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Evolution of an enzyme activity: Crystallographic structure at 2-Å resolution of cephalosporinase from the *ampC* gene of *Enterobacter cloacae* P99 and comparison with a class A penicillinase

(β-lactamase/protein folding/bacterial β-lactam resistance)

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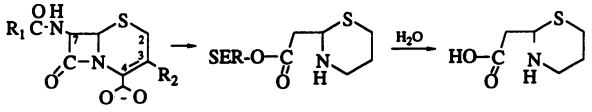
ABSTRACT **The structure of the class C *ampC* β-lactamase (cephalosporinase) from *Enterobacter cloacae* strain P99 has been established by x-ray crystallography to 2-Å resolution and compared to a class A β-lactamase (penicillinase) structure. The binding site for β-lactam antibiotics is generally more open than that in penicillinases, in agreement with the ability of the class C β-lactamases to better bind third-generation cephalosporins. Four corresponding catalytic residues (Ser-64/70, Lys-67/73, Lys-315/234, and Tyr-150/Ser-130 in class C/A) lie in equivalent positions within 0.4 Å. Significant differences in positions and accessibilities of Arg-349/244 may explain the inability of clavulanate-type inhibitors to effectively inactivate the class C β-lactamases. Glu-166, required for deacylation of the β-lactamoyl intermediate in class A penicillinases, has no counterpart in this cephalosporinase; the nearest candidate, Asp-217, is 10 Å from the reactive Ser-64. A comparison of overall tertiary folding shows that the cephalosporinase, more than the penicillinase, is broadly similar to the ancestral β-lactam-inhibited enzymes of bacterial cell wall synthesis. On this basis, it is proposed that the cephalosporinase is the older of the two β-lactamases, and, therefore, that a local refolding in the active site, rather than a simple point mutation, was required for the primordial class C β-lactamase to evolve to the class A β-lactamase having an improved ability to catalyze the deacylation step of β-lactam hydrolysis.**

Only since the late 1970s have the *Enterobacter* species become resistant to β-lactam antibiotics (penicillins and cephalosporins), so that now they are the third greatest cause of Gram-negative nosocomial infections in the United States (1). The primary cause of this resistance is the constitutive or β-lactam-inducible overproduction of β-lactamase (EC 3.5.2.6), which catalyzes, via a serine-bound acyl intermediate, the hydrolysis of the β-lactam to an inactive acid:



The β-lactamases have been grouped into classes A through D (2) or groups 1 through 4 (3). Most studied have been the class A β-lactamases (group 2), whose members are commonly called penicillinases and use a non-classical serine mechanism (4). The body of data on the class C enzymes, the so-called cephalosporinases or group 1 β-lactamases, is considerably smaller than that for class A. Because these β-lactamases can destroy the once-dependable third-generation cephalosporins such as cefotaxime, the clinical significance of this class is growing rapidly (5). Their rather low catalytic activity against these cephalosporins is offset by their high overproduction. Especially troublesome is the class C β-lactamase from the *ampC* gene of *Enterobacter cloacae*, as it is only weakly inactivated by mechanism-based inhibitors such as clavulanic acid, and it is expected to develop activity against a currently effective carbapenem, imipenem (1).

To provide structural details for mechanistic proposals, and hopefully for the design of inhibitors and new cephalosporins, we present here the crystal structure of the class C β-lactamase from *Ent. cloacae* strain P99, with a comparison of the class A and class C β-lactamases at the levels of polypeptide folding and active site architecture.

RESULTS

Crystallization and Data Collection. The *ampC* β-lactamase from *Ent. cloacae* strain P99 (6) was crystallized at room temperature from 11% (vol/vol) polyethylene-glycol 8000 at pH 6.5–8.5 in 50 mM sodium cacodylate buffer containing 1 mM $MgCl_2$ and 1 mM NaN_3. The crystals have space group $P2_1$ with two molecules in the asymmetric unit. Cell dimensions are a = 46.50 Å, b = 83.47 Å, c = 95.46 Å, and β = 90.0°. Diffraction data (Table 1) were collected at room temperature on a Siemens area x-ray detector with a Rigaku 12-kW rotating anode source with graphite-monochromatized Cu Kα radiation. Intensities were reduced and scaled with the Xengen programs (7).

Structure Determination. The structure was solved by the multiple isomorphous replacement method with the inclusion of anomalous scattering (MIRAS). Protein phases calculated from one heavy atom derivative were used to refine parameters of the other (35). With the mercury derivative contributing usefully only to 4.5-Å resolution, the figure-of-merit for 13,749 reflections to 3-Å resolution is 0.80. Fitting of maps with the sequence of 361 residues (6) was done with FRODO (8) on an Evans and Sutherland PS330 graphics system. The two molecules of the asymmetric unit were traced and fitted independently. Later, a map to 2-Å resolution was calculated with model-based phases and coefficients $2mF_o - DF_c$ (9).

