Penicillin Target Enzyme and the Antibiotic Binding Site

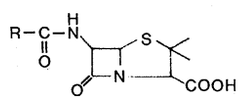
Abstract. *The three-dimensional structure of a penicillin-sensitive D-alanyl-carboxypeptidase-transpeptidase has been determined by x-ray crystallography to a resolution of 2.8 angstroms. The site of binding of the β-lactam antibiotics penicillin and cephalosporin has been located. These findings constitute direct observation of the interaction of β-lactams with a transpeptidase enzyme and establish the feasibility of defining the molecular stereochemistry of this interaction for purposes of drug design.*

Antibiotics of the β-lactam family (penicillins and cephalosporins) exert their bactericidal effects by disrupting bacterial cell wall synthesis through inhibition of enzymes that cross-link D-alanyl peptides on peptidoglycan strands of the growing cell wall (*1*). The cross-linking process has two steps: carboxypeptidation followed by transpeptidation

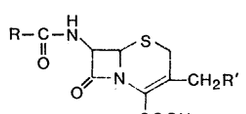
$$\text{Enzyme} + \text{R-D-Ala-D-Ala-COOH} \rightarrow \text{enzyme-CO-Ala-R} + \text{D-Ala-COOH} \quad (1)$$

$$\text{Enzyme-CO-Ala-R} + \text{R'-NH}_2 \rightarrow \text{R'-NHCO-Ala-R} + \text{enzyme} \quad (2)$$

It has been proposed that a β-lactam antibiotic inhibits these enzyme-catalyzed reactions because it is a structural analog of the D-alanyl-D-alanine portion of the nascent peptidoglycan; the β-lactam CO–N bond corresponds to the peptide bond and the D-center carboxyl group to the carboxyl of the terminal D-alanine (*1–3*). Thus, the antibiotic is capable of acylating the enzyme by a reaction similar to that of step 1.



Penicillin



Cephalosporin

Unfortunately, the inhibition is generally reversible and the β-lactam–enzyme intermediate is slowly converted back to active enzyme. For this reason, it is desirable to develop new β-lactam antibiotics that irreversibly inhibit cell growth of a wider range of bacterial species at lower therapeutic doses or when applied over shorter time periods (*4*). An additional factor determining the clinical effectiveness of new β-lactams is their susceptibility to hydrolysis by the β-lactamase enzymes, which are being encountered with increasing frequency. Active-site mapping of both types of enzyme is needed for rational design of new β-lactams, and x-ray analyses of both are under way (*5–8*).

A β-lactam–sensitive, bifunctional carboxypeptidase-transpeptidase from *Streptomyces* R61 has been characterized chemically and kinetically (*9–12*). This peptidase catalyzes hydrolysis of the terminal D-alanine from a donor analog with a turnover number of 55 sec^{-1} at 37°C and *p*H 7.5 (step 1) and transpeptidation with a suitable acceptor (step 2). Covalent catalysis proceeds by the transitory formation of a serine ester–linked acyl–enzyme intermediate. The peptidase also binds β-lactams, but the ester-linked penicilloyl- and cephalosporoyl-enzyme intermediates thus formed have long half-lives, ranging up to 180 hours. Both catalysis and β-lactam binding involve the same serine residue (*11, 13*). We previously described the crystallization of the transpeptidase and showed that the orthorhombic unit cell of the crystals is in space group $P2_12_12_1$; has dimensions a = 51.1 Å, b = 67.4 Å, and c = 102.8 Å; and has one 37,400-dalton molecule in the asymmetric unit (*14*). The crystals diffract to 1.8-Å resolution. We now describe first the tertiary structure of the enzyme and then its complexes with two β-lactams and a substrate analog.

The molecule is generally ellipsoidal, with dimensions 60 by 45 by 35 Å. Figure 1A shows a stereoscopic view of the α-carbon positions of 300 fitted residues. Figure 1B is a schematized ribbon tracing of the molecular backbone. The secondary structure is clustered into two regions, one all helical and the other a combination of helices with β structure (α + β) (*15, 16*). The elements of secondary structure comprise at least 40 percent of the molecule (see legend to Fig. 1B for details).

