Structural Basis of Extended Spectrum TEM β-Lactamases

CRYSTALLOGRAPHIC, KINETIC, AND MASS SPECTROMETRIC INVESTIGATIONS OF ENZYME MUTANTS*

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The E166Y and the E166Y/R164S TEM-1 β-lactamase mutant enzymes display extended spectrum substrate specificities. Electrospray mass spectrometry demonstrates that, with penicillin G as substrate, the rate-limiting step in catalysis is the hydrolysis of the E166Y acyl-enzyme complex. Comparison of the 1.8 Å resolution x-ray structures of the wild-type and of the E166Y mutant enzymes shows that the binding of cephalosporin substrates is improved, in the mutant enzyme, by the enlargement of the substrate binding site. This enlargement is due to the rigid body displacement of 60 residues driven by the movement of the Ω-loop. These structural observations strongly suggest that the link between the position of the Ω-loop and that of helix H5, plays a central role in the structural events leading to extended spectrum TEM-related enzymes. The increased Ω-loop flexibility caused by the R164S mutation, which is found in several natural mutant TEM enzymes, may lead to similar structural effects. Comparison of the kinetic data of the E166Y, E166Y/R164S, and R164S mutant enzymes supports this hypothesis.

Bacterial resistance to penicillins and cephalosporins represents an increasing risk in the chemotherapy of Gram-negative bacterial infections. This resistance often arises from the emergence and the dissemination of the plasmid-encoded extended spectrum β-lactamases (EC 3.5.2.6) (Blazquez et al., 1995; Palzkill et al., 1995; Venkatachalam et al., 1994; Viadu et al., 1995). Most of these proteins are derived from the Escherichia coli TEM enzyme, via the combination of a few point mutations, which have led, so far, to 27 TEM-related enzymes (Morosini et al., 1995). The parent TEM-1 is a very efficient enzyme and hydrolyzes penicillin substrates through an acylation and a deacylation step (Swarén et al., 1995). Extended spectrum TEM-related enzymes hydrolyze third generation cephalosporin substrates. Based on the kinetic data, two common characteristics of these enzymes are (i) a severalfold reduction in the catalytic turnover toward penicillins, and (ii) an increase in the catalytic efficiency against cephalosporin substrates (Raquet et al., 1994; Sowek et al., 1995).

At the molecular level, it appears that high catalytic efficiencies against the third generation cephalosporin oxyimino-β-lactams, such as cefotaxime and ceftazidime, are primarily linked, in natural TEM mutants, to the mutation of Arg164 (Naumovski et al., 1992) or to the mutation of Gly238 (Jacoby et al., 1991). In the x-ray structure of the TEM-1 enzyme (Brookhaven DataBank entry 1BTL) (Jesch et al., 1993), Arg164 forms two salt bridge interactions. These are important for the conformation and the stability of the Ω-loop region (residues 161–180). Some residues from this loop define the active site topology, while others, such as Glu166, are essential for catalysis. Site-directed mutagenesis studies demonstrated the requirement for an acidic side chain in position 166 (Delaire et al., 1991) and the involvement of Glu166 in the deacylation reaction (Adachi et al., 1991). Both the acid-base properties and the position of this proton acceptor group are reflected in the value of the deacylation rate constant (Swarén et al., 1995). Gly238 residues at the C-terminal edge of strand S3, which borders the substrate binding site cavity, and is in van der Waals contact with Asn170 main-chain atoms in the Ω-loop. Detailed kinetic and mass spectrometric investigations on the G238S mutant enzyme demonstrated a significant decrease of the deacylation rate constant, likely related to a perturbation of the deacylation machinery (Saves et al., 1995a).

The E166Y mutant displays one of the characteristic features of extended spectrum enzymes: similar activities toward penicillin and cephalosporin substrates (Delaire et al., 1991). The high resolution structure of this mutant shows unexpected structural differences compared with the wild-type enzyme. Comparison of the kinetic data of the E166Y, E166Y/R164S, and R164S (Sowek et al., 1991; Raquet et al., 1994) mutant enzymes suggests that these structural differences may play a key role in extending the substrate specificity of the TEM-related enzymes.

