A 1.2-Å snapshot of the final step of bacterial cell wall biosynthesis

Wenlin Lee*, Michael A. McDonough†, Lakshmi P. Kotra*, Zhi-Hong Li*, Nicholas R. Silvaggi†, Yoshifumi Takeda*, Judith A. Kelly†‡, and Shahriar Mobashery*‡

†Department of Molecular and Cell Biology and Institute of Materials Science, University of Connecticut, Storrs, CT 06269-3125; and *Institute for Drug Design and the Department of Chemistry, Wayne State University, Detroit, MI 48202-3489

Edited by Gregory A. Petsko, Brandeis University, Waltham, MA, and approved November 14, 2000 (received for review August 11, 2000)

The cell wall imparts structural strength and shape to bacteria. It is made up of polymeric glycan chains with peptide branches that are cross-linked to form the cell wall. The cross-linking reaction, catalyzed by transpeptidases, is the last step in cell wall biosynthesis. These enzymes are members of the family of penicillin-binding proteins, the targets of β-lactam antibiotics. We report herein the structure of a penicillin-binding protein complexed with a cephalosporin designed to probe the mechanism of the cross-linking reaction catalyzed by transpeptidases. The 1.2-Å resolution x-ray structure of this cephalosporin bound to the active site of the bifunctional serine type D-alanyl-D-alanine carboxypeptidase/transpeptidase (EC 3.4.16.4) from *Streptomyces sp.* strain R61 reveals how the two peptide strands from the polymeric substrates are sequestered in the active site of a transpeptidase. The structure of this complex provides a snapshot of the enzyme and the bound cell wall components poised for the final and critical cross-linking step of cell wall biosynthesis.

Since their clinical introduction more than 50 years ago, β-lactam antibiotics have been essential drugs in combating bacterial infections. To date, β-lactams constitute approximately 60% of all clinically used antibiotics. These antibiotics interfere with cell wall biosynthesis. All bacteria, with very few exceptions, have a cross-linked cell wall (1, 2). The cross-linking takes place as the last step in biosynthesis of cell wall, a reaction that is catalyzed by bacterial transpeptidases (1, 3–6), enzymes that rely on an active-site serine for their reactions. The activated serine of transpeptidase attacks the carbonyl of the penultimate D-Ala of the peptidoglycan precursor, with concomitant departure of the terminal D-Ala, to give rise to an acyl-enzyme intermediate (Fig. 1*A*). An amine from the side chain of another peptidoglycan (diaminopimelate, modified lysine, or ornithine derivative) reacts with the ester of the acyl-enzyme intermediate to give the cross-linked species.

It was suggested by Tipper and Strominger (7) that a β-lactam antibiotic, such as a penicillin or a cephalosporin, mimics the conformation of the acyl-D-Ala-D-Ala portion of the bacterial peptidoglycan. It also was found that β-lactam antibiotics covalently modify the active-site serine of transpeptidase via the β-lactam carbonyl group, which spatially corresponds to the carbonyl of the penultimate D-Ala of the pentapeptide. The penicillin structure in its entirety remains tethered to the enzyme as an integral part of the long-lived acyl-enzyme species, presenting a steric barrier to the approach of the second strand of peptidoglycan at the ester carbonyl (8). With the active site covalently modified by the antibiotic, the enzyme is no longer available for its normal function, transpeptidation is prevented, and bacterial death ensues.