Structure Refinement. The 722-residue model was built by using energy minimization and simulated annealing with XPLOR (10). Refinement was done with the restrained least-squares crystallographic program PROLSQ (11). The resulting R factor for a model excluding water molecules and terminal residues Thr-1 and Gln-361 is 0.217 for 41,710 reflections ($F \geq 3\sigma F$) from 10.0- to 2.0-Å resolution. Covalent bonds and angles in the model deviate 0.018 Å and 3.2° from ideal values. Coordinate errors in the model are estimated (12) to be 0.25 Å. The two molecules in the asymmetric unit differ by

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Table 1. Data from native and derivative crystals

Parameter	Native	$K_3UO_2F_5$	$Hg(SO_3CH_3)_2$
d_{min} ($d_{3\sigma(I)}$), Å	1.86 (2.0)	2.26 (2.3)	2.33 (2.6)
No. observations	170,149	125,514	23,843
No. unique reflections	51,415	30,676	15,574
$R_{sym}(I)$, $I/\sigma(I)$	0.040, 24	0.050, 20	0.051, 27

only 0.21 Å (rms) in the positions of backbone atoms of all residues, even though a twofold noncrystallographic symmetry was never imposed during the refinement. The largest differences approach 2 Å in a protruding loop region from residue 203 to residue 207.§

Description of the Structure. The β-lactamase is a mixed α–β structure with an all-helical domain on the left and a mixed α/β domain on the right (Fig. 1*A*). Residues in the helical and β conformations total 34% and 22% of the polypeptide, respectively. The class C β-lactamase from *Citrobacter freundii* has 75% sequence homology with the title enzyme, and a brief report of its crystal structure has appeared (14). However, its atomic coordinates are not available for a comparison.

Comparison with a Class A Penicillinase. The folding of this 39,200-Da class C cephalosporinase is compared in Fig. 1 *B* and *C* with the folding of a class A penicillinase, the 29,500-Da β-lactamase from *Bacillus licheniformis* (ref. 15, Protein Data Bank ref. 4BLM). Four additional β-strands in the class C molecule (Fig. 2*A*) form a small antiparallel sheet which is considerably twisted (60–90°) with respect to the major five-stranded sheet common to both types of β-lactamase. For more detailed comparison of secondary structures, we use the unified nomenclature for penicillin-recognizing enzymes (16), in which the class A numbers are expanded as necessary with letters a, b, c, . . . to denote additional secondary structure elements not present in smaller class A β-lactamases. The result is seen in Fig. 2*B*, where the *spatial* correspondence between the elements of the two molecules is diagrammed, based on Fig. 1*C*.

An important difference between the tertiary structures of the two types of β-lactamase occurs at the bottom of the catalytic site in the vicinity of helix H7 (Fig. 3). This

§The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference 1BLT). This information is embargoed for 1 year (coordinates) from the date of publication.

