β-bromopenicillanic acid (**2**) was reacting at the active site of β-lactamase I with the formation of a covalent bond. The next step was to test this idea. For this, a radioactive reagent was required.

The site labelled by 6-β-*bromopenicillanic acid*

6-β-Aminopenicillanic acid labelled with ^{3}H in its 2-β-methyl group (Usher *et al.* 1975) was used to prepare radioactive 6-α-bromopenicillanic acid, which was then converted into the mixture containing some of the β-epimer. This was used to inactivate the enzyme; after gel filtration the radioactivity of the protein corresponded to the incorporation of about one molar proportion of radioactivity (Knott-Hunziker *et al.* 1979*a*). These results suggested that inactivation was a consequence of covalent modification. The next step was to identify the site that was labelled.

The labelled enzyme was treated with trypsin, and the tryptic digest fractionated by paper chromatography and paper electrophoresis. The radioactivity was largely associated with one peptide, which was identified as peptide T7 from the work of Thatcher (1975). The sequence of this peptide is: Phe-Ala-Phe-Ala-Ser-Thr-Tyr-Lys and it comprises residues 40–47. This peptide has several residues capable of being modified by the reagent. Digestion with aminopeptidase M provided evidence on which residue had been labelled. The digest contained a radioactive compound which, after acid hydrolysis, liberated serine as the sole amino acid. Hence the site labelled is serine-44 (Knott-Hunziker *et al.* 1979*b*). Since serine-44 is a conserved residue in the sequences of the β-lactamases that have been studied it must be regarded as a good candidate for an active site group in these enzymes.

Serine 44 in β-lactamase I may be regarded as the counterpart of the serine residue in the DD-carboxypeptidase–transpeptidase from *Streptomyces* R61 (Frère *et al.* 1976), and that in the D-alanine carboxypeptidase of *Bacillus subtilis* (Georgopapadakou *et al.* 1977); these serine residues react with benzylpenicillin. When more of the sequences around these serine residues have been determined, it will be interesting to see whether there are similarities to the sequence around serine-44 in β-lactamase I.

There is little evidence about other active site groups that might cooperate with serine-44. As mentioned above, carboxyl groups are a possibility but imidazole groups seem unlikely. Serine proteinases, of course, have both carboxyl and imidazole groups that cooperate with the serine residue in the active site. It seems likely to be more than a coincidence that benzeneboronic acid is an inhibitor of β-lactamases I (Kiener & Waley 1978), as it is of several serine proteinases.

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Mechanism of inactivation

The main clue to the nature of the reaction between β-lactamase I and 6-β-bromopenicillanic acid lay in a new peak in the ultraviolet absorption spectrum (figure 2). The position of the maximum shifted from 326 to 314 nm when the protein was denatured (Knott-Hunziker *et al.* 1979*b*). The absorption at 314 nm, and the structure of the reagent, call to mind the conversion of 6-α-chloropenicillanic acid into a dihydrothiazine (**3**) (McMillan & Stoodley 1968).

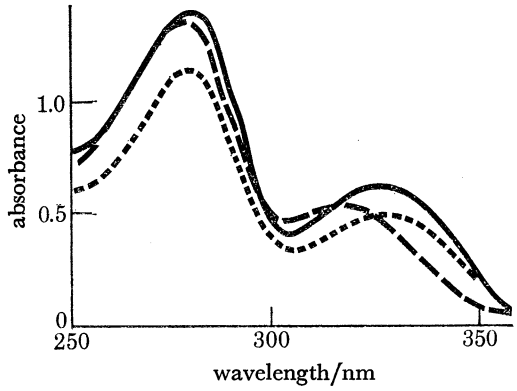
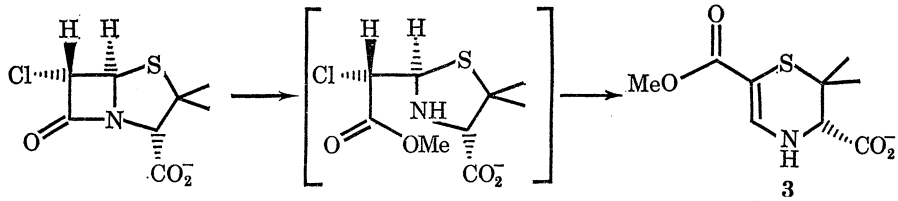
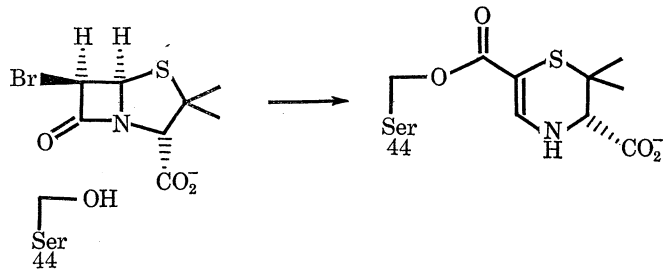


