Comparison of β-lactamases of classes A and D: 1.5-Å crystallographic structure of the class D OXA-1 oxacillinase

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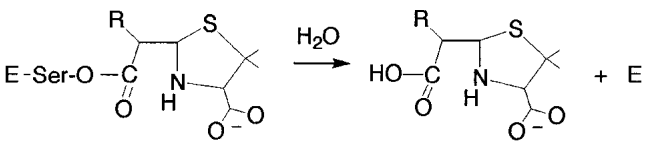
Abstract

The crystallographic structure of the *Escherichia coli* OXA-1 β-lactamase has been established at 1.5-Å resolution and refined to $R = 0.18$. The 28.2-kD oxacillinase is a class D serine β-lactamase that is especially active against the penicillin-type β-lactams oxacillin and cloxacillin. In contrast to the structures of OXA-2, OXA-10, and OXA-13 belonging to other subclasses, the OXA-1 molecule is monomeric rather than dimeric and represents the subclass characterized by an enlarged Ω loop near the β-lactam binding site. The 6-residue hydrophilic insertion in this loop cannot interact directly with substrates and, instead, projects into solvent. In this structure at pH 7.5, carboxylation of the conserved Lys 70 in the catalytic site is observed. One oxygen atom of the carboxylate group is hydrogen bonded to Ser 120 and Trp 160. The other oxygen atom is more exposed and hydrogen bonded to the Oγ of the reactive Ser 67. In the overlay of the class D and class A binding sites, the carboxylate group is displaced ca. 2.6 Å from the carboxylate group of Glu 166 of class A enzymes. However, each group is equidistant from the site of the water molecule expected to function in hydrolysis, and which could be activated by the carboxylate group of Lys 70. In this ligand-free OXA-1 structure, no water molecule is seen in this site, so the water molecule must enter after formation of the acyl-Ser 67 intermediate.

Keywords: Enzyme mechanism; antibiotic resistance; x-ray crystallography; sedimentation

β-Lactams block bacterial cell wall synthesis by inhibiting a D-alanyl-D-alanine peptidase (DD-peptidase), which forms peptide cross-links within the peptidoglycan network (Jamin et al. 1995; Massova and Mobashery 1998). To be effective, a β-lactam antibiotic must be resistant to hydrolysis by bacterial β-lactamases (Frere 1995; Livermore 1995; Kotra et al. 2001), thought to share a common ancestor with the DD-peptidases (Kelly et al. 1986). Currently, more than 250 β-lactamases have been identified, many the result of point mutations in response to new β-lactams (Knox 1995; Medeiros 1997). The enzymes are grouped into four classes (A, B, C, and D) based on amino acid sequence (Bush et al. 1995). The serine-reactive β-lactamases (classes A, C, and D) rapidly hydrolyze the β-lactam bond via an acyl intermediate:



Mechanistically, the β-lactamases are similar to the DD-peptidases in the acylation step (Knox et al. 1996; Pratt

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Abbreviations: HEPES, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); MPD, 2-methyl-2,4-pentanediol; PEG, polyethylene glycol; rmsd, root of the mean of the squared deviations; rpm, revolutions per minute.

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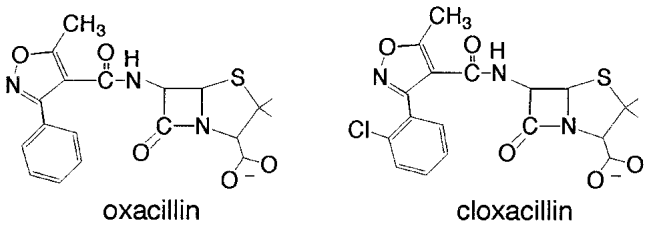
2002). But the acyl intermediate of the β-lactamase undergoes deacylation much more rapidly, the result being a very efficient destruction of the antibiotic (up to 1500 sec^{-1}) and protection of peptidoglycan synthesis.

The class D β-lactamases are called oxacillinases because of their ability to hydrolyze oxacillin and cloxacillin two to four times faster than classical penicillins



such as penicillin G (Ledent et al. 1993). Almost 30 class D enzymes are known. They are designated OXA-1, OXA-2, etc., and fall into at least five subgroups on the basis of phylogeny analysis (Sanschagrin et al. 1995; Barlow and Hall 2002). They have <20% amino acid identity with the more prevalent and better-understood β-lactamases of classes A and C (Couture et al. 1992; Naas and Nordmann 1999). Only in class D have dimeric enzymes been found (Dale and Smith 1976; Danel et al. 2001). OXA-1, shown in this work to be monomeric in solution and in the crystal, is the most common of the class D enzymes and is found in up to 10% of *Escherichia coli* isolates, in *Pseudomonas aeruginosa* and in epidemic strains of *salmonellae* (Medeiros 1984). As a result of point mutations and plasmid transfer, natural OXA variants (e.g., OXA-15, OXA-18, OXA-19) have arisen with an expanded substrate spectrum that includes imipenem and third-generation cephalosporins such as cefotaxime, ceftriaxone, and aztreonam (Naas and Nordmann 1999).