We designed and synthesized cephalosporin **1**, which incorporates components of the cell wall in its structure (Fig. 1*B*). We envisioned that cephalosporin **1** would modify the active-site serine of the transpeptidase, as would other β-lactams, by mimicking the acyl-D-Ala-D-Ala portion of the peptidoglycan (the portions of **1** in red in Fig. 1*B*). But, the portion to the right of the acylated species with our cephalosporin (in green; complex **2**, Fig. 1*B*) would mimic the approaching nucleophile, diaminopimelate, from the second strand of the peptidoglycan. Hence, cephalosporin **1** was expected to be an inhibitor of transpeptidases, albeit a unique one with structural characteristics of the two strands of peptidoglycan. Complex **2** represents a snapshot of the two peptidoglycan strands just before the cross-linking reaction, when diaminopimelate from the second strand approaches the ester of the acyl-enzyme intermediate formed by the transpeptidase bound to the first strand of peptidoglycan. Although there are some variations in the structures for the cross-links of peptidoglycans in different types of bacteria, the structure of peptidoglycan that is incorporated into cephalosporin **1** is seen for all Gram-negative bacteria and occurs in many Gram-positive organisms (1). Therefore, the mechanistic conclusions drawn from complex **2** would be valid for the majority of bacteria. We hasten to add that the two "strands of peptidoglycan" would be tethered to each other in complex **2**. It turns out that this reduction in the degrees of freedom for the two strands is absolutely necessary for the structure determination, in light of the fact that numerous attempts at binding derivatives of peptidoglycan into the PBP active sites have so far failed to reveal any structural information. We report herein the synthesis of cephalosporin **1**, the complex of a transpeptidase modified by **1** and its mechanistic implications for the cross-linking of the bacterial cell wall.

Methods

The synthetic procedures for the preparation of cephalosporin **1** and each of the intermediates, and their characterization by high-field ^{13}C and ^{1}H NMR, IR, and high-resolution mass spectra can be found in the supplemental material and Tables 2 and 3, which are published on the PNAS web site, www.pnas.org. All compounds containing the oxazolidine moiety exhibited two sets or broadening of NMR signals at room temperature, due to the presence of a dynamic equilibrium between two conformers in solution in the absence of enzyme (9, 10). Additionally, compounds with peptide chains attached to the cephalosporin moiety also exhibited additional conformers. These conformational states were studied either by recording the spectra at higher temperatures or by reducing the concentration to minimize such conformational states that may be induced in an intermolecular set of interactions.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PBP, penicillin binding protein; DAP, diaminopimelate; NAM, *N*-acetylmuramic acid.

Data deposition: The coordinates of the complex reported in this paper have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID code 1HVB).

See commentary on page 1319.

‡To whom reprint requests should be addressed. E-mail: som@chem.wayne.edu or kelly@uconnvm.uconn.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