FIGURE 2. Absorption spectra of β-lactamase I inactivated by 6-β-bromopenicillanic acid. About 30 mg of enzyme in 3 ml of 0.5 M NaCl, 1 mM EDTA was treated with 1.5 mg of a mixture containing 50% of 6-β-bromopenicillanic acid: inactivation was virtually complete in a few minutes. The solution was dialysed and freeze-dried. The absorption spectrum was measured on a solution (1.5 mg/ml) in 1% NH_4HCO_3 (——), on the solution made 3 M in guanidinium chloride (– – –), and finally on the solution dialysed to remove the denaturant (- - -).



We suspect that a dihydrothiazine is also formed when 6-β-bromopenicillanic acid reacts with β-lactamase I:



This suggestion accommodates the finding that the modified peptide T 7 was neutral (zero net charge on paper electrophoresis at pH 6.5) when the tryptic digest was submitted directly to electrophoresis. The suggested rearrangement involves a nucleophilic displacement and has well defined stereochemical requirements. The ring-expansion might entail the thiazolidine moiety's swinging out of the site to permit rotation about C-5–C-6. Another possibility, which

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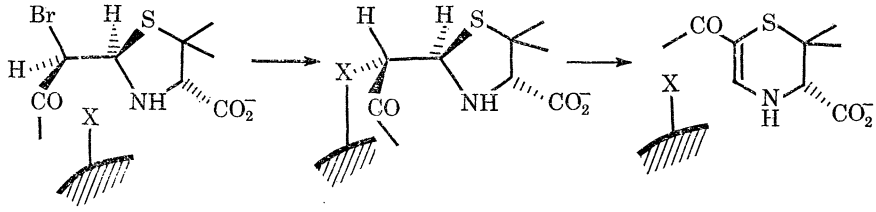
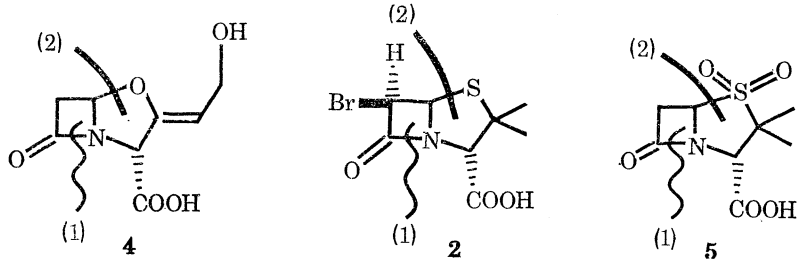
cannot be proved as yet, is that the stereochemical requirements might be met by a double displacement. Moreover, the speed of the reaction suggests the catalytic intervention of an enzyme group X (e.g. a carboxylate):



These considerations bring us to the question of what it is that determines whether a β-lactam is a substrate or an inactivator of a β-lactamase. The key to inactivation may lie in a rearrangement of the acyl-enzyme. A two-step process may be envisaged. The acyl-enzyme formed by fission (1) reacts further by fission of the C-5–S or C-5–O bond, fission (2): the altered compound can no longer undergo hydrolysis. These processes seem likely for clavulanic acid (**4**) (Reading & Cole 1977; Charnas *et al.* 1978), and for the sulphone (**5**) (English *et al.* 1978), and were explicitly formulated for 6-β-bromopenicillanic acid above.



