An X-ray-crystallographic study of β -lactamase II from *Bacillus cereus* at 0.35 nm resolution

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Crystals of β -lactamase II (EC 3.5.2.6., ‘penicillinase’) from *Bacillus cereus* were grown with Cd(II) in place of the natural Zn(II) cofactor and stabilized by cross-linking with glutaraldehyde. Their space group is C2, the cell dimensions are $a = 5.44$ nm, $b = 6.38$ nm, $c = 7.09$ nm and $\beta = 93.6^\circ$, and there is one molecule in the asymmetric unit. Diffraction data were collected from cross-linked crystals of the Cd(II)-enzyme, the apoenzyme and six heavy-atom derivatives. The electron-density map calculated at 0.35 nm resolution reveals the essential Cd(II) ion surrounded by three histidine residues and one cysteine residue. The position of a glutamic acid residue, modification of which destroys activity [Little, Emanuel, Gagnon & Waley (1986) *Biochem. J.* 233, 465–469], suggests the probable location of the active site of the enzyme. Two minor Cd(II) sites not essential for activity were also located. The structure of the apoenzyme at this resolution appears to differ from that of the Cd(II)-enzyme only in the orientation of two of the histidine residues and the cysteine residue that surround the metal ion.

INTRODUCTION

The β -lactamases are a family of enzymes (EC 3.5.2.6, ‘penicillinase’) that hydrolyse the β -lactam ring of penicillins and cephalosporins and thereby destroy their antibiotic activity. The production of β -lactamases by bacteria can therefore confer resistance towards the β -lactam antibiotics. The development of bacterial resistance to penicillin was first observed by Abraham & Chain (1940), who attributed this to the production of an enzyme that they called ‘penicillinase’. Today β -lactamase-mediated resistance is a widespread clinical problem.

β -Lactamase II is one of two extracellular β -lactamases produced by *Bacillus cereus* 569/H/9. Unlike β -lactamase I, which is active mainly against penicillins, β -lactamase II has a very broad spectrum of activity encompassing most β -lactams and, indeed, owes its discovery to its ability to hydrolyse cephalosporins. It also has the unusual requirement of zinc for activity (Sabath & Abraham, 1966). Its molecular mass was originally reported as 22000 Da (Davies *et al.*, 1974), but the sequence determination revealed a protein of 227 residues with a molecular mass of 24946 Da (Hussain *et al.*, 1985; Ambler *et al.*, 1985).

The enzyme has alone been assigned to class B, according to a classification of β -lactamases based upon amino acid sequence (Ambler, 1980; Jaurin & Grundström, 1981). As a metalloenzyme it is clearly distin-

guished from members of class A (which includes β -lactamase I) and class C, all of which contain an active serine residue and require no cofactors. In fact β -lactamase II remains the sole member of class B, for although a second zinc-dependent β -lactamase has been characterized from the pathogen *Pseudomonas maltophilia* (Saino *et al.*, 1982), its properties and limited sequence data suggest that there must be more than one class of zinc β -lactamase (Bicknell *et al.*, 1985).

The Zn(II) ion of β -lactamase II may be replaced by Co(II), Cd(II), Mn(II) or Hg(II) to yield an active enzyme (Davies & Abraham, 1974), although the Zn(II)-enzyme is the most active. Furthermore, the enzyme has been shown to bind two Zn(II) or Co(II) ions (Davies & Abraham, 1974; Baldwin *et al.*, 1978). The dissociation constant at the first site, occupation of which is essential for activity, is about $1 \mu\text{M}$, whereas the second site exhibits much weaker binding, with a dissociation constant of about 24 mM. ^1H n.m.r. studies of the Zn(II)- and Co(II)-enzyme have identified three histidine residues as ligands at the first site, and a further histidine residue at the second, weaker, site (Baldwin *et al.*, 1978; Galdes *et al.*, 1980). These histidine residues were subsequently located in the sequence by differential ^3H exchange and tryptic digestion (Baldwin *et al.*, 1979). Involvement also of the sole cysteine residue at the first site has been inferred from absorption spectroscopy of the Co(II)- and Cd(II)-enzymes (Davies & Abraham, 1974; Bicknell & Waley, 1985; Bicknell *et al.*, 1986) and

Abbreviations used: PEG, poly(ethylene glycol); CPase, carboxypeptidase; TPase, transpeptidase.