The class D β-lactamases differ significantly from class A and C enzymes by use of a carboxylated lysine side chain for catalysis (Golemi et al. 2001) and exhibiting biphasic burst kinetics (Ledent et al. 1993; Danel et al. 2001; Golemi et al. 2001). The deacylation step of catalysis is slower than the acylation step, possibly because the oxacillinases less effectively activate a molecule of water for hydroylsis. With the exception of OXA-18 (Philippon et al. 1997), Class D enzymes are not inhibited by mechanism-based class A inhibitors such as clavulanic acid (Payne et al. 1994; Page 2000), but they are unique in being readily inhibited by NaCl (Yamagishi et al. 1969; Dale 1971). Here, we describe the high-resolution structure of the monomeric, carboxylated OXA-1 oxacillinase and compare it with the structures of dimeric OXA-2, OXA-10, and OXA-13 from other subclasses. Examination of these oxacillinases, some carboxylated at Lys 70 and some not, may clarify questions about the relationship of carboxylation to water activation and chloride inhibition.

Results

Solution behavior

Analysis of sedimentation data was performed with NONLIN (Johnson et al. 1981), in which a number of molecular models (momoner, dimer, etc.) are assumed, and all of the data is fitted globally in to determine the best fit. The criteria of fitting is that the rms noise be low relative to that of the blanks and that there be little or no systematic error. The global fit of all six data sets to the monomeric molecule was good (0.016 fringes rms noise, as compared with the blank noise between 0.01 and 0.02 fringes) with very little systematic noise, returning a molecular weight (MW) of 29.0 ± 0.2 kD. A slightly better fit (significant by f-test, Fig. 1) was obtained (0.0153 fringes rms noise) from the monomer–dimer association model, returning a monomer MW of 28.4 ± 0.2 kD. The dimerization constants from this fit were quite small and varied with concentration and rotor speed. The fact that the dimerization constants tended to decrease with concentration, rather than remain constant, indicates the presence of either molecular or associative heterogeneity and not simple equilibria. The most common cause of the former kind of heterogeneity would be the presence of a small quantity of irreversible aggregate. Assuming that the aggregate is a dimer, the quantity present can be estimated to be ca. 2% by weight. The two highest concentrations did not reach equilibrium, in that the concentration versus radius curves slowly decreased with time, indicating a gradual

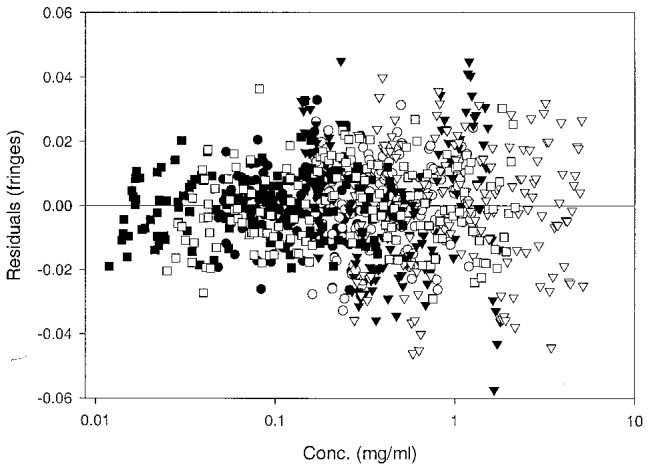


Figure 1. Residuals of sedimentation data (in fringes) from fit to monomer-dimer model vs log concentration. (*Closed circles*) 0.3 mg/mL; (*open circles*) 1 mg/mL, 20 krpm; (*closed squares*) 0.3 mg/mL; (*open squares*) 1 mg/mL, 28 krpm; (*closed triangles*) 2 mg/mL; (*open triangles*) 4 mg/mL, 25 krpm.

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loss of material. This is understandable, as the highest concentration reached in the cell with the highest loading concentration was >5 mg/mL, a concentration in which insolubility was observed previously.

Crystal structure determination

A self-rotation search with AMORE (Navaza 1994) showed that the two molecules in the cell are related by a 180° rotation about an axis along the short bc diagonal. In the molecular replacement search, the structure of OXA-10 (1FOF; Paetzel et al. 2000) or 1E4D (Maveyraud et al. 2000) was used as the model. The search was effective only after the model was abbreviated to its secondary structure elements by eliminating 55 residues in loops and chain termini. The program EPMR (Kissinger 1999) with 15-4-Å data produced a solution with a 36-Å translation of the second molecule parallel to the diad rotation axis, ruling out a dimeric form of the enzyme. The crystallographic *R*-factor and correlation coefficient for this initial solution were rather poor (0.59 and 0.22).

Structure refinement

A number of cycles of simulated annealing were performed using the protocol in CNS (Brunger et al. 1998). The two molecules were at first restrained by the screw-like noncrystallographic symmetry. Solvent flattening and torsion angle refinement were used. Initial temperatures of 5–10,000 K were gradually decreased in later cycles to 300 K. A bulk solvent correction and maximum likelihood target were applied. All the data with $F > 0\sigma(F)$ were used, 1.8% of which were randomly selected for cross validation and *R*-free calculation. Program CHAIN (Sack and Quiocho 1997) was used for manual fitting and display.

In the first cycles, the electron density was reasonably clear for most of the core secondary structure. The *R* factor improved to 0.38, but did not continue to change significantly. Assuming that there were still large differences between the probe and target structures, an omit-type molecular replacement technique was used (MacRee 1993). A peptide segment of three residues was systematically omitted along the entire length of the probe, and the rotation function peak height for each altered model was plotted along the probe sequence. This deletion procedure identified ~10 suspect segments that were examined and refitted. With more cycles of simulated annealing refinement and the inclusion of high resolution data, the *R* factor went down to 0.30 at 2.2-Å resoluton. A final rotation peak-versus-sequence plot indicated that five peptide segments needed refitting, after which the *R* factor decreased to 0.27 for 2.0-Å data.

