Articles

Crystallographic Structure of a Phosphonate Derivative of the *Enterobacter cloacae* P99 Cephalosporinase: Mechanistic Interpretation of a β-Lactamase Transition-State Analog[†,‡]

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ABSTRACT: The crystal structure of a complex formed on reaction of the *Enterobacter cloacae* P99 cephalosporinase (β-lactamase) with a phosphonate monoester inhibitor, *m*-carboxyphenyl [[*N*-[(*p*-iodophenyl)acetyl]amino]methyl]phosphonate, has been obtained at 2.3-Å resolution. The structure shows that the inhibitor has phosphonylated the active site serine (Ser64) with loss of the *m*-carboxyphenol leaving group. The inhibitor is positioned in the active site in a way that can be interpreted in terms of a transition-state analog. The arylacetamido side chain is placed as anticipated from analogous β-lactamoyl complexes of penicillin-recognizing enzymes, with the amido group hydrogen-bonded to the backbone carbonyl of Ser318 (of the B3 β-strand) and to the amides of Gln120 and Asn152. There is support in the asymmetry of the hydrogen bonding of this side chain to the protein and in the 2-fold disorder of the benzyl group for the considerable breadth in substrate specificity exhibited by class C β-lactamases. One phosphonyl oxygen atom is in the oxyanion hole, hydrogen-bonded to main-chain NH groups of Ser318 and Ser64, while the other oxygen is solvated, not within hydrogen-bonding distance of any amino acid side chain. The closest active site functional group to the solvated oxygen atom is the Tyr150 hydroxyl group (3.4 Å); Lys67 and Lys315 are quite distant (4.3 and 5.7 Å, respectively). Rather, Tyr150 and Lys67 are more closely associated with Ser64Oγ (2.9 and 3.3 Å). This arrangement is interpreted in terms of the transition state for breakdown of the tetrahedral intermediate in the deacylation step of catalysis, where the Tyr150 phenol seems the most likely general acid. Thus, Tyr150, as the phenoxide anion, would be the general base catalyst in acylation, as proposed by Oefner et al. [*Nature* (1990) *343*, 284–288]. The structure is compared with that of a similar phosphonate derivative of a class A β-lactamase [Chen et al. (1993) *J. Mol. Biol. 234*, 165–178], and mechanistic comparisons are made. The sensitivity of serine β-lactamases, as opposed to serine proteinases, toward inhibition by phosphonate monoanions is supported by electrostatic calculations showing a net positive potential only in the catalytic sites of the β-lactamases.

The threat posed by bacterial infections to human health remains powerful because of the ever-increasing resistance of bacteria to the antibiotics that we deploy against them. A major source of resistance to the β-lactam antibiotics is the ability of bacteria to produce enzymes, the β-lactamases, that specifically catalyze the hydrolysis of β-lactams (Neu, 1992; Liu et al., 1992). The most widespread and clinically important β-lactamases, of classes A and C (Waley, 1992), are serine hydrolases that catalyze β-lactam hydrolysis by a double displacement mechanism involving an acyl-enzyme intermediate.

Our knowledge of these enzymes has been greatly advanced by the publication of crystal structures of both class A (Dideberg et al., 1987; Herzberg & Moult, 1987; Moews et al., 1990; Strynadka et al., 1992; Jelsch et al., 1993) and class C β-lactamases (Oefner et al., 1990; Lobkovsky et al., 1993). Although, from these studies and from amino acid sequence analysis (Ghuysen, 1991), the nature of the conserved functional groups at the active sites of these enzymes is now clear, there is no consensus as to the actual mechanism(s) of catalysis beyond the existence of the acyl-enzyme intermediate described above (Oefner et al., 1990; Gibson et al., 1990; Knap & Pratt, 1991; Lamotte-Brasseur et al., 1991; Strynadka et al., 1992; Fink, 1992). The analogies with serine proteinases and with bacterial D-Ala-D-Ala peptidases (DD-peptidases) have been considered (Kelly et al., 1986; Herzberg & Moult, 1987, 1991; Knox & Kelly, 1989; Ghuysen, 1991; Strynadka et al., 1992), but no unambiguous assignment of the catalytic role of functionally analogous participating groups has been achieved, with the exception of the serine hydroxyl nucleophile and an oxyanion pocket.

Under these circumstances, one logical approach to the mechanism would be through the crystal structure of a complex of a β-lactamase with a transition-state analog inhibitor. In such a structure one would expect to find the functional groups of the active site clustered about the inhibitor in just the way they would about a normal transition state, thus allowing a

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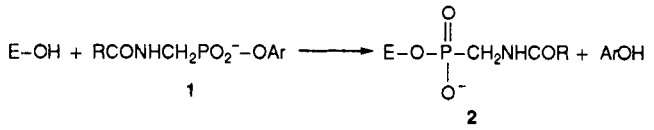
Scheme 1

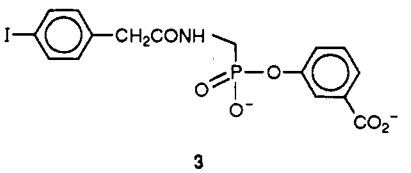


more direct extrapolation to the catalytic roles of these groups than is possible from the structure of the enzyme alone or even of an acyl-enzyme intermediate.

Although no tightly binding β-lactam-derived transition-state analogs are yet available (Pratt, 1992), a series of phosphonate monoesters, **1**, have been introduced (Pratt, 1989) which phosphonylate the active site serine of both class A (Rahil & Pratt, 1991) and class C (Pratt, 1989; Rahil & Pratt, 1992) β-lactamases, as shown in Scheme 1. The anionic phosphonyl derivative **2** should be structurally analogous to the tetrahedral intermediates and associated transition states involved in turnover of analogous acyclic esters, $RCONHCH_2CO_2Ar$, which are in fact β-lactamase substrates (Pratt & Govardhan, 1984; Govardhan & Pratt, 1987).

The crystal structure of a complex of one such phosphonate with a class A β-lactamase, that of *Staphylococcus aureus* PC1, has been obtained (Chen et al., 1993). This complex has also been shown to have the thermodynamic properties of a transition-state analog structure (Rahil & Pratt, 1994). Although the structure was, in many ways, interpretable in terms of the interaction of a substrate with the enzyme, details of the mechanism of normal catalysis were still ambiguous. The recently published crystallography of a class C β-lactamase, that of *Enterobacter cloacae* P99 (Lobkovsky et al., 1993), afforded the potential for further clarification. The active site functionalities of class A and class C β-lactamases have been compared crystallographically (Oefner et al., 1990; Lobkovsky et al., 1993) and were seen to have their reactive serine and oxyanion pocket similarly positioned with respect to other residues in the binding pocket. An important difference was the identity and placement of potential general acid/base catalysts in the active sites. It is hoped that the crystal structure of a phosphonylated class C enzyme might identify the common features essential to β-lactamase catalysis. Further, it might become possible to discover the structural basis for the observed greater effectiveness of phosphonate monoesters as inhibitors of serine β-lactamases than of serine proteinases (Rahil & Pratt, 1992) and of class C β-lactamases than of class A (Rahil & Pratt, 1991).