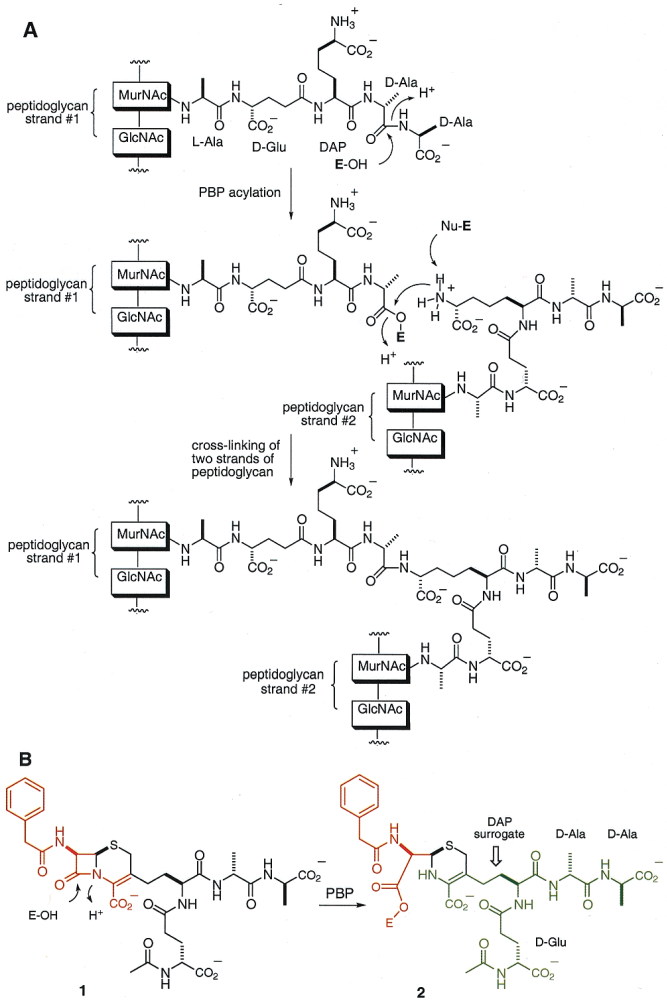


Fig. 1. (*A*) A representative transpeptidation reaction for Gram-negative bacteria and some Gram-positives catalyzed by certain penicillin-binding proteins (PBPs), beginning with acylation of the active-site serine of the enzyme by the peptide of one strand of peptidoglycan (E denotes the enzyme). Reaction of the second strand of the peptidoglycan with the ester of the acyl-enzyme intermediate results in the cross-linked cell wall. (*B*) The backbone of cephalosporin **1** (in red) mimics the terminal acyl-D-Ala-D-Ala portion of the peptide branch of the first strand of the peptidoglycan. The C-7 acyl moiety, the phenylacetyl group, is that seen in penicillin G. Cephalosporin **1** acylates the active site serine of the transpeptidase, as would the peptide from the first strand of peptidoglycan, concomitant with the departure of the terminal D-Ala. The β-lactam nitrogen and its adjacent carbon and carboxylate collectively serve as a surrogate for the departing D-Ala, and the same atoms constitute a portion of the incoming DAP surrogate in strand 2 in complex **2**. The acyl-enzyme species **2** depicts the first enzyme-bound ''peptidoglycan'' strand (shown in red) poised to receive the amine of DAP from the second strand of peptidoglycan (shown in green).

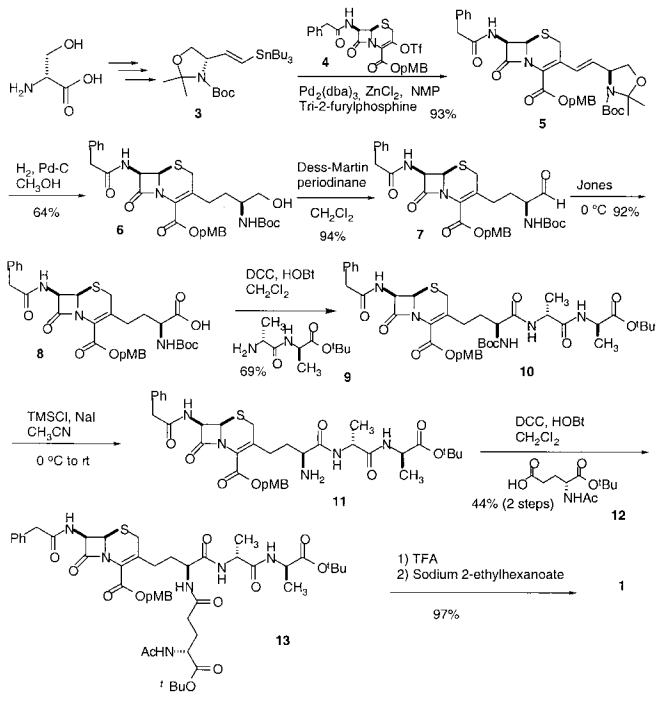
Crystallization and Refinement. A 0.30 × 0.20 × 0.15-mm native R61 DD-peptidase/transpeptidase crystal (space group $P2_12_12_1$, cell dimensions a = 51.8, b = 66.4, and c = 100.1 Å, one 37.5-kDa molecule per asymmetric unit) was soaked for 4 days in a 30 mM solution of cephalosporin **1**, 28% PEG-8000, 50 mM sodium phosphate at pH 6.8. The crystal then was dipped in cryoprotectant, made up of the soak solution plus 20% glycerol, and flash-cooled in a nitrogen gas stream. Data were collected at beamline X12C of the National Synchrotron Light Source at Brookhaven National Laboratory, Brookhaven, New York and processed by using HKL (11). Initial refinement was carried out by using CNS (12). The refined model was fitted to $2F_o-F_c$ and F_o-F_c electron density maps by using CHAIN (13). Several rounds of refinement and fitting were performed. Solvent molecules, with reasonable hydrogen bond distances and geometry, were added except in the region of the ligand-binding site. The agreement between the model of the complex and the diffraction data are measured by the R factor, which converged in CNS to 0.214. After CNS refinement, SHELX-97 (14) was used to refine the molecule anisotropically, which resulted in a 0.035 drop in R_{free}, an independent measure of the progress of the refinement. The program O (15) was used to analyze and fit electron density maps from SHELX-97. At this stage, the ligand and alternate side-chain conformations were added. When the R factor converged in SHELX-97, hydrogen atoms were added that appeared as 3-σ difference density peaks.