After adding water molecules, using group *B* factors for main chain and side chain atoms, releasing the noncrystallographic symmetry restriction, and changing from torsion angle to cartesian refinement, the *R* factor improved dramatically to 0.20 (R_{free} 0.23). At 1.9-Å resolution, extra density on the ε-nitrogen of Lys 70 was very clear in both molecules and was modeled as a covalently bonded carboxylate group (Fig. 2). At 1.8 Å, MPD molecules were fit to elongated regions of difference electron density. For the last cycles of refinement, individual atom *B* factors were used. The resulting crystallographic *R* factor is 0.183 for data with $F > 0\sigma(F)$ to 1.5-Å resolution (Table 1).

The final model comprises two protein molecules, 552 water molecules (B <60 Å^2) and 10 MPD molecules. Side-chain density is not seen for the amino-terminal Ser 18 and Thr 19 in each molecule. Lys 150 in the Ω loop is omitted from both molecules, and two neighboring residues are truncated to glycines or alanines. Initially, no density was seen for the side chain of Arg 128. It was suspected that the

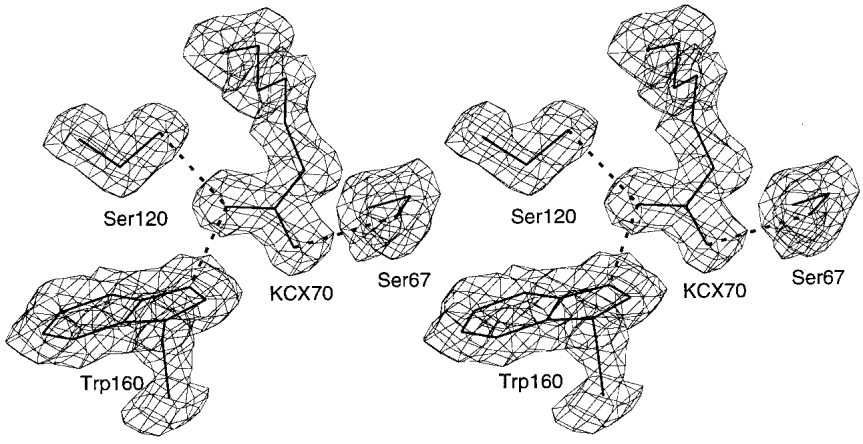

Figure 2. 2*Fo-Fc* electron density map showing groups in the catalytic site near the carboxylated Lys 70 side chain. Contoured at 1.2σ. Hydrogen bonds are indicated by *broken lines*. The map was drawn by CHAIN (Sack and Quiocho 1997).

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Table 1. *Structure refinement*

Resolution range (highest shell, Å)	75–1.5 (1.55–1.50)
No. of reflections used [$F > 0\sigma(F)$]	67,388
R-factor	0.183 (0.215)
R_{free}-factor[a]	0.204 (0.222)
Deviations from ideality (rms)	
Bond lengths (Å)	0.005
Bond angles (deg.)	1.4
Dihedrals (deg.)	22.1
Planarity (deg.)	0.81
Mean β-factors (Å^2)	
Protein molecule A, B	13.4, 14.8
MPD	39.6
Water	29.5
All atoms	16.4

[a] Calculated from 1396 reflections (1.8%) omitted from the penultimate cycle of refinement. The standard *R*-factor for last cycle includes all reflections.

published DNA sequence of the gene (Ouellette et al. 1987) is in error, and this was subsequently confirmed by the original authors (P. Roy, unpubl.). The corrected amino acid sequence (Fig. 3) now conforms to all other OXA sequences in having glycine at this position. Ramachandran analysis showed that 87.1% of the residues lie in most-favored regions, with no nonglycine residues in disallowed regions. Two conformations are assigned to the side chain of Met 99 in both molecules.

Discussion

Common features among the serine β-lactamases of classes A and D are a reactive serine and an oxyanion hole to stabilize tetrahedral transition states in both acylation and deacylation. Two conserved lysine residues exist in the binding site. However, only in class D is the lysine in the Ser 67-X-X-Lys 70 motif modified by CO_2 in a pH-dependent manner to provide full activity (Maveyraud et al. 2000; Golemi et al. 2001). Discussion has focused on how the Ser 67 is activated for nucleophilic attack on the β-lactam, what residue(s) activates a water molecule for deacylation of the acyl intermediate, what causes the unusual biphasic kinetics noted for these enzymes, and how anions such as chloride lead to inhibition (Dale 1971; Paetzel et al. 2000; Golemi et al. 2001; Maveyraud et al. 2002). Of continuing interest is the influence of dissociation on the activity of the dimeric oxacillinases (Danel et al. 2001), an effect not present in the monomeric class A enzymes.

Molecular species in solution

Unlike other oxacillinases of known crystallographic structure, the OXA-1 enzyme is found here to exist largely as a monomer over a very wide concentration range from ca. 10 μg/mL to 5 mg/mL.