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is consistent with the earlier observation that the presence of Zn(II) at the first site protects the cysteine against chemical modification (Davies & Abraham, 1974).

Recently an essential glutamic acid residue has been identified by chemical modification (Little *et al.*, 1986), and further investigation into the mechanism has led to spectroscopic characterization of intermediates at sub-zero temperatures (Bicknell & Waley, 1985) and by rapid-scanning stopped-flow techniques above zero (Bicknell *et al.*, 1986). These experiments have revealed a branched kinetic pathway and confirm that the metal ion is directly involved in catalysis. Such a distinctly different mechanism may explain why β -lactamase II is not susceptible to the inhibitors of the class-A and class-C enzymes. Indeed, no inhibitor of either zinc-dependent β -lactamase is known, and an important future aim of the three-dimensional structure determination described in here will be to facilitate the design of such a molecule.

EXPERIMENTAL

Crystal preparation

β -Lactamase II was purified from *Bacillus cereus* 569/H/9 culture supernatant as described previously (Davies *et al.*, 1974). Crystals of the apo-, Zn(II)-, Cd(II)- and Hg(II)-enzyme were grown under various different conditions (B. J. Sutton, unpublished work), but crystals of the Cd(II)-enzyme were the most suitable for X-ray analysis. The enzyme was dissolved to 5 mg/ml in 13.5% (w/v) poly(ethylene glycol) (PEG) 4000 (BDH, Poole, Dorset U.K.) and 2.5 mM- CdCl_2 . Portions (0.4 ml) were placed in silicone-treated glass wells and sealed in the presence of 0.4 ml of 18% (w/v) PEG 4000. Crystals grew during the course of 2–3 weeks at room temperature.

The crystals were cross-linked with glutaraldehyde (Sigma Chemical Co., St. Louis, MO, U.S.A.) at a concentration of approx. 0.02% (v/v) in the mother liquor (see Quiocho & Richards, 1964). After a period of at least 24 h, the crystals could be transferred to solutions of the heavy-atom compounds, in the absence of PEG, for derivatization. Soaking conditions were as follows: K_2PtBr_4 , 0.05 mM, 1 day; K_2PtCl_4 , 0.1 mM, 4 days; *p*-chloromercuribenzoysulphonate, 0.1 mM, 7 days; Baker's dimercurial reagent [mercury *m*-(2,3-dimethoxytetramethylene)bisacetate], 0.1 mM, 5 days; ethylmercury phosphate, 0.1 mM, 23 days; NaAuCl_4 , 0.05 mM, 2 days. Crystals of the apoenzyme were prepared by soaking in repeatedly refreshed solutions of 1 mM-EDTA for 4 days.

X-ray data collection and processing

Measurements were made on a Hilger and Watts four-circle diffractometer modified for the quasi-simultaneous measurement of five reflections (Artymiuk & Phillips, 1985). Ni-filtered CuK_α radiation was used from a sealed tube operated at 40 kV and 30 mA. The peaks were recorded with an omega-scan, and the two equivalents, *hkl* and $-\bar{h}-\bar{k}-\bar{l}$, were measured for each reflection close together in time, so that anomalous differences could be determined under conditions of constant absorption and radiation decay. The data were collected in overlapping shells of resolution, beginning at the highest resolution, and radiation damage was monitored by repeated measurements of four reflections. Absorption

curves were collected appropriate to each shell of data, and the correction was applied by the method of North *et al.* (1968). Each set of data was collected from a single crystal.

After application of the Lorentz, polarization and absorption corrections, each derivative data set was scaled by the method of Matthews & Czerwinski (1975) to the apoenzyme data set. The mean fractional isomorphous difference was calculated as a function of resolution, and the difference in structure factor amplitude was analysed as a function of reciprocal space in order to monitor any lack of isomorphism. Within the resolution limits of the data sets (shown in Table 1), the effect was not considered significant. The identification of heavy-atom positions from isomorphous difference Patterson maps and their subsequent refinement is described below.

RESULTS

Crystallization and derivative preparation

The crystals of Cd(II)- β -lactamase II grew typically to a size of 1.00 mm $\times$ 0.3 mm $\times$ 0.3 mm, in space group C2 with cell dimensions $a = 5.51$ nm, $b = 6.15$ nm, $c = 7.00$ nm and $\beta = 98.3^\circ$. The consistent morphological features were elongation along the direction [001], and rhombic cross-section with the forms {110} and $\{-110\}$ best developed. The ratio, V_m , of the volume of the asymmetric unit (nm^3) to the molecular mass (in Da) is 2.35×10^{-3} , indicative of a single molecule in the asymmetric unit and a fractional solvent content of the unit cell of 0.48 by volume, assuming a value of $0.74 \text{ cm}^3/\text{mg}$ for the partial specific volume of the protein (Matthews, 1968).

