The subclass B3 FEZ-1 β-lactamase produced by Fluoribacter (Legionella) gormanii is a Zn(II)-containing enzyme that hydrolyzes the β-lactam bond in penicillins, cephalosporins, and carbapenems. FEZ-1 has been extensively studied using kinetic, computational modeling and x-ray crystallography. In an effort to probe residues potentially involved in substrate binding and zinc binding, five site-directed mutants of FEZ-1 (H121A, Y156A, S221A, N225A, and Y228A) were prepared and characterized using metal analyses and steady state kinetics. The activity of H121A is dependent on zinc ion concentration. The H121A monozinc form is less active than the dizinc form, which exhibits an activity similar to that of the wild type enzyme. Tyr156 is not essential for binding and hydrolysis of the substrate. Substitution of residues Ser221 and Asn225 modifies the substrate profile by selectively decreasing the activity against carbapenems. The Y228A mutant is inhibited by the product formed upon hydrolysis of cephalosporins. A covalent bond between the side chain of Cys221 and the hydrolyzed cephalosporins leads to the formation of an inactive and stable complex.

Metallo-β-lactamases are bacterial enzymes that hydrolyze antibiotics of the β-lactam family. They are classified as class B (1) or group 3 (2) β-lactamases. In the last decade, the discovery of an increasing number of new metallo-β-lactamases resulted in a subdivision into three molecular subclasses: B1, B2, and B3. Thereafter, a standard numbering scheme (3) was adopted that identifies conserved residues involved in the catalytic activity. Subclass B3 β-lactamases are broad spectrum enzymes that require one or two Zn(II) ions for activity (4). These enzymes are produced by various environmental species, of which some can cause opportunistic infections (such as S. maltophilia (5) and F. gormanii (6)), whereas others are not pathogenic such as Janthinobacterium lividum (7) and Caulobacter crescentus (8, 9). The structures of several subclass B1 β-lactamases have been solved by x-ray crystallography (BclI (10, 11), CcrA (12), BlaB (13), and IMP-1 (14)). To date, no structure of a subclass B2 enzyme is available. In subclass B3, the crystal structures of the metallo-β-lactamases L1 from S. maltophilia (15) and FEZ-1 from F. gormanii (16) have been solved. Comparison of the tertiary structures of the different enzymes highlighted similar organizations of the secondary structure elements; they all contain an αβα sandwich with two central β-sheets and α-helices on the external faces (10). The active site with the binuclear zinc center is located at the bottom of the β-sheet core. Zn1 is tetrahedrally coordinated by three histidines, His116, His118, His196, and a water molecule. In the subclass B1 enzymes, Zn2 is coordinated by His663, Asp210, Cys221, and two water molecules to form a trigonal bipyramid. In subclass B3 β-lactamases, a Ser residue replaces Cys221, and His121 is the third ligand of Zn2. Our studies were performed on the FEZ-1 β-lactamase. FEZ-1 is a monomeric enzyme. The sequence of the mature protein is easily aligned with that of the L1 enzyme with 33% of isology (17). The two subclass B3 β-lactamases exhibit a broad activity spectrum against β-lactam antibiotics, but FEZ-1 shows a preference for cephalosporins (18), whereas the L1 β-lactamase seems to be more active against penicillins (19, 20). Comparison of the x-ray structures reveals similar zinc binding sites in FEZ-1 and L1. In L1, Ullah et al. (15) postulate that the carbonyl oxygen of the β-lactam substrate interacts with an oxinolone hole formed by Zn-1 and the side chain of Tyr228. This tyrosine is conserved and could play the same role in FEZ-1. In L1, the β-substituent on C-6 or C-7 of the β-lactam substrate generally fits in a hydrophobic pocket formed by the “flap” connecting β3 and β4, and by the loop between β3 and β5, which is considerably longer in subclass B3 enzymes than in subclass B1 (21, 22). In this pocket, the hydrophobic residues Phe156 and Ile162 of L1 are replaced by a Tyr and a Ser residue, respectively, in FEZ-1. These substitutions, together with Asn225, should influence the substrate specificity, with a facilitated interaction between FEZ-1 and β-lactams bearing a less hydrophobic β side chain (15). With the exception of GOB-1, all of the subclass B3 enzymes have a serine at position 221 (23). The x-ray structures of L1 (15) and FEZ-1 (16) indicate that this residue interacts with a water molecule (Wat2) linked to Zn2. Modeling studies suggest that Wat2 might play the role of proton donor to the nitrogen of the β-lactam ring. The interaction with Ser221 should help its positioning in the catalytic cavity. A typical structural aspect of L1 when compared with the other class B β-lactamases is the presence of an intramolecular disul-
fide bridge between Cys256 and Cys296. These residues are conserved in FEZ-1, but this enzyme contains an additional Cys200 close to the active site. Finally, L1 and FEZ-1 are a homotetramer and a monomer, respectively. In the L1 structure, one main interaction between two subunits involves the N terminus (15, 24). However, this structural motif is missing in FEZ-1 in this paper, we have studied the role of His121, Tyr156, Ser221, and Tyr228 in the catalytic activity of FEZ-1.