**EXPERIMENTAL PROCEDURES**

Protein Production—Site-directed mutagenesis, protein expression, and purification was performed as described (Saves et al., 1995b). For site-directed mutagenesis, the following oligonucleotides were used: E166Y, 3'-CTA GCA ACC ATA GGC CTC GAC TTA CTT-5'; and E166Y/R164S, 3'-CAT TGA GAA CTA ACC ATG GGC CTC GAC TTA CTT-5'.

Determination of Kinetic Parameters of Substrate Hydrolysis and Determination of Acylation and Deacylation Rate Constants by Electrospray Mass Spectrometry (ESMS)1—The Michaelis-Menten kinetic parameters and the elementary rate constants by ESMS were determined as described previously (Saves et al., 1995a, 1995b).

Crystal Preparation—A solution of the E166Y mutant enzyme (14.5 mg/ml) in 45 mM potassium phosphate buffer (pH 7.8), containing 7.5% (v/v) saturated ammonium sulfate solution, was equilibrated, at 6 °C, against 100 mM potassium phosphate buffer (pH 7.8), containing 42%

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1 The abbreviation used is: ESMS, electrospray mass spectrometry.
The overall structure of the E166Y \(\beta\)-lactamase is very similar to the 1.8-Å wild-type enzyme structure (Jelsch et al., 1993). However, the E166Y mutation leads to significant structural differences in two areas: the region 165–170 (WYPELN), which is part of the \(\Omega\)-loop, and residues 85–142, which move as a rigid body. This region contains helices H3 (109–111), H4 (119–128), and H5 (132–142) and two coil regions (86–108 and 112–118). It is part of the helical domain (residues 62–212) of class A \(\beta\)-lactamases (secondary structure assignment from Jelsch et al. (1993)).

The WYPELN Region—The movement of residues 165–170 (Fig. 1A) is directly related to the point mutation at position 166 and is restricted to these residues by the salt bridges Arg\(^{164}\)-Glu\(^{171}\) and Arg\(^{164}\)-Asp\(^{179}\). In order to accommodate the bulkier phenyl group, 166 C-\(\alpha\) moves by 0.7 Å from its position in the wild-type structure, away from the protein core. Tyr\(^{166}\) OH is now at hydrogen bond distance (2.9 Å) from Ser\(^{70}\) O-\(\gamma\) compared with the 4.2 Å distance between Glu\(^{166}\) O-\(\varepsilon\)-1 and Ser\(^{70}\) O-\(\varepsilon\) in the wild-type enzyme. This movement drives the displacement of Trp\(^{165}\) C-\(\omega\) and Pro\(^{167}\) C-\(\alpha\), found at 0.3 and 1.0 Å, respectively, from their positions in TEM-1. Movement of the Trp\(^{165}\) main-chain atoms, without significant modifications of its \((\phi,\psi)\) angles, forces the rotation of the indol ring that occupies the same steric volume. The C-\(\alpha\) atoms of Glu\(^{168}\) and Leu\(^{159}\) move 0.5 and 0.2 Å, respectively, from their positions in the wild-type structure, but their side-chain conformations are little affected. More important changes occur for Asn\(^{170}\), whose C-\(\alpha\) is displaced by 0.5 Å and its side chain by 1 Å. There is no hydrogen bond exchanged between this side-chain amide group and Tyr\(^{166}\) OH, in contrast to the important interaction, formed in the wild-type structure, with the Glu\(^{166}\) carboxylate group. These structural modifications induce minimal effects on the main-chain atoms of Ser\(^{70}\) and Lys\(^{73}\), which are shifted by 0.15 Å. However, Ser\(^{70}\) O-\(\gamma\) and Lys\(^{73}\) N-\(\varepsilon\) move by 0.3 and 0.7 Å, respectively, and the amino group is now at hydrogen bond distance to Tyr\(^{166}\) OH (3.0 Å) (Fig. 1A).