The fresh Cd(II)-enzyme crystals diffracted well to a resolution of at least 0.24 nm, but subsequently were found to undergo a progressive deterioration over a period of weeks, this being manifested both by a reduced extent of X-ray diffraction and development of a slightly opaque appearance. These changes were observed whether or not the crystallization well was opened, and were not halted by transfer to a solution of constant PEG concentration. A more extreme form of this behaviour had been observed with the Zn(II)-enzyme crystals, which quickly converted into a gel-like phase and often redissolved.

Chemical cross-linking of the Cd(II)-enzyme crystals was therefore performed as described above. The process clearly affected the crystal packing, for the cell dimensions were subsequently found to be $a = 5.44$ nm, $b = 6.38$ nm, $c = 7.09$ nm and $\beta = 93.6^\circ$. Interestingly, these are virtually isomorphous with the metastable crystals of the Zn(II)-enzyme, and also of the apoenzyme (B. J. Sutton, unpublished work). After cross-linking, the Cd(II)-enzyme crystals suffered a detectable, but very slight, reduction in the intensity of diffraction at about 0.24 nm resolution. However, in marked contrast with the attempts at derivatization of the uncross-linked crystals, which caused cell-dimension changes, particularly in b and β , it proved possible to prepare sufficiently isomorphous heavy-atom derivatives and also apoenzyme crystals by soaking as described above.

Heavy-atom refinement and phasing

Three-dimensional isomorphous difference Patterson syntheses were calculated between each of the potential

Table 1. Details of the data collection, processing and refinement of heavy-atom parameters for the isomorphous phasing of β -lactamase IIAbbreviations used: EMP, ethylmercury phosphate; PCMBs, *p*-chloromercuribenzoysulphonate.

Data set	Resolution limit (nm)	No. of terms measured	No. of unique terms	R_{sym}^*	$R_{\text{iso}}^\dagger$	Heavy-atom sites‡	Relative occupancy§	Temperature factor (nm ²)	$R_K^\parallel$
Apo-Cd(II)	0.30	10411	4608	0.045	—	—	—	—	—
	0.35	6947	5733	0.019	0.22	Cd1	2.26	0.56	0.17
						Cd2	0.76	0.16	
						Cd3	0.44	0.49	
K ₂ PtBr ₄	0.35	6199	5307	0.035	0.19	Cd4	0.83	0.72	0.12
						Cd1	2.50	0.20	
						Cd2	0.76	0.18	
						Cd3	0.44	0.48	
						Pt2	0.90	1.8	
						Pt3	1.26	1.2	
						Pt3'	0.52	1.3	
K ₂ PtCl ₄	0.35	4714	3962	0.042	0.26	Pt4	0.91	1.5	0.20
						Pt1	2.04	0.80	
						Pt1'	1.56	1.9	
						Pt2	1.45	1.7	
						Pt3	1.25	0.98	
						Pt3'	1.56	1.9	
						Cd1	1.77	0.04	
PCMBs	0.35	6301	5300	0.039	0.16	Cd2	0.29	−0.19	0.11
						Hg6	0.45	0.85	
Baker's	0.45	3843	2735	0.052	0.25	Hg1	0.78	0.20	0.19
						Hg2	0.71	0.46	
						Hg3	0.51	0.16	
						Hg4	0.44	0	
						Hg5	0.27	0	
EMP	0.60	1716	1182	0.040	0.17	Cd1	1.57	0.18	0.13
						Cd2	0.50	0.16	
						Cd3	0.25	0	
						Cd4	0.93	0.72	
						Hg6	0.24	0	
						Hg7	0.55	0.49	
						Cd1	2.34	0.20	
NaAuCl ₄	0.60	1520	1143	0.044	0.18	Cd2	0.30	0.20	0.11
						Cd3	0.37	0.20	
						Au1	0.60	0.20	
						Au2	0.60	0.20	
						Au3	0.39	0.20	
						Au4	0.30	0.20	
						Au5	0.28	0.20	

* $R_{\text{sym}} = \sum_n [\sum_m |F_m - \langle F \rangle_n|] / \sum_n [m \langle F \rangle_n]$ where n is the number of unique reflections and m is the number of equivalent measurements of a reflection.† $R_{\text{iso}} = \sum |F_{\text{PH}} - F_{\text{P}}| / \sum |F_{\text{P}}|$

‡ Identical names indicate coincident sites in different derivatives.