The differences between different β-lactamases in their responses to inactivators is at least as marked as the differences in their actions on substrates. For example, β-lactamase I is inactivated by clavulanic acid (Durkin & Viswanatha 1978; Abraham & Waley 1979), but only poorly by the sulphone (**5**). Understanding of these idiosyncrasies awaits knowledge of the three-dimensional structure of β-lactamase I (Aschaffenburg *et al.* 1978).

Substrate-induced inactivation

β-Lactamase I shows biphasic kinetics in the hydrolysis of methicillin or cloxacillin, and there is evidence for an alteration in the conformation of the enzyme (Samuni & Citri 1975; Citri *et al.* 1976; Kiener & Waley 1977). These changes seem to be correlated with the enzyme action, i.e. catalysis of the hydrolysis of the substrate. This is easier to understand if it is the acyl-enzyme whose conformation is altering. Thus a possible (partial) explanation of substrate-induced inactivation is that, for some substrates, the acyl-enzyme is prone to unfolding. Instability of the acyl-enzyme could be a feature in common with the inactivations discussed above. In substrate-induced inactivation by methicillin or cloxacillin, the fate of the moiety derived from the substrate is not known.

β-Lactamase II

The discovery of β-lactamase II was a consequence of its ability to catalyse the hydrolysis of cephalosporin C (Newton & Abraham 1956); the key to further progress lay in the finding that the enzyme required Zn II for activity (Sabath & Abraham 1966). In this respect β-lactamase II

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is, as far as we know, unique among β-lactamases. And since the metal ion is believed to be in the active site, its ligands represent active site groups. β-Lactamase II is not inactivated by 6-β-bromopenicillanic acid (Pratt & Loosemore 1978), nor apparently by the other β-lactams that we have tested.

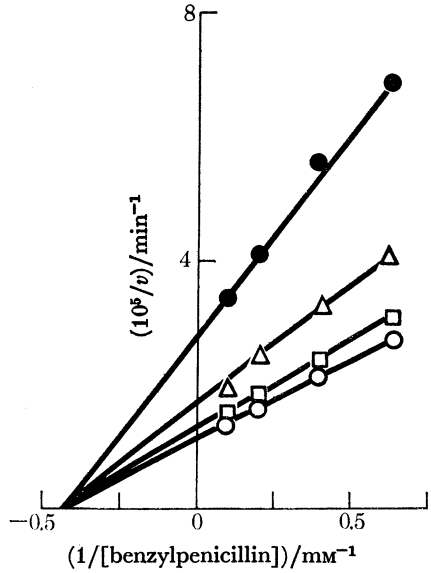

FIGURE 3. Variation of rate of hydrolysis of benzylpenicillin by β-lactamase II at different concentrations of Zn II. The hydrolysis was carried out in 10 mM *N,N-bis*(2-hydroxyethyl)glycine, 1 M NaCl, pH 6, at 30 °C in the pH-stat. The concentration of free Zn II were: ●, 0.95 μM; △, 2.38 μM; □, 4.77 μM; ○, 9.59 μM.

Kinetic mechanism

A metal ion is required for the activity of β-lactamase II; the apoenzyme, after the removal of Zn II, had less than 0.001 % of the original activity. Several metal ions, notably Co II and Cd II, could replace Zn II, and gave a less active enzyme (Davies & Abraham 1974). The steady-state kinetics of the hydrolysis of benzylpenicillin has been measured in metal buffers; *N,N-bis*(2-hydroxyethyl)glycine was used to obtain a known concentration of free Zn II in the micromolar range. The results (figure 3) exclude an ordered pathway in which combination of the apoenzyme with metal precedes combination with the substrate: this mechanism would give lines intersecting on the ordinate. The alternative is a random pathway, in which a ternary complex (EMS) of enzyme (E), metal (M) and substrate (S) can be reached by alternative pathways. In its simplest form the following scheme would apply:

$$\begin{array}{ccc} \mathrm{E} & \rightleftharpoons & \mathrm{ES} \\ \Downarrow\Uparrow & & \Downarrow\Uparrow \\ \mathrm{EM} & \rightleftharpoons & \mathrm{EMS} \xrightarrow{k} \mathrm{EM}+\mathrm{P}. \end{array}$$