This paper therefore presents the crystal structure of a transition-state analog complex **2** derived from reaction of the class C β-lactamase of *E. cloacae* P99 with the phosphonate **3**. Comparisons of this structure with that of the analogous



class A structure referred to above and that of a monoanionic phosphoryl derivative of γ-chymotrypsin (Harel et al., 1991) are also offered. The mechanism of β-lactamase catalysis is discussed in the light of these comparisons.

EXPERIMENTAL PROCEDURES

Synthetic Methods. Triethylammonium m-Carboxyphenyl [[N-[(p-Iodophenyl)acetyl]amino]methyl]phosphonate. (*p*-Iodophenyl)acetyl chloride, bp 90 °C/0.05 torr, was prepared from reaction of the carboxylic acid with thionyl chloride (Leffler & Wilson, 1960). Triethylamine, 128 mg, 1.26 mmol, was added to a stirred solution of the acid chloride, 153 mg, 0.54 mmol, and sodium *m*-carboxyphenyl (aminomethyl)-phosphonate (Rahil & Pratt, 1992), 53 mg, 0.21 mmol, in methanol, 5 mL, at room temperature. After a reaction time of 6 h, volatiles were removed under reduced pressure. The residue was extracted with methylene chloride, leaving the required product as an insoluble salt. This solid was purified by QAE-Sephadex ion-exchange chromatography as previously described (Rahil & Pratt, 1992). The identity and purity of the product, as the triethylammonium salt, was established by its ^{1}H NMR spectrum: (H_2O) δ 1.3 (t, J = 7.5 Hz, 18, CH_3), 3.2 (q, J = 7.5 Hz, 12, CH_2), 3.55 (s, 2, CH_2CO), 3.65 (d, J = 12 Hz, 2, CH_2P), 7.05 (d, J = 10 Hz, 2, IArH), 7.15–7.65 (m, 4, ArH), 7.75 (d, J = 10 Hz, 2, IArH).

Crystallographic Methods. Crystals of the native enzyme were grown from poly(ethylene glycol) (M_r 8000) in 50 mM sodium cacodylate buffer at pH 6.5 in space group $P2_1$ with two molecules in the asymmetric unit. The phosphonate complex was prepared by soaking the native crystal in 1.5 mM phosphonate at pH 6.5 for 36 h. X-ray diffraction data (Cu Kα, λ = 1.54 Å) were collected from a single crystal measuring 0.35 × 0.4 × 0.6 mm at room temperature over a 46-h period. [The half-life of the complex in solution is approximately 86 h (Pratt, 1989).] A Siemens area X-ray detector was used on a Rigaku rotating anode generator operated at 40 kV and 180 mA. An 0.25° ω scan was used with a count time of 100 s per frame. Cell dimensions for the crystal of the complex are 46.5, 83.5, and 95.5 Å and β = 90.0° and differ less than 0.5% from the native cell constants. A total of 84 494 observations to 2.32-Å resolution were reduced by the Xengen program (Howard et al., 1987) to 28 780 unique reflections with an $R_{sym}(I)$ = 0.044 (Table 1). When scaled against the native structure amplitudes, the amplitudes from the complex produced an R_{isom} equal 0.056, where $R_{isom} = \Sigma|F_{complex} - F_{native}|/\Sigma|F_{complex} + F_{native}|$. Fitting of electron density maps was done with FRODO (Jones, 1985) on an Evans & Sutherland PS330 graphics system.

Protein Models. The crystallographic model of the native *E. cloacae* P99 β-lactamase at 2-Å resolution (Lobkovsky et al., 1993) is available from the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, as entry 1BLT. The expanded native model used here for initial phasing of the data from the complex includes 801 water molecules and has a crystallographic R factor equal to 0.175 at 1.86-Å resolution. Refinement of the phosphonate complex is discussed below. Other crystallographic models used are the class A β-lactamase of *Bacillus licheniformis* (Protein Data Bank, entry 4BLM; Knox & Moews, 1991), a phosphonate complex of the class A β-lactamase of *S. aureus* (Chen et al., 1993), α-chymotrypsin (entry 4CHA), and a γ-chymotrypsin phosphate monoester complex (Harel et al., 1991).

Electrostatic Potential Calculation. Electrostatic potential surfaces (Gilson & Honig, 1987) were calculated using DELPHI version 2.40 and INSIGHT version 2.2.0 (Biosym Technologies, Inc.) on a Silicon Graphics 4D/70GT running IRIX 4.0.5. Hydrogen atoms were added to the crystallographic structures at the pH of maximum enzyme activity. All DELPHI computations used 64 grid points along the longest molecular dimension. Focusing grids had a 10-Å border on all sides of the enzyme, and the final computations had a 1-Å border. The final grid sizes were 0.80, 0.90, and 0.96 Å for α-chymotrypsin, the *B. licheniformis* β-lactamase,

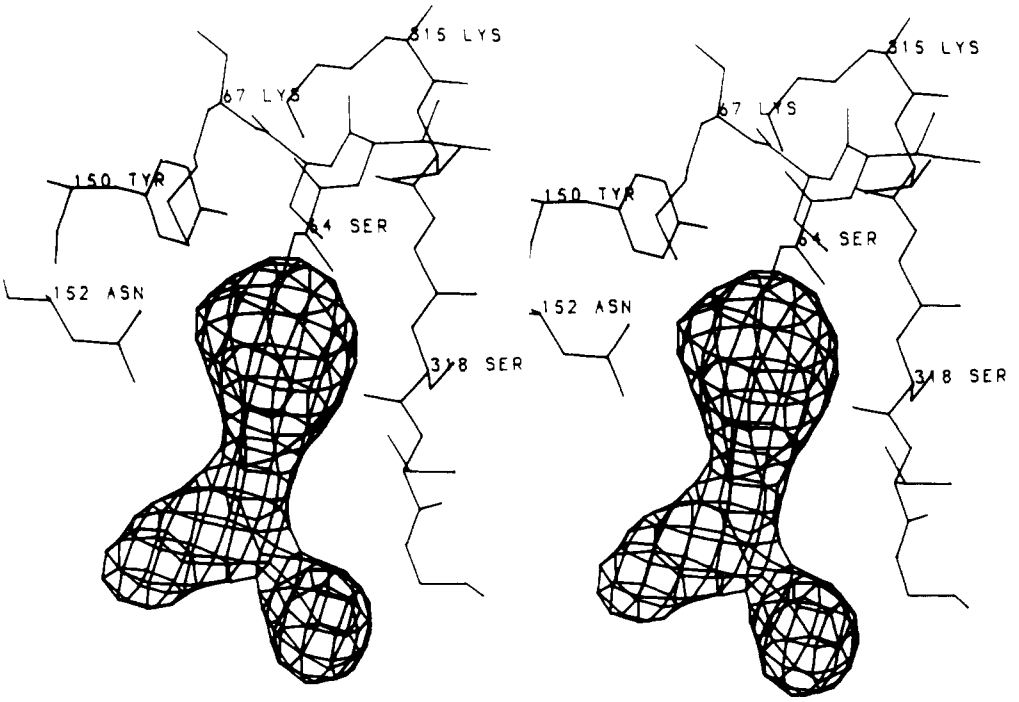


FIGURE 1: Stereoview of difference electron density in the phosphonate complex of the *E. cloacae* P99 cephalosporinase (molecule 1) calculated with native MIR phases and coefficients $|F_{o,complex}| - |F_{o,native}|$ at 3-Å resolution. The contour level is 4σ.

and the *E. cloacae* β-lactamase, respectively. Dielectric constants of 2 and 80 were used for the interior and exterior of the solvent-accessible surface, defined by a probe radius of 1.8 Å. The reaction field was calculated with an ionic strength of 0.145 M, a Debye length of 8 Å, and an ion exclusion radius of 2 Å.