These structural modifications within the \(\Omega\)-loop affect the solvatation and the hydrogen bond network within the substrate binding site. Four water molecules are lost, and two water molecules and the sulfate ion are shifted compared with the wild-type enzyme (Fig. 1B). The water molecule Wat297,
**Fig. 1.** A, stereo view of residues 70–73 and 165–170 in the wild-type (thin lines) and E166Y (thick lines) structures. B, stereo view of the substrate binding sites in the wild-type (thin lines) and E166Y (thick lines) structures. The crystallographic water molecules in the TEM-1 β-lactamase are represented by crosses. Those in the E166Y enzyme are represented by dots. The location of the sulfate ion is also represented. C, stereo view of the H5 helix (residues 132–142) and of residues 165–166 of the β-loop. The hydrogen bonds between the Asn146 side-chain atoms and the main-chain atoms of residue 166, and between Thr140 O-γ and Trp165 Nα are shown by dotted lines. The wild type is in thin lines, and the E166Y enzyme is in thick lines. The shifts in position for 166 Cα, 136 Cα and 140 O-γ are, respectively, of 0.7, 0.4, and 0.6 Å.
considered to be the nucleophile group in the deacylation step (Strynadka et al., 1992; Jelsch et al., 1993; Swarén et al., 1995), is shifted by 1.5 Å but maintains hydrogen bonds to Ser70 nitrogen, Ser70 O-γ, Asn170 side-chain amide group, and Wat391. The 1-Å motion of the sulfate ion has several consequences: (i) it leads to the exclusion of Wat323 from the oxyanion hole, and of Wat404, found in the vicinity of Ser130 main-chain oxygen atom in the wild type structure, (ii) one of its oxygen atoms is now at hydrogen bond distance (2.9 Å) from Ala237 nitrogen, and (iii) it provides more space in the vicinity of the Ser130, Ser235, and Arg244 side chains in the E166Y structure, where a new water molecule (Wat518) is found. All other water molecules occupy nearly the same positions in both structures (Fig. 1B). Solvent molecules that are bound to residues of the Ω-loop move accordingly and preserve their interactions, except for the two water molecules (Wat422 and Wat472) that are expelled by the previously described Asn170 side-chain motion. However, the 1.0-Å displacement of Pro467 generates a cavity that is filled by a new water molecule.

The 85–142 Region—The rigid body motion of the region 85–142 is significant when comparing the wild-type and the E166Y structures. Asn136, Thr140, located at three consecutive turns of helix H5, Thr141 O-γ moves by about 0.6 Å toward Trp165 N-ε, to which it is now hydrogen-bonded (Fig. 1C).

At the edges of this moving domain, residues Arg83 and Thr141, at the C termini of helices H2 and H5, respectively, and Glu89 and Arg93, in the connecting loop between helices H2 and H3, are clustered within a 4-Å radius sphere (Fig. 2). The movement of residue 141 induces the reorientation of the Arg93 side chain and the expulsion of Wat380, which was bridging Arg83 N-ε-1, Glu89 O-ε-1, and Thr141 O-γ in the wild-type structure. In the mutant enzyme, Arg83 N-ε-1 is now hydrogen-bonded to the Thr141 main-chain oxygen atom.