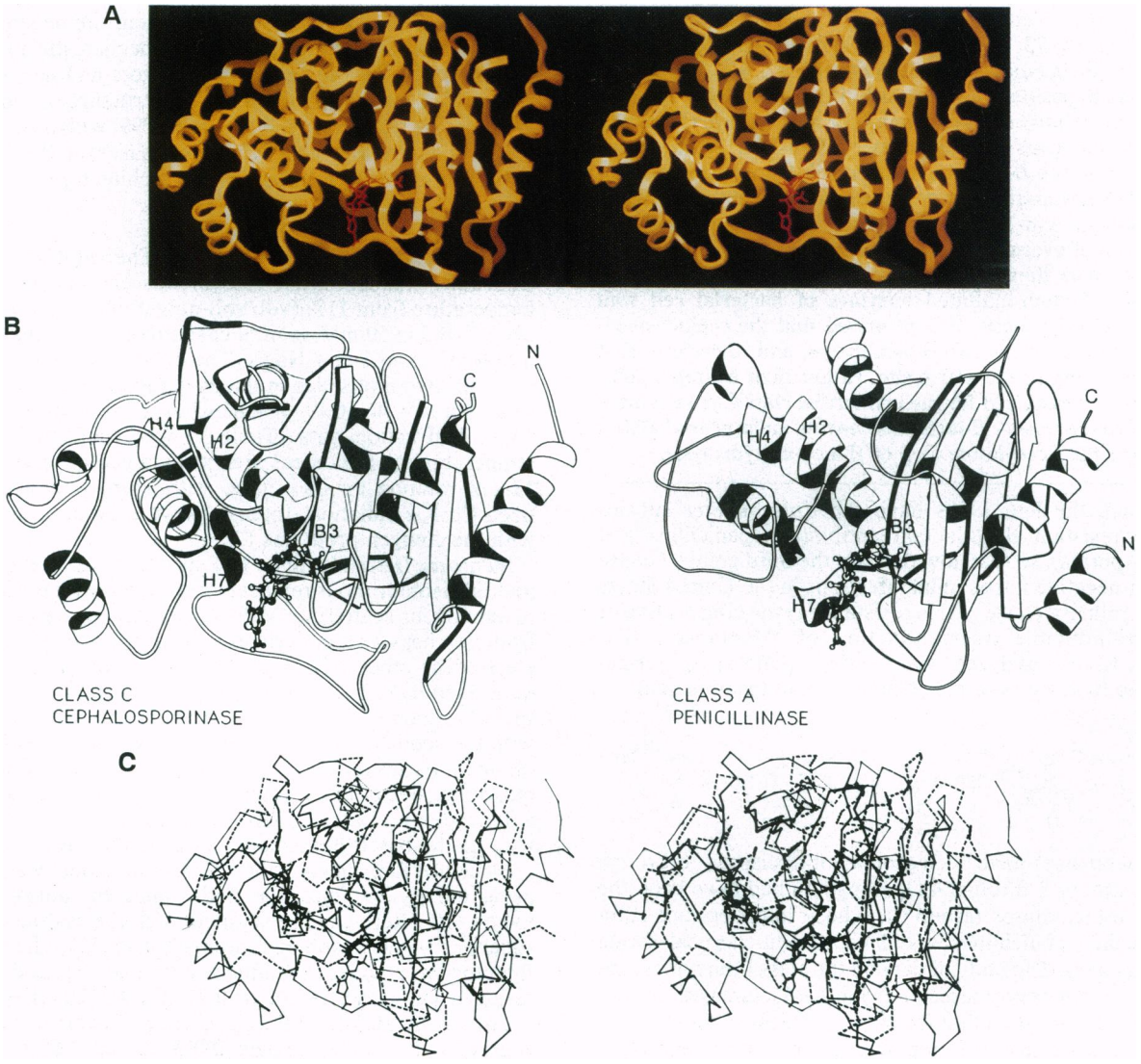


FIG. 1. (*A*) Stereoview of a ribbon representation of the *ampC* cephalosporinase of *Ent. cloacae* P99. The N terminus is on the helix at right, and the cephalosporin binding site is in lower center. (*B*) Molscript (13) drawings of the class C cephalosporinase (*Left*) and the smaller class A penicillinase of *Bacillus licheniformis* (Protein Data Bank ref. 4BLM). Cefotaxime is positioned in the binding site. (*C*) Superposition of the α-carbon traces of the cephalosporinase (solid line) and penicillinase (broken line). They are oriented as in *B*.

