The crystal structure of β -lactamase from *Staphylococcus aureus* at 0.5 nm resolution

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The preparation, crystallization and low-resolution structure determination of β -lactamase (EC 3.5.2.6, 'penicillinase') from *Staphylococcus aureus* is described. The enzyme crystallizes in space group I222 with 1 molecule per asymmetric unit and cell dimensions $a = 5.45(1)$, $b = 9.39(1)$ and $c = 13.87(2)$ nm. The structure was determined at 0.5 nm resolution by using phases calculated from $(\text{NH}_4)_2\text{Pt}(\text{CN})_4$ and $\text{KAu}(\text{CN})_2$ derivatives. The mean figure of merit $\langle m \rangle$, for the 1106 reflexions used was 0.70. Difference Fourier syntheses for data collected from crystals soaked in platinum D-methionine and in 6-(4-hydroxy-3,5-di-iodobenzamido)penicilloic acid revealed the likely position of the active site of the enzyme.

Some bacteria are able to protect themselves from the lethal effects of β -lactam antibiotics by hydrolysing the cyclic amide bond characteristic of these compounds. The hydrolytic enzymes conferring this protection are known as β -lactamases (EC 3.5.2.6, 'penicillinase' or 'cephalosporinase') and are of particular clinical importance because their genes can be readily transferred via plasmids, even between organisms of different species. Their presence in a population of pathogens may seriously limit the therapeutic value of particular antibiotics, and hence an understanding of the structure of the proteins and their interactions with β -lactams should assist in the rational design of new compounds in which good antibiotic properties are combined with stability towards the enzymes.

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These enzymes may also provide useful models for the interactions of the antibiotics with their lethal targets. The target enzymes, which are involved in cell-wall biosynthesis, are generally less readily prepared than are the extracellular β -lactamases. Gene sequences now becoming available suggest that there may be significant homology with the β -lactamases, at least in the immediate neighbourhood of the active site (Coulson, 1984).

A classification scheme for the β -lactamases, based largely on the functional properties of the enzymes, has been proposed (Richmond & Sykes, 1973). More recently protein and nucleic acid sequence data have provided the basis for a structural classification that is more appropriate for enzymological purposes. As originally proposed (Ambler, 1980), this scheme had two classes. Class A included all those enzymes whose sequence was then known (from *Bacillus cereus*, *Bacillus licheniformis*, *Staphylococcus aureus* and the R_{TEM} plasmid of *Escherichia coli*). These sequences are clearly homologous, though the single Gram-negative example is more distantly related to the others than these are to one another (30% identity of sequence compared with 50–60%). Since the ' β -II' enzyme of *Bacillus cereus* requires Zn^{2+} ions for its activity, it was assigned to Class B. This judgement has been confirmed by the partial sequence

information now available for this enzyme (R. P. Ambler, unpublished work; J. O. Lampen, personal communication), which shows no detectable homology with the Class A proteins. However, determination of the sequence of the gene coding for the chromosomal cephalosporinase from *E. coli* ('ampC'; Jaurin & Grundstrom, 1981) has required the creation of Class C. This class has grown rapidly with demonstrations of the homology of the *ampC* gene with cephalosporinase genes from a wide range of Gram-negative species (Bergstrom *et al.*, 1982). Several β -lactamases are amenable to analysis by X-ray crystallography, and, since significant changes in physical and functional properties are associated with changes in amino acid sequence, determination of the structures of all of these enzymes should be particularly valuable in understanding the molecular basis of these functional properties.

Mechanistic studies have proceeded furthest with the Class A enzymes. Experiments with active-site-directed inhibitors and with poor substrates (Knott-Hunziker *et al.*, 1979; Cartwright & Coulson, 1980; Kemal & Knowles, 1981) have thoroughly established that the mechanism of Class A enzymes involves the transient acylation of a serine residue [residue 70 in the standard numbering scheme (Ambler, 1979)]. A similar mechanism has been established for the Class C enzymes (Knott-Hunziker *et al.*, 1982). The absence of a conserved histidine residue in the β -lactamase sequences means that the enzymes are almost certainly not structurally related to either of the known types of serine proteinase. Functional and chemical studies have failed to provide convincing evidence for any other components of the active-site apparatus.