In such a scheme one route may predominate. In fact, the pathway via EM seems to be greatly favoured, because experiments with the use of equilibrium dialysis have shown that the apoenzyme binds at least two substrates too weakly (if at all) for the combination to be detectable. Methicillin, for example, was found to have a dissociation constant greater than 120 mM. On the other hand, the penicilloic acid from benzylpenicillin was bound by the apoenzyme, the dissociation constant being 40 mM; it also behaved as a competitive inhibitor.

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Although the kinetics of the random pathway are simple if the binding steps can be regarded as at equilibrium, this condition is not fulfilled here. From nuclear magnetic resonance (n.m.r.) experiments (see later) the rate constant for $EM \rightarrow E + M$ is less than 10^4 min^{-1} (slow exchange), but k is about 10^5 min^{-1} for benzylpenicillin. The equation for the non-equilibrium steady-state velocity deduced for the scheme given above by Whitehead (1976) is complicated, but under the limiting conditions of substrate saturation the lines in figure 3 can be used to give values for the dissociation constant for metal dissociating from the ternary complex. However, it is by no means unlikely that the situation is more complicated than is represented by the simple scheme given above.

β-Lactamase II catalyses the hydrolysis of several penicillins and cephalosporins at fairly similar rates (Kuwabara & Abraham 1967; Davies *et al.* 1974). In fact, the specificity is different for the Zn II and Co II enzymes: the spread of values for k_{cat}/K_m (for the substrates that we have tested) was considerably greater for the Zn II enzyme than for the Co II enzyme (G. S. Baldwin, unpublished experiments).

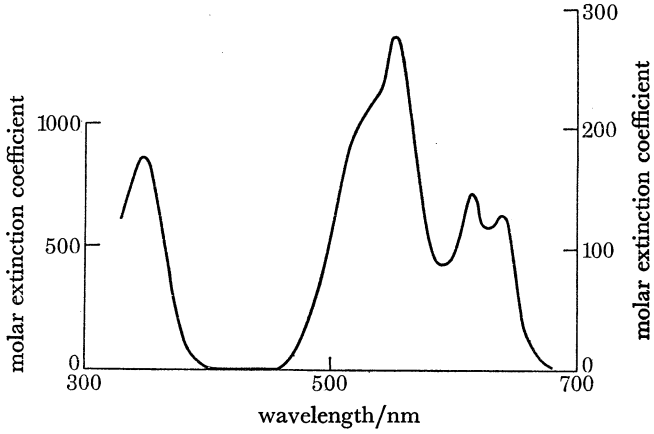

FIGURE 4. Absorption spectrum of Co II β-lactamase II. The spectrum was recorded at pH 5.98, 30 °C, in 200 mM sodium succinate, 1 M NaCl with a Cary 17 spectrophotometer. The concentration of enzyme was 1.45 mM, and the concentration of Co II was 10.4 mM.