Energy Minimizations and Molecular Dynamics Simulations. Energy minimizations and molecular dynamics simulations were performed by using the AMBER 5.0 suite of software (16, 17). Graphical and molecular database analyses were performed by using the SYBYL software (version 6.5, Tripos Associates, St. Louis). For cephalosporin **1** and for all other residues, MNDO electrostatic potential charges and standard AMBER parameter set were used, respectively. Carbohydrate residues, *N*-acetylglucosamine and *N*-acetylmuramic acid (NAM), were treated by using the GLYCAM force field parameter set (18). The 1.2-Å resolution crystal structure of the *Streptomyces* R61 PBP complexed with the cephalosporin **1** was used for the starting coordinates. Initially, the protein molecule and its crystallographic symmetry counterpart, including the crystallographic water molecules, were solvated with 25,692 TIP3 waters and subjected to energy minimization for 5,000 iterations. Subsequently, molecular dynamics simulations were carried out on the assembly at constant volume for 10 ps at 300 K in the time steps of 2 fs by using periodic boundary conditions. The system was then further equilibrated at constant pressure of 1 atm for 40 ps at 300 K by using periodic boundary conditions with nonbonded cut-off of 12 Å. After 50 ps, snapshots of the molecular dynamics simulations were collected at every picosecond for up to 248 ps. The average rms deviation of C_α atoms was 1.28 ± 0.18 Å. Coordinates of the energy-minimized inhibitor in the presence and absence of the symmetry-related protein molecule were extracted and overlapped to compare the conformational differences.

Results and Discussion

Compound **1** was synthesized according to Scheme 1. The key intermediate **5** was obtained by a Stille coupling between stannane **3** and triflate **4.** Several other attempts, including the Wittig reaction, Suzuki coupling, Heck coupling, and Zn-catalyzed Reformatsky-type coupling, to form the C—C bond between cephalosporin derivatives and appropriate oxazolidines were not successful. Subsequent hydrogenation of **5** in excess Pd/C gave the alcohol **6**. The isopropylidene moiety was removed presumably by the slight acidity present in the commercial Pd/C. A two-step Dess-Martin/Jones oxidation of **6** afforded the acid **8** in high yield. Compound **8** then was coupled to the protected D-Ala-D-Ala **9** by dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) to give **10**. The Boc group of **10** was removed in the presence of chlorotrimethylsilane (TMSCl)/NaI. Another DCC coupling of the resultant **11** with protected D-Glu yielded the desired **13**. Subsequent global deprotection of all of the acid-sensitive protecting groups and conversion of the resultant carboxylic acids into sodium salts gave the desired **1**.

Cephalosporin **1** acylated the bifunctional serine type D-alanyl-D-alanine carboxypeptidase/transpeptidase (EC 3.4.16.4)



Scheme 1.

from *Streptomyces sp*. strain R61 readily, because incubation of cephalosporin **1** and the enzyme in crystalline form gave a stable acyl-enzyme species that was detectable by using x-ray crystallographic techniques. The position of cephalosporin **1** in the active site was determined by fitting 1.2-Å difference electron density maps. Statistics for the data collection and refinement show that this is a well-determined structure (Table 1).

Fig. 2*A* shows the details of binding of cephalosporin **1** in the active site pocket of the enzyme, for which a schematic is presented in Fig. 2*B*. Cephalosporin **1** is bound between the all-helical and the α/β domains of the PBP. PBPs have three signature sequences with eight conserved residues: Ser_{62}–x–x–Lys, which contains the catalytic serine residue; Ser/Tyr_{159}–x–Asn, on a loop below the active site; and Lys/His_{298}–Thr–Gly–Thr/Ser, which occurs on the inner strand of the β-sheet that flanks the active site. Both strands 1 and 2 of cephalosporin **1** are oriented antiparallel to the inner β-strand of the antiparallel β sheet of the enzyme. The catalytic serine residue is somewhat buried in a pocket formed below the grooves on the surface of the enzyme, where the bulk of the peptidoglycan analogs bind. The strands are anchored by 11 hydrogen bonds in the acylated enzyme species, five associated with strand 1 and six associated with strand 2. Seven of these interactions involve residues that are conserved in all known PBPs (see Fig. 2).