§ Arbitrary scale of occupancy factors.

|| $R_K = \sum |F_{\text{PH(obs.)}} - F_{\text{PH(calc.)}}| / \sum |F_{\text{PH(obs.)}}|$.

derivative data sets and both the apo- and Cd(II)-enzyme data, after local scaling of all data sets to the apoenzyme data set. The resolution limits and mean fractional isomorphous differences (R_{iso}) for all derivatives that proved to be useful are shown in Table 1. Non-isomorphism limited the contribution of certain derivatives to relatively low resolution.

The Cd(II)-apoenzyme difference Patterson map revealed, as expected, a single dominant peak ($0.25 \times$ origin peak) and also a number of much weaker features consistent with further Cd(II) sites with considerably lower occupancy. This pattern of peaks was found in

most, but not all, of the other difference Patterson maps.

The K₂PtBr₄-apoenzyme difference Patterson map was readily interpreted in terms of two Pt sites (Pt2 and Pt3) in addition to the principal Cd(II) site (Cd1). Although the K₂PtCl₄ Patterson map indicated the same two Pt sites (Pt2 and Pt3), a third peak was found, close to, but not coincident with, the position of the Cd1 peak, which was absent in this map. The refined occupancy factors later confirmed that in this derivative the Cd(II) had been displaced by K₂PtCl₄ (site Pt1).

None of the other difference Patterson maps was so

clearly interpretable, and therefore the three Pt sites of the K_2PtCl_4 derivative, after refinement using lower estimates of the heavy-atom structure factor amplitudes, F_{HLE} (Blundell & Johnson, 1976), were taken as the starting point for phase calculation. This choice minimized the extent of bias in the refinement of the other derivatives which contained the common Cd1 site. A phased refinement procedure was then applied to the other derivatives in turn following the inspection of difference Fourier maps. Isotropic temperature factors were set at 0.20 nm^2 for each heavy atom in the initial refinement at 0.60 nm resolution, and only later refined when the higher-resolution data were included. The final occupancy and temperature factors are given in Table 1.

The handedness of the structure was established by means of the anomalous difference measurements. Difference Fourier maps for all derivatives calculated with single derivative (K_2PtCl_4) phases based upon both hands were inspected, and the mean figure of merit for double derivative phase calculations compared. Later inspection of protein electron-density maps confirmed the initial choice.

Phased refinement and calculation of difference Fourier maps revealed three minor sites for Cd(II) which were detected in most of the other heavy-atom derivatives (see Table 1). The existence of these, and other sites common to several derivatives, dictated that it was impossible to eliminate the possibility of bias when phased refinement was carried out with joint phases calculated from two or more derivatives. However, parallel refinements with phases based upon different combinations of derivatives were made in order to monitor and minimize this effect.

The refinement of the K_2PtCl_4 and K_2PtBr_4 derivatives at 0.35 nm resolution and inspection of difference Fourier maps indicated that both of the Pt3 sites, and Pt1 in the K_2PtCl_4 derivative, were better modelled as double sites separated by approx. 0.50 nm (listed as Pt3 and Pt3', and Pt1 and Pt1' in Table 1). Two sites in the $NaAuCl_4$ derivative, Au1 and Au2, are also separated by only 0.55 nm . It is presently unclear whether these represent alternative binding sites, or merely an approximation to the modelling of the complex ions. In the Baker's derivative, however, four sites (Hg1–4) lie close together in the region originally occupied by Cd1. On the assumption that this represents two molecules of the dimercury compound, and pairing the sites according to occupancy, the intramolecular Hg–Hg separations are 0.50 nm and 0.48 nm .

It is interesting that the two derivatives in which the Cd1 ion is displaced, K_2PtCl_4 and Baker's, are the only derivatives in which no other minor Cd(II) sites are found. This suggests that derivative preparation and/or the presence of these heavy-atom compounds in solution assisted Cd(II) removal, since no candidates for direct displacement are found at the positions of these secondary Cd sites.