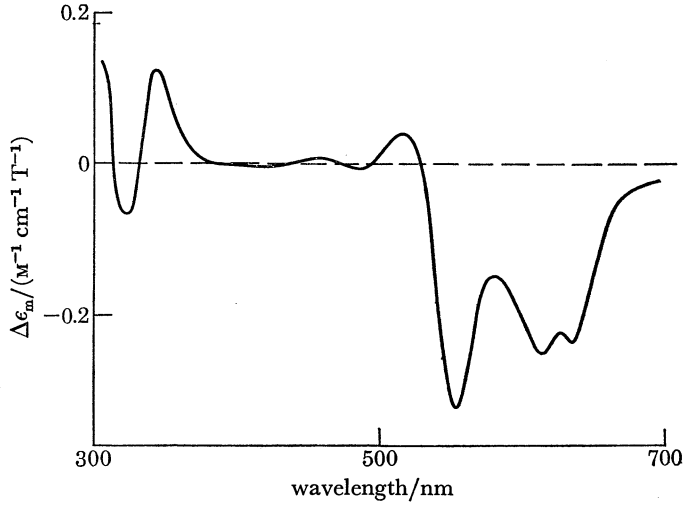

FIGURE 5. Magnetic circular dichroic spectrum for Co II β-lactamase II. The spectrum was recorded at pH 6.05 in 50 mM sodium succinate–1 M NaCl. The concentration of enzyme was 0.2 mM and the concentration of Co II was 0.45 mM.

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Groups that bind the metal ion

There are two sites in β-lactamase II that bind Zn II, and their affinities for the metal ion differ greatly, the dissociation constants being in the micromolar range for the first site and in the millimolar range for the second site (Davies & Abraham 1974; Baldwin *et al.* 1978). It is the first site whose occupancy is correlated with catalytic activity. The nature of the ligands is now discussed. The evidence is mainly derived from spectroscopy.

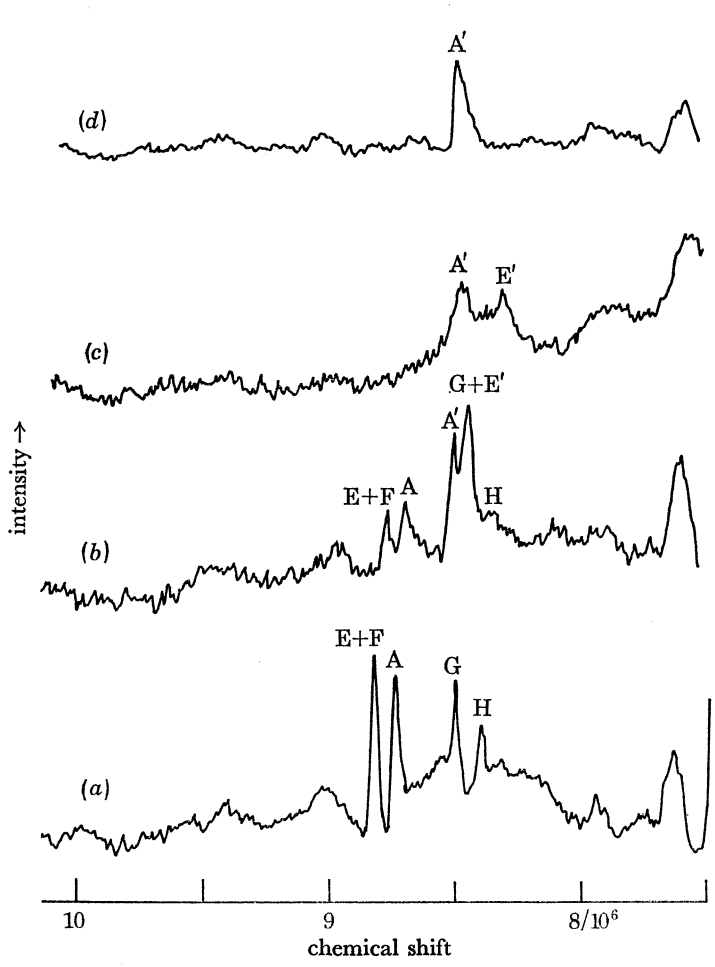

FIGURE 6. Nuclear magnetic resonance spectrum of β-lactamase II at 270 MHz. The region containing the C-2 proton resonances of histidine is shown for the apoenzyme (*a*), at pH 5.70, and for solutions containing 0.96 molar proportions of Co II at pH 5.74 (*b*), 1.36 molar proportions at pH 6.06 (*c*) and 2 molar proportions of Co II at pH 5.75 (*d*). The enzyme (4.5 mM) was in 20 mM succinate–1 M NaCl. The labels A–H refer to resonances assigned to histidine residues. The chemical shift was relative to 2,2-dimethyl-2-silapentane-5-sulphonate.

