Studying enzyme-β-lactam interactions using X-ray diffraction

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The interaction of representative β-lactam antibiotics with a bacterial enzyme target has been mapped in three dimensions using X-ray diffraction data to 2.25 Å resolution. Examination of complexes of cephalosporin C, benzylmonobactam, and α-(2,3)-methylenepenicillin G with the D-alanyl-D-alanine transpeptidase-carboxypeptidase from Streptomyces *R61 shows that the enzyme's reactive serine has acylated the β-lactam ring of each inhibitor. The known half-lives of the three acyl complexes can be correlated with the distance of the drug's carboxylate (or sulfonate) group from complementary groups on the DD-peptidase.*

Keywords: β-lactam antibiotics, X-ray diffraction, molecular recognition

When an antibiotic molecule binds to its target, molecular recognition is an essential factor in determining the effectiveness of a drug molecule. In the case of β-lactam antibiotics (e.g., penicillins, cephalosporins and monobactams), the targets are the D-alanyl-D-alanine transpeptidases/carboxypeptidases (D-D peptidases). These bacterial enzymes are responsible for cross-linking the bacterial peptidoglycan during the final stages of cell wall biosynthesis. D-D peptidases catalyze the cleavage of the terminal D-alanyl-D-alanine peptide bond, functioning as a transpeptidase if an appropriate amino acceptor on a neighboring peptidoglycan strand is available for cross-linking, or as a carboxypeptidase if the acceptor is a water molecule (Figure 1).

It has long been appreciated that the binding of β-lactams by the D-D peptidases is a case of mistaken recognition. In 1965, Tipper and Strominger[1] proposed that the β-lactam bond of penicillins was an analog of the peptide bond of the natural D-alanyl-D-alanine substrate.

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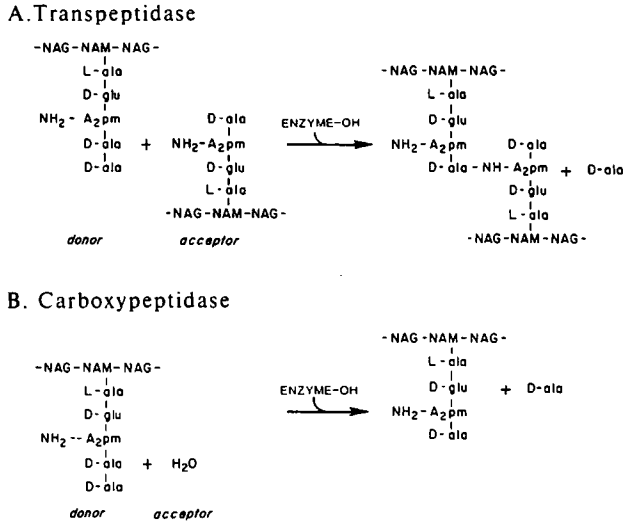

Figure 1. Two reactions catalyzed by an active-site serine D-D peptidase with glycopeptide substrate (NH_2-A_2 pm is diaminopimelic acid). In each reaction sequence, (a) and (b), the first reactant represents the carbonyl donor and the second represents an appropriate amino acceptor for the transpeptidation reaction. In the carboxypeptidation reaction, water acts as the acceptor molecule. Both reactions occur via the formation of an acyl-enzyme intermediate using the catalytic serine residue's side chain

During the more than twenty years since the substrate analog hypothesis was made, researchers have tried to understand the wide variability observed in the binding affinity and potency of β-lactams. Efforts have been made to identify the critical parameters of β-lactams that correlate well with their ability to act as effective inhibitors of the D-D peptidases, but exceptions always arise.

CRYSTALLOGRAPHIC METHODS

We have attempted to gain insight into understanding why one drug molecule has a long half-life on a target

enzyme and another has a short half-life on the same enzyme by making use of our three-dimensional (3D) model of the D-D peptidase from *Streptomyces* R61 that was determined using X-ray diffraction techniques.[2] The method we used was to diffuse the antibiotics of interest into preformed, native crystals of the R61 enzyme; to collect the X-ray diffraction data for the complexes; to subtract the native data from that of the complexes; and to image the differences between the native structure and the derivatized forms using difference Fourier synthesis to calculate difference electron density maps. When successful, this approach reveals the position of the bound antibiotic and any shifts in the enzyme induced by binding the drug.