Kinetics and Mass Spectrometry—Electrospray mass spectrometry experiments were used to determine the molecular weight of the E166Y mutant protein and of its molecular complexes. This soft ionization technique generates gas phase multicharged ions directly from the solution, and it allows characterization of the covalent intermediates of the reaction (Aplin et al., 1990). The E166Y enzyme readily formed an acyl-enzyme intermediate with penicillin G. This complex (measured molecular mass of 29,318 ± 3 Da) was the only enzyme species that could be detected during the steady state of the reaction. Its formation was extremely fast, and no free mutant enzyme could be detected 10 s after the reaction was started, even at 2.5 °C. The difference between the molecular mass of this complex and that of the free mutant enzyme is in excellent agreement with the molecular mass of penicillin G (334 Da). The acyl-enzyme formation was equal to the initial E166Y protein concentration, and the determined kcat value at 37 °C (0.36 s−1) is identical to the kcat value measured from steady-state enzyme kinetics. In the mass range of the antibiotic, peaks corresponding to penicillin G and to the penicilloic acid reaction product (18 mass units higher) were followed during the time course of the reaction. After 5 min of reaction time at 37 °C, the only molecular species observed by ESMS were the acyl-enzyme complex (Fig. 3A), unreacted substrate, and the reaction product, penicilloic acid (Fig. 3B). When similar experiments were conducted with cephaloridin as substrate, we found that most of the enzyme was present in its free state (data not shown). The acyl-enzyme complex represented less than 10% of the total protein species. Its molecular mass indicated that the only reaction was the β-lactam cycle cleavage upon acyl-enzyme formation and that no elimination of the C-3’ substituent on the dihydrothiazine ring occurred. As previously discussed (Saves et al., 1995a), a precise quantitation cannot be achieved on species amounting to 2–10% of the total ESMS signal.

The kinetic data of the E166Y and the E166Y/R164S mutant enzymes are reported in Table III. The single E166Y mutation led to improved Kcat values for all substrates compared with the wild-type enzyme. However, compared with the E166Y enzyme, the E166Y/R164S double mutant enzyme discriminates between penicillin substrates (decreased Km values) and cephalosporin substrates (increased Km values). When compared with the wild-type enzyme, mutant proteins bearing the E166Y mutation display highly reduced kcat values, although they are higher for cephalosporin than for penicillin substrates. These effects in Km and kcat led to modified substrate spectra in both mutant enzymes, a characteristic of extended spectrum TEM-related enzymes, as exemplified by the R164S TEM-1 mutant (Table III) (Sowek et al., 1991; Raquet et al., 1994). Taken together, these data suggest that the extended substrate spectra of these mutants arise from structural events common to all of these proteins.
DISCUSSION

Effect of the E166Y Mutation on the Enzyme Structure and Consequences for the Binding of Cephalosporin Substrates—The structural modifications observed in the E166Y TEM-1 mutant are of two types. First, the mutation at position 166 induces the optimal short-range effect required to accommodate the larger side chain. Second, it induces an unexpected long-range effect, in which a large part of the protein helical domain that bears important residues for catalysis is relocated.

The short range effect on the 165-170 $\Omega$-loop region is restricted by the preceding Arg$^{164}$ and the following Glu$^{171}$. Their side chains are engaged in two salt bridges that are important for the conformation and the stability of the $\Omega$-loop and for the correct location of residue 166 within the active site. The salt bridge between Arg$^{164}$ and Asp$^{179}$ is buried and inaccessible to solvent molecules, which increases the strength of this interaction.

Three hydrogen bonds are exchanged between the Asn$^{136}$...
side-chain and Glu\(^{166}\) main-chain atoms and between Thr\(^{140}\) O-\(\gamma\) and Trp\(^{190}\) N-\(\epsilon\). Thus, the movement of the 165–170 region leads to the concerted relocation of Asn\(^{126}\), Asn\(^{136}\), and Thr\(^{140}\) (Fig. 1C). This rigid body motion of the H5 helix drives the displacement of the whole 85–142 region, which represents one-third of the enzymatic domain (Fig. 2). This movement requires only a few small main-chain dihedral angle rotations and preserves all of the interactions that occur within this protein domain.

The 85–142 region bears residues that delineate one side (residues 104–105, 130, 132) of the substrate binding cavity. The active site bottleneck is found between the hydroxyl groups of Ser\(^{130}\) and Ser\(^{235}\), and is precisely the binding site of the thiazoline ring of penicillins and of the dihydrothiazine ring of cephalosporins (Fig. 4). The distance between Ser\(^{130}\) O-\(\gamma\) and Ser\(^{235}\) O-\(\gamma\) is 5.4 Å in TEM-1 (Jeshc et al., 1993). The movement of residues 85–142 in the E166Y enzyme increases this distance to 6.0 Å. As the \(K_m\) of cefotaxime, measured by competition procedures, is lowered 1500-fold compared with TEM-1 (Delaire et al., 1991), we suggest that the increased active site aperture favors the better binding of cephalosporins. This is because the dihydrothiazine ring has a larger steric volume than the thiazoline ring.