Bacterial Strains and Vectors—The Escherichia coli XL-1 Blue (Stratagene Inc., La Jolla, CA) and E. coli BL21(DE3) pLysS (Novagen Inc., Madison, WI) strains were used as hosts for the construction of vectors for the expression of all of the studied proteins. The pDML1810 plasmid described previously by Mercuri et al. (18) was used as template in the PCR site-directed mutagenesis amplifications. The expression vector pET28b+ (Novagen) was used for the construction of the Tet-based expression plasmid.

Chemicals and Antibiotics—The primers used for the mutagenesis were synthesized by Amersham Biosciences. The QuikChange™ site-directed mutagenesis kit was from Stratagene (La Jolla, CA), and the isopropyl-β-thiogalactopyranoside was purchased from Eurogentech (Liege, Belgium). Chloramphenicol, ampicillin (Δε370 = −820 M⁻¹ cm⁻¹), cefalotin (Δε370 = −6500 M⁻¹ cm⁻¹), cefotaxime (Δε370 = −7500 M⁻¹ cm⁻¹), and cefuroxime (Δε365 = −7600 M⁻¹ cm⁻¹) were purchased from Sigma, and benzylpenicillin (Δε260 = +15,000 M⁻¹ cm⁻¹) was purchased from Unipath OXoid (Basingstoke, UK). 7-Aminopenicillanic acid (7-ACA) (Δε350 = −7500 M⁻¹ cm⁻¹) was a gift from GlaxoSmithKline.

Site-directed Mutagenesis—The FEZ-1 mutants were obtained according to the instruction manual of the QuikChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA). The oligonucleotides (forward and reverse) used for the generation of the mutants are shown in Table I. PCR conditions were as follows: incubation at 95 °C for 30 s and 20 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 68 °C for 10 min). After amplification, the PCR fragments were digested by the DpnI restriction enzyme in order to eliminate all nonmutated DNA. The digested plasmids were used to transform E. coli XL-1 Blue competent cells, and colonies were isolated on Luria Bertani (25) agar plates containing 50 μg/ml each ampicillin and kanamycin.

The nucleotide sequences of the desired mutants were verified by DNA sequencing. Thereafter, the plasmids were digested by the Neo and Cm restriction enzymes, and the fragments were ligated into the pET28b+ vector previously digested by the same enzymes to yield pDML1817-H121A, pDML1819-C200A, pDML1820-S219A, pDML1825-Y156A, pDML1826-S162A, pDML1827-N225A, and pDML1828-Y228A. Finally, E. coli BL21 (DE3) pLysS cells were transformed with the plasmids containing the different mutations.

The mutant proteins were produced and purified as described for the wild type enzyme (18) in two purification steps. After the second step, the fractions that exhibited β-lactamase activity were collected and concentrated on a YM-10 membrane (Amicon, Beverly, MA) to a final concentration of about 1 mg/ml. Protein concentrations were determined using the BCA assay (Pierce), and the absorbance at 280 nm was also measured. The purity and the molecular masses of all purified proteins were confirmed by determining the molecular mass values with the help of an electrospray mass spectrometer (VG Bio-Q) upgraded with a Platform source (Micromass, Altrincham, UK). The samples (100 pmol) were dissolved in 0.05% formic acid, 50% acetonitrile in water and injected into the source of the mass spectrometer with a syringe pump (Harvard Instruments, South Natick, MA) at a flow rate of 6 μl/min.