The replacement of the diamagnetic Zn II by the paramagnetic Co II permits a wider range of properties to be studied. The ultraviolet absorption spectrum of the Co II enzyme shows a maximum at 348 nm (Davies & Abraham 1974) (figure 4). Experiments with the use of equilibrium dialysis with $^{60}CoCl_2$ showed that there are two sites for Co II and that the increase in absorption at 348 nm was correlated with occupancy of the first site. The absorption at 348 nm in Co II proteins is usually ascribed to a charge-transfer transition, the ligand being the anion

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derived from the thiol group of a cysteine residue. Similarly, the magnetic circular dichroism spectrum shows a positive contribution at 345 nm, associated with the nearby absorption band (figure 5). Moreover, the Cd II enzyme shows an absorption band at 245 nm, attributed to charge-transfer transitions involving the same ligand. β-Lactamase II contains one residue of cysteine (Davies *et al.* 1974), which is reactive in the apoenzyme but unreactive in the Zn II enzyme. This difference is consistent with the thiol's being a ligand to Zn II.

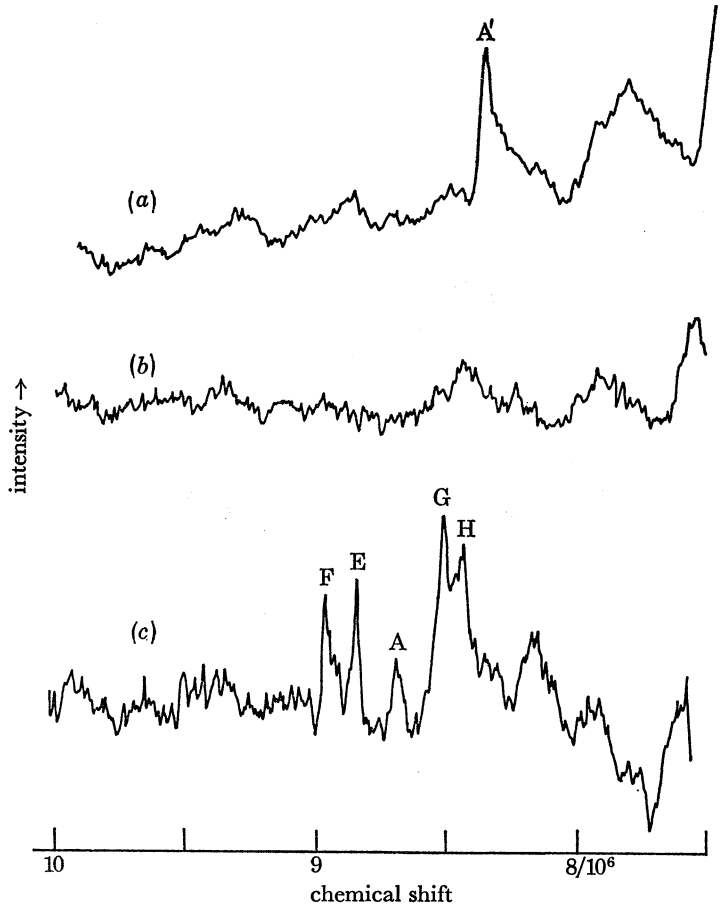

FIGURE 7. Deuterium exchange of histidine C-2 protons of dicobalt β-lactamase II. The enzyme (5 mM) in 20 mM succinate–20 mM triethanolamine–1 M NaCl in ^2H_2O was incubated at pH 8.07, 37 °C. The spectrum at the start of the experiment (*a*) had changed after 5 days (*b*). After 7 days, the enzyme was converted into apoenzyme, and the n.m.r. spectrum was recorded at pH 5.27 (*c*).