Over many years, some unusual properties of Class A enzymes have been attributed to conformational isomerization. The relationships between these phenomena remain uncertain. However, it is well established that there is an intermediate in the folding pathway of the *S. aureus* enzyme that is inactive, but that contains almost all the secondary structure of the native state (Carrey & Pain, 1978; Creighton & Pain, 1980). The intermediate is somewhat less compact than the native state, and it has been proposed that this is due to a loosening of the relationship of the domains of a three-domain structure (Adams *et al.*, 1980). Conformations analogous to this 'I'-state are the stable forms of the two point-mutant enzymes 'P2' and 'P54' (Novick, 1963; A. F. W. Coulson & R. P. Ambler, unpublished work; R. H. Pain, personal communication). Reaction of substrates such as quinacillin and cloxacillin with Class A proteins induces the formation of conformational states that are enzymically inactive and more susceptible to de-

naturation than is the native enzyme, but that may persist in solution after the inactivating substrate has left the protein (Virden *et al.*, 1978; Citri *et al.*, 1976).

The *S. aureus* β -lactamase of strain PC1 is borne on a plasmid that lacks a replication origin. Timmis *et al.* (1975) inserted the gene into pSC101, and McLaughlin *et al.* (1981*a,b*) transferred it from there to the relaxed replicon pMB9. This isolated DNA directed the synthesis of a protein that was at least partially active and of higher M_r than that normally isolated. Sequencing of the early part of the gene revealed a 24-residue leader sequence, which is presumably concerned with the excretion of the enzyme and is removed in *S. aureus* itself.

Preliminary crystallographic data for the Class A β -lactamase I from *Bacillus cereus* 569 have been published (Aschaffenburg *et al.*, 1978). A clear electron-density map, based on the use of three heavy-atom derivatives, has been obtained at low resolution, but the corresponding map at 0.25 nm resolution is not readily interpretable in detail (D. C. Phillips, personal communication). A low-resolution structure has been described for the *E. coli* R_{TEM} enzyme (DeLucia *et al.*, 1980). Similar problems to those encountered with *B. cereus* have also prevented this work being carried to high resolution (J. Knox, personal communication).

In the present paper we describe the determination of the structure of the extracellular enzyme from *Staphylococcus aureus* PC1 at 0.5 nm resolution.

Experimental

Materials

The reagents used throughout were normal good commercial grades and were not especially purified. Procedures not specified below were those described by Ambler (1975).

Syntheses

4-Hydroxy-3,5-di-iodophenylacetic acid. The method of synthesis was essentially that of Matsuura & Cahnmann (1959). Tris (42 g, 0.35 mol) and hydroxyphenylacetic acid (5 g, 0.033 mol) were dissolved in water (100 ml). A solution of I₂ (16.7 g, 0.066 mol) and KI (16.5 g) in water (75 ml) was added slowly, with stirring, to maintain a pale-yellow colour. After 25 min, the mixture was cautiously acidified with conc. HCl. The dense white precipitate of 4-hydroxy-3,5-di-iodophenylacetic acid was collected by filtration and dried in a vacuum desiccator. The yield was 12.7 g (96%).

4-Acetoxy-3,5-di-iodophenylacetic acid. The material from the previous reaction (0.031 mol) was dissolved in a solution of KOH (3.5 g, 0.062 mol) in

water (6ml). Crushed ice (30g) was added, followed immediately by acetic anhydride (2.96ml). The mixture was stirred until the ice melted; a viscous white oil separated. The mixture was extracted with chloroform, and the chloroform extract was dried over Na_2SO_4 and evaporated under reduced pressure. The resulting oil was reduced to a solid by dissolving it in a small volume of chloroform, precipitating with *n*-hexane, decanting and drying under vacuum. In this way 6g of white powder was obtained.