At this time, we see no structure ho-

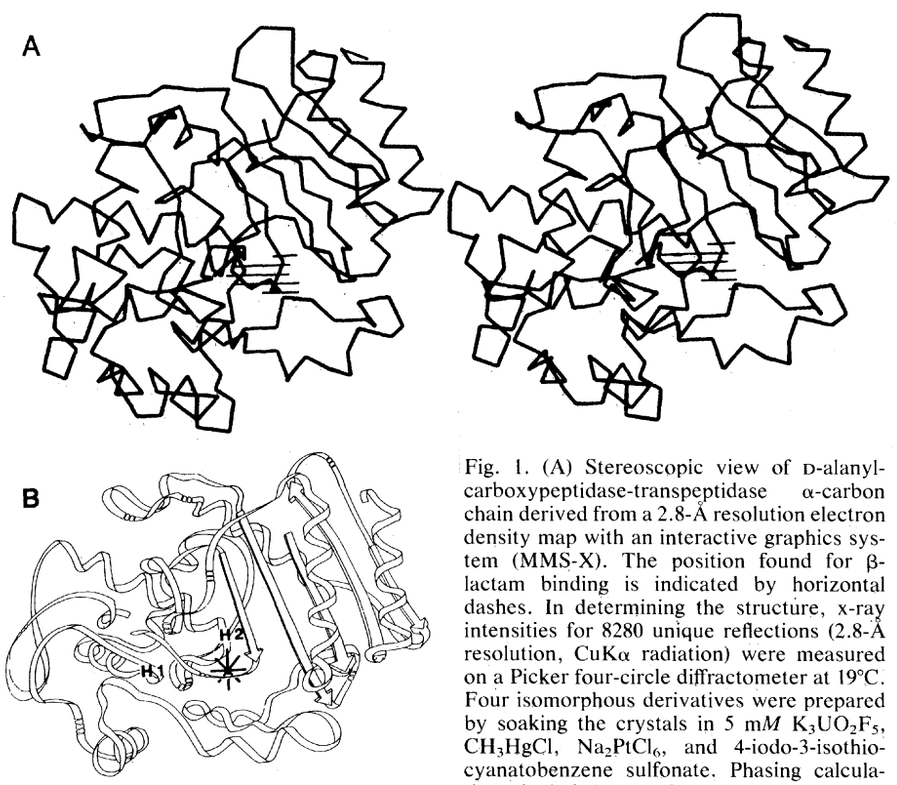


Fig. 1. (A) Stereoscopic view of D-alanyl-carboxypeptidase-transpeptidase α-carbon chain derived from a 2.8-Å resolution electron density map with an interactive graphics system (MMS-X). The position found for β-lactam binding is indicated by horizontal dashes. In determining the structure, x-ray intensities for 8280 unique reflections (2.8-Å resolution, CuKα radiation) were measured on a Picker four-circle diffractometer at 19°C. Four isomorphous derivatives were prepared by soaking the crystals in 5 m*M* $K_3UO_2F_5$, CH_3HgCl, Na_2PtCl_6, and 4-iodo-3-isothiocyanatobenzene sulfonate. Phasing calculations included use of anomalous scattering differences and resulted in an overall mean figure-of-merit of 0.72. The amino acid sequence is unknown (*9*), and there is one break in the connectivity of our chain tracing at this resolution. (B) Ribbon representation of transpeptidase backbone chain, with view direction as in (A). The amino terminus begins the α helix on the right. Arrows indicate the directions of β strands. Dashed segments are areas of weak electron density in the current map. The β-lactam binding site is marked near helix NH_2-termini H1 and H2. The molecule consists of 30 percent α helix (eight segments ranging in length from 10 to 15 residues) and 10 percent β structure (five strands). The β-sheet has a slight left-handed twist (*15, 16*) (overall Ω = −30°). Three of the eight helices protect both faces of the β-sheet. Four helices, including the single helix on the back face of the sheet, form a group whose members are unidirectional, but with rather large neighbor-to-neighbor twist angles of 40° (see Fig. 3).