Several key features are revealed by the structure of the complex. Serine-62 is positioned on a helix dipole, and it is likely that the pKa of its side-chain hydroxyl is somewhat lowered. The side chain of Lys-65 is in contact with the Ser-62 hydroxyl (3.04 Å) in the absence of any ligand and it is believed to activate it for the enzyme acylation event (5, 8). The Nε of the diaminopimelate (DAP) surrogate in the inhibitor is located on the β-side of the β-lactam, 2.6 Å from the carbonyl of the ester bond linking the compound to the enzyme. This positioning is ideally suited for the subsequent addition to the ester carbonyl that would result in the natural cross-linked cell wall. Tyrosine-159, assuming that its side chain is deprotonated, is the likely active-site base positioned for activation of the incoming DAP amine (at 4.0 Å) for the addition reaction. The DAP amine, Tyr-159, and Lys-65 could function as a relay mechanism for transfer of a proton from the incoming peptidoglycan to the departing Ser-62.

Crystallographic studies have shown little change in the enzyme structure on binding β-lactams. One exception is the side chain of Thr-301, which rotates to hydrogen bond with a side-chain nitrogen in bulky cephalosporins (8). This rotation also is observed with cephalosporin **1**. Interestingly, with cephalosporin **1** there is also an unusual rotation of the β-sheet carbonyl of Thr-301, moving it 3.3 Å away from the active site. This movement is no doubt necessary for steric reasons to allow proper positioning of the peptidoglycan analog. The only other shift of the enzyme to accommodate the ligand is a movement of the side chain of Asn-161, which moves into a hydrogen-bonding orientation, with Nδ2 positioned 2.9 Å from the oxygen of the C-7 side-chain amide of cephalosporin **1**.

Crystal packing positions a second copy of the enzyme molecule near the active site of the first, and three interactions occur between the bound ligand and the symmetry-related copy of the enzyme. One interaction is a strong hydrogen bond (2.7 Å) between oxygen of the side chain in D-Glu of cephalosporin **1** and the main-chain amide of Asp-98 on the symmetry-related molecule. The other two contacts are at distances of 3.7 and 3.3 Å, representing weak interactions. The effect of this symmetry-related copy of the enzyme on binding of cephalosporin **1** was investigated by using molecular dynamics simulations. No difference in binding of cephalosporin **1** was observed with or without the inclusion of the symmetry-related copy of the enzyme molecule. Within the simulation period of 198 ps (after equilibration), the inhibitor in both situations behaved similarly. The average rms deviations of all of the atoms of the cephalosporin inhibitor in the presence and absence of the symmetry-related enzyme molecule were 1.62 ± 0.41 and 1.49 ± 0.80 Å, respectively. This shows that the movement of cephalosporin **1** after acylation of the active site serine residue is not influenced by the inclusion of the symmetry-related enzyme molecule. Therefore, the structure depicts a realistic conformation, unaffected by crystallographic packing.

In solving the structure of this complex, we were able to consider the position of the polysaccharide portions of the cell wall. We have extended the structures of the peptidoglycan portions from the crystallographically determined positions for cephalosporin **1** up to NAM–*N*-acetylglucosamine (NAG) res-

Table 1. Crystallographic data

Resolution limit	1.2 Å
Number of reflections	
Measured	2,333,719
Unique	111,373
Reflections, $F > 4\ \sigma$	93,339
Completeness	
All data	96%
Highest resolution shell (1.21–1.17 Å)	73%
R_{sym}* (on I)	0.040
$\langle I/\text{Sigma}\ (I)\rangle$ overall	25.0
Refinement	
Resolution	∞ − 1.17 Å
R factor (no cutoff)	0.115
R free (no cutoff)	0.154
Data in test set	5,560
Nonhydrogen atoms	3,337
rms	
Bond lengths (Å)	0.015
Angle distances (Å)	0.030
Average B(iso) of ligand	33.3 Å^2

*$R_{sym} = \Sigma|\text{Iobs} - \langle I\rangle|/\Sigma\ \langle I\rangle$.