6-(4-Acetoxy-3,5-di-iodobenzamido)penicillanic acid. A 1g portion of the material from the previous stage was mixed with thionyl chloride (0.8ml) in a round-bottomed flask. After 10min, volatile material was removed by rotary evaporation under high vacuum at room temperature. A suspension of 6-aminopenicillanic acid (0.48g) in a mixture of 10% (w/v) NaHCO_3 solution (4.2ml) and acetone (4.2ml) was added. The pH of the resulting clear solution was returned to 8 by cautious addition of 10% NaHCO_3 . The mixture was extracted with isobutyl methyl ketone (4-methylpentan-2-one), acidified with conc. H_3PO_4 and re-extracted with the same solvent. The non-aqueous layer was dried over Na_2SO_4 . To the dry solution was added a solution of potassium 2-ethylhexanoate (0.5g) in butan-1-ol (1ml). The mixture was left overnight, and the resulting fine white precipitate was collected by centrifugation and dried. The powder was redissolved in 2ml of acetone/water (1:1, v/v) and precipitated by addition of diethyl ether (100ml). The precipitate was collected by decantation and dried in a vacuum desiccator with acetone/chloroform/acetic acid (10:9:1, by vol.; Manni *et al.*, 1959) showed a single component with mobility 0.6 (as expected for penicillins) and staining positively for the penicillin nucleus (blue spots on a yellow background on spraying the plate with a mixture of aq. 10% (w/v) FeCl_3 (20ml), 5% (w/v) $\text{K}_3\text{Fe}(\text{CN})_6$ (10ml) and 20% (v/v) H_2SO_4 (70ml) (McGilveray & Strickland, 1967). The n.m.r. spectrum in deuteriochloroform at 100MHz showed six singlets. The shifts in p.p.m. from tetramethylsilane, the relative intensities and assignments of these peaks were as follows: 8.25, 2.1, aromatic; 6.05, 1.9, C-6 and C-7 protons; 4.93, 1.0, C-3 proton; 4.07, 1.8, $\text{Ar}-\text{CH}_2-\text{CO}-$ methylene; 2.90, 3.2, $\text{CH}_3-\text{CO}-$ methyl; 2.10, 6.0, C-2 methyl groups.

This penicillin is not very soluble in water; addition of a small volume of concentrated penicillinase solution to a saturated solution in water brought about a rapid hydrolysis, as judged by t.l.c., but no attempt was made to measure the kinetics of the reaction.

Solutions of 6-(4-hydroxy-3,5-di-iodobenzamido)penicilloic acid for soaking the crystals

were prepared from this material by mixing the penicillin (3.5mg) with 0.1M-NaOH (0.15ml). The suspension cleared after a few seconds; after 15min, the pH was re-adjusted to 5.5 (indicator paper) by cautious additions of 1M-HCl or 1M-NaOH. I_2 titration and observation of the u.v. spectrum and behaviour on t.l.c. indicated that this treatment brought about complete hydrolysis, as expected, of the β -lactam ring and the phenyl acetate ester.

Protein purification and crystallization

A constitutive mutant strain of *S. aureus* (PC1) (Richmond, 1963) was grown to the end of the exponential phase in a 50-litre Biotec fermenter, with the use of CY medium (Novick, 1963) with the β -glycerophosphate replaced by Na_2HPO_4 (16.3g/l) + KH_2PO_4 (3.3g/l). The cells were removed by centrifugation and the basic extracellular proteins were adsorbed on cellulose phosphate (Whatman P11; washed and de-fined in distilled water), which was subsequently packed into a column. The protein was eluted with 75%-saturated $(\text{NH}_4)_2\text{SO}_4$, and the fractions containing β -lactamase activity were pooled, dialysed against tap water and freeze-dried. In current preparations, dialysis and freeze-drying at this stage are omitted. The freeze-dried material was redissolved in 50mM-ammonium acetate/acetic acid buffer, pH 5, and applied to a Sephadex G-100 column, from which it was eluted with the same buffer. The peak fractions were again pooled, dialysed and freeze-dried. Redissolved protein was tested for purity by comparison of u.v. absorption at 280 and 260nm (useful since the protein contains no tryptophan), by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and by specific activity (at least 20000 μmol of benzylpenicillin cleaved/h per mg (Pollock & Torriani, 1953).