Sequence alignment

An alignment of OXA-1, OXA-2 (1K38; P. Charlier, unpubl.), and OXA-10 (Maveyraud et al. 2000; Paetzel et al. 2000), representing crystal structures from three subclasses of class D oxacillinases (Sanschagrin et al. 1995; Barlow and Hall 2002), is shown in Figure 3. The longer sequence of OXA-1 (Ouellette et al. 1987) necessitates a more inclusive numbering scheme than those derived from the shorter sequences. This alignment is anchored at Ser 67 to follow convention and differs slightly from previous alignments

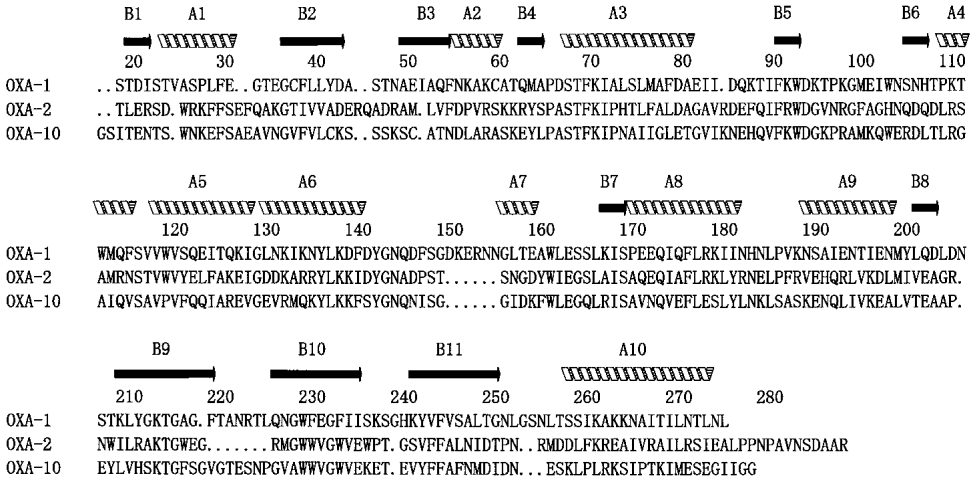

Figure 3. Structure-based amino acid sequence alignment of OXA-1, OXA-2, and OXA-10. With this alignment, amino acid identities for OXA-1/OXA-2 and OXA-1/OXA-10 pairs are 26.4% and 28.5%, respectively. Numbering and secondary structure are indicated for OXA-1.

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(Maveyraud et al. 2000; Paetzel et al. 2000; Pernot et al. 2001), especially for the carboxy-terminal 20 residues.

A notable feature of β-lactamases in the OXA-1 subgroup is the absence on the carboxy-terminal helix of an arginine directed to the active site. OXA-1 contains Ser 258 in the position spatially equivalent to the guanidinium group of Arg 261 in other subgroups. Some enzymes in the OXA-1 subgroup have Gly 258. Serine and glycine make impossible an electrostatic interaction with the C3(4) carboxylic acid group of β-lactams, as is seen in the crystal structure of the OXA-13 complex with meropenem (Pernot et al. 2001), and in the OXA-10 complex with a 6β-hydroxy penicillanate inhibitor, in which a sulfate anion bridges between the inhibitor and Arg 261 (Maveyraud et al. 2002). Clearly, a positively charged side chain in this region is not absolutely required for activity in the oxacillinase family.

Folding

The OXA-1 β-lactamase has two domains, one helical and the other a mixed domain containing a six-stranded antiparallel β-sheet with helices on both sides (Fig. 4). The reactive Ser 67 and the β-lactam binding sites lie between the two domains. Generally, the folding of the class D oxacillinase resembles that of TEM-type class A β-lactamases (Fig. 5A). The class D enzyme, however, has much more room at the top of the binding site compared with the class A enzyme, and the carboxy-terminal helix on the front of the β-sheet is positioned ~3 Å closer to the binding site.

OXA-1 differs from the other OXA structures in the Ω-loop (143–166), which is longer by six residues in OXA-1 (Fig. 5B,C). OXA-1 also differs from other OXA structures in the position of the short B8 β-strand above the binding site, presumably because OXA-1 does not utilize this strand for dimerization, as do all other oxacillinases of known structure. Here, the strand drops down in the direction of the carboxy-terminal helix, blocking the end of the channel extending up from the binding site and proposed to be a vestage of a substrate-binding channel of an ancestral protein (Maveyraud et al. 2000).

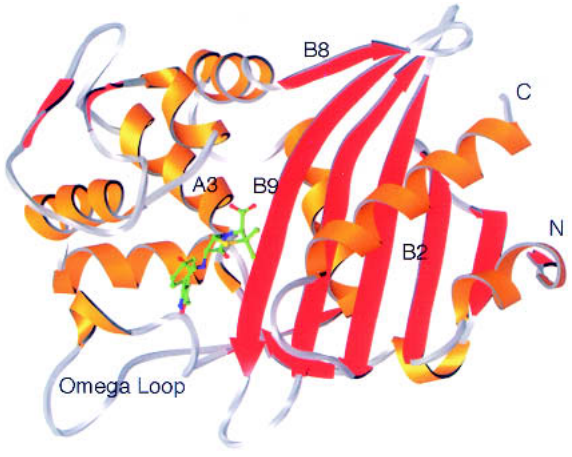

Figure 4. Tertiary structure of the monomeric OXA-1 β-lactamase. Oxacillin is modeled in the β-lactam binding site. The figure was drawn by RIBBONS (Carson 1991).