The mean figure of merit for phases calculated from the seven derivatives with the inclusion of anomalous phasing was $0.91\text{--}0.60 \text{ nm}$ and $0.78\text{--}0.35 \text{ nm}$ resolution. These values undoubtedly reflect the considerable similarities between several of the derivatives with regard to the major sites of heavy-atom substitution, and indeed the decision to include all seven was based largely upon a comparative assessment of the quality of the electron-density maps calculated with phases based upon different

combinations of the derivatives. Inspection of these maps was also important in order to monitor any distortion of the map, particularly in the region of the common Cd1 site. At this position in the apoenzyme map a negative peak was found and, although indicative of bias in the phases, this peak was smallest when all seven derivatives were included in the phase calculation. No peaks were detected at any of the other heavy-atom positions.

Electron-density-map interpretation

Electron density maps of the apo- and Cd(II)-enzyme (coefficients $|F(\text{apo})|$ and $|2F(\text{Cd}) - F(\text{apo})|$ respectively) were calculated and analysed at 0.60 , 0.45 and 0.35 nm resolution. Each was contoured in multiples of the root-mean-square (r.m.s.) error in the electron density, with the lowest contour equal to this value.

In the 0.60 nm -resolution maps, the regions of protein density and solvent were clearly distinguished, and a molecular boundary was drawn, defining a protein of approximate extent $5.0 \text{ nm} \times 3.5 \text{ nm} \times 4.5 \text{ nm}$ in a , b and c . No subdivision into separate domains was apparent, and only a single short rod-like feature presented the appearance characteristic of α -helical polypeptide conformation at this resolution. The Cd(II) ion (Cd1), the highest feature in the map, was found to lie in a fold of the protein density, at the surface, with access to a wide solvent channel in the crystal.

At 0.45 nm resolution it was clear that the Cd(II) ion lay in a pocket formed by three distinct segments of the protein, and indeed the quality of the map was such that extensive stretches of polypeptide chain could be traced. Evidence of slight differences in the protein electron density between the apo- and Cd(II)-enzyme were detected in the region surrounding the Cd1 site, but not elsewhere.

Initial interpretation at 0.35 nm resolution focused on the region of Cd1 in the Cd(II)-enzyme map. Two metal ligands were clear (indicated by A and B in Fig. 1), each with a shape consistent with the side chain of a histidine residue. The polypeptide chain to which one of them was connected clearly made a further approach to the metal through another electron-density feature (indicated by C in Fig. 1), also with a shape indicative of a histidine residue. Earlier n.m.r. and ^3H -exchange studies (Baldwin *et al.*, 1978, 1979) had shown, once the sequence was known, that three histidine residues 86, 88 and 210 were involved in metal binding. When the polypeptide chain was traced on each side of the electron-density features A, B and C, fitting of the sequence established that A and C represented the side chains of residues 88 and 86 respectively, and B represented residue 210 (see Fig. 1).

The complete polypeptide chain was then traced in both the Cd(II)- and apoenzyme maps (which were of similar quality and interpretability), although in several regions the exact course of the chain was unclear. α -Carbon atomic positions were located initially in a map-stack, and then transferred to a molecular graphics system (Evans and Sutherland PS330) running the program FRODO (Jones, 1982). Despite the uncertainties in some parts of the map, other regions, such as that surrounding the Cd(II) ion, were sufficiently clear for side chains to be fitted to the electron density.

Two important results concerning residues previously identified by chemical-modification studies were immediately apparent in this initial model. Firstly, the position

Fig. 1. Region surrounding the metal ion in the Cd(II)-enzyme electron-density map

Six sections perpendicular to b are shown, each separated by 0.1 nm and contoured in multiples of the r.m.s. error in electron density (broken contour level = r.m.s. error). The scale bar represents 0.2 nm. Numbers indicate the α -carbon atom positions as located in the map-stack, and the letters A, B and C indicate electron-density features that correspond to the side chains of histidine residues 88, 210 and 86 respectively (see the text). A region of solvent surrounding one of the crystallographic 2-fold axes may clearly be seen (lower right).

of the α -carbon atom of cysteine residue 168 was found to lie adjacent to Cd1 (see Fig. 1). As mentioned above, spectroscopic evidence and the ability of metal to protect this residue from chemical modification had implicated cysteine in metal binding. The electron density for the side chain of this residue is relatively weak in the Cd(II)-enzyme map, but it is considerably stronger, consistent with the nature of this residue, in the apoenzyme map. Secondly, glutamic acid residue 37 was found to lie at the end of a shallow cleft that extends away from the metal ion. Chemical modification of this residue is known to destroy enzyme activity (Little *et al.*, 1986).