Crystals were grown by the addition of a solution of unbuffered protein to $(\text{NH}_4)_2\text{SO}_4$ solution, with NaOH added dropwise to adjust the pH to 8.0. Final $(\text{NH}_4)_2\text{SO}_4$ concentrations varied from 60 to 75% saturation, and the protein concentration was 10–20mg/ml. Large platy needles, up to 5mm long $\times$ 0.5mm $\times$ 0.2mm thick, grew within 4 weeks in the presence of precipitate. The crystals diffract to about 0.25nm resolution, and are of space group I222 (distinguished from $\text{I}2_12_1$, by subsequent heavy-atom-binding analysis) with cell dimensions 5.45(1)nm $\times$ 9.39(1)nm $\times$ 13.87(2)nm. The crystal density was measured by flotation to be 1.33g/ml, indicating one protein molecule of M_r 28800 per asymmetric unit, and a V_m of $3.12 \times 10^{-3} \text{ nm}^3/\text{relative atomic mass unit}$. This value is at the upper end of the range for crystals of globular proteins (Matthews, 1968), implying that 65–70% of the crystal volume is occupied by solvent.

Repeating the crystallization has been difficult, and all of the crystallographic work described in the present paper was carried out with the initial batch of crystals. The poor reproducibility of crystallization does not appear to be due to the presence or absence of a major component from the protein preparations, since repeated analytical and sequence studies have shown that protein samples, judged to be homogeneous by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, consist of a single polypeptide chain of the expected composition and with no detectable carbohydrate (see below). The solubility of the protein (freeze-dried or as an amorphous precipitate) in $(\text{NH}_4)_2\text{SO}_4$ has been found to depend on the way in which the protein solutions are prepared. It is also higher for samples of the enzyme that had been bound to CM-cellulose, a treatment that increases the specific activity of the enzyme. It seems possible that some long-lived conformational state of the enzyme, induced by low ionic strength, may be necessary for crystallization. The crystals used in the present study, however, were positively identified as being β -lactamase by both enzymic and amino acid analyses.

X-ray data collection

Data for the native and derivative crystals were collected on a Hilger and Watts four-circle diffractometer at the University of Bristol kindly made available to us by Dr. H. C. Watson, and on a Nonius CAD4 diffractometer at the University of Edinburgh. Ni-filtered Cu $K\alpha$ -radiation from a sealed tube running at 40 kV and 20 mA was used. Peaks were measured with an ω scan, typically of 1° including the background. Two equivalent reflexions were collected, in order to allow use of the anomalous scattering differences from the heavy-atom derivatives. There were approximately 3000 reflexions in the 0.5 nm set, and each data set was collected from one crystal with less than 10% radiation decay. Integrated intensities were obtained by ordinate analysis (Watson *et al.*, 1970), by using an algorithm similar to that of Tickle (1975). A linear decay correction based on the behaviour of monitor reflexions was applied, and the absorption effects were corrected for by the method of North *et al.* (1968). After Lorentz and polarization corrections had been applied, the derivative data sets were scaled to the native by a scale factor of the form $k = a \cdot \exp(-b \cdot \sin^2 \theta / \lambda^2)$, where a and b are constants determined by least-squares analysis of the mean difference in intensity between derivative and native, as a function of $\sin^2 \theta / \lambda^2$. The differences in structure factor amplitudes were examined as a function of resolution, native amplitude and the h , k and l indices, to detect trends indicative of non-isomorphism: none was observed.

Carbohydrate analysis

The absence of neutral sugars, amino sugars and sialic acids from penicillinase was shown by the following procedures, which are based on the methods described by Spirò (1972).

Neutral sugars. Samples (20.5 mg) of the protein were sealed into glass tubes with 2 ml of 1 M-HCl and incubated at 108°C for 6 h. A 50 μg portion of glucose, corresponding to about 1.3 mol of sugar/mol of protein, was added to one sample before hydrolysis. The tubes were cooled and opened, and each hydrolysate was diluted with 8 ml of water. The solution was passed successively through a column containing the cation-exchanger Zeo-Karb 225 in its H^+ form (2.5 ml) and a second column containing a similar volume of the anion-exchanger Dowex 1X8 (400 mesh) in its formate form. The columns were washed with 25 ml of water, and the combined washings were rotary-evaporated. The residue was dissolved in 100 μl of water. Portions (10 μl) of this solution were applied to strips of Whatman no. 1 chromatography paper. Chromatograms were developed overnight with butan-1-ol/ethanol/water (10:1:2, by vol.). Samples (20 μg) of various neutral, mono- and disaccharides were run in parallel. After being dried, the papers were dipped in an AgNO_3 solution prepared by mixing 0.1 ml of a saturated aqueous solution with 20 ml of acetone and the minimum amount of water to dissolve the precipitate formed. The papers were dried and sprayed with 0.5 M-NaOH. No mono- or di-saccharides were observed in the protein hydrolysates; glucose was easily seen in the samples to which it had been added. Quantities of about 0.1 mol/mol of protein would have been detected by this procedure.