RESULTS

Inactivation Kinetics. The *E. cloacae* P99 β-lactamase, 0.2 μM, was inactivated by the phosphonate, 8.3 μM, in 20 mM MOPS buffer at 25 °C. The reaction was followed by the loss of β-lactamase activity, assayed against cephalothin, as a function of time. The second-order rate constant for the inactivation was 840 s^{-1} M^{-1}. This value is very similar to that obtained for the unsubstituted (phenylacetyl)phosphonate under the same conditions—817 s^{-1} M^{-1} (Rahil & Pratt, 1992)—presumably indicating the absence of any strongly positive or negative interactions between the iodo substituent and the enzyme active site.

Crystallographic Mapping of the Complex. The difference electron density map at 3-Å resolution calculated with coefficients $|F_{o,complex}| - |F_{o,native}|$ and unbiased native phases from multiple isomorphous replacement (MIR) is shown in Figure 1. Highest density occurs in the β-lactam binding site adjacent to Ser64 and presumably represents a covalently bound phosphonate group devoid of the *m*-carboxyphenoxide leaving group. Bimodal density at the bottom can be modeled by two positions of the *p*-iodobenzyl substituent resulting from a 45° rotation about the CH_2–NH bond. Difference density was similar in both molecules of the asymmetric unit.

Refinement of the Complex. A model of the phosphonate complex was built from coordinates of the native enzyme and energy minimized by XPLOR (Brünger, 1990). Crystallographic refinement of the complex was done with PROLSQ (Konnert & Hendrickson, 1980) using CONEXN (Pahler & Hendrickson, 1990) for inclusion of the side chain, the two positions of which were assigned equal occupancies. Phosphonate occupancy was not refined, but because the final temperature factor for the phosphorus atom is 17% greater than that of the Oγ of the attached Ser64, total phosphonate occupancy may be less than 1.0. No water molecules in the binding site were included in early cycles of the refinement and were obtained from an $|F_o| - |F_c|$ difference map near the end of the refinement. Final parameters from the refinement of the complex are shown in Tables 1 and 2. Deviations of covalent bonds and angles from ideality are 0.008 Å and 1.1°, respectively. The two molecules in the asymmetric unit differ by only 0.18 Å (rms) in the positions of Cα atoms, even though

Table 1: Crystal Data and Refinement Parameters

no. of observations to 2.32 Å	82 494
no. of unique data (% of possible)	
∞–2.32 Å	28 780 (91)
2.47–2.32 Å	7904 (76)
$I/\sigma(I)$, $R_{sym}(I)$	
∞–2.32 Å	21, 0.044
2.47–2.32 Å	4, 0.158
no. of atoms (protein, phosphonate, water)	6242, 28, 797
mean *B* (Å²) (protein, phosphonate, water, all)	21, 47, 40, 23
rms coordinate shift in final cycle (Å)	0.016
estimated coordinate error (Å)	0.25,[a] 0.16[b]
R factor (all 27 532 data 8–2.3 Å)	0.192

[a] Luzzati, 1952. [b] Read, 1986.

Table 2: Deviations of Model from Ideal Geometry

model	rms deviation	weighting parameters
distances (Å)		
bonds (1–2 neighbor)	0.008	0.020
angles (1–3 neighbor)	0.020	0.030
planar 1–4 distance	0.017	0.040
chiral volume (Å³)	0.113	0.150
torsion angles (deg)		
planar	1.5	3.0
staggered	21.0	30
transverse	21.8	30
thermal factors (Å²)		
main chain (1–2 neighbor)	0.6	1.5
main chain (1–3 neighbor)	0.9	2.0
side-chain bond	1.3	3.0
side-chain angle	2.0	4.5

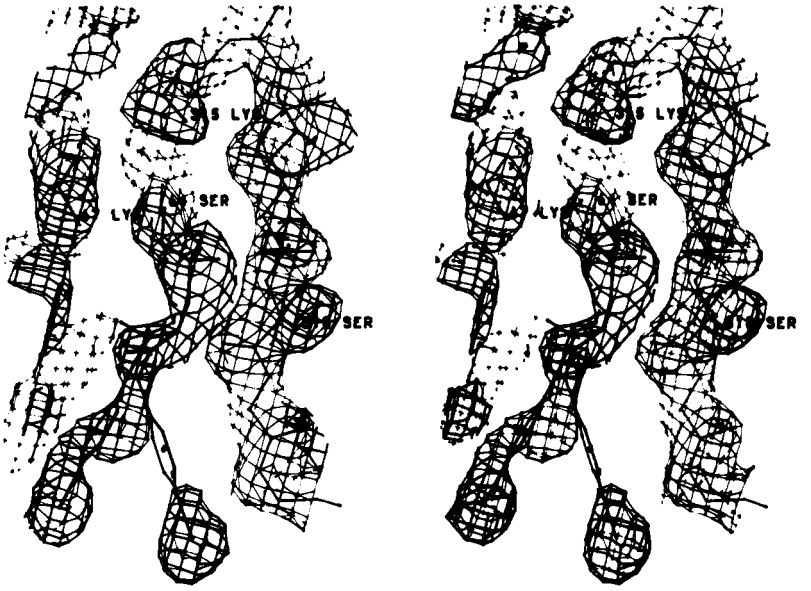


FIGURE 2: Electron density map of the phosphonate complex at 2.3-Å resolution with Read (1986) coefficients $|2mF_{o,complex}| - D|F_{c,complex}|$. The contour level is 1$\sigma$. The *p*-iodobenzyl group occupies two positions. Atoms in this view are also shown in Figure 4a.

FIGURE 3: Molscript drawing (Kraulis, 1991) of the *E. cloacae* cephalosporinase bound at Ser64 with the monoester phosphonate.

a 2-fold pseudosymmetry was never imposed during the refinement. Atomic coordinates have been deposited in the Protein Data Bank as entry 1BLS. A portion of the resulting 2.3-Å Read map (Read, 1986) with coefficients $|2mF_{o,complex}| - D|F_{c,complex}|$ is shown in Figure 2. Incomplete density is seen for the benzyl group, though the iodine atom in the *para* position is quite clear and served to define two positions for the group. Continuous density from Ser64 to the phosphorus atom confirms the covalency of the complex, and the volume of the density surrounding the phosphorus atom shows that a five-atom tetrahedral cluster, rather than a six-atom trigonal bipyramidal cluster, is present.