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mology between the bifunctional penicillin-sensitive R61 carboxypeptidase-transpeptidase and the smaller penicillin-insensitive D-alanyl-carboxypeptidase from *Streptomyces albus* G (*17, 18*). Monofunctional D-alanyl-carboxypeptidases, although able to cleave the terminal D-alanine from peptidoglycan (step 1), are unable to catalyze transpeptidation (step 2). Their significance in the cell wall biosynthetic process is unclear. Some D-alanyl-carboxypeptidases are inhibited by β-lactams, others are not. The insensitive carboxypeptidase from *S. albus* G is a two-lobed zinc-requiring molecule with an α-helical NH_2-terminal domain and an α + β COOH-terminal domain with five strands of β structure located near the peptide-binding site. The suggestion that the D-alanyl-carboxypeptidases are ancestors of the penicillin-destroying β-lactamases (*1, 2*) is supported by similarities in primary (*19*) and secondary (*20*) structure. It is therefore conceivable that the penicillin binding site (see below) in this D-alanyl-carboxypeptidase-transpeptidase is broadly similar to the penicillin hydrolysis site in the β-lactamases.

Binding of a cephalosporin and a penicillin to the transpeptidase was observed crystallographically by the Fourier difference synthesis method. Figure 2 shows the electron density difference at 2.8-Å resolution resulting from soaking the enzyme crystal in 15 m*M* cephalosporin C (Lilly) at *p*H 6.8 at room temperature for 46 hours before x-ray data collection. The density is commensurate with the size of the nucleus of the antibiotic, as can be seen by the placement of the cephalosporin structure within the density. Density at the same location was observed after soaking the transpeptidase crystal in 40 m*M* *o*-iodophenylpenicillin.

The β-lactam binding site in relation to the enzyme's secondary structure is shown schematically in Fig. 3. The β-lactam's position near the NH_2-terminal ends of two α helices is in accord with the generalization of Hol *et al.* (*21*), who proposed that the large dipole moment of the helix, equivalent to one-half positive charge at its NH_2-terminus, may be a significant factor in stabilizing intermediates in a catalytic pathway. In view of this proposal, the attraction of a β-lactam to two helix NH_2-termini in a transpeptidase could explain the long known fact that all β-lactams contain a negative carboxylate group, or in the case of some new monocyclic β-lactams, a sulfonate group. Final alignment of the β-lactam within this binding region depends on more subtle interactions with nearby amino acid side groups; recent chemical modification studies (*13*) indicate a role for an arginine residue.

The hypothesis of Tipper and Strominger (*2*) for the mode of action of β-lactam antibiotics is that substrate and β-lactam bind at the same location on the transpeptidase. We have done a crystal soaking experiment to determine the enzymic location of the R-D-alanyl-D-alanine substrate binding site. Using a substrate analog, α-*tert*-butyloxycarbonyl-ε-(trifluoroacetyl)-L-lysyl-D-glutamyl-D-alanine, we produced a 4-Å resolution difference map with significant electron density at the β-lactam site (not shown). This result suggests that the site on the enzyme responsible for the catalysis of peptidoglycan cross-linking in the growing bacterial cell wall overlaps the antibiotic binding site.

This x-ray analysis has revealed the structural organization of a β-lactam–sensitive transpeptidase and has shown that both penicilloyl- and cephalosporyl-enzyme intermediates can be observed within the time required for x-ray data collection. We have established the feasibility of using high-resolution crystallographic methods to visualize details of the binding of a series of β-lactams to a target enzyme. The geometric fit of each β-lactam to the receptor site can be correlated with its inhibitory strength and antibiotic activity. Description of the enzymic architecture at the receptor site will provide heretofore unknown stereochemical parameters for the design of β-lactam antibiotics (or non-β-lactam antibiotics) and will increase our understanding of the molecular mechanism of drugs that inhibit bacterial cell wall biosynthesis.