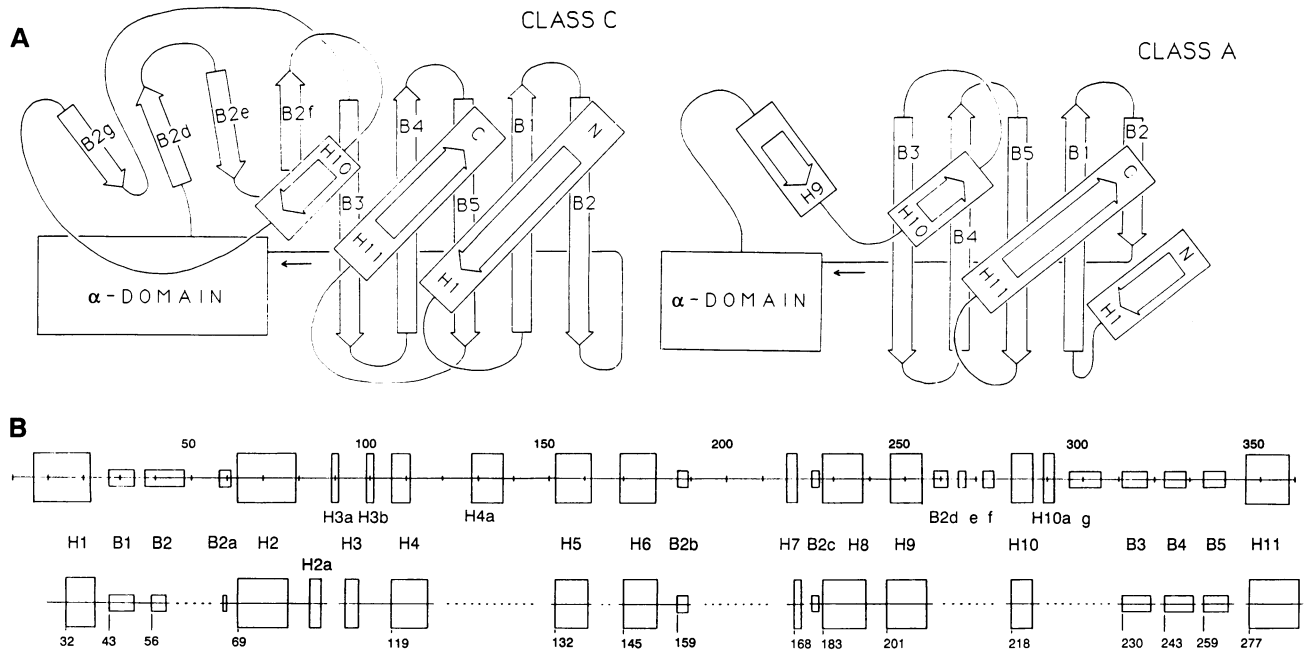


FIG. 2. (*A*) Comparison of the secondary structure domains of cephalosporinase (*Left*) and penicillinase (*Right*). Rectangles and arrows are α-helices and β-strands, respectively. (*B*) Spatial equivalence of secondary structure in the class C cephalosporinase (*Upper*) and class A penicillinase of *B. licheniformis* (*Lower*) based on the three-dimensional alignment in Fig. 1*C*. Helices (H), β-strands (B), and deletions are indicated by large rectangles, small rectangles, and dotted lines, respectively. Numbering for the smaller *B. licheniformis* enzyme is discontinuous; the first residue in each element is numbered. The class C enzyme contains four major insertions in the minimal class A motif at residues 120–150, 190–215, 264–277, and 290–310. Helices H10 run in opposite directions in the folded structures.

difference arises because the paths of the two polypeptide chains diverge after the common β-strand B2b, with the class C enzyme taking a much longer path from the rear of the molecule to the catalytic site. Here, each chain forms a short helix (H7) which contains an invariant acid residue, Glu-166 and Asp-217 in class A and C enzymes, respectively. The chains come together again at β-strand B2c. These two loops are approximately related by a 180° twist which places the two acid residues in very different positions relative to the three catalytic residues common to the two enzymes.

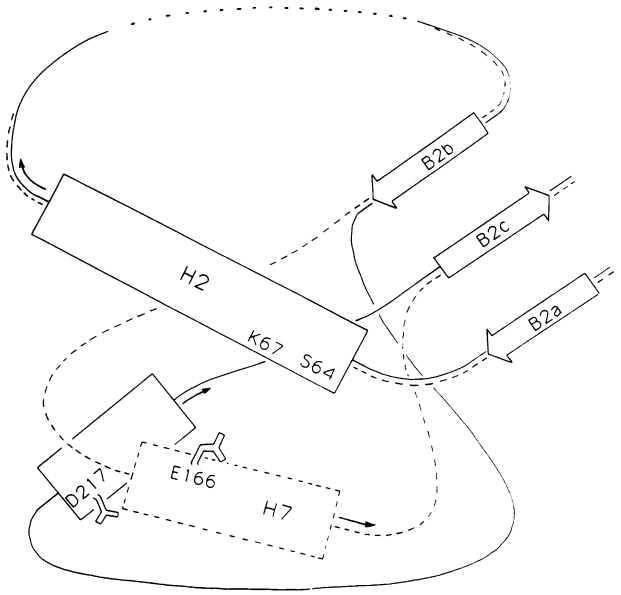


FIG. 3. A difference in polypeptide folding in the β-lactam binding sites of class A (broken line) and class C (solid line) β-lactamases. The two loops at bottom are related by an approximately 180° twist.