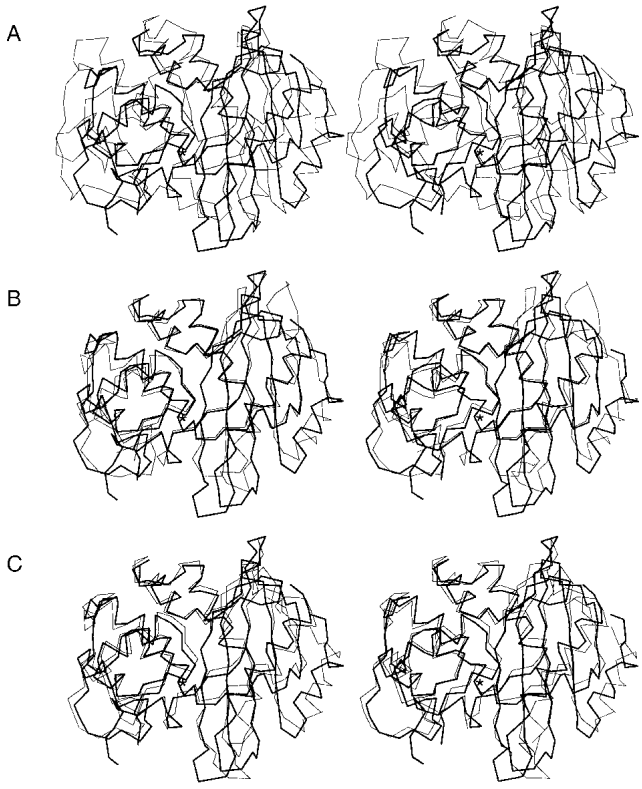

Figure 5. Stereoview of the Cα trace of OXA-1 (*bold line*) over (*A*) class A TEM-1, (*B*) OXA-2, and (*C*) OXA-10. The position of the reactive Ser 67 is marked by *star*.

Differences occur also in the lower part of the B9 strand, which is pushed out by the atypically large Asp 66 side chain. The loop connecting the B9 and B10 strands is six residues longer than in OXA-2. The loop is about the same length as in OXA-10 and OXA-13, but lies closer to the binding site, extending in the direction of the Ω-loop. In some way, these loop differences may be determinants in the specificity of OXA variants for various β-lactams.

In a study of the OXA-13 structure, the 91–104 loop on the outer left side of the binding site was found to be disordered, yet ordered in an acyl-serine intermediate with meropenem. A more internal 114–116 loop containing a conserved Ser 115 moved by 3.5 Å and reoriented upon binding the inhibitor, leading to the suggestion that complexation with a ligand is nesssary for loop ordering (Pernot et al. 2001). In this apo OXA-1 structure, we find no disorder in these two regions, and none was reported in the apo structures of OXA-2 and OXA-10. It therefore appears that dis-

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order of these two loops is not intrinsic to all ligand-free oxacillinases.

The β-lactam binding site in classes A and D

It is immediately apparent that the β-lactam binding site of OXA-1 (Fig. 6A) is much more hydrophobic than binding sites in class A (and C) β-lactamases. Trp 102 and Val 117 overlay the Tyr 105 and Asn 132, respectively, in class A enzymes (Fig. 6B). OXA-1 has no polypeptide near the 104 position normally occupied by hydrogen-bonding side chains in class A. The 104 and 132 side chains of class A are thought to be involved in binding β-lactam substrates (Knox 1995), but these seemingly important interactions are not possible in this class D enzyme. Both classes have a conserved hydrophobic side chain on the Ω-loop in the binding site. In class A enzymes, this residue is Leu 169. In class D enzymes, it is Leu/Ile 161, but this side chain is shifted ca. 2 Å to the virtual position of the Glu 166-H_2O-Asn 170 triad, which activates the catalytic water molecule in class A β-lactamases (Fig. 6). Thus, a hydrophobic group in class D has replaced a critical hydrophilic array in the class A catalytic site.

Unlike class A β-lactamases, most oxacillinases have large hydrophobic aromatic residues at postion 215 just after the conserved KTG motif on the B9 strand. The OXA-1 subgroup has a smaller alanine or threonine (Ala 215 in OXA-1). Leu 255 on the carboxy-terminal helix projects toward the 215 position, and apparently compensates for the large hydrophobic side chains often present on the B9 strand. Further down the B9 strand, a Phe 218 lies where solvent-exposed hydrophilic side chains are found in class A β-lactamases (at position 240).

Lysine carboxylation in the catalytic site

Carboxylation of the conserved Lys 70 is clearly seen at pH 7.5 in both copies of the OXA-1 molecule in the unit cell (Fig. 2). A hydrogen bond exists between the carboxylate group and the side-chain amide group of the conserved Trp 160 on the Ω-loop, an interaction common to all known carbamylated oxacillinase structures, and one which would