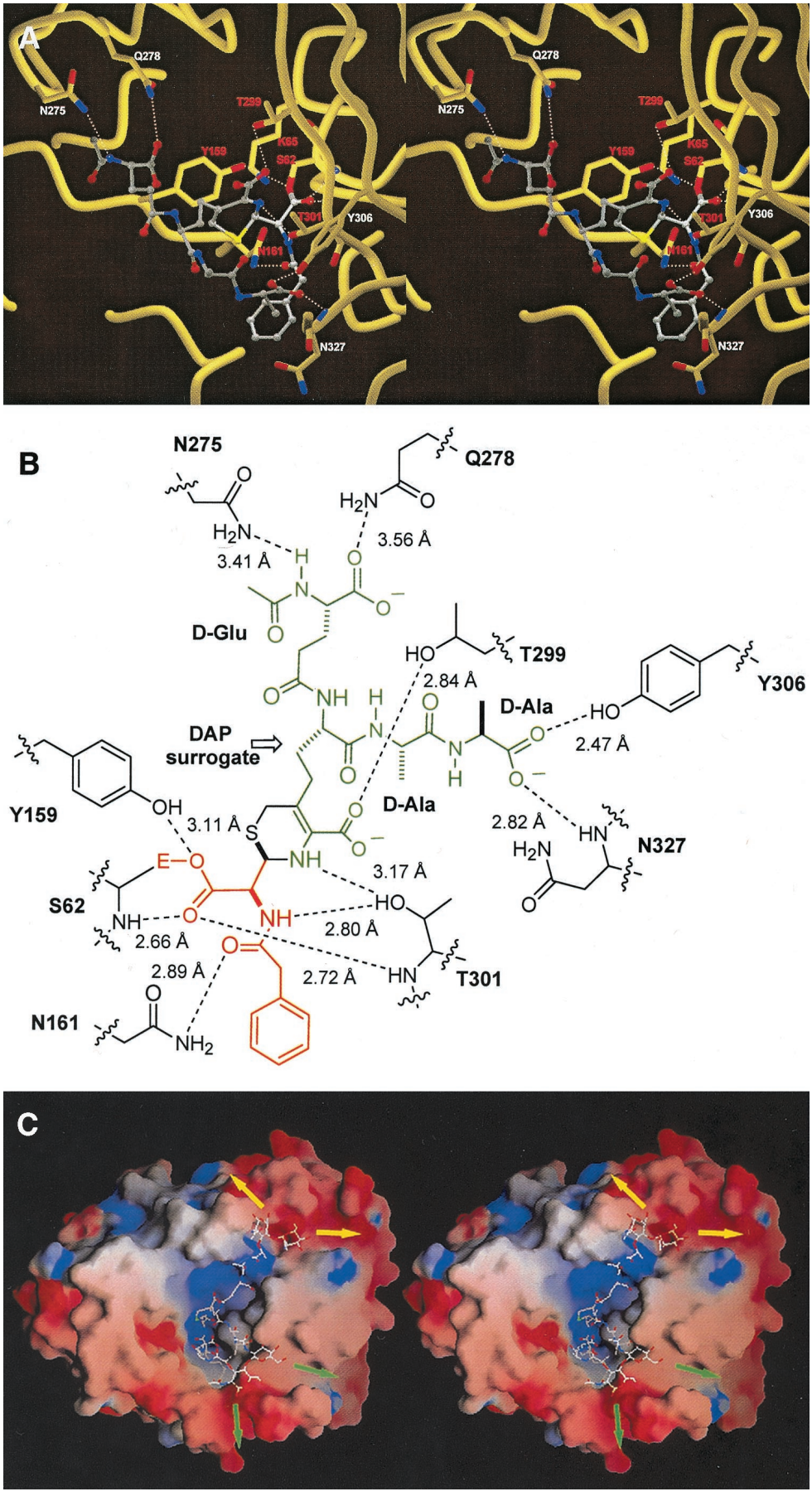


Fig. 2. (*A*) Stereoview of the active site of *Streptomyces* R61 DD-carboxypeptidase/transpeptidase acylated by cephalosporin **1**, generated by using MOLSCRIPT (22) and RASTER3D (23, 24). The ligand is rendered as ball and stick with CPK atom colors. Hydrogen bonds between the enzyme and the ligand are shown as dotted white lines. Residues labeled in red are conserved among PBPs and β-lactamases. (*B*) A schematic of interactions shown in *A*; the red and green segments correspond with those of the same colors for species 2 shown in Fig. 1*B*. (*C*) Energy-minimized computational model of peptidoglycan strands bound to *Streptomyces* R61 PBP extended from the x-ray structure of species 2. The orientation of the enzyme is the same as in *A*. The electrostatic enzyme surface was computed in GRASP (25) with blue representing positive surface charge and red representing negative surface charge. The peptidoglycan model is shown in the ball-and-stick representation, color-coded according to the atom type (N, blue; C, white; O, red; S, yellow). The positions of polymer extensions of the *N*-acetylglucosamine–NAM chains are shown by green arrows for the first strand and by yellow arrows for the second strand.

idues of the peptidoglycan strands. Eight crystallographic water molecules were removed in building the extensions onto the x-ray structure of cephalosporin **1.** The model of the crystallographic structure with the NAG–NAM extensions was solvated and energy-minimized by using the AMBER 5.0 package. Energy minimization showed stable glycan strands aligned in well-defined grooves on the surface of the enzyme (Fig. 2*C*). These hydrophilic grooves, which are 18 Å apart at the peptide branching points in NAM, would accommodate the polymeric backbone of the sugar residues in peptidoglycan. We note that Pares *et al.* (19) built a model of the peptidoglycan into the active site of their 3.5-Å resolution structure of the PBP2x from *Streptococcus pneumoniae*. Although a direct comparison of their model and ours is not entirely possible, it is clear that the putative saccharide-binding grooves in that report do not agree with the positioning developed in our work.