Structure of the Complex. Figure 3 shows the tertiary structure of the cephalosporinase and the location of the phosphonyl binding site adjacent to the B3 β-strand of the β-sheet. Figure 4 shows amino acid residues which neighbor the tetrahedral phosphonyl species. Covalent bonds (restrained in the refinement) and dihedral angles involving the seryl–phosphorus group are compared in Table 3 with other phosphon(or)yl complexes, and important interatom distances are listed in Table 4. The terminal P–O_1 group binds in the oxyanion binding pocket formed by the backbone NH groups of Ser64 and Ser318 and clearly corresponds to the C–O group of a β-lactamoyl species. The P–O_1 interaction with the oxyanion hole is quite asymmetrical, with the P–O_1 group binding preferentially to the main-chain Ser318NH (2.3–2.4 Å, distance L2). The main-chain nitrogen atom of Ser64NH is 2.8–3.0 Å (L1) from the phosphoryl oxygen atom, but the hydrogen-bonding angle P–O_1···N is unfavorable, being near 90°.

The P–O_2 group is surrounded by three potentially cationic residues, but at rather long distances, viz., 5.4, 4.4, and 6.7 Å to Lys67, Lys315, and Arg349, respectively. Its nearest contacts are with the hydroxyl group of Tyr150 (3.4–3.5 Å, distance C′) and especially with a water molecule which bridges to the hydroxyl group of Ser318 in molecule 1 or to Thr316 in molecule 2. Three remaining interactions (J, K_1, and K_2) bind the acylamide linkage of the phosphonate to the CO of Ser318 (2.7 Å) and to the NH_2 groups of Gln120 and Asn152, as in β-lactamoyl complexes. Here, however, the CO group binds either to one or the other of the amide groups, but not symmetrically to both, and 2-fold disorder in the benzyl side chain results. As seen in β-lactamoyl complexes with the *Streptomyces* sp. R61 DD-peptidase (Kelly et al., 1989), the hydrophobic substituent of the acylamide is exposed, though an edge of the benzyl group is near (3.1 Å) the face of the aromatic ring of Tyr221.

Comparison with the Native Cephalosporinase Structure. Overlay of the coordinates of the phosphonate complex with the native coordinates produces a 0.12-Å rms difference for protein atoms within 10 Å of Ser64Oγ, indicating that very little change has occurred in the binding site due to phosphonylation. The largest atom shifts are about 0.25 Å in the positions of Ser64Oγ, Lys67Nε, and Tyr150O, resulting in distances to Ser64Oγ (A and C in Figure 4b) which are changed by 0.2 Å upon formation of the complex (Table 4).

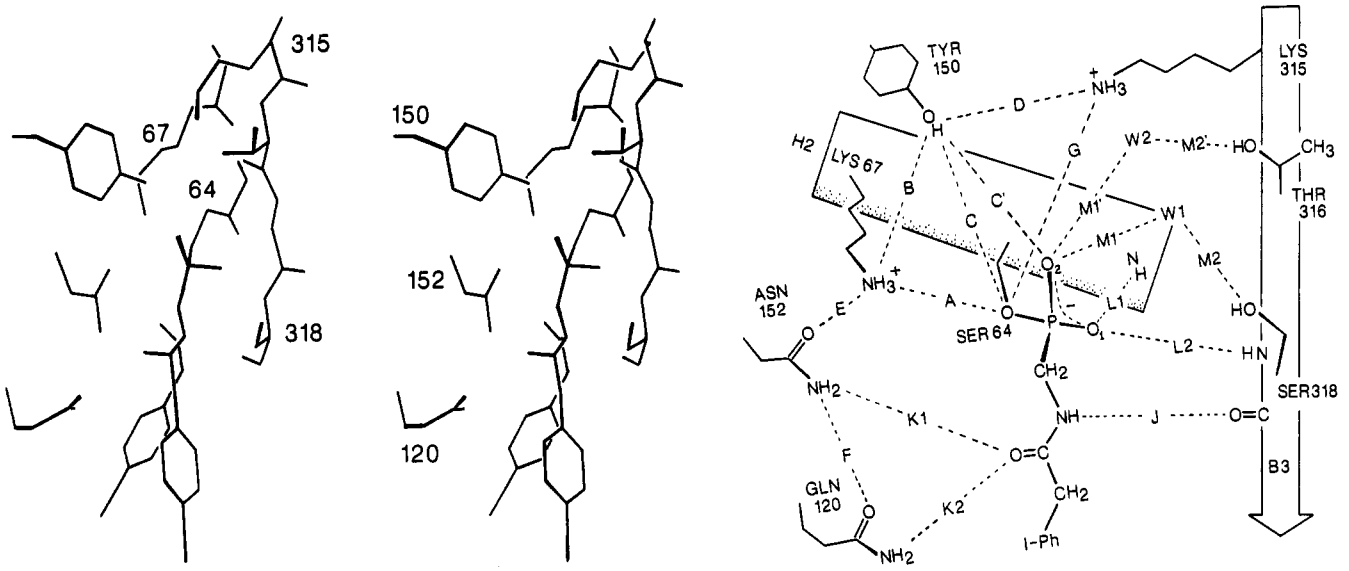


FIGURE 4: (a, left) Stereoview and (b, right) schematic drawing of interactions between the Ser64-bound phosphonyl intermediate and groups in the binding site. Labeled distances are given in Table 4.

Table 3: Phosphon(or)yl–Serine Geometry in Related Complexes

	class C β-lactamase			
	molecule 1	molecule 2	class A β-lactamase[a]	γ-chymotrypsin[b]
distance (Å)				
SerOγ–P	1.65	1.65	1.6	1.55
P–O_1	1.55	1.57	1.4	1.43
P–O_2	1.56	1.56	1.4	1.55
P–C (or O)	1.55	1.55	1.8	1.52
dihedral angle (deg)				
SerCβ–Oγ–P–O_1	–60	–64	–24	–41
SerCβ–Oγ–P–C (or O)	–165	–170	–155	–162
SerOγ–P–C (or O)–N (or C)	36	65	177	67

[a] β-Lactamase of *S. aureus* PC1 (Chen et al., 1993). [b] Monoisopropyl phosphate complex (Harel et al., 1991).

Table 4: Distances (Å) in Phosphonylated and Native β-Lactamases[a]

	class C β-lactamase					
	molecule 1		molecule 2		class A β-lactamase[c]	
distance[b]	complex	native	complex	native	complex	native
A	3.32	3.12	3.39	3.10	2.8	2.5
B	2.77	2.68	3.35	3.20	4.0	3.7
C	3.01	3.26	2.92	3.14	3.2	3.3
C′	3.40		3.46		3.3	
D	3.46	3.11	3.23	2.99	3.0	3.0
E	2.57	2.73	2.31	2.52	2.6	2.7
F	2.91	3.05	3.08	*d*		
G	4.70	4.78	4.72	4.71	4.6	4.7
J	2.71		2.73		3.6	
K1	3.24		2.98		2.6	
K2	3.23		3.71			
L1	3.05	3.64 Wat	2.82	3.54 Wat	2.9	3.2 Wat
L2	2.29	2.96 Wat	2.38	2.70 Wat	2.9	3.0 Wat
M1	3.22					
M1′			2.33			
M2	2.68					
M2′			2.63			

[a] Equivalences for the class A/C residues are Ser70/64, Lys73/67, Ala104/Gln120, Ser130/Tyr150, Asn132/152, Lys234/315, Ser235/Thr316, Gly236/317, and Gln237/Ser318. [b] See Figure 4b. [c] β-Lactamase of *S. aureus* PC1 (Chen et al., 1993). [d] Gln120 shows two-position disorder in molecule 2.