Amino sugars. A similar procedure was used for protein hydrolysis, except that 4 M-HCl was used, and glucosamine was added to one sample instead of glucose. After hydrolysis the tubes were cooled and opened, and the acid was removed on a rotary evaporator. The residue was dissolved in 5 ml of water, and the basic components were adsorbed on a column (2.5 ml) of the cation-exchanger in its H^+ form. The column was washed with 15 ml of water, and the basic components were eluted with 10 ml of 2 M-HCl. The combined eluates were rotary-evaporated, and the residue was dissolved in 100 μl of water. Samples (10 μl) were applied to Whatman no. 1 paper for electrophoresis at pH 2, 3.5 and 6.5. The electrophoretograms were stained with ninhydrin. At all three pH values glucosamine had a mobility close to that of arginine, but resolved from it. It was easily detected in the sample to which it had been added, but no components other than the expected amino acids were found in the protein hydrolysates. The sensitivity was similar to that for the neutral sugars.

Sialic acids. The colorimetric procedure for the detection of sialic acids in proteins described by Spiro (1966) was employed, with 5mg samples of protein. No significant colour developed, under conditions in which about 0.1 mol of sugar/mol of protein would have been detected.

Results and discussion

Search for heavy-atom derivatives

About 30 heavy-atom compounds were screened under various conditions to try to obtain useful heavy-atom derivatives. For the cases where the crystals did not crack or dissolve on soaking, precession photographs were taken to assess the degree of substitution. This screening was carried out first at the pH of crystallization, and later, to lessen the likelihood of interference by NH_4^+ and OH^- ions (Sigler & Blow, 1965), the pH was lowered to 5.6 by transferring the crystals to 90%-saturated $(\text{NH}_4)_2\text{SO}_4$ solution in the presence of 50mM-acetate buffer. Under these conditions a number of derivatives looked promising, and two, 1mM- $(\text{NH}_4)_2\text{Pt}(\text{CN})_4$ and 2mM- $\text{KAu}(\text{CN})_2$, gave interpretable Patterson maps and have been used for phase determination.

An attempt was also made to develop specific heavy-atom derivatives by utilizing the function of the enzyme. This approach was based on the observations that the crystals are enzymically active and that penicillin G diffused into the crystals produced small changes in the diffraction pattern. A di-iodinated penicilloic acid [6-(4-hydroxy-3,5-di-iodobenzamido) derivative] was prepared and diffused into the crystals. This compound caused a characteristic cracking of the crystals and 'smearing' of the diffraction pattern, which had been observed to a lesser extent with penicillin G. For the *B. cereus* enzyme conformational changes are associated with the interactions of substrates with bulky side chains on the enzyme (Citri *et al.*, 1976). This problem was overcome by cross-linking the crystals with glutaraldehyde. Evidence that some D-amino acids are inhibitors of the enzyme (Saz *et al.*, 1961) suggested that such compounds might also provide useful derivatives. Pt-D-methionine was examined together with Pt-diglycine. All three compounds gave rather small changes in the diffraction pattern and uninterpretable three-dimensional Patterson syntheses. However, subsequent examination of protein-phased difference-Fourier maps did show essentially the same, single-site, binding, presumably in the active site, for the penicilloic acid and D-methionine derivatives.

Determination of heavy-atom positions

Three-dimensional Patterson syntheses with

coefficients $(|F_{\text{PH}}| - |F_{\text{P}}|)^2$ were calculated, omitting a few suspect large differences, and the three Harker sections were examined for consistent sets of self peaks (see Fig. 1). One derivative, the $(\text{NH}_4)_2\text{Pt}(\text{CN})_4$, was readily interpretable in terms of a major site, and protein phases derived from this were used to calculate a difference-Fourier map, from which an additional minor site was identified. Three sites for the $\text{KAu}(\text{CN})_2$ derivative were obtained from the Pt derivative. None of the other derivatives was wholly satisfactory.