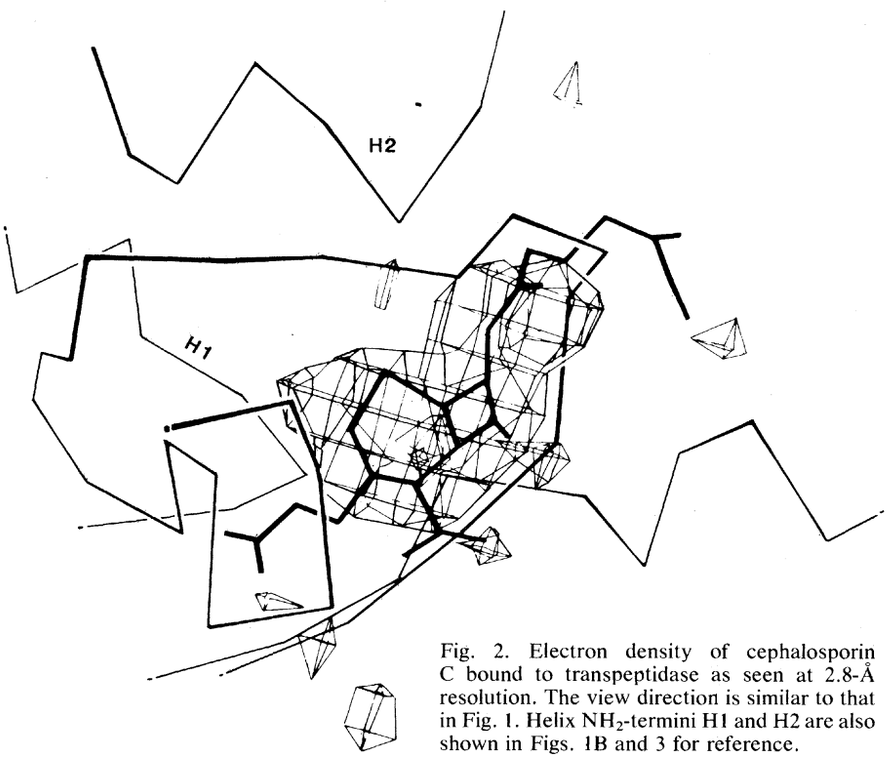

Fig. 2. Electron density of cephalosporin C bound to transpeptidase as seen at 2.8-Å resolution. The view direction is similar to that in Fig. 1. Helix NH_2-termini H1 and H2 are also shown in Figs. 1B and 3 for reference.

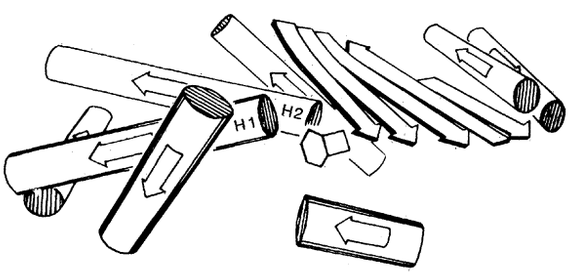

Fig. 3. Relation of β-lactam site to secondary structure elements in transpeptidase. Helices and β-strands are represented by cylinders and arrows, respectively. Directions indicated on each are from amino to carbonyl group. The view direction is approximately as in Fig. 1.