There is mechanistic and structural evidence that β-lactamases, enzymes that confer resistance to β-lactam antibiotics, evolved from PBPs (5, 20, 21). The two classes of enzymes have similar overall structural folds, yet neither can effectively catalyze the reaction of its kin. The structure of Fig. 2*C* reveals why this is the case. The groove that accommodates peptidoglycan strand 2 is obliterated by an insertion in β-lactamases (residues 214–224 and 285–296 in class A and class C β-lactamases, respectively) at that site. It is interesting that the local folds for the two peptide insertions are distinct in the two classes of β-lactamases. Hence, it would appear that nature adopted this ploy on two separate occasions in evolution of β-lactamases to prevent interactions with strand 2 of peptidoglycan. Class A and C β-lactamases are known to have descended from different PBPs (5), so these independent evolutionary adaptations are plausible. Furthermore, the surface of the transpeptidase that interacts with peptidoglycan strand 1 (at six o'clock in Fig. 2*C*) is entirely absent in the two classes of β-lactamases. Therefore, β-lactamases have lost the structural surface for binding to both peptidoglycan strands, so as to be able to specialize exclusively as enzymes of antibiotic resistance.

These structural studies show the positions of the peptidoglycan components poised for the cross-linking step of cell wall biosynthesis, providing a unique glimpse of how this critical step takes place in bacteria. These data, together with the extension of the crystallographic structure to model the glycan positions, provide valuable information to aid in the design of novel antibacterials that would bind to these enzymic subsites.