Heavy-atom positions and occupancies were refined for each derivative separately, by using the lowest estimates of heavy-atom structure amplitudes (FHLE) (Harding, 1962; Blundell & Johnson, 1976), with programs written by E. Dodson. Temperature factors were not refined, but were fixed at approximately that of the native data set, $B = 0.25 \text{ nm}^2$. Approximately half the reflexions in each set had sufficiently reliable FHLEs for this purpose (see Table 1 for details). The resultant parameters were used for several cycles of conventional phase refinement by using a program written by H. Muirhead [see Blundell & Johnson (1976) and Dodson (1976) for a discussion of these approaches]. Tests with simulated data showed that this produced a better set of parameters than did FHLE refinement alone (Herzberg, 1982). The refinement statistics are shown in Table 1. Centroid protein phases and figures of merit resulting from this step were used to calculate an electron-density map (Blow & Crick, 1959). The mean figure of merit for the 1106 reflexions of the 0.5 nm data set that were included was 0.70.

Electron-density map

The map, which was calculated in sections of constant x , was contoured with the lowest level at 0.2 of the maximum density and upwards in intervals of 0.1 of this. Hard-copy output was obtained on a Versatec electrostatic plotter and copied on to transparent acetate sheets on a scale of 0.632nm/cm. Fig. 2 shows parts of the map. The twofold axes running in the y and z directions in the $x = 0$ and $x = \frac{1}{2}$ planes are the main constraints to the molecular packing. The map shows that the molecules pack as tight dimers around the crystallographic twofold axes parallel to the x direction, each dimer spanning half a unit cell in that direction. The boundary of the dimer shown is very clear, with almost empty sections of map at $x = 0$ and $x = \frac{1}{2}$ and clear solvent channels in the y and z directions, along the dimer faces. The main molecular contacts outwith the dimer are at its corners, with other dimers related to it by the body centring. The most obvious division of density within the dimer itself is in the x - z plane at $y = \frac{1}{2}$, where a

Fig. 1. (a) The three Harker sections of the difference Patterson map for the $(\text{NH}_4)_2\text{Pt}(\text{CN})_4$ derivative and (b) the three Harker sections of the difference Patterson map for the $\text{KAu}(\text{CN})_2$ derivative

Table 1. Statistics for the multiple isomorphous replacement phasing of β -lactamase

Data set	Native	$(\text{NH}_4)_2\text{Pt}(\text{CN})_4$		$\text{KAu}(\text{CN})_2$		
Unique reflexions (1.2–0.5 nm)	1562	1562		1562		
R_{iso}^*		0.15		0.16		
Reflexions used in FHLE†		873		873		
$R_w(\text{FHLE})^\ddagger$		0.40		0.51		
Number of sites		2		3		
Relative occupancy§		1.4	0.3	1.2	0.6	0.4
Reflexions used for phasing	1106	1106		1106		
Weight in phasing		1		1.25		
$R_s^\P$		0.54		0.57		
R_k^{**}		0.07		0.08		
$R_{\text{anom}}^{\dagger\dagger}$		0.89		0.90		
Resolution shells (10/nm)	m	$e/f^{\ddagger\dagger}$		$e/f^{\ddagger\dagger}$		
12.0–8.0	0.71	0.53		0.63		
8.0–7.0	0.74	0.53		0.51		
7.0–6.0	0.72	0.51		0.63		
6.0–5.5	0.70	0.53		0.60		
5.5–5.0	0.66	0.63		0.65		
Overall	0.70	0.55		0.61		

$$^* R_{\text{iso}} = \frac{\sum_h ||F_{\text{PH}} \text{ obs.}| - |F_{\text{P}} \text{ obs.}||}{\sum_h |F_{\text{P}} \text{ obs.}|}$$

† Rejection criteria as in Evans (1983).

$$^\ddagger R_w^2(\text{FHLE}) = \frac{\sum_h \omega |F_{\text{HLE}} - F_{\text{H}} \text{ calc.}|^2}{\sum_h \omega F_{\text{HLE}}^2}$$