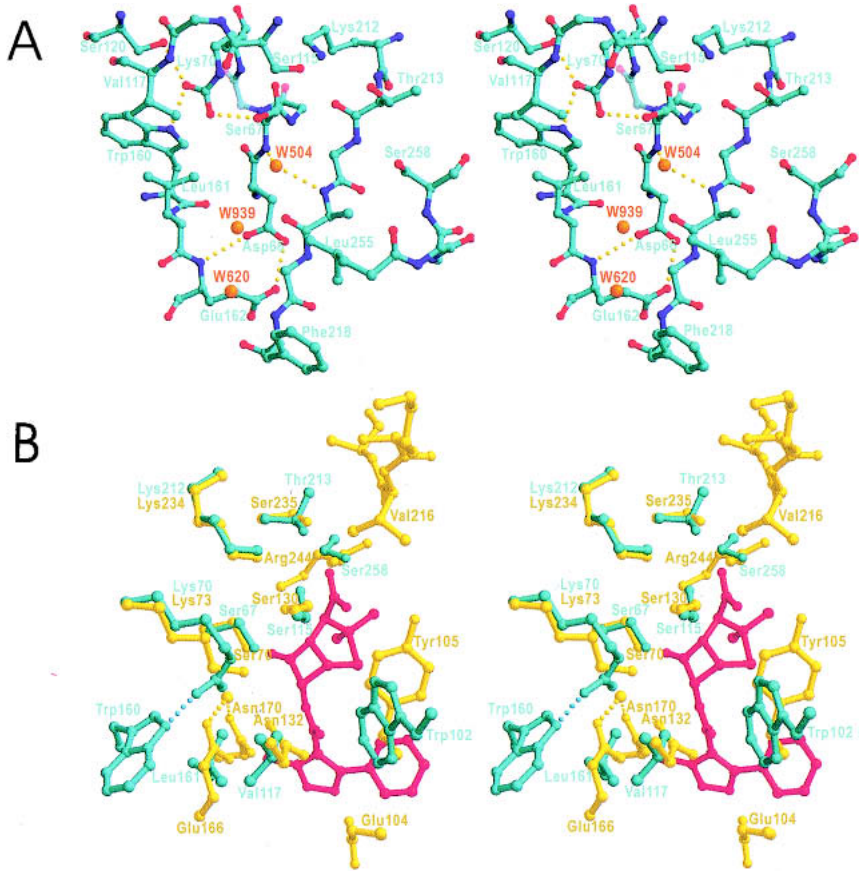

Figure 6. (*A*) Stereoview of the β-lactam binding site in OXA-1. Hydrogen bonds are drawn as *dotted lines*. Some side chains are omitted for clarity. (*B*) Overlay of the binding sites of OXA-1 (blue) and TEM-1 (orange). Oxacillin is shown in red. View angle of *B* is ~90° from *A*. Figure was drawn with MOLSCRIPT (Kraulis 1991) and Raster3D (Merritt and Bacon 1997).

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stabilize the deprotonated form of the carboxylate group. The carboxylate group forms two other hydrogen bonds with the reactive Ser 67 and Ser 120, leaving unfilled a fourth hydrogen-bonding position (Fig. 7).

Carboxylation of Lys 70 was first observed in a ligand-free OXA-10 crystal structure at pH 8.5 (Maveyraud et al. 2000). In another structure of ligand-free OXA-10 at pH 6.5 (Paetzel et al. 2000), in which Lys 70 carboxylation was apparently not considered at the time, a water molecule is said to be tightly bound to Nε of Lys 70 (2.5 Å). Further studies of the OXA-10 enzyme have shown that full carboxylation of the lysine requires pHs above 6, and that a deprotonated carboxylate group is required for activity (Golemi et al. 2001). It has been proposed that the carboxylate deprotonates the reactive Ser 67 prior to attack on the β-lactam carbonyl group (Maveyraud et al. 2002; Fig. 8). A conserved Ser 115 (Ser 130 in class A) may assist in transfering the proton to the nitrogen atom of the thiazole leaving group (Paetzel et al. 2000). However, given the 4–5-Å distance between Ser 115 and the carboxylate group, it may also be necessary for the Nε of Lys 70 to participate in the proton transfer.

Acylserine reaction intermediates have been isolated crystallographically at several pHs. An intermediate of OXA-10 with a 6β-hydroxyisopropyl-penicillanate inhibitor was mapped at pH 7.5 (Golemi et al. 2001) and pH 8.5 (Maveyraud et al. 2002). At pH 5.5, in which lysine carboxylation (and activity) is disfavored, another group was nevertheless able to demonstrate acylation of the OXA-13 enzyme using the inhibitor meropenem (Pernot et al. 2001). They mention no carboxylation of Lys 70, and, instead, report a water molecule in the expected carboxylate position. We note that this water molecule is only 2.2 Å from the

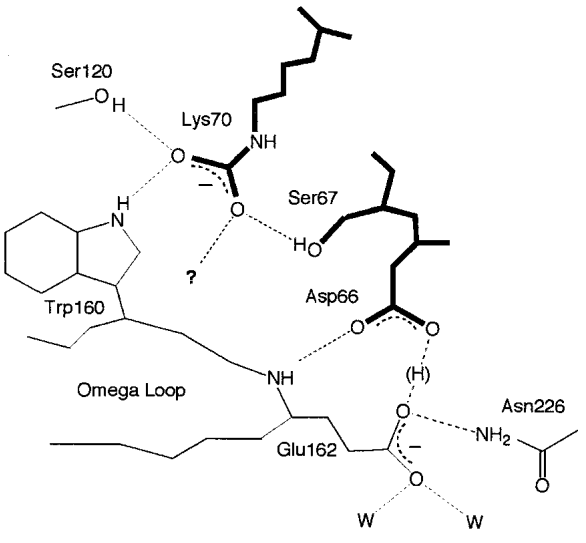

Figure 7. Hydrogen bonding in the binding site and around the Asp 66 unique to OXA-1.

ε-nitrogen of Lys 70, so that the water may in fact be a low-occupancy carboxylate group.