The β-Lactam Binding Site. Between the α domain on the left and the α/β domain on the right lies the site of β-lactam binding (Fig. 4*A*), as observed in crystallographic studies of β-lactam binding in β-lactamases (14, 17–19) and in the β-lactam-inhibited *Streptomyces* R61 D-Ala-D-Ala-peptidase (20). The reactive Ser-64 is at the N terminus of the H2 helix and is adjacent to invariant Lys-67. A second conserved lysine, Lys-315, lies on β-strand B3 and is part of a signature K(H)T(S)G tripeptide seen in all penicillin-recognizing enzymes (21, 22). Modeling of β-lactam binding to β-lactamases (14, 23, 24) suggests that a β-lactam substrate is likely to be oriented with the carbonyl group of the β-lactam ring hydrogen-bonded in an oxyanion binding pocket between helix H2 (NH of Ser-64) and β-strand B3 (NH of Ser-318). The variable 318 residue also supplies a carbonyl group to hydrogen bond to the amide of the β-lactam's acylamide linkage. Hydrogen bonds are possible from the acylamide carbonyl group to the amides of Gln-120 and Asn-152. The carboxylic acid group of a dihydrothiazine ring of cephalosporins would lie near Thr-316 and conserved Asn-346 from the C-terminal helix H11. R_2 substituents on the ring would lie at the top of the binding site near residues 287–289, which are hydrophilic in all class C sequences. Significantly, a rather open space exists at the bottom for R_1 substituents.

Comparison of β-Lactam Binding Sites in Class A and C Enzymes. In Fig. 4 *A* and *B* it is seen that the three conserved functionalities common to both classes, Ser-64/70 and Lys-67/73 on helix H2 and Lys-315/234 on β-strand B3, lie in equivalent positions. Tyr-150 exists in the position occupied by Ser-130 in class A enzymes. The functional atoms of the four side chains are within 0.4 Å of their counterpart on the other enzyme; simple alteration of their crystallographic conformation would put them even closer. Only the inter-residue distance *A* is significantly different in the two active sites. Two other residues with counterparts in class A enzymes are Gln-120 and Asn-152. As in the class A enzymes, Asn-152 is strongly hydrogen bonded to Lys-67 on the H2 helix. It is significant that the residue required for deacylation

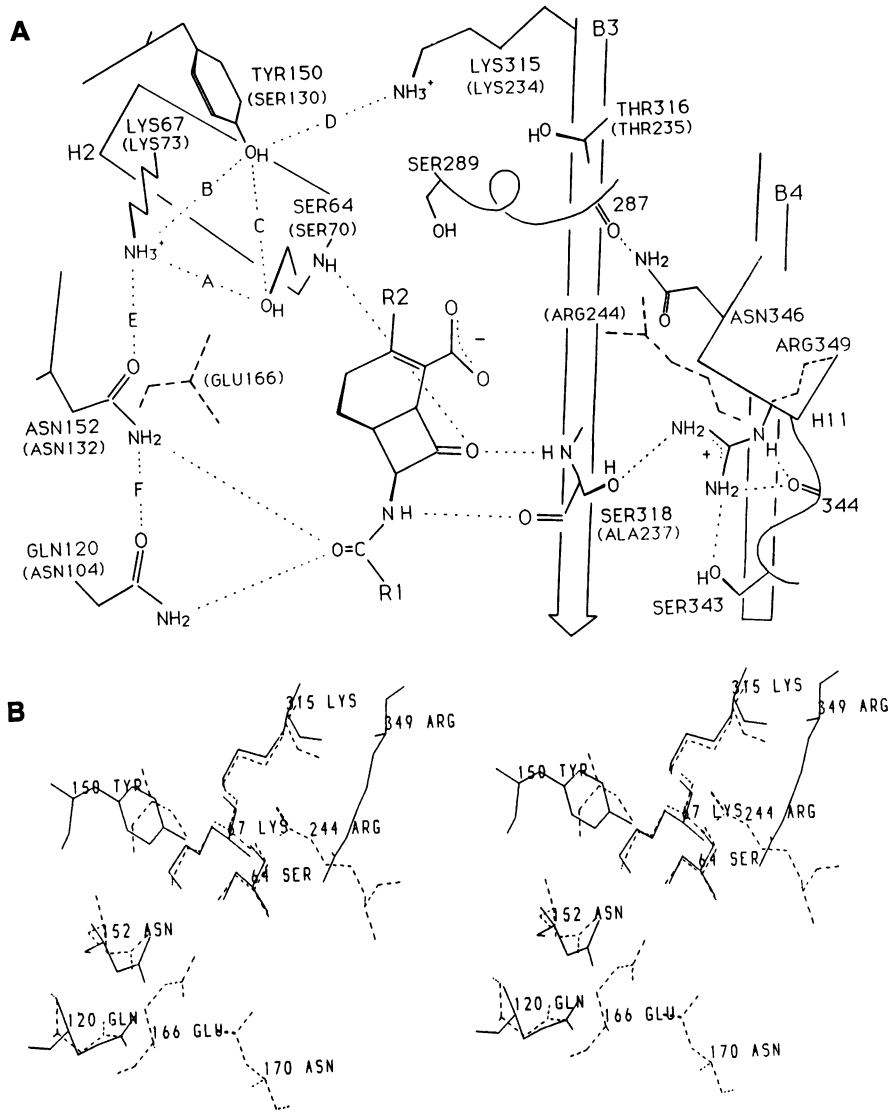
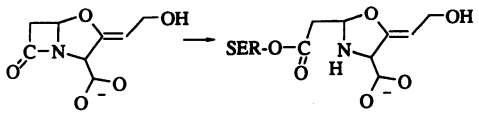