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Echolocation in Bats: The External Ear and Perception of the Vertical Positions of Targets

Abstract. *Echolocating bats (*Eptesicus fuscus*) can perceive changes of as little as 3° of arc in the vertical angles separating pairs of horizontal rods. This acuity depends upon modification of sounds entering the external ear canal by the structures of the external ear. Deflection of the tragus degrades the acuity of vertical-angle perception from 3° to about 12° to 14°. The pinna-tragus structure produces a strong secondary echo of sounds entering the external ear canal, and the delay of this echo after the time when the sound directly enters the ear canal apparently encodes the vertical direction of a sound source.*

Bats (Microchiroptera) use a form of biological sonar called echolocation for orientation in the environment (*1*). They can perceive the size, shape, direction, distance, and velocity of objects by using acoustic images as a substitute for vision (*2, 3*). Acoustic information in sonar echoes (intensity, frequency, and time of occurrence) represents various target features to the bat (*3*) and the bat's auditory system processes these echoes to reconstitute spatial images of objects within the bat's brain (*4*). The perceptual operating range of echolocation by various species of insectivorous bats extends out to roughly 1 to 10 m for targets comparable in size to the flying insects on which these bats prey (*5*). Because perception of auditory space is such an important part of the bat's use of hearing, the mechanisms of sound localization in echolocation are likely to be highly developed and correspondingly more clearly observed in bats than in other kinds of animals where spatial perception is only one aspect of the use of hearing. We report here the results of experiments which show that echolocating bats can perceive the locations of sonar targets in the vertical plane with exceptional accuracy. Furthermore, the acoustic cues for vertical localization by bats may be more distinguishable and more experimentally separable than has been demonstrated in other mammals, including humans, perhaps as a consequence of their heightened significance in the bat's life.

Echolocating bats of the species *Eptesicus fuscus* (the big brown bat; family Vespertilionidae) were trained with the use of methods described in (*6*) to discriminate between two simultaneously presented pairs of horizontal brass rods (4 mm in diameter and 22 cm long) separated by vertical angles of different sizes. The rods were suspended in a plane located 44 cm from the bat by nylon monofilament wires 0.15 mm in diameter and the rods in the pairs were oriented to be perpendicular to the bat's "line of sight" from its observing position on an elevated platform. Each bat was trained to approach the pair of horizontal rods separated by the smaller vertical angle (positive stimulus) by moving forward a few centimeters onto one of two other elevated platforms located in the same directions as the pairs of rods. The bat received a piece of a mealworm (*Tenebrio* larva) offered in forceps as a food reward for its correct choice. Responses to the other pair of rods, separated by the larger vertical angle (negative stimulus), were not rewarded.

The smaller vertical angle was kept constant at 6.5°, and the larger angle was varied from an initial 40° down to 35°, 30°, 25°, 20°, 16°, 13°, 11°, 9.5°, 8.5°, 7.5°, and 6.5°. When each bat had learned to respond correctly to the 6.5° angle at a criterion of better than 90 percent correct responses for 50 consecutive trials, the larger angle was reduced to 35° and the experiment begun. For the larger vertical angles (negative stimuli) down to 13°, 30 trials were conducted for each bat before the arrangement was changed to the next smaller angle. For negative stimuli of 11° and smaller, 50 trials were conducted for each value. By pairing an angle of 6.5° with successively smaller angles from 40° to 6.5°, we could assess psychophysically the acuity of the bat's discrimination of vertical angles by the descending method of limits (*6*). Of the two bats which completed the entire experiment, one (bat a) had been blinded (enucleated) to prevent the possible use of visual cues in the discrimination.

The graph in Fig. 1 shows the performance of two bats on discriminations of a vertical angle of 6.5° versus angles from 16° to 7.5° (angular differences from 9.5° to 1.0°). The bats readily discriminated larger angular differences and proceeded smoothly through the descending method-of-limits procedure to the region near threshold performance indicated in Fig. 1. If we use 75 percent correct responses as an index of the limits of the bat's performance, we find that *Eptesicus fuscus* can discriminate vertical angles with an acuity of 3° to 3.5°. This compares well with an acuity of about 1.5° for discrimination of horizontal angles in a similar experimental procedure (*7*).

The mechanisms for perception of the vertical position of a sound source by humans appear to involve modifications imposed upon sounds entering the external ear canal by the external ear acting as a receiving antenna (*8*). Sound reflects in different ways from the ridges and folds of the external ear, depending upon the vertical angle of incidence of the sound.

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