Water molecules in the catalytic site

In the low-pH crystal structures of OXA-10 (pH 6.5; Paetzel et al. 2000) and OXA-13 (pH 5.5; (Pernot et al. 2001), a water molecule is said to bridge from the Lys 70 ε-nitrogen atom to Trp 160. This water molecule, if correctly identified (see above), is claimed to be the catalytic water molecule for deacylation. However, if the Lys 70 is carboxylated in these structures, the water molecule may be positioned elsewhere. One would expect the hydrolytic water to be activated by the proximal (front) oxygen atom of the carboxylate group, as this proximal oxygen is directed into the catalytic site (Figs. 6, 7). A water molecule hydrogen bonded to the carboxylate group would overlay the water molecule activated by a glutamic acid group (Glu 166) in the class A enzymes. In this apo OXA-1 structure, the proximal oxygen has only one hydrogen bond, a strong 2.7-Å interaction with the Oγ of Ser 67. The second hydrogen-bonding site of the proximal oxygen atom, 120° from the first, is devoid of a water molecule. Similarly, the clearly carboxylated apo OXA-2 (pH 9.0) and high-pH OXA-10 (pH 8.5; Maveyraud et al. 2000) structures are devoid of a water molecule positioned for attack on an acylserine intermediate. Therefore, unlike the situation in the class A β-lactamases, in which the water molecule required for hydrolysis is well ordered in all ligand-free crystal structures, in native class D enzymes, the hydrolytic water molecule is apparently absent and binds at a later step in the catalytic mechanism. It is significant that in the crystal structure of the penicillanate inhibitor bound to the OXA-10 enzyme (Maveyraud et al. 2002), the 6β hydroxyl group of the stable acylserine intermediate is hydrogen bonded to the proximal carboxylate oxygen atom.

This carboxylated OXA-1 structure, unlike the carboxylated OXA-2 and OXA-10 structures, does not contain a water molecule bridging between the distal oxygen atom of the Lys 70 carboxylate group and the side chain of residue 73. Rather, OXA-1 and its subgroup mates OXA-18 and OXA-22 have Leu 73 replacing the hydrogen-bonding residues Asn, Ser, and His found in the other subgroups. Thus, the postulated role of this water as a structural water molecule (Maveyraud et al. 2000) is without generality to all class D enzymes.

A water molecule is seen in the oxyanion hole formed by the backbone amide groups of Ser 67 and Ala 215, and it would be displaced by an incoming β-lactam carbonyl group. The water is conserved in all apo class D crystal structures. Also common is another water molecule hydrogen bonding to the Oγ of Ser 115. Interestingly, the side chain rotamer of this serine (chi1 = −61°), here and in OXA-2, differs from that in the corresponding Ser 130 in class A enzymes (−140°±20°), with one exception (Lim et

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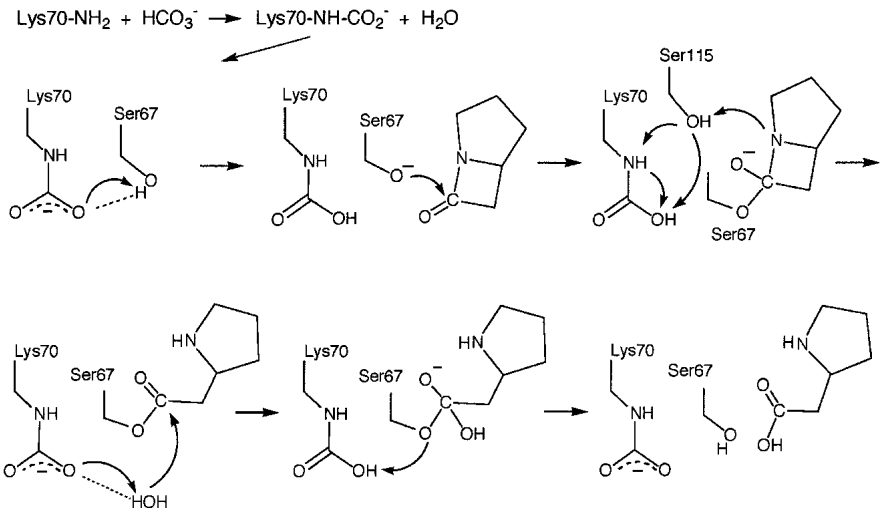

Figure 8. Carboxylation of Lys 70, and a β-lactam hydrolysis mechanism for class D β-lactamases, adapted from Maveyraud et al. (2002). Proton transfer to the thiazole leaving group via Ser 115 could proceed by either of two paths.

al. 2001). A conformational rotation would be necessary for the hydroxyl group of Ser 115 to act as a relay for proton donation to the nitrogen atom of the β-lactam ring during acylation (Fig. 8).

Why Asp 66?

OXA-1 is quite unique among all known class D β-lactamases in having a potentially charged side chain at position 66 adjacent to the reactive serine (Ser 67). As in all class A β-lactamases, the residue here has a strained main-chain conformation ($\phi = 47°$, $\psi = -135°$). The side chain of Asp 66 is behind the B9 strand and just below the oxyanion hole. Its carboxylic acid group is very near (2.6 Å) an oxygen atom of another carboxylic acid group from Glu 162 (Fig. 7). Charge dispersal could occur if the two acidic groups share a proton. Further charge dispersal results via a hydrogen bond from the other oxygen atom of the Asp 66 carboxylic acid group to the backbone amide of Glu 162, and via hydrogen binding between Glu 162, Asn 226, and two water molecules. The strong Asp 66–Glu 162 linkage helps to keep the reactive Ser 67 properly positioned relative to Trp 160, which must orient the important carboxylate group on Lys 70.