FIG. 4. (*A*) β-Lactam binding sites of class A and class C β-lactamases. Residue numbers in parentheses and side chains represented by broken lines refer to the class A *B. licheniformis* enzyme. Averaged distances (Å) in the class A/C enzymes are A = 2.67/3.11, B = 3.18/3.08, C = 3.21/3.13, D = 2.91/3.09, E = 2.57/2.53, and F = 2.91/3.01. (*B*) Stereoview of side chains in the β-lactam binding sites of the *Ent. cloacae* and *B. licheniformis* (broken lines) β-lactamases.

in class A enzymes (4, 25, 26), Glu-166, is without a counterpart in this and other (14) class C β-lactamases. The nearest acid residue, Asp-217, is 10 Å away from Ser-64, and its carboxylate group is directed away from Ser-64 (Fig. 3). Asn-152 lies about 3 Å from the virtual Glu-166 and 4.8 Å from Ser-64. Nor is there a counterpart for Asn-170, which is thought to assist hydrolysis, albeit poorly, in class A mutants lacking Glu-166 (18). On the right side of the binding site is a buried arginine (Arg-349) conserved in the class C β-lactamases. The side chain is well stabilized by multiple interactions with Ser-318 and Ser-343. This Arg-349 is unlikely to be the counterpart of the class A Arg-244 (27–29), however, because the two guanidinium groups are separated 4.5 Å and the side chains differ greatly in solvent accessibility (2.5 $Å^3$ vs. 24.5 $Å^3$ for Arg-244). It is more likely that nearby Asn-346 (Fig. 4*A*) is a hydrogen-bonding counterpart of Arg-244 and helps to bind the carboxylic acid group of β-lactams.

Interaction with Clavulanic Acid Inhibitors of β-Lactamases. Clavulanic acid irreversibly inactives most class A β-lactamases, but it is generally ineffective in binding to and inhibiting the class C β-lactamases (1, 30). In the class A enzymes, an initial acylation by the reactive serine



is followed by protonation of the double bond, which leads to opening of the oxazolidine ring followed by various rearrangements. From modeling, it has been proposed that the protonation step is inadvertently enzyme catalyzed (31, 32). A crystallographic water molecule, held by Arg-244, was said to be the source of the proton necessary for oxazolidine ring opening. For this class C β-lactamase, acylation is probably very slow (30). Nevertheless, once acylation is achieved, a similar modeling of the acylated intermediate (Fig. 5) shows that Arg-349 is too distant for interaction with the clavulanate carboxylic acid group. Instead, the clavulanate acid group hydrogen bonds to Asn-346 and also to a water molecule (W11). Unlike the water molecule in the class A case, W11 is distant from the nascent carbanion in the double bond of the

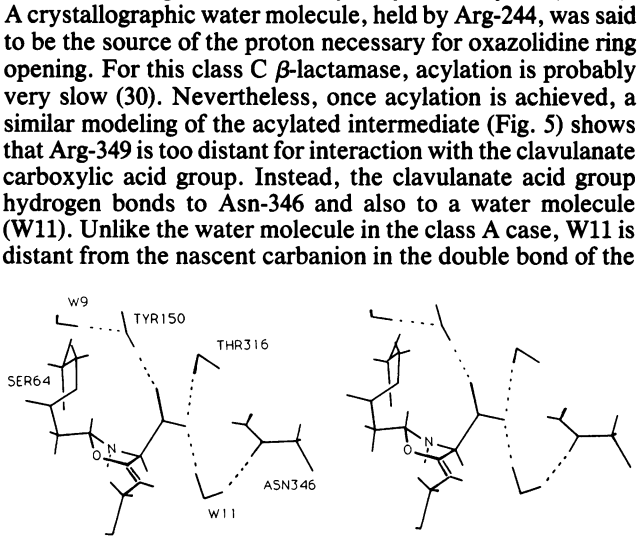


FIG. 5. Modeled Ser-64-bound clavulanic acid intermediate in the binding site of the *Ent. cloacae* β-lactamase. Its position was optimized by energy minimization.

intermediate, and it is unlikely to provide a proton for the ring-opening step. Thus, the inefficiency of the clavulanate inactivation process appears to rest both on the low acylation rate and on a deficient protonation of the double bond.