We have looked at three β-lactam molecules—one cephalosporin, one penicillin and one monobactam—that exhibit a wide range of half-lives when complexed with the R61 enzyme in solution (see Table 1). Specifically, the three drug molecules used in these studies were cephalosporin C, provided by Eli Lilly; the monobactam analog of penicillin G, provided by Squibb; and (2,3)-α-methylene benzyl-penicillin, provided by Hoffman-La Roche. Their structures are shown in Figure 2.

ANALYSIS OF ELECTRON DENSITY MAPS

When the difference Fourier maps for each of the three complexes were examined, it was evident that the drug molecules all bound at a similar site on the R61 enzyme. The enzyme has two distinct clusters of secondary structure elements: one region is all helical, and a second is made up of a five-stranded antiparallel β-sheet flanked on both faces by helices. The active site where the catalytic serine residue (Ser 62) is located is found between these two regions of the enzyme (Figure 3).

To interpret the details of binding of the drug molecules, models of each antibiotic were docked into the experimentally observed electron density maps that resulted from our difference Fourier analyses. It was immediately evident that the intact drug molecules, as shown in Figure 2, could not be fitted into the individual electron density maps. The drugs had, indeed, reacted with the enzyme, and marked changes to their structures had been recorded in our X-ray diffraction experiments.

Table 1. The three β-lactam molecules exhibit a wide range of half-lives when complexed with the R61 enzyme in solution

β-lactam	Half-life of drug-enzyme complex in solution at 30°C
Cephalosporin C	7 days[7]
Monobactam analog of penicillin G	6 days[8]
(2,3)-α-Methylene-benzyl penicillin	13 hours[8]

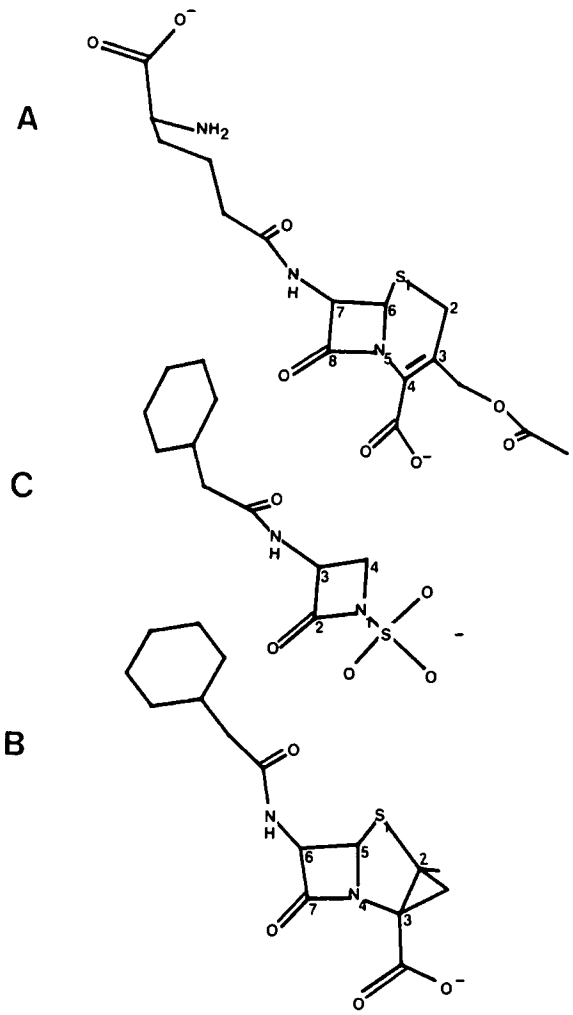

Figure 2. (a) Cephalosporin C (Lilly); (b) (2,3)-α-methylene-benzylpenicillin (Hoffmann-La Roche, R023-6829); (c) monobactam analog of penicillin G (Squibb, SQ26,324)