The relative positions of Cd1, the three histidine residues 86, 88 and 210, cysteine 168 and glutamic acid residue 37 can be seen in Fig. 2. Also shown is one of the minor Cd(II) sites (Cd3), description of which will be deferred until the Discussion section.

Inspection of the electron-density map of the apoenzyme at 0.35 nm resolution revealed evidence of conformational differences in the vicinity of the site of

Cd1, but not elsewhere. Fig. 3 shows that these changes involve principally the side chains of residues 88, 168 and 210. Clearly some caution must be exercised in the interpretation of differences in this region, where both maps are susceptible to distortion caused by the heavy-atom substitution. [The relative weakness of the electron density for the cysteine residue side chain in the Cd(II)-enzyme map referred to above may well be a result of this effect.] However, single derivative maps, of considerably poorer quality but free from bias, were also calculated, and they confirmed the result presented in Fig. 3.

The definitive trace of the complete polypeptide chain must await the completion of crystallographic refinement. However, a striking feature of the present model, even at this preliminary stage, is the very limited extent of secondary structure. There is only a single, short, region of α -helix (this is the feature identified in the low-resolution maps), and although stretches of extended polypeptide chain lie within hydrogen-bonding distance of each other, there are no three-stranded β -sheet structures extending for more than a few residues.

DISCUSSION

Crystallographic analysis at 0.35 nm resolution of the enzyme β -lactamase II, with Cd(II) in place of the natural cofactor Zn(II), has revealed the overall three-dimensional structure of the molecule and in particular the disposition of residues around the essential metal ion. The enzyme is known to have a further binding site for a non-essential Zn(II) or Co(II) ion (Davies & Abraham, 1974; Baldwin *et al.*, 1978), and presumably therefore also for Cd(II). Minor Cd(II) sites have indeed been identified in the present study.

Major Cd(II) site (Cd1)

The discovery of three histidine residues, 86, 88 and 210, surrounding the metal ion (Fig. 2) confirms the earlier conclusions from n.m.r. and ^3H -exchange studies. Continuity in the electron density between the Cd(II) ion and the side chains of residues 88 and 210 clearly indicates that they are ligands for the metal, but the electron density for residue 86, a residue albeit close enough to be considered as a potential ligand, is not continuous with that of the metal. In the crystal structure it is also clear that although the sole cysteine residue 168 lies adjacent to the Cd(II) ion, it is not a strong ligand, for the Cd-S distance is approx. 0.45 nm. Nevertheless, there is continuity between the electron density for the side chain and that of Cd(II). The location of this residue is certainly consistent with the observation that the presence of metal would protect it from chemical modification (Davies & Abraham, 1974), and more particularly with the recent spectroscopic evidence that the metal-to-sulphur distance is relatively long in the free enzyme (Bicknell & Waley, 1985; Bicknell *et al.*, 1986).

The affinity of β -lactamase II for Zn(II) at this site is remarkably low, approx. $1\ \mu\text{M}$. Although the affinity for Cd(II) is uncertain, the apparently rather 'loose' arrangement of ligands would appear to account for the low Zn(II) affinity and indeed allow for substitution of the several cations that yield an active enzyme. Furthermore, it is easy to envisage the changes in metal co-ordination number entailed in a mechanism recently proposed for the enzyme (Bicknell *et al.*, 1986).

Fig. 2. Structure of β -lactamase II in the region of the principal metal-ion site, Cd1, viewed in the same orientation as in Fig. 1 (parallel with *b*)

One of the minor sites, Cd3, is also shown. The course of segments of the polypeptide chain is represented by the positions of the α -carbon atoms, and side-chains of residues involved in metal binding, as well as that of glutamic acid residue 37, are shown (see the text).

Fig. 3. Structure of the apoenzyme in the same region as that represented in Fig. 2

Changes in the conformation of the side chains of residues 88, 168 and 210 may be seen.