DISCUSSION

This comparison of the topology and catalytic geometry of two distantly related β-lactamases has shown broad anatomical similarities but also localized differences in folding which confer catalytic uniqueness to each enzyme. When the active site of the class C *Ent. cloacae* β-lactamase is overlaid with that of a representative class A β-lactamase, four residues implicated in β-lactam binding or catalysis easily coincide. All interresidue distances are equivalent except that between the reactive Ser-64/70 and its adjacent Lys-67/73 on helix H2. The longer Ser · · · Lys separation in this class C enzyme would reduce any influence of Lys-67 on the activation of Ser-64 prior to acylation. For the class C *C. freundii* enzyme, an acylation and deacylation mechanism was proposed (14) which utilizes an anionic Tyr-150, activated by nearby Lys-67 and Lys-315. However, the rather long distances seen here (>3 Å) involving Tyr-150, and the very strong binding of Lys-67 with Asn-152 (2.5 Å), make questionable this role for Tyr-150. Without a Glu-166 counterpart, perhaps hydrolytic deacylation of the acyl intermediate is simply not enzymatically catalyzed in class C β-lactamases.

Another residue in class A enzymes, a generally conserved Arg-244 thought to assist substrate binding and turnover (27, 28), clearly does not have an exact counterpart in the class C enzyme. In the class A β-lactamases, this Arg-244 is adventitiously placed to assist the enzyme's inactivation by mechanism-based clavulanic acid inhibitors (31, 32). We find an arginine (Arg-349) near the binding site, but it is improperly oriented for strong hydrogen bonding to the β-lactam carboxylate group. A better positional counterpart of Arg-244, Asn-346, might weakly bind the carboxylic acid groups of β-lactam substrates and clavulanic acid. However, modeling indicates that Asn-346 does not adequately position a water molecule for interaction with the acylated clavulanate intermediate. It appears, therefore, that an enzymatic catalysis of the protonation step of the clavulanate mechanism, as proposed for the clavulanate-sensitive class A enzymes, is unlikely in this class C binding site.

The class A and C tertiary structures differ markedly at the bottom of the binding site, where acylamide substituents of β-lactams would lie. From an analysis of mutant class A β-lactamases, Huletsky *et al.* (33) proposed how a displacement of the B3 β-strand, to produce a larger space at the bottom of the binding site, helps account for the extended activity of recent SHV- and TEM-type mutants toward third-generation cephalosporins such as cefotaxime and ceftazidime. This class C β-lactamase, with its inherently larger space near the bottom of the β-strand, is already better configured to interact with β-lactams with rigid and branched R_1 substituents.

Comparison of β-lactamases with an ancestral penicillin-sensitive D-Ala-D-Ala carboxypeptidase-transpeptidase (DD-peptidase) has been discussed in terms of function (34) and secondary structure (16), and now a detailed comparison of the three-dimensional structures of all three types of β-lactam-recognizing enzymes will be possible. Preliminary comparison reveals the folding of the class C β-lactamase is much closer to that of the parent DD-peptidase than is the folding of the class A β-lactamase. This difference in folding now becomes the basis for a conjecture that a catalytic general base, the Glu-166 unique to the class A enzymes, had to arise not by simple point mutation in the precursor class C enzyme but by a local refolding (Fig. 3) which moved an existing acid residue, perhaps Asp-217, to an optimal position to activate a water molecule for a catalyzed deacylation of the acyl intermediate. This *mutational refolding* produced a modified β-lactamase (class A) having the ability to hydrolytically deacylate β-lactamoyl intermediates much more rapidly than the primordial class C β-lactamase.