Metal Content Analysis—The zinc content of the enzyme was measured by atomic absorption in the flame mode using a PerkinElmer 2100 spectrometer. Before the metal analyses, 1 ml of a 40 μM protein solution was dialyzed three times during 8 h at 4 °C against 1 liter of double-distilled metal-free water containing 10 mM cacodylate buffer, pH 6. The zinc content of this dialysis buffer was about 400 nm. The final dialysis buffers were used as blanks. Protein concentration was determined spectrophotometrically by using ε260 = 26,000 M⁻¹ cm⁻¹. The metal content values reported for each sample are averages of results from three independent experiments.

Kinetic Studies—The hydrolysis of all antibiotics was monitored by following the absorbance variation resulting from the opening of the β-lactam ring, using a UV/vis 860 spectrophotometer equipped with thermostatically controlled cells and connected to a Copam PC 85C microcomputer. Cells with 0.2–1.0-cm path lengths were used depending on the substrate concentration. The kinetic parameters were determined either under initial rate conditions, using both Hanes’ linearization of the Henri-Michaelis equation and a direct nonlinear regression with the hyperbolic equation or by analyzing the complete hydrolysis time courses, as described by De Meester et al. (26). The reported kinetic parameters values are the means of at least three experiments in which the different enzymes were added to the substrate solutions prepared in buffers containing the stated Zn²⁺ concentrations. All experiments were performed at 30 °C in 10 mM cacodylate, pH 6.0. Bovine serum albumin (20 μg/ml) was added to diluted β-lactamase solutions in order to prevent enzyme denaturation.

The heat stability of the different proteins was characterized. The enzymes (0.1 mg) were incubated at 50 °C in 10 mM cacodylate, pH 6.0. Aliquots were withdrawn after increasing periods of time, and the residual activity was measured against 1 mM benzylpenicillin.

Molecular Modeling—The S221A mutant model was based on the three-dimensional structure of FEZ-1 enzyme (16). The molecular model was built using the Homology module of the Insight program (Molecular Simulations, San Diego, CA), running on a Silicon Graphics workstation. After model building, energy minimization was achieved using the Discover module of the same package to avoid bad molecular contacts. Finally, the geometric features were analyzed with the Insight II program.

Inhibition of Y228A Mutant by the Hydrolysis Cephalosporin Products—The progressive inactivation of the enzyme was monitored by analyzing the hydrolysis time course of different nitrocefin or cefuroxime concentrations in 10 mM sodium cacodylate, pH 6.0. In 10 ml of 100 μM cefuroxime, cefotaxime, or cefalotin were hydrolyzed by 15 μg of FEZ-1 β-lactamae in 1 mM cacodylate buffer, pH 6. The hydrolysis of the cephalosporins was followed by recording the absorbance variation at 280 nm. After completion, the hydrolyzed antibiotics were extracted from the enzyme solution on a YM-10 membrane (Amicon, Beverly, MA). The filtrates were collected and dried.

The powders were resuspended in water to a final concentration of hydrolyzed product of 1 mM. The activity of the Y228A mutant was measured in the presence of different concentrations of the hydrolyzed cephalosporins. The variations of the pseudo-first-order inactivation
They were monoclinic and belonged to space group P2_1, with unit cell
parameters of a = 78.50 Å, b = 77.43 Å, c = 101.70 Å, \( \alpha = 90^\circ, \beta = 101.70^\circ \), with two molecules per asymmetric unit. A data set at 2.0 Å

## Table II

### Kinetic parameters of the wild type enzyme (WT) and the H121A, Y156A, S221A, N225A, and Y228A FEZ-1 mutant enzymes

The measurements were performed in 15 mM sodium cacodylate/cacodylic acid, pH 6.0, with 1 Å vapor diffusion in hanging drops at 8 °C. Crystals of the Y228A mutant were obtained by mixing 1 mM benzylpenicillin with 20% glycerol. Data were processed in Oxford Cryosystems liquid nitrogen cryostat) after a short soaking in 20% polyethylene glycol MME 5000, 0.2 M ammonium sulfate, 0.1 M sodium cacodylate/cacodylic acid, pH 6.0, and crystallization solution added with 20% glycerol. Data were collected using a Nonius FR-591 rotating anode x-ray generator (15 m in 15 mM cacodylate buffer, pH 6.0, at 30 °C. S.D. values were between 10 and 20%. ND, not determined.