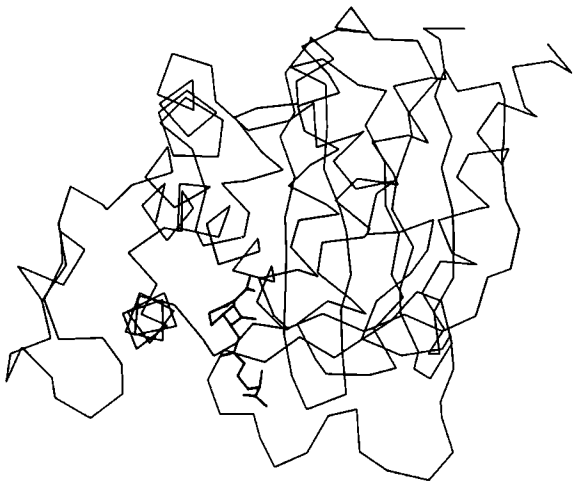

Figure 3. Alpha carbon trace of R61 enzyme. Antiparallel β-sheet with helices on both faces found on right, all-helical region on left. Hydrolyzed cephalosporin C (represented with bold lines) at experimentally determined binding site between the β-sheet and all-helical region

THE CEPHALOSPORIN C MAP

In the case of cephalosporin C, there was no density for the C3 acetate group present on the six-membered dihydrothiazine ring of the intact drug molecule. The ability of the C3 substituent to act as a good leaving group is correlated with the ease of opening the β-lactam bond and the formation of a stable acyl-enzyme complex.[3,4]

Further evidence that the β-lactam bond had been broken and the strain of the four-membered β-lactam ring had been released was the fact that tetrahedral carbon atoms at C6 and C7 of cephalosporin C fit the density observed while bridgehead carbons with the 90-degree angle imposed by an intact β-lactam ring did not. An additional major change to the structure was a rotation of approximately 50 degrees about the C6–C7 bond, taking the dihydrothiazine ring from its position when fused to the β-lactam ring in the intact structure to a staggered configuration with respect to the C7–C8 bond. Figure 4 shows the difference between the structures of the intact cephalosporin C and its form in the acyl-enzyme complex as isolated in these X-ray diffraction studies. Note that the carbonyl group of the β-lactam is being shown as a carboxylate in the open-ring form indicating its covalent attachment to the catalytic serine, the second oxygen on C8 being the gamma oxygen of the serine side chain. This configuration is clearly indicated in our difference map. The shift of the gamma oxygen of Ser 62 from its native position to that in the acyl-enzyme complex is the only major protein shift observed on binding the inhibitory drug molecule.

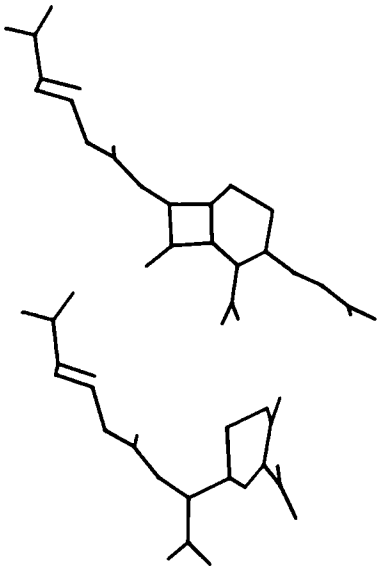

Figure 4. Top—intact cephalosporin C molecule; bottom—hydrolyzed form of drug as determined by fitting drug into difference electron density map

Figure 5 shows the region of the active site of the R61 enzyme with the cephalosporin C bound. Several notable features are apparent on examining the disposition of the drug molecule with respect to the topology of the enzyme's active site. On the right side of Figure 5, one can see the inner strands of the antiparallel β-sheet. Recalling Tipper and Strominger's substrate analog hypothesis, the C7 amide substituent and the β-lactam of the drug are said to be comparable to a D-D dipeptide. Examining the directionality of the pseudopeptide, one sees its sense is antiparallel to the innermost strand of the β-sheet, thereby extending the motif of the antiparallel sheet. This provides a generalized bonding pattern that would be appropriate for normal peptide substrates and would also be consistent with the high tolerance for variation in the side chains of the four-membered ring in β-lactams.