Mechanistic Aspects of the E166Y Mutation—Mass spectrometry measurements showed that during the steady state, the E166Y acyl-enzyme complex is the sole enzyme species with penicillin G as substrate. This indicates that the acylation rate is larger than the deacylation rate. Indeed, the determined \(k_3\) value (0.36 s\(^{-1}\)) is 4000-fold smaller than the corresponding wild-type enzyme deacylation rate constant (1500 s\(^{-1}\)) (Christensen et al., 1990). Since residue 166 is chemically involved in the deacylation step, a large decrease in \(k_3\) could arise from the different acid-base properties of Tyr compared with Glu as a proton acceptor.

Within experimental errors, \(k_3\) is identical to the \(k_{cat}\) value determined from steady-state kinetic measurements, which prevents calculation of \(k_2\). However, examination of the \(k_{cat}/K_m\) values suggests slower acylation rates with the E166Y mutant enzymes compared with the wild-type enzyme. The role of Lys\(^{73}\) in the acylation reaction was recently described (Swarén et al., 1995). Removal of the negative charge provided by Glu\(^{166}\) significantly decreases the basicity of the unprotonated Lys\(^{73}\) in the E166Y Michaelis complex, in line with the decrease of \(k_2\) suggested by the steady-state kinetic data (detailed electrostatic calculations will be presented elsewhere).

ESMS experiments using cephaloridin showed that there is no elimination of the C-3' substituent of the substrate forming the acyl-enzyme complex. Thus, the lower \(K_m\) values with the E166Y mutant protein for cephalosporin substrates do not arise from a change in the rate-limiting step resulting from a different kinetic pathway, as was shown to occur with the PC1 enzyme (Faraci and Pratt, 1985, 1986). The rate-limiting step for cephalosporin hydrolysis by the wild-type enzyme is acylation (Saves et al., 1995a). A similar conclusion could be drawn for the E166Y mutant, as ESMS shows that more than 90% of the protein is found as free enzyme during the course of the reaction. However, the detection of a small amount of acyl-enzyme complex would suggest that the \(k_2\) and \(k_3\) values are of similar magnitude.

Kinetic Effects upon Mutation of \(\Omega\)-Loop Residues—The crys-

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**Table III**

Kinetic data of the TEM-1, E166Y, and E166Y/R164S enzymes

| Substrate | TEM-1 | E166Y | E166Y/R164S |
|-----------|-------|-------|-------------|
|           | \(k_{cat}K_m\) | PG     | \(k_{cat}K_m\) | PG     | \(k_{cat}K_m\) | PG     |
|           | \(s^{-1} M^{-1}\) | %     | \(s^{-1} M^{-1}\) | %     | \(s^{-1} M^{-1}\) | %     |
| PG        | 2.1 \times 10^4 | 100   | 3.0 \times 10^4 | 100   | 1.8 \times 10^4 | 100   |
| AMX       | 2.8 \times 10^4 | 133   | 1.8 \times 10^4 | 60    | 8.5 \times 10^4 | 47    |
| CD        | 1.9 \times 10^6 | 9     | 4.9 \times 10^3 | 163   | 4.6 \times 10^4 | 25    |
| CFP       | 3.0 \times 10^6 | 14    | 3.2 \times 10^5 | 106   | 2.9 \times 10^5 | 161   |
| CAZ       | 1.7 \times 10^3 | 8.5 \times 10^{-5} | —     | 3.3 \times 10^4 | 18    |
| CTX       | 8.8 \times 10^{-5} | 8 \times 10^{-4} | —     | 1.0 \times 10^4 | 100   |
| AZT       | 8.8 \times 10^{-4} | —     | —     | 4.28 \times 10^{-2} | 0.02  |
| NCF       | 2.1 \times 10^7 | 100   | 1.5 \times 10^4 | 60    | 1.5 \times 10^4 | 8     |

a Not measurable.