Minor Cd(II) sites (Cd2 and Cd3)

Of the three minor sites for Cd(II), Cd2 and Cd3 are found to lie adjacent to potential cation-binding residues: aspartic acid residue 56 for Cd2, and aspartic acid residues 81 and 90 for Cd3 (Fig. 2). One minor site was identified by n.m.r. (Baldwin *et al.*, 1978) through its effect upon an adjacent histidine residue. Cd2 is approx. 0.9 nm from the side chain of histidine-221 in the crystal structure and may represent this site. It must be remembered, however, that the n.m.r. experiments were performed with Zn(II) and Co(II), not Cd(II), and also do not preclude the existence of other minor sites. Cd3 would not have been detected because it lies close to Cd1 which would dominate the effect upon the resonances of the three adjacent histidine residues. However, the observation that saturation of the minor sites affects the activity of the enzyme towards certain cephalosporin substrates (Davies & Abraham, 1974) may well relate to Cd3, for this site is only 1.0 nm away from Cd1, whereas Cd2 is 2.5 nm away.

Active-site region

Direct evidence for the involvement of the metal ion in catalysis has been obtained recently (Bicknell & Waley, 1985; Bicknell *et al.*, 1986), and certainly the location of the Cd1 site in a shallow cleft at the surface of the enzyme might well allow for direct interaction with substrate. This cleft, lined with residues of a predominantly hydrophobic nature, extends away from the metal ion towards the side chain of glutamic acid residue 37, which lies at a distance of approx. 2.3 nm. Chemical modification of this residue prevents activity (Little *et al.*, 1986), a result consistent with its location if substrate interacts with the enzyme in this cleft (Fig. 2). The distance of glutamic acid-37 from Cd1 is clearly too

great, however, for this residue to play the role proposed by Little *et al.* (1986), namely to act as a general base to abstract a proton from a metal-co-ordinated water molecule. Glutamic acid residue 212, considerably closer to the metal at a distance of approx. 1.1 nm, is perhaps an alternative candidate, although it too is not close enough, at least in the free enzyme without substrate present.

Apoenzyme structure

The only significant conformational differences between the apo- and Cd(II)-enzyme structures detected at this resolution are shown in Figs. 2 and 3, and involve the side-chains of residues 88, 210 and 168. Yet n.m.r. (Baldwin *et al.*, 1978) and c.d. studies (Davies & Abraham, 1974) had earlier suggested that conformational changes extending through the protein occurred upon metal binding. N.m.r., however, is particularly sensitive to very small conformational changes, and detection of these will have to await crystallographic analysis at higher resolution.

Evolutionary origin

The suggestion that the β -lactamases are evolutionarily related to the carboxypeptidase/transpeptidase (CPase/TPase) enzymes involved in the final stages of bacterial cell-wall biosynthesis (Pollock, 1971), gained considerable support recently with the elucidation of the tertiary structures of two β -lactamases of class A. β -Lactamase I from *Bacillus cereus* (Samraoui *et al.*, 1986) and the β -lactamase from *Bacillus licheniformis* (Kelly *et al.*, 1986) were both found to resemble closely the CPase/TPase from *Streptomyces* R61 with respect to the spatial arrangement of secondary-structural elements, implying a common evolutionary origin.

β -Lactamase II, and the L1 enzyme from *Pseudomonas*

maltoophilia (Saino *et al.*, 1982), are to date the best-known metallo- β -lactamases, and both require zinc. Two other β -lactamases that are probably zinc enzymes have been reported, namely those from *Flavobacterium odoratum* (Sato *et al.*, 1985) and from *Bacteroides fragilis* (TAL2480 and TAL3636) (Cuchural *et al.*, 1986). A zinc-dependent bacterial CPase is also known, from *Streptomyces albus* G, and its three-dimensional structure has been determined (Dideberg *et al.*, 1982). It has no sequence similarity with either of the metallo- β -lactamases for which sequence data are available, and indeed has little affinity for β -lactams. It is already clear from the present study, however, that there is no tertiary-structural similarity with β -lactamase II either, for the CPase structure contains a high percentage of α -helical conformation. The evolutionary origin of β -lactamase II therefore remains unknown.

Future prospects

These first results of the crystallographic analysis of β -lactamase II have revealed the structure at the active centre, and indicate that a complete description of the three-dimensional structure of the molecule will be possible. In particular, the existence of a stable, cross-linked, form in which the enzyme retains activity (B. J. Sutton, I. E. Crompton & S. G. Waley, unpublished work), will allow investigation of the mechanism of action. Detailed cryoenzymological studies have been carried out (Bicknell & Waley, 1985), and these experimental conditions may now be applied to the crystalline enzyme in order to investigate intermediates in the hydrolysis of both penicillin and cephalosporin substrates.

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