In the center of Figure 5 and to the left, one sees the N-terminal portions of the two alpha helices that begin at the active site area and traverse the all-helical region of the enzyme. The catalytic Ser 62 is found at the N terminus of the central helix. Associated with helices in proteins are dipole moments that result in partial positive charges on the amino termini of the helices. This positive environment is, no doubt, important in attracting to the active site the carboxyl-terminated substrate or β-lactams that have a negatively charged group (carboxylate or sulfate) adjacent to the β-lactam nitrogen. Also contributing to the positive environment of the active site is a lysine residue that is invariant in all penicillin-binding proteins and that occurs three residues after the catalytic serine.[5] The necessity of this spacing is now apparent from our crystallographic studies of the R61 enzyme. If this con-

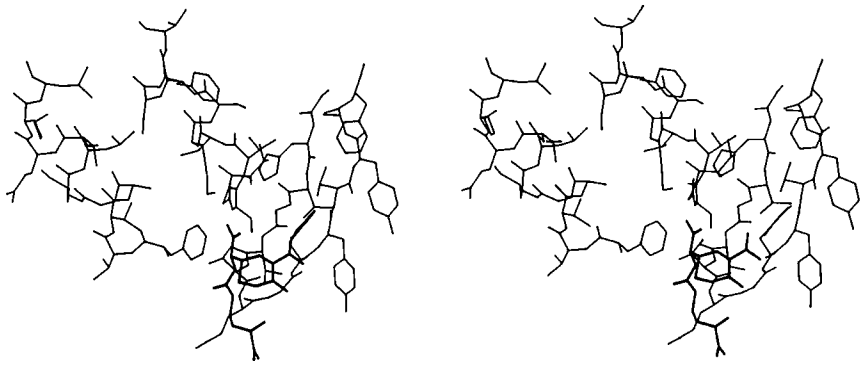

Figure 5. Stereoscopic view of active site of R61 enzyme with hydrolyzed cephalosporin C bound. For clarity, covalent link of acyl-enzyme complex (between Ser 62 gamma oxygen and C8 of drug) is not shown

served lysine is to play a role in binding and/or catalysis, two residues must follow the reactive serine to complete the first turn of helix following the serine in order to bring the lysine side chain back into the region of the active site. An additional basic residue is found at the top of the active site in Figure 5, a histidine located on the innermost strand of the β-sheet. When other penicillin-binding protein sequences are examined, we see that there is a conserved triad that is found approximately 60 residues from the carboxyl termini of the sequences.[5,6] In all sequences (except the R61 enzyme), one finds lysine (histidine)-threonine or serine-glycine, creating a pattern of basic-hydroxyl-glycine. The basic residue is adding to the positive environment, the hydroxyl is a potential hydrogen-bonding residue, and the absolutely conserved glycine must be important for steric considerations because it is the residue closest to the catalytic serine side chain.

THE MONOBACTAM AND PENICILLIN MAPS

When the difference Fourier maps for the monobactam and the penicillin were fitted with models of these drugs, it was again necessary to break the β-lactam bond, tetrahedralize the bridgehead carbon atoms and rotate approximately 50 degrees about the bond between those two atoms. For these compounds, there is no leaving group comparable to the C3 acetate of cephalosporin C. The intact monobactam and its acyl-enzyme form are shown in Figure 6, and the same two forms for the penicillin are shown in Figure 7. When one examines the binding position of the monobactam and the penicillin in the active site of the R61 enzyme (Figures 8 and 9), one finds they are analogous to that of cephalosporin C. However, when one compares in detail the configurations by superimposing these structures on the ring-open cephalosporin, an interesting difference is noted. For the open-ring monobactam/cephalosporin pair (Figure 10), we see good overlay of the nucleus of the two structures, with close positioning of the cephalosporin ring carboxylate and the monobactam sulfate. Some variation in the side-chain positions is observed, but this is not unexpected because these portions of the molecules are on the surface of the enzyme. The superposition of the open-ring penicillin with the cephalosporin structure shows a quite different position for the ring carboxylates (Figure 11). In the cephalosporin, C4 is a planar carbon because of a double bond in the ring. In the penicillin, this atom's counterpart, C3, is a tetrahedral carbon. This change of geometry results in a position for the carboxylate 2 Å from the position of the cephalosporin C4 carboxylate. This is the major difference between the three acyl-enzyme forms of the drug molecules. It is interesting to note that the half-lives of the cephalosporin and the monobactam are comparable, while that of the penicillin is shorter by a factor of more than 20.