This manuscript is dedicated to Koji Nakanishi on the occasion of his 75th birthday as a testimonial of my esteem for his numerous contributions to the field of chemistry (S.M.). We thank Robert T. Vinopal and James R. Knox for their critical reading of the manuscript. A sample of the cephalosporin starting material for preparation of triflate **4** was generously provided by the Otsuka Chemical Co. (Osaka, Japan). Jean-Marie Frère of the Université de Liège generously provided the purified R61 DD-peptidase/transpeptidase for crystallization. Diffraction data were collected at Brookhaven National Laboratory at beamline X12-C in the National Synchrotron Light Source, supported by the U.S. Department of Energy (DE-AC02–98CH10886), National Science Foundation, and National Institutes of Health (1P41 RR12408–01A1). This research was supported by a grant from the State of Connecticut Critical Technologies Program in Drug Design (to J.A.K.) and a National Institutes of Health grant (to S.M.).

1. Schleifer, K. H. & Kandler, O. (1972) *Bacteriol. Rev.* **36,** 407–477.
2. Labischinski, H. & Maidhof, H. (1994) in *Bacterial Cell Wall,* eds. Ghuysen, J.-M. & Hakenbeck, J. (Elsevier, Amsterdam), pp. 23–39.
3. Bugg, T. D. H. & Walsh, C. T. (1992) *Nat. Prod. Rep.* **9,** 199–215.
4. Kelly, J. A., Kuzin, A. P., Charlier, P. & Fonze, E. (1998) *Cell. Mol. Life Sci.* **54,** 353–358.
5. Massova, I. & Mobashery, S. (1998) *Antimicrob. Agents Chemother.* **42,** 1–17.
6. Ghuysen, J. M. (1997) *Int. J. Antimicrob. Agents* **8,** 45–60.
7. Tipper, D. J. & Strominger, J. L. (1965) *Proc. Natl. Acad. Sci. USA* **54,** 1133–1141.
8. Kuzin, A., Liu, H., Kelly, J. A. & Knox, J. R. (1995) *Biochemistry* **34,** 9532–9540.
9. Garner, P. & Park, J. M. (1987) *J. Org. Chem.* **52,** 2361–2364.
10. Delle Monache, G., Di Giovanni, M. C., Maggio, F., Misiti, D. & Zappia, G. (1995) *Synthesis* 1155–1158.
11. Otwinowski, Z. & Minor, W. (1997) *Methods Enzymol.* **276,** 307–326.
12. Brügner, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., *et al*. (1998) *Acta Crystallogr. D* **54,** 905–921.
13. Sack, J. S. (1988) *J. Mol. Graphics* **6,** 224–225.
14. Sheldrick, G. M. & Schneider, T. R. (1997) *Methods Enzymol.* **277,** 319–343.
15. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991) *Acta Crystallogr. A* **47,** 110–119.
16. Case, D. A., Pearlman, D. A., Caldwell, J. W., Cheatham, T. E., III & Ross, W. S. (1997) AMBER 5 (Univ. of California, San Francisco).
17. Pearlman, D. A., Case, D. A., Caldwell, J. W., Ross, W. S., Cheatham, T. E., Debolt, S., Ferguson, D., Seibel, G. & Kollman, P. (1995) *Comp. Phys. Commun.* **91,** 1–41.
18. Woods, R. J., Dwek, R. A., Edge, C. J. & Fraser-Reid, B. (1995) *J. Phys. Chem.* **99,** 3832–3846.
19. Pares, S., Mouz, N., Pétillot, Y., Hakenbeck, R. & Dideberg, O. (1996) *Nat. Struct. Biol.* **3,** 284–289.
20. Kelly, J. A., Moews, P. C., Knox, J. R., Frére, J. M. & Ghuysen, J. M. (1982) *Science* **218,** 479–481.
21. Bush, K. & Mobashery, S. (1998) in *Resolving the Antibiotic Paradox: Progress in Understanding Drug Resistance and Development of New Antibiotics*, eds. Rosen, B. P. & Mobashery, S. (Plenum, New York), pp. 71–98.
22. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24,** 946–950.
23. Bacon, D. J. & Anderson, W. F. (1988) *J. Mol. Graphics* **6,** 219–220.
24. Merritt, E. A. & Murphy, M. E. P. (1994) *Acta Crystallogr. D* **50,** 869–873.
25. Nicholls, A., Sharp, K. & Honig, B. (1991) *Proteins Struct. Funct. Genet.* **11,** 281–296.