Tyr150 moves closer to the Ser64Oγ, and its interactions with Lys67 and Lys315 weaken (B and D). The hydrogen bonding between Lys67 and Asn152 (E), quite strong in the native state, is only slightly weakened by complexation.

A water molecule weakly bound in the oxyanion pocket of the native enzyme was replaced by the P–O_1 oxygen atom of the inhibitor. It is notable that in the native enzyme this water molecule was also asymmetrically bound to the main-chain NH groups, as is the oxygen atom of P–O_1. Another water molecule bonded to both Gln120 and Asn152 was replaced by the acylamide CO group of the phosphonate.

Comparison with the Phosphonate Complex of a Class A β-Lactamase. The crystal structure of the covalent tetrahedral complex of the class A β-lactamase of *S. aureus* PC1 with *p*-nitrophenyl [*N*-(benzyloxycarbonyl)amino]phosphonate has recently been determined to 2.3-Å resolution (Chen et al., 1993). Covalent bonds and conformations involving the phosphorus center are compared in Table 3. Table 4 lists distances to groups in the class A and class C binding sites. A similar dihedral angle SerCβ–Oγ–P–C places the acylamide group of each phosphonate antiparallel to the B3 β-strand. The hydrogen bond from the NH group of the phosphonate to the CO of Gln237/Ser318 (distance J) is much stronger in the class C complex, presumably because the class C site, in contrast to the class A site, affords more space near the bottom of the β-strand to acylamide substituents (Lobkovsky et al., 1993). In both β-lactamase complexes, the oxyanion pocket is occupied by the O_1 oxygen atom of the phosphonate, but the binding of the P–O_1 bond is considerably more symmetric, and slightly weaker, in the class A β-lactamase than in the class C enzyme (distances L1 and L2). The P–O_2 group is solvated in both complexes, and the O_2 atom is equally distant (3.3 and 3.4 Å) from Ser130/Tyr150.

Comparison with a Phosphate Complex of a Serine Proteinase. Both classes of β-lactamase, as well as proteinases of the chymotrypsin type, employ a reactive serine and an oxyanion pocket. Whether further structural analogy exists in the two catalytic sites, especially whether β-lactamase contains a counterpart for the His57 general acid/base, has been discussed for the class A (Herzberg & Moult, 1991; Chen et al., 1993) and class C (Oefner et al., 1990) β-lactamases. We show in Figure 5 a superimposition of the reactive serine and oxyanion pocket of the class C β-lactamase

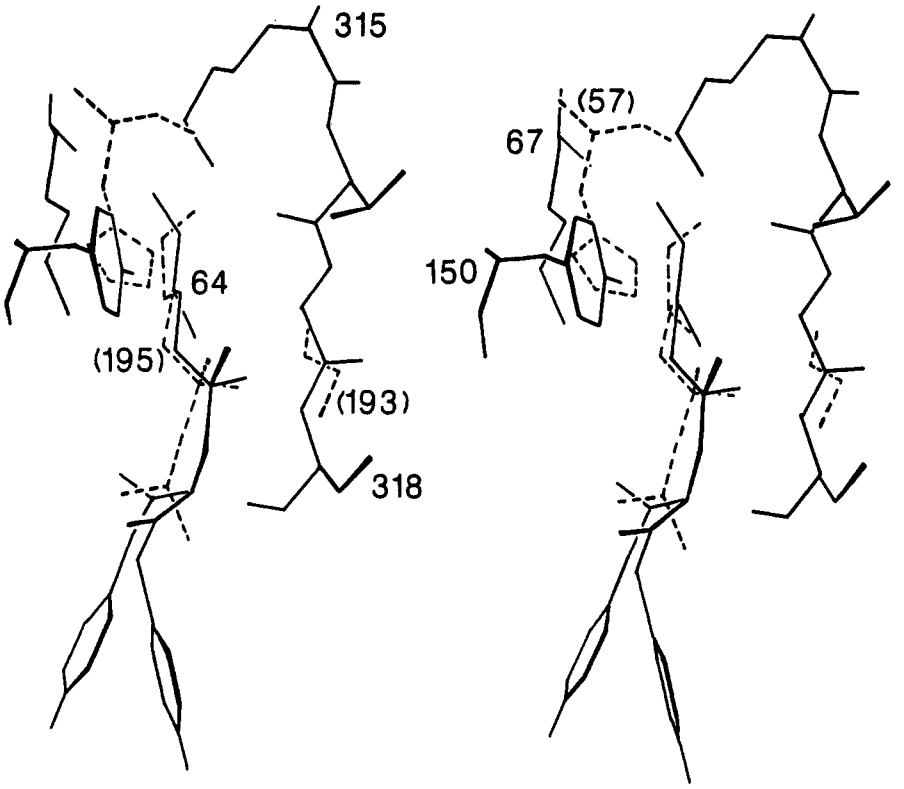


FIGURE 5: Overlay of the catalytic sites in the phosphonate complex of cephalosporinase and in the monoisopropyl phosphate complex of γ-chymotrypsin (Harel et al., 1991). Ser195 and the main-chain NH groups at 193 and 195 (dashed lines) are respectively near Ser64 and the NH groups at 64 and 318 in the β-lactamase. The hydroxyl oxygen atom of Tyr150 is approximately 0.7 Å from Nε of His57. Chymotrypsin residue numbers are in parentheses.

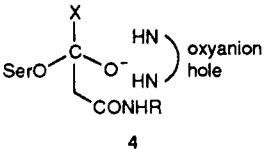
complex over a γ-chymotrypsin complex with monoisopropyl phosphate (MIP; Harel et al., 1991).

In the chymotrypsin complex, the binding of the P–O_1 group in the oxyanion pocket has an asymmetry which is similar to, but less pronounced than, that seen in the class C β-lactamase. The exposed P–O_2 bond in chymotrypsin binds much more strongly with His57Nε (2.6 Å, distance C′) than it binds in the β-lactamases with the hydroxyl oxygen atom of Ser130/Tyr150, the apparent counterparts of His57. The separation of Nε of His57 from SerOγ (distance C) is 3.1 Å, between the distances in the two β-lactamase complexes.

Comparison of Electrostatic Potential in the Catalytic Sites. Figure 6 shows the calculated electrostatic potential in the catalytic sites of the two class of serine β-lactamases and in the serine proteinase, α-chymotrypsin. Potentials were determined at the pH of optimum activity for each enzyme and were plotted at −4 and 4 kT. Surprisingly, little electropositive density is centered at the oxyanion pocket of any of the enzymes, though all β-lactamase sites contain positive density near the region where the carboxylic acid group of β-lactams is expected to bind. Comparison of panels a and b of Figure 6 reveals that the calculated potential within the catalytic site of the class C β-lactamase depends very strongly on the protonation state of Tyr150. The unprotonated state of the class C site (Figure 6b) produces a density more like the density seen in the class A site (Figure 6c), though the class C site is somewhat more electropositive. The two types of β-lactamase clearly contain more electropositive density than does α-chymotrypsin (Figure 6d).