§ Occupancies are on an arbitrary scale.

|| Higher weight has been given to the gold derivative because otherwise the platinum derivative tends to dominate the phasing.

$$^\P R_s = \frac{\sum_h ||F_{\text{PH}} \text{ obs.}| - |F_{\text{PH}} \text{ calc.}||}{\sum_h ||F_{\text{PH}} \text{ obs.}| - |F_{\text{P}} \text{ obs.}||}$$

$$^{**} R_k = \frac{\sum_h ||F_{\text{PH}} \text{ obs.}| - |F_{\text{PH}} \text{ calc.}||}{\sum_h |F_{\text{PH}} \text{ obs.}|}$$

$$^{\dagger\dagger} R_{\text{anom}} = \frac{\sum_h |\Delta_{\text{anom}} \text{ obs.} - \Delta_{\text{anom}} \text{ calc.}|}{\sum_h \Delta_{\text{anom}} \text{ obs.}}$$

$$^{\ddagger\dagger} e/f = \frac{\langle |F_{\text{PH}} \text{ obs.} - F_{\text{PH}} \text{ calc.}|^2 \rangle^{1/2}}{\langle F_{\text{H}} \text{ calc.}^2 \rangle^{1/2}}$$

Fig. 2. Three regions of the β -lactamase electron-density map

Twofold axes are shown, and the molecular boundary is outlined in (b) and (c). (a) Superposition of three sections around $x = 0$. This is the region around the three intersecting twofold axes where the dimers above and below these sections end, creating a large solvent channel. Density in the corners of the Figure belongs to the molecules related to the central dimer by body centring, and is shown more fully in (c). (b) Superposition of thirteen sections from $x = 0.341$ nm to $x = 2.39$ nm, showing the bulk of the density for a pair of molecules forming a tight dimer. (c) Three sections around $x = \frac{1}{2}$, showing the packing between a dimer, the bulk of the density of which is between $x = 0$ and this region (outlined), and a dimer related to it by the twofold axes in this plane along $y = \frac{1}{2}$ and $z = \frac{1}{2}$. The crosses mark the binding sites for the active-site-directed compounds on the two molecules of the dimer.

clear impression of low density is obtained (see Fig. 2b). Such a partitioning, however, implies a very thin elongated molecule with approximate dimensions $3\text{ nm} \times 3\text{ nm} \times 8\text{ nm}$. An alternative division approximately in the x - y plane at $z = \frac{1}{2}$ gives a more globular molecule approx. $3\text{ nm} \times 6\text{ nm} \times 4\text{ nm}$. Solution studies for the related *E. coli* enzyme, where a similar ambiguity of boundary has been found at low resolution (Knox *et al.*, 1976), support the more globular shape. The division along $y = \frac{1}{2}$ would then indicate a rather loose association of structural domains. Physico-chemical studies have suggested a three-domain structure for the molecule, with denaturation behaviour consistent with a lack of compactness at domain interfaces (Adams *et al.*, 1980). The present electron-density map is not inconsistent with a three-domain interpretation.

Difference electron-density maps were calculated for two of the active-site-directed compounds

originally intended to provide heavy-atom derivatives. These Fourier maps had terms of the form $m(|F_D| - |F_P|) \cdot \exp(i\alpha_P)$ where m is the figure of merit, α_P is the native protein phase, and $|F_D|$ and $|F_P|$ are the structure factor amplitudes of the derivative and native respectively. The compounds were 6-(4-hydroxy-3,5-di-iodobenzamido)penicilloic acid and Pt-D-methionine, both of which might be expected to be substrate analogues. Fig. 3 shows parts of these difference electron-density maps. Both have a single peak in essentially the same position. Lower-level features nearby, which are more pronounced for the penicilloic acid compound, suggest local conformational changes accompanying binding, consistent with the crystal instability experienced with this compound. It seems very probable that this binding is at the active site of the enzyme, and if so this will be very useful as a marker for chain tracing from a higher-resolution map.

Fig. 3. Sections at $x = 15/32$ from the difference electron-density maps for (a) 6-(4-hydroxy-3,5-di-iodobenz-amido)penicilloic acid and (b) Pt-D-methionine. Negative contours are broken.

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