b Raquet et al. (1994) data for TEM-1.
In the TEM-1 enzyme, the R164X (except for lysine) mutation, by removing two out of the four salt bridges of the V-loop, will release some, but not all, of its conformational constraints. This will likely affect the relative positions of the partners involved in the deacylation step. A slight displacement of the V-loop residue Glu166 will decrease the deacylation rate of good TEM-1 substrates. Indeed, this rate is related both to the direction of the electrostatic potential gradient between the Glu166 carboxylate and the ester carbonyl carbon of the acyl-enzyme complex, and to its magnitude, which is very sensitive to atomic positional differences (Swaren et al., 1995). The re-duction in \( k_{\text{cat}} \) is of 10–50-fold in the TEM-related enzymes bearing the Arg164 mutation (R164S, R164H, R164S/E104K, and R164S/E240K) but is only marginally affected in the E104K and E240K single mutants (Sowek et al., 1991; Raquet et al., 1994, Petit et al., 1995). This proposal is consistent with the situation observed in the PC1 \( \beta \)-lactamase, where a single salt bridge (Arg164→Asp179) stabilizes the V-loop conformation. The substantial disorder of the loop resulting from the D179N mutation (Herzberg et al., 1991), led in that case to a 600-fold decrease in the \( k_{\text{cat}} \) value for penicillin G.

The movement of the 85–142 region, in the E166Y mutant enzyme structure, explains why binding is improved for cephalosporin substrates compared with the wild-type enzyme. A similar movement of the 85–142 region can be assumed to occur in the E166Y/R164S mutant protein. Interestingly, the additional R164S mutation in the E166Y enzyme has no effect with respect to the substrate spectrum (\( k_{\text{cat}}/K_m \)) for cephalosporin substrates, whereas the single R164S mutation in TEM-1 led to major kinetic differences. This paradox is explained if one assumes that the R164S mutation allows structural perturbations similar to those observed, and already achieved, in the E166Y protein. It explains why the additional R164S mutation in the E166Y enzyme is kinetically silent and offers a structural explanation of the consequences of the Arg164 mutation in the wild-type enzyme.

We propose that the conformational constraints of the V-loop, partly controlled by residue 164, and the position of the 85–142 region are interdependent in the TEM-1 enzyme. However, in the R164X TEM-1 enzymes, this structural link would only be kinetically discernible when large substituents on the substrate molecule reach the V-loop residues, as is the case with third generation cephalosporin substrates (Raquet et al., 1994). The release of short contacts, achieved by the V-loop movement that drives the 85–142 region, accounts for the kinetic effects that are consistently found in extended spectrum TEM-related enzymes bearing the Arg164 mutation. First, the improvement of the binding of large cephalosporin substrates (i.e. ceftazidime) should increase the catalytic efficiencies for such molecules relative to penicillin substrates. \( k_{\text{cat}}/K_m \) are, indeed, 1–3 orders of magnitude larger in the R164S mutant than in the wild-type enzyme (Table II). Second, the large \( k_{\text{cat}}/K_m \) differences found in TEM-1 within the cephalosporin substrates should level out in the R164X mutant enzymes. Indeed, the \( k_{\text{cat}}/K_m \) of cefaloridin versus ceftazidime is decreased from \( 3 \times 10^4 \) in the TEM-1 enzyme to 33 in the R164S mutant enzyme. This property is fulfilled in all TEM mutant proteins bearing the R164X mutation (Sowek et al., 1991; Raquet et al., 1994).

Mutations that occur in the vicinity of the V-loop residues, such as G238S, were shown to drastically reduce the deacylation rate constant (Saves et al., 1995a). Natural mutants in this position also display extended substrate spectra, and work is in progress that will further illustrate the involvement of the V-loop region in the molecular evolution of the TEM-1 \( \beta \)-lactamase.

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