DISCUSSION

The structure of the inert complex formed on reaction of the phosphonate monoester **3** with the *E. cloacae* P99 cephalosporinase (class C β-lactamase) possesses many of the features expected of a tetrahedral intermediate in β-lactamase catalysis, as would be inferred from structural studies of β-lactamoyl derivatives of both class A and class C β-lactamases (Oefner et al., 1990; Strynadka et al., 1992; Chen et al., 1993) and of the β-lactam-inhibited DD-peptidase of *Streptomyces* sp. R61 (Kelly et al., 1989; Knox & Kelly, 1989). The phosphonyl group is covalently bonded to the active site seryl–Oγ, as depicted in **2**, and as anticipated from chemical studies (Pratt, 1989; Rahil & Pratt, 1992), one phosphonyl oxygen (O_1) occupies a previously identified oxyanion hole, and the amido side chain is hydrogen-bonded to the B3 β-strand. The complex therefore will be assumed, for the purposes of the present discussion, to be a close analog of the acyl-transfer tetrahedral intermediate **4** and associated



4

transition states, where X, discussed further below, may be a leaving group (as in an acylation transition state) or a hydroxyl group (as in deacylation).

A striking feature of the placement of the inhibitor-derived moiety, however, is its asymmetry and side-chain positional disorder. This is seen in the asymmetry of placement of the phosphonyl oxygen O_1 in the oxyanion hole (Figure 4, Table 4, distance L1 *vs* L2), the asymmetry of hydrogen bonding of the amido side chain (distance J *vs* K1 and K2), and the two-position binding of the aryl group of the side chain. This apparent nonideality of binding might well reflect the attributes of an active site designed to accommodate the broadest possible

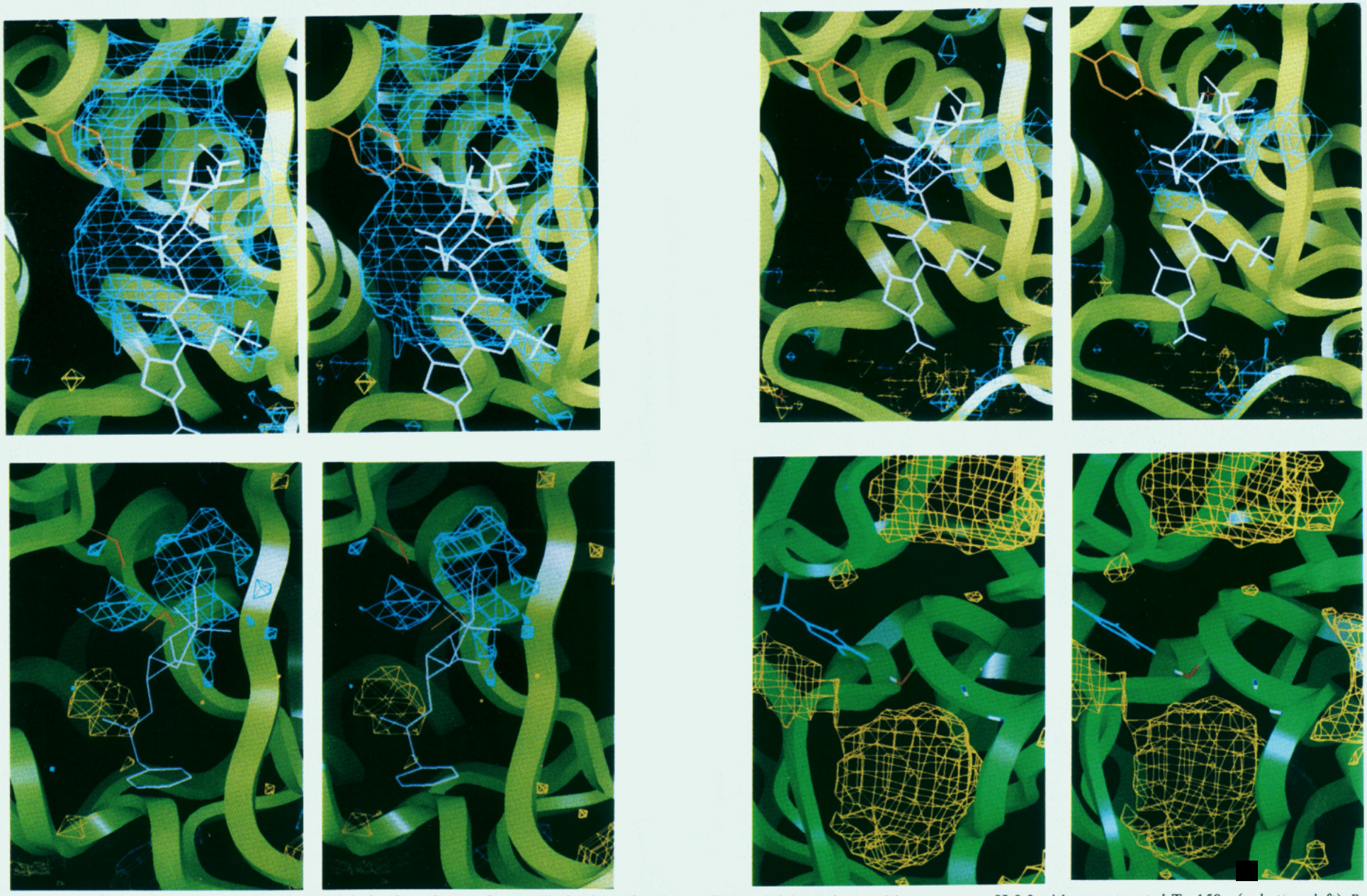


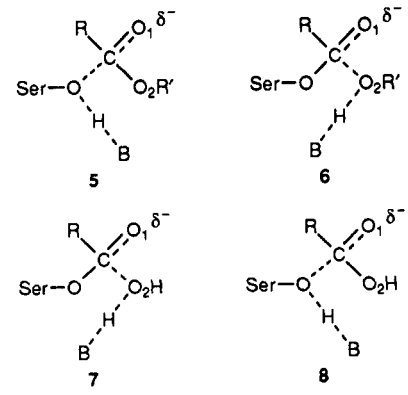
FIGURE 6: Comparison of electrostatic potentials in the substrate-free catalytic sites of two β-lactamases and a serine proteinase, each at their optimum catalytic pH. The yellow and blue grid surfaces are drawn with DELPHI at –4 and 4 kT. (a, top left) *E. cloacae* P99 β-lactamase (class C) at pH 8.0 with protonated Tyr150; cefotaxime is shown for positional reference only. (b, top right) *E. cloacae* β-lactamase at pH 8.0 with unprotonated Tyr150. (c, bottom left) *B. licheniformis* β-lactamase (class A) at pH 6.5 with penicillin G. (d, bottom right) α-Chymotrypsin at pH 7.8 with unprotonated His57; the reactive Ser195 and the two main-chain NH groups (193 and 195) are oriented similarly to the serine and NH groups in the two β-lactamases.

range of (β-lactam) substrates, an important feature of the role of this enzyme in bacterial resistance to β-lactam antibiotics. To this purpose, various compensatory or fail-safe devices might be expected in the active site. This idea has also arisen from consideration of the effects of site-specific mutations on class A β-lactamase activities (Juteau et al., 1992).