Ω *loop*

The Ω-loop at the bottom of the binding site is six to seven residues shorter than the Ω-loop in class A β-lactamases, and the carboxy-terminal portion of the loop does not extend under the β-sheet as much (Fig. 5). None of the acidic side chains along the Ω-loop are positioned for a role as a catalytic base, similar to that of Glu 166 in class A enzymes. The path of the Ω-loop in OXA-1 deviates considerably from Ω-loops in OXA-2 and OXA-10. The loop extends toward the B9 strand because of the link between Asp 66 and Glu 162, described above. In other OXA structures, this link is absent, and the Glu 162 side chain is exposed in solvent. The Ω-loop is longer by six residues in the OXA-1 subgroup relative to other OXA enzymes. The insertion in OXA-1 is completely hydrophilic (-Asp-Lys-Glu-Arg-Asn-Asn-), extends into the solvent, and is somewhat disordered. Kinetic data show that this insertion does not allow the enzyme to turnover third-generation cephalosporins with large C6(7) side chains (Ledent et al. 1993), as proposed for an Ω-insertion mutant of a class C β-lactamase (Crichlow et al. 2001).

Still unclear is how chloride inhibits this oxacillinase (Yamagishi et al. 1969; Dale 1971). Repeated attempts to locate a chloride-binding site in OXA-1 by crystal soaking experiments using NaCl up to 200 mM have so far failed to produce consistent electron density changes within the β-lactam binding site, and these studies are continuing. Another remaining question is the structural basis of the resistance of most class D β-lactamases to inhibition by mechanism-based class A inhibitors such as clavulanic acid. One might think that the lack of a conserved arginine residue at position 258 (Figs. 3, 6B) could explain the general resistance of this class to this inhibitor (Imtiaz et al. 1993, 1994; Wang et al. 2002), yet OXA-18 with Gly 258 is known to be effectively inhibited by clavulanic acid (Philippon et al. 1997).

Materials and methods

Isolation and purification of OXA-1 β*-lactamase*

The OXA-1 gene was cloned from the RGN238 plasmid into pET12a-KM, as described previously (Sun et al. 2001). An error in

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Table 2. *X-ray data collection*[a]

Cell constants (Å, deg.)	36.0, 51.6, 72.9 70.2°, 84.1°, 81.5°
d_{min} (Å)	1.50 (1.55–1.50)
Observations	183,270 (4561)
Reflections	69,838 (2979)
Redundancy	2.62 (1.53)
Completeness	86.5% (36.9%)
Av. I/σ(I)	20.8 (5.3)
R_{sym} (I)[b]	4.9% (12.3%)

[a] Data for the highest shell are in parentheses.
[b] $R_{sym} = \sum | I_{av} - I_i | / \sum I_i$, in which I_{av} is the average of all individual observations, I_i. The space group is *P*1.

the published amino acid sequence of this gene (Ouellette et al. 1987) was found at residue 128 after examination of the electron density map (see Results). Crude enzyme was purified to at least 95% purity as estimated from SDS–polyacrylamide gels.

Sedimentation data

Approximately 100 uL of the solution (0.3 and 1 mg/mL protein) were loaded into the solution channels of an exterior-loading analytical ultracentrifugation cell (12-mm optical path) with sapphire windows, resulting in a solution height of 2.5 mm. The last milliliter of filtrate (100 mM sodium phosphate buffer at pH 7.0) from a Centricon10 membrane (Amicon) that was used to concentrate the protein (therefore being in approximate dialysis equilibrium with the solution) was loaded into the solvent channels of the cell. The cell was centrifuged in a Beckman XL-I analytical ultracentrifuge utilizing interference optics, at 20°C and 20 krpm. Interferograms of the solution-solvent pairs were made every 3 h to record the concentration gradient. The attainment of centrifugal equilibrium was determined by means of the program MATCH (D.A. Yphantis, unpubl.). After equilibrium was achieved (ca. 28 h), the rotor speed was increased to 28 krpm. The new equilibrium was achieved in 14 h. After the run was completed, blanks (to correct for systematic errors involving window distortion) were made by centrifuging water versus water at the same speeds and temperature. These blanks were subtracted from the equilibrium scans to produce the final data. A similar experiment was run using protein solutions of 2 and 4 mg/mL. The centrifuge was run at 25 krpm for >100 h without equilibrium being attained for either concentration. Program SEDNTERP (Laue et al. 1992) was used to calculate parameters including MW (28.4 kD), specific volume (0.739 mL/g), and solvent density at 20° (1.0027g/mL).

Crystallization

The vapor diffusion method was used, in which a 10-uL sitting drop [2.5–3 mg/mL protein, 10% PEG 8000, 0.05 M HEPES (pH 7.5)] was equilibrated over a buffered reservoir solution containing 20% PEG. With streak and macro seeding at 1.2–1.5 mg/mL, prismatic crystals grew to 0.6 × 0.1 × 0.05 mm. Twinning was common. The crystals have space group *P*1 with a = 36.0 Å, b = 51.6 Å, c = 72.9 Å, α = 70.2°, β = 84.1°, γ = 81.5°, and Z = 2 (Sun et al. 2001). The VM and solvent content are calculated to be 2.24 $Å^3$/Da and 44%, respectively.

X-ray diffraction data

Data to 1.5-Å resolution were collected at 100 K at MacCHESS station A1 at Cornell University (λ = 0.935 Å). The crystals were cryoprotected by soaking for less than 3 min with 25% MPD in the PEG buffer. Two crystals were used to collect high and low resolution data sets. Merging of the two data sets with *HKL* (Otwinowski and Minor 1997) showed a data redundancy of 2.6 (Table 2).

Accession number

Coordinates and structure factors have been deposited in the Protein Data Bank at the Research Collaboratory for Structural Bioinformatics, Rutgers University, as entry 1M6K.

Acknowledgments

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