CONCLUSIONS

At this stage of our studies, we now know that we can isolate drug-enzyme complexes and examine the binding in detail. By systematically studying families of drugs with varying kinetic properties, we may begin to recognize the essential features responsible for molecular recognition and better understand the inhibition of D-D peptidases by this important class of antibiotics.

ACKNOWLEDGEMENT

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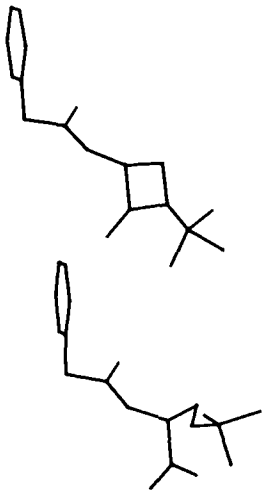

Figure 6. Top—intact monobactam analog of penicillin G; bottom—hydrolyzed form of drug as determined by fitting drug into difference electron density map

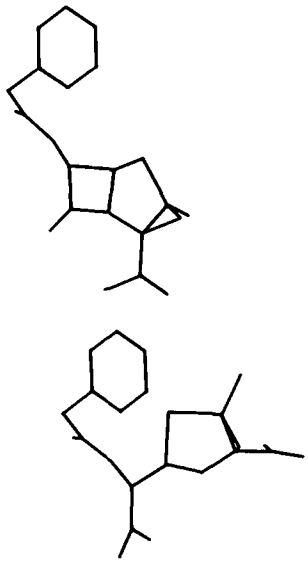

Figure 7. Top—intact (2,3)-α-methylene-benzylpenicillin; bottom—hydrolyzed form of drug as determined by fitting drug into difference electron density map

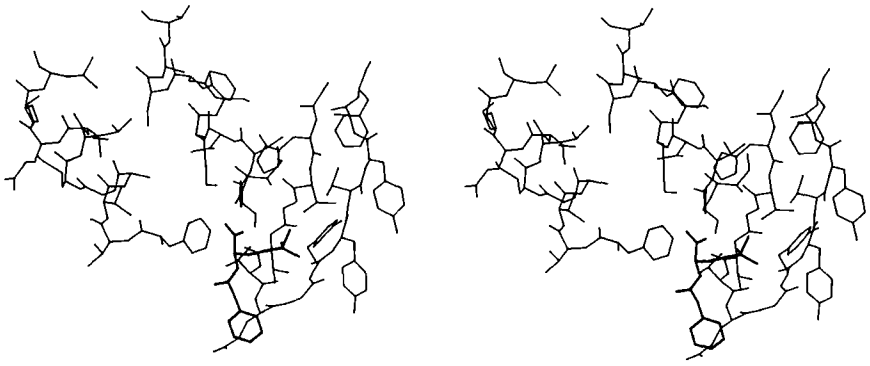

Figure 8. Stereoscopic view of active site of R61 enzyme with hydrolyzed monobactam bound. For clarity, covalent link of acyl-enzyme complex (between Ser 62 gamma oxygen and C2 of drug) is not shown