It is possible, for example, that the asymmetry of the oxyanion binding is compensated for by the very short distance from the phosphonyl oxygen O_1 to one NH hydrogen bond donor of the oxyanion pocket, that from the B3 β-strand (distance L2). This might permit sufficiently strong stabilization of the oxyanion, i.e., of tetrahedral intermediate **4**, by means of a single low-barrier hydrogen bond (Cleland, 1992; Gerlt & Gassman, 1993). It should be noted, however, that in the native enzyme a water molecule occupying the oxyanion hole is also asymmetrically placed (Table 4), although it is clearly not as firmly bound as the phosphonate oxygen. In the class A *S. aureus* PC1 β-lactamase, the analogous water molecule of the native structure and O_1 of a phosphonate complex (Chen et al., 1993) both appear more symmetrically placed in the oxyanion hole. Occupation of the hole may then be dictated as much by local electrostatics as by substrate structure. The asymmetry of the amide hydrogen bonding, with the appearance of much stronger hydrogen bonding to the B3 β-strand than to Asn152 contrasts noticeably with the situation in the complex of a phosphonate with the class A *S. aureus* β-lactamase (Chen et al., 1993) where the amide group is more closely associated with Asn132 (comparably placed to Asn152 of the class C enzyme) than with the β-strand. Compensation between these two hydrogen bonds that hold the amide group is thus suggested. Certainly in β-lactam substrates, a strong hydrogen bond to the side-chain amide carbonyl is not essential for efficient catalysis in either class A or class C β-lactamases (Pratt et al., 1992); it is possible that, in cases where that hydrogen bond is not possible, a stronger hydrogen bond from the side-chain amide NH to the β-strand carbonyl is formed, as in the present phosphonate complex. Finally, the positional disorder of the side chain likely reflects the more spacious side-chain binding site present in class C than in class A β-lactamases (Lobkovsky et al., 1993); a unique side-chain position is observed in the comparable class A complex (Chen et al., 1993). This difference probably also relates to the broader specificity of the class C enzyme. It is interesting to note that discrete electron density for the crystallographic binding of deiodo-**3** to the class C β-lactamase could not be found, perhaps because of its even greater mobility than that of **3** (J. R. Knox, unpublished data).

Another specificity issue is that of various β-lactamases toward the phosphonate monoester inhibitors **1**. The class C P99 β-lactamase is much more susceptible to inhibition by these molecules than are class A β-lactamases and serine proteinases (Rahil & Pratt, 1991, 1992). This may simply be a matter of the steric fit of the pentacoordinated transition state into these active sites and possibly, therefore, a situation modifiable by variations in side-chain and leaving group geometry. Or, as suggested previously (Rahil & Pratt, 1992), it may reflect a more general property of these active sites, the relative positive electrostatic potential available to stabilize the transition state for phosphonylation where charge on the phosphonyl group may approach −2, depending on how concerted the phosphonyl-transfer mechanism is in this case. Electrostatic calculations were performed on three native serine hydrolases: γ-chymotrypsin, the class A β-lactamase of *B. licheniformis*, and the class C β-lactamase of *E. cloacae* P99, in what is likely to be the active form of each. The results (Figure 6) show that the positive electrostatic potential is clearly greater in the case of the β-lactamases than chymotrypsin. This difference may therefore indeed explain the susceptibility of β-lactamases to phosphonate monoester monoanions. The difference in positive potential between the class A and class C β-lactamases in less marked, and thus the reason for the greater susceptibility of the class C β-lactamase to inhibition by phosphonate monoanions is less evident from these results. It is possible that Tyr150 is protonated in the active form of the class C enzyme (compare panels a and b of Figure 6), but this would leave the active site without an obvious basic catalyst. It seems more likely that this difference between the class A and C active sites represents a further example of the greater promiscuity of the class C active site. In accord with this interpretation, it has been observed that the P99 β-lactamase is a better catalyst of the hydrolysis of acyclic substrates in general than are class A enzymes (Govardhan & Pratt, 1987; Murphy & Pratt, 1991).

Scheme 2



The distribution of protein side-chain functional groups around the tetrahedral phosphonyl group of the inhibitor is shown in Figure 4. The immediately striking feature is that none of them is very close (hydrogen-bonded) to either phosphonyl oxygen, O_1 or O_2—the closest to either of these is the hydroxyl oxygen of Tyr150 which is 3.4 Å from O_2. Although the active site contains two conserved lysine residues, Lys67 and Lys315, these are 5.4 and 4.4 Å distant, respectively, from O_2 (and further from O_1). This situation is similar to that found in the class A β-lactamase–phosphonate complex (Chen et al., 1993), discussed further below, but contrasts strongly with that in the MIP derivative of γ-chymotrypsin (Harel et al., 1992) and β-trypsin (Kossiakoff & Spencer, 1980) where the general base catalyst of the active site, the histidine imidazole group of the "catalytic triad", in protonated form, is strongly hydrogen-bonded (2.6 Å) to the phosphoryl oxygen corresponding to O_2 (Figure 5). Since the latter observation is readily interpretable in terms of the accepted serine proteinase mechanism and in terms of the MIP derivative as a good transition-state analog, the qualitatively very different structure of Figure 4 or 5 requires careful analysis.

First, there is the question as to just which transition state of the normal acyl-transfer reaction the present crystal structure corresponds. A symmetric, serine proteinase-like double displacement mechanism may involve, consecutively, the four transition states of Scheme 2. Of these, two fundamental classes are distinguished by the position of the catalytic base B. In **5** and **8**, the "outer" transition states, B is more closely associated with the active site SerOγ than with the leaving/entering group oxygen (O_2), and the reverse

is true in the "inner" transition states **6** and **7**. Less fundamentally, at least to a symmetric mechanism, **5** and **6** are distinguished from **7** and **8** by the presence of the leaving group substituent R.

The MIP derivatives of γ-chymotrypsin and β-trypsin have been interpreted as mimics of transition state **7**, that for formation of the tetrahedral intermediate in deacylation—no R group is present and BH^+ (the protonated imidazole of His57) is hydrogen-bonded (2.6 Å) to the leaving/entering group O_2. The distance between Nε of His57 and the SerOγ in the MIP complex is significantly greater (3.1 Å).

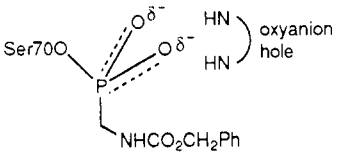
The structure of Figure 4, lacking an R group, would initially appear to be best interpreted in terms of the transition states **7** and **8** of Scheme 2. The closest functional groups to the phosphonyl moiety, Lys67 and Tyr150, are closer to the SerOγ than to O_2 (Table 4). Hence transition state **8** would seem to be the closest analog, where the base B would therefore be either the neutral amine group of Lys67 or the phenoxide anion of Tyr150. Of these, the latter appears to be the more likely because the distance C decreases on formation of the phosphonate derivative from the native enzyme, as might be expected as it became protonated, while, conversely, the distance A increases. The distances B and D also increase as would be expected if the tyrosyl phenoxide (or the Lys67 amine) became protonated. It is noticeable, however, that although the Tyr150 hydroxyl oxygen, presumably protonated in the phosphonate, is the closest functional group to the serine oxygen of **8**, there may only be a moderately strong hydrogen bond between them—the distance C is about 3 Å. It may well be, however, that the positions of the protein functional groups, particularly that of Tyr150, are somewhat distorted with respect to those in a normal transition state **8** by the presence of the partial negative charge on O_2 (3.4 Å from the Tyr150 hydroxyl oxygen) and by the formally neutral SerOγ; in transition state **8**, O_2 would be closer to neutral and SerOγ would carry a partial negative charge—both of these differences would bring the Tyr150 hydroxyl group closer to SerOγ.