In the visible region, the spectra of the Co II enzyme have the features that, in other proteins, are associated with the geometry about the Co II ion's being related to that of a distorted tetrahedron (Holmquist *et al.* 1975). The absorbance in both the ultraviolet and visible regions is abolished by the addition of one molar proportion of Zn II. Clearly, the Zn II competes successfully with the Co II and displaces it from the first site.

Granted, then, that the cysteine residue is one ligand, what are the others? Here the evidence comes from measurements of the n.m.r. spectrum at 270 MHz. Five resonances in the region 7.8–8.8/10⁶ (measured downfield from sodium 2,2-dimethyl-2-silapentane-5-sulphonate) could be assigned to histidine residues. These were referred to as resonances A–E in the Zn II enzyme.

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Three of these resonances, B, C and D, moved upfield by about $0.6/10^6$ when the Zn II was removed; they were referred to as F, G and H in the apoenzyme. The positions of these resonances varied with pH (titrated) in the manner characteristic of histidine resonances when the apoenzyme was studied. In the Zn II enzyme, however, these three resonances did not titrate. Moreover, the same three residues exchanged their C-2 protons for solvent (^2H_2O) much more slowly when Zn II was present (Baldwin *et al.* 1978). This difference has been utilized in experiments directed towards identifying these histidine residues in the polypeptide chain (Baldwin *et al.* 1979). Further evidence that these three histidine residues are ligands has been provided by the n.m.r. spectrum of the Co II enzyme. The Co II will shift the resonances of nearby protons. Addition of Co II to the apoenzyme brought about a progressive decrease in

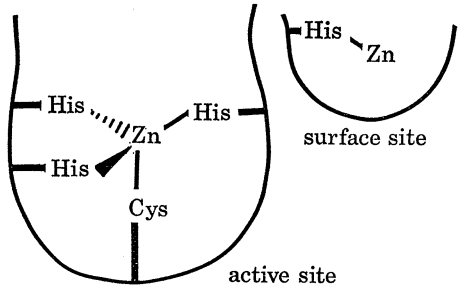

FIGURE 8. Diagrammatic representation of the metal-binding sites of β-lactamase II. The ligands believed to bind Zn II (or Co II) are shown. The active site is regarded as internal, and as having four ligands disposed more or less tetrahedrally about the metal ion.

the intensity of resonances assigned to the histidine residues and the appearance of new resonances (labelled A′ and E′) (figure 6). Eventually only resonance A′ remained; this is a resonance assigned to the histidine residue that is not involved in binding Co II. Exchange with solvent deuterium was also investigated. Initially, the spectrum of the Co II enzyme (with two molar proportions of Co II showed resonance A′, but after keeping the solutions in ^2H_2O for 5 days this resonance was no longer seen (figure 7). This is attributed to exchange of the C-2 proton of the histidine residue with solvent deuterium. After 7 days the Co II enzyme was converted into apoenzyme and its n.m.r. spectrum measured at a pH where the histidine resonances were well resolved: resonance A was diminished in intensity, but resonances F, G, H (and E) were present with undiminished intensity: exchange had been prevented by the presence of Co II. Thus resonance A′ in the Co II enzyme is assigned to histidine A. Other experiments showed that resonance E′ in the Co II enzyme could be assigned to histidine E, which was a ligand at the second (weaker) site. Hence resonances F, G and H in the apoenzyme correspond to the histidine residues that are ligands for Co II (as for Zn II).

Our conclusions about the nature of the metal-binding sites in β-lactamase II are summarized diagrammatically in figure 8. The first site may be regarded as internal, as many protons are affected by the initial addition of Co II to apoenzyme. The ligands are three histidine residues and the thiol group. The differences in catalytic activity between the Zn II and Co II enzymes point to the metal's playing a catalytic rather than merely a structural role. Whether the substrate binds to the enzyme-bound metal and becomes a fifth ligand (the coordination geometry perhaps being that of trigonal bipyramid) or whether a molecule of water does, or whether instead there is reorganization of the enzyme and one of the other ligands becomes detached from the metal, are questions for future work to resolve.

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