| Enzymes | Antibiotics | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) |
|---------|-------------|---------------|---------|----------------|---------------|---------|----------------|
| WT      | Benzylpenicillin | 70 590 | 0.11 | 50 280 | 0.18 |
|         | Cefuroxime  | 320 50 | 6.6 | 330 35 | 9.4 |
|         | Cefotaxime | 170 50 | 2.4 | 430 70 | 6.1 |
|         | Cephalothin | 300 120 | 2.5 | ND ND ND |
|         | Nitrocefin  | 100 90 | 0.9 | 600 190 | 3.2 |
|         | 7-ACA     | >200 1000 | 0.2 | >2000 1000 | 2 |
|         | Meropenem  | 45 85 | 0.5 | ND ND ND |
|         | Biapenem  | >70 1000 | 0.07 | ND ND ND |
| H121A   | Benzylpenicillin | 15 500 | 0.03 | 600 2200 | 0.27 |
|         | Cefuroxime  | 27 55 | 0.5 | 110 55 | 2.1 |
|         | Cefotaxime | 60 100 | 0.6 | 120 60 | 2 |
|         | Nitrocefin  | 7 100 | 0.07 | 220 130 | 1.7 |
|         | Imipenem >7 1000 | 0.007 | >70 1000 | 0.07 |
| Y156A   | Benzylpenicillin | 25 950 | 0.026 | 125 930 | 0.13 |
|         | Cefuroxime  | 550 60 | 9.2 | 820 125 | 6.5 |
|         | Cefotaxime | 660 150 | 4.4 | 750 150 | 5 |
|         | Nitrocefin  | 190 130 | 1.5 | 50 20 | 2.4 |
|         | Imipenem ND | ND | ND | ND ND ND |
| S221A   | Benzylpenicillin | 12 960 | 0.013 | 10 700 | 0.013 |
|         | Cefuroxime  | 160 85 | 1.9 | 480 360 | 1.3 |
|         | Cefotaxime | 90 85 | 1.1 | 480 440 | 1.1 |
|         | Nitrocefin  | 8 80 | 0.1 | 85 240 | 0.35 |
|         | Imipenem >2 1000 | 0.002 | >2 1000 | 0.002 |
|         | Meropenem  | 0.3 115 | 0.005 | ND ND ND |
|         | Biapenem >5 1000 | 0.005 | ND ND ND |
| N225A   | Benzylpenicillin | 25 800 | 0.04 | 20 800 | 0.03 |
|         | Cefuroxime  | 350 50 | 8 | 600 30 | 19 |
|         | Cefotaxime | 800 120 | 7 | 1800 125 | 12 |
|         | Nitrocefin  | 16 20 | 0.8 | 60 180 | 3 |
|         | Imipenem 11 | 1250 | 0.01 | 5.5 800 | 0.007 |
|         | Meropenem  | 35 800 | 0.04 | ND ND ND |
|         | Biapenem >4 1000 | 0.004 | ND ND ND |
| Y228A   | Benzylpenicillin | 125 3600 | 0.035 | 40 1300 | 0.03 |
|         | Cefuroxime  | 5900 160 | 35 | 6800 130 | 50 |
|         | Cefotaxime | 1250 100 | 12.8 | 7600 660 | 11 |
|         | Cephalothin | 2350 70 | 32 | ND ND ND |
|         | Nitrocefin  | 200 140 | 1.4 | 3250 65 | 50 |
|         | 7-ACA     | 20 880 | 0.0024 | ND ND ND |
|         | Imipenem >20 1000 | 0.02 | 14 1000 | 0.014 |

1 The abbreviations used are: MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; ESI, electrospray ionization; MS, mass spectrometry; 7-ACA, 7-aminocephalosporanic acid.

2 The mutant enzyme FEZ-1 Y228A and FEZ-1 WT (30 μM) were incubated with cefuroxime in a 1:1 ratio. The reaction was stopped after 15 min by denaturing the enzyme in 50% acetonitrile, 0.1% formic acid. The enzyme-cefuroxime complex was measured using a ESI-Q-TOF mass spectrometer (4700 Medical Systems). Mass spectra were processed using the Micromass Z Tide version 3.1 software of Micromass.