If the structure of Figure 4 does indeed mimic **8**, O_2 represents the oxygen of the water molecule involved in hydrolysis of the acyl-enzyme. This water molecule, whose attack might also be catalyzed by the Tyr150 phenoxide ion (transition state **7**) would then most likely come from solvent. This situation can be contrasted with that thought to obtain in class A β-lactamases where the hydrolytic water has been proposed to approach from the opposite face of the acyl group, occluded on substrate binding between the substrate and the Glu166 carboxylate (Knox & Moews, 1991; Knox et al., 1993) which is believed to be the general base catalyst in deacylation (Herzberg & Moult, 1987, 1991; Escobar et al., 1991). The P99 β-lactamase has no comparably placed carboxylate, and no occluded water has been observed (Lobkovsky et al., 1993); the nucleophile may therefore, perhaps necessarily, be derived directly from solution. This difference may explain why class C β-lactamases more readily than class A permit deacylation of acyl-enzyme intermediates by nucleophiles other than water—small alcohols (Knott-Hunziker et al., 1982; Govardhan & Pratt, 1987) and D-amino acids (Pratt & Govardhan, 1984; Pazhanisamy & Pratt, 1989), for example.

Alternatively, there is some chance, if the mechanism were asymmetric for example (see below), that the transition state analogous to Figure 4 is **5**, in which case O_2 represents the position of the leaving group in acylation. A leaving group extending from that point could favorably interact with the side chains of Ser318, Thr316, and Lys315.

The position reached above from interpretation of the present crystal structure, that the most likely base catalyst in the P99 β-lactamase active site is the Tyr150 phenoxide, agrees with the proposal of Oefner et al. (1990), made on the basis of the crystal structure of an acyl-enzyme formed on interaction of the class C β-lactamase of *Citrobacter freundii* with a poor substrate. Inasmuch as the present structure is that of a transition-state analog, the justification for that conclusion is now stronger. As also proposed by Oefner et al. (1990), an important role of the two lysine ammonium groups of the active site would be to depress the pK_a of Tyr150 to yield a credible general base at close to neutral pH—the pH of maximal activity (k_{cat}/K_m) of the P99 β-lactamase is around 7.5 (Rahil & Pratt, 1993). The positive charges of the lysines may also serve to electrostatically assist in the stabilization of the tetrahedral oxyanion intermediate (Knap & Pratt, 1991)—both Nε's are less than 6 Å from O_1 and, depending on the effective dielectric constant of the medium, could provide several kilocalories per mole in transition-state stabilization. The electrostatic calculations on the native β-lactamases (Figure 6), using positive Lys67 and Lys315, produced a net positive potential displaced somewhat from the oxyanion pocket but in the region later occupied by the tetrahedral phosphoryl group, even when Tyr150 was unprotonated. It is quite striking, in retrospect, that early β-lactamase models, employed by Schwartz and co-workers (Schwartz, 1965; Schwartz & Pflug, 1967; Kinget & Schwartz, 1968) before anything was known of any β-lactamase active site, consisted of 3,6-bis[(dimethylamino)methyl]catechols, where a cationic amine side chain promoted phenolic dissociation at neutral pH; these models contained an oxygen nucleophile adjacent to a phenol and two ammonium ions!

As mentioned above, the crystal structure of the phosphonyl derivative **9** of a class A β-lactamase, that of *S. aureus* PC1 (Chen et al., 1993), is similar in many ways to that observed here. The positioning of the side chain and phosphonyl group



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is very similar. The significant differences lie in the presence of the conserved (in class A β-lactamases) Ser130 hydroxyl group in place of that of Tyr150 and the existence of an occluded water molecule, described above, hydrogen-bonded between the carboxyl(ate) side chain of Glu166, Asn170, and the Ser70Oγ. Structure **9** has been interpreted (Chen et al., 1993) in terms of transition state **5**, where the position of O_2 represents that of the leaving group of the substrate. (It was not interpreted in terms of **8** because of the strong feeling that the deacylating water should be delivered by Glu166, in which case the latter residue should therefore be hydrogen-bonded to the corresponding phosphonyl oxygen.) Under this scenario, the base B would be either the neutral Lys73 (equivalent to Lys67 in the P99 β-lactamase) amine (Strynadka et al., 1992) or the occluded water molecule, aided by Glu166 (Lamotte-Brasseur et al., 1991). Since it seems unlikely that the Lys73 ammonium ion, directly adjacent to a carboxyl group, would be neutral at the below pH 7 (Fink, 1992), the latter possibility at this stage seems more likely (Rahil & Pratt, 1994). Direct employment of the Ser130 hydroxyl group in the manner proposed above for that of Tyr150 of the P99 enzyme has been mooted (Oefner et al., 1990), but it is still not clear that

its pK_a could be sufficiently reduced to permit it to act, in the anionic form, as a general base. Thus, in class A β-lactamases, Glu166 *with its attendant water molecule* may be the direct analog of the class C Tyr150, but placed more appropriately to catalyze turnover, and particularly the deacylation step, of penicillins, to which the class A β-lactamases seem better adapted.

No data on Tyr150 mutants of class C β-lactamases appear yet to have been published. Mutations of the analogous Tyr159 of the *Streptomyces* sp. R61 DD-peptidase (Wilkin et al., 1993) and of Ser130 of class A β-lactamases (Jacob et al., 1990; Juteau et al., 1992) lead to substrate-dependent decreases in activity but not, in general, to the complete loss of activity. Compensation mechanisms involving water molecules may be involved.

In summary, it appears that the most direct interpretation of the structure presented in this paper, in terms of a transition-state analog, would favor the Tyr150 hydroxyl group as the most likely candidate for the general acid/general base catalyst of the class C β-lactamase active site. A mechanism of catalysis similar to that proposed by Oefner et al. (1990) might therefore reasonably obtain. The intriguing question remains, however, as to why the structure (in common with the analogous class A structure) more closely resembles transition states **5** and **8** rather than, as in the serine proteinases, **6** and **7**. A direct interpretation of these facts might be that β-lactamases are required to stabilize **5** and **8** more strongly than **6** and **7** while the reverse is true of serine proteinases. This could reflect the relative leaving group abilities of their natural substrates. It is interesting in this regard to notice from Table 3 that the SerOγ–P bond distances for the β-lactamases are greater than that for γ-chymotrypsin, as would be expected for analogs of **5** and **8** *vs* **6** and **7**, respectively; that the distances were restrained during refinement should be noted as a *caveat*, however. The crystal structure of a phosphonate derivative of the *Streptomyces* DD-peptidase, closely analogous in structure to the P99 β-lactamase, even to the existence of an active site tyrosine (Ghuysen, 1991; Wilkin et al., 1993), but where the natural substrate is a peptide, would clearly be of interest.

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