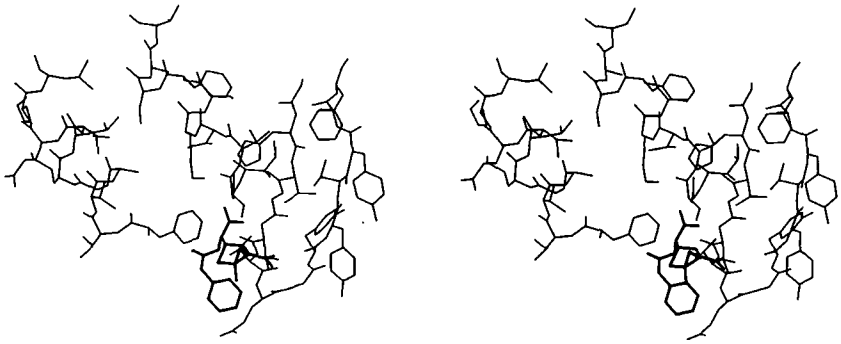

Figure 9. Stereoscopic view of active site of R61 enzyme with hydrolyzed penicillin bound. For clarity, covalent link of acyl-enzyme complex (between Ser 62 gamma oxygen and C7 of drug) is not shown

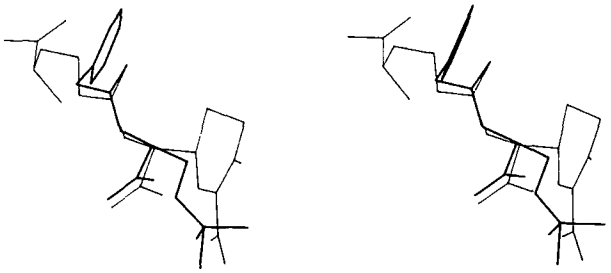

Figure 10. Stereoscopic view of the positions of the acyl-enzyme forms of cephalosporin C and monobactam (bold lines) as determined by Fourier difference analyses

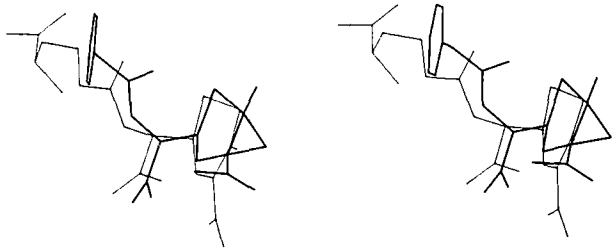

Figure 11. Stereoscopic view of the positions of the acyl-enzyme forms of cephalosporin C and penicillin (bold line) as determined by Fourier difference analyses

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REFERENCES

1 Tipper, D.J., and Strominger, J.L. *Proc. Natl. Acad. Sci.* USA 1965, **54**, 1133–1141

2 Kelly, J.A., Knox, J.R., Moews, P.C., Hite, G.J., Bartolone, J.B., and Zhao, H. *J. Biol. Chem.* 1985, **260**, 6449–6458

3 Faraci, W.S., and Pratt, R.F. *J. Am. Chem. Soc.* 1984, **106**, 1489–1490

4 Boyd, D.B. *J. Org. Chem.* 1985, **50**, 886–888

5 Kelly, J.A., Boyington, J.C., Moews, P.C., Knox, J.R., Dideberg, O., Charlier, P., Libert, M., Wery, J.P., Duez, C., Joris, B., Dusart, J., Frere, J.M., and Ghuysen, J.M. In *Frontiers of Antibiotic Research,*

H. Umezawa (Ed.) Academic Press, New York, 1987, 327–337
6 Joris, B., Ghuysen, J.M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frere, J.M., Kelly, J.A., Boyington, J.C., Moews, P.C., and Knox, J.R. *Biochem. J.* 1988, **250**, 313–324
7 Ghuysen, J.M., Frere, J.M., Leyh-Bouille, M., Coyette, J., Dusart, J., and Nguygen-Disteche, M. *Annu. Rev. Biochem.* 1979, **48**, 73–101
8 Georgopapadakou, N.H., Russo, D.A., Liebman, A., Burger, W., Rossman, P., and Keith, D. *Antimicrob. Agents Chemother.* 1987, **31**, 1069–1074