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1. Neu, H. C. (1992) *Science* **257**, 1064–1072.
2. Ambler, R. P., Frere, J.-M., Ghuysen, J.-M., Joris, B., Levesque, R. C., Tiraby, G., Waley, S. G. & Coulson, A. F. W. (1991) *Biochem. J.* **276**, 269–272.
3. Bush, K. (1989) *Antimicrob. Agents Chemother.* **33**, 264–270.
4. Escobar, W. A., Tan, A. K. & Fink, A. L. (1991) *Biochemistry* **30**, 10783–10787.
5. Jacoby, G. A. & Archer, G. L. (1991) *N. Engl. J. Med.* **324**, 601–612.
6. Galleni, M., Lindberg, F., Normark, S., Cole, S., Honore, N., Joris, B. & Frere, J.-M. (1988) *Biochem. J.* **250**, 753–760.
7. Howard, A. J., Gilliland, G. L., Finzel, B. C., Poulos, T. L., Ohlendorf, D. H. & Salemme, F. R. (1987) *J. Appl. Crystallogr.* **20**, 383–387.
8. Jones, T. A. (1985) *Methods Enzymol.* **115**, 157–170.
9. Read, R. J. (1986) *Acta Crystallogr.* Sect. A **42**, 140–149.
10. Brünger, A. T. (1990) X-PLOR Manual, Version 2.1, Yale University, New Haven, CT.
11. Konnert, J. H. & Hendrickson, W. A. (1980) *Acta Crystallogr.* Sect. A **36**, 344–349.
12. Luzzati, V. (1952) *Acta Crystallogr.* **5**, 802–810.
13. Kraulis, P. (1991) *J. Appl. Crystallogr.* **24**, 946–950.
14. Oefner, C., D'Arcy, A., Daly, J. J., Gubernator, K., Charnas, R. L., Heinze, I., Hubschwerfen, C. & Winkler, F. K. (1990) *Nature* **343**, 284–288.
15. Knox, J. R. & Moews, P. C. (1991) *J. Mol. Biol.* **220**, 435–455.
16. Joris, B., Ledent, P., Dideberg, O., Fonze, E., Lamotte-Brasseur, J., Kelly, J. A., Ghuysen, J.-M. & Frere, J.-M. (1991) *Antimicrob. Agents Chemother.* **35**, 2294–2301.
17. Strynadka, N. C. J., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betzel, C., Sutoh, K. & James, M. N. G. (1992) *Nature (London)* **359**, 700–705.
18. Knox, J. R., Moews, P. C., Escobar, W. A. & Fink, A. L. (1993) *Protein Eng.* **6**, 11–18.
19. Chen, C. C. H. & Herzberg, O. (1992) *J. Mol. Biol.* **224**, 1103–1113.
20. Kelly, J. A., Knox, J. R., Zhao, H., Frere, J.-M. & Ghuysen, J.-M. (1989) *J. Mol. Biol.* **209**, 281–295.
21. 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., Frere, J.-M. & Ghuysen, J.-M. (1987) in *Frontiers of Antibiotic Research*, ed. Umezawa, H. (Academic, New York), pp. 327–337.
22. Herzberg, O. & Moult, J. (1991) *Curr. Opin. Struct. Biol.* **1**, 946–953.
23. Lamotte-Brasseur, J., Dive, G., Dideberg, O., Charlier, P., Frere, J.-M. & Ghuysen, J.-M. (1991) *Biochem. J.* **279**, 213–221.
24. Juteau, J.-M., Billings, E., Knox, J. R. & Levesque, R. C. (1992) *Protein Eng.* **5**, 693–701.
25. Delaire, M., Lenfant, F., Labia, R. & Masson, J.-M. (1991) *Protein Eng.* **4**, 805–810.
26. Adachi, H., Ohta, T. & Matsuzawa, H. (1991) *J. Biol. Chem.* **266**, 3186–3191.
27. Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P. & Frere, J.-M. (1990) *Proteins Struct. Funct. Genet.* **7**, 156–171.
28. Zafaralla, G., Manavathu, E. K., Lerner, S. A. & Mobashery, S. (1992) *Biochemistry* **31**, 3847–3852.
29. Delaire, M., Labia, R., Samama, J.-P. & Masson, J.-M. (1992) *J. Biol. Chem.* **267**, 20600–20606.
30. Reading, C. & Farmer, T. (1981) *Biochem. J.* **199**, 779–787.
31. Imtiaz, U., Billings, E., Knox, J. R., Manavathu, E. K., Lerner, S. A. & Mobashery, S. (1993) *J. Am. Chem. Soc.* **115**, 4435–4442.
32. Knox, J. R. (1993) in *Recent Advances in the Chemistry of Anti-infective Agents*, eds. Bentley, P. H. & Ponsford, R. (R. Soc. Chem., London), Spec. Publ. no. 119, pp. 37–49.
33. Huletsky, A., Knox, J. R. & Levesque, R. C. (1993) *J. Biol. Chem.* **268**, 3690–3697.
34. Pazhanisamy, S. & Pratt, R. F. (1989) *Biochemistry* **28**, 6875–6882.
35. Furey, W. & Swaminathan, S. (1990) *Am. Crystallogr. Assn. Mtg. Abstr.* **18**, 73.