The expression of all proteins was done in E. coli BL21(DE3) pLysS. Production of β-lactamases was tested at three temperatures, 37, 28, and 18 °C, in presence of 0.5 or 1 mM isopropyl-β-thiogalactopyranoside. Enzyme production was estimated by measuring the activity of crude extracts against cefuroxime and by SDS-PAGE. For the S162A and C200A variants, no
β-lactamase production was found either in soluble or insoluble form. These substitutions appeared to impede the correct folding of the protein and yielded an unstable protein. In the case of Ser162, the comparison of the L1 and FEZ-1 structures reveals the presence of a two-residue insertion (Asn109–Asp110) in the FEZ-1 sequence (16). The insertion generates a very short 3_10 helix stabilized by a hydrogen bond formed by the side chain interaction between Asp110 and Ser162. In addition, Cys200 is close to the active site and involved in a large network of hydrogen bonds.

For all of the other mutants, the maximal production in E. coli BL21(DE3) pLYS S was obtained at 28 °C 6 h after the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The mutants were purified as described for the wild type FEZ-1 (17). The yields after purification could be estimated to be 4–8 mg/liter for the different enzymes.

**Thermal Stability of Wild Type and Mutants of FEZ-1**

At 30 °C the apparent stabilities of the WT and mutant enzymes were similar. At 50 °C, the Y156A, S221A, N225A, and Y228A mutants were rapidly inactivated (t1/2 < 15 min). In the case of mutant H121A, the enzyme was inactive after 15 min of incubation at 50 °C, but the addition of 100 μM zinc (final concentration) yielded an enzyme as stable as the wild type.

**Zinc Content of the Different Mutants**

Metal analyses of wild type FEZ-1 demonstrated that the enzyme binds 1.8 ± 0.2 Zn(II) ions per molecule. The Y156A, S221A, N225A, and Y228A mutants were found to also bind two zinc ions at an external zinc concentration of 0.4 μM. Under the same conditions, one zinc ion was found per H121A molecule. Thus, the substitution markedly reduced the affinity of the β-lactamase for the second zinc ion. Unexpectedly, despite the fact that all of the proteins but H121A appeared to contain a full complement of two zinc ions at an external zinc concentration of 0.4 μM, the addition of 100 μM Zn to the incubation medium significantly modified the kinetic parameters in a few cases. This observation remains presently unexplained unless one assumes that the opportunities for the second zinc might be specifically decreased by the substrate.

**Substitution of a Zinc Ligand: The H121A Mutant**

With the exception of benzylpenicillin, all of the K_m values were not dependent on the zinc ion concentration and were similar to those computed for the wild type enzyme (Table II). By contrast, at low [Zn^{2+}], the catalytic constant k_{cat} and the catalytic efficiencies k_{cat}/K_m of H121A were reduced 3–30-fold compared with the WT. The substitution of His^{121} allowed the production of a monozinc form at low external concentration of metal ions. The addition of zinc to the reaction mixture increased the catalytic efficiency of H121A mainly by increasing the k_{cat} value. This behavior reflects the appearance of a dizinc H121A at high [Zn^{2+}], which was nearly as active as the wild type FEZ-1. The [Zn^{2+}] dependence of k_{cat} and K_m was measured with nitrocefin. As expected, K_m values remained constant for all of the tested [Zn^{2+}], but the k_{cat} values increased up to 5 μM. Further changes were observed at high [Zn^{2+}] (k_{cat} = 220 s^{-1} at [Zn] > 5 μM) (Fig. 1). Based on these data, we can postulate that the apparent dissociation constants for zinc of the monozinc and dizinc forms of H121A FEZ-1 are lower than 0.4 and 2.5 μM, respectively. The modification of a ligand for the second zinc ion thus had a strong impact on the catalytic properties of the enzyme. A similar although more important phenomenon was observed in the case of the B. cereus metallo-β-lactamase (BcII). In that enzyme, the replacement of Cys^{221} by alanine or serine yields a poorly active monozinc enzyme, whereas the dizinc form is nearly as active as the wild type BcII (30, 31). PAC and NMR experiments indicated that the cadmium ion can be found independently in both sites when less than one metal ion equivalent is added to the BcII apoenzyme (36). In addition, EXAFS experiments indicated that the same phenomenon is observed for the monozinc BcII (30). On the basis of all of the available data on FEZ-1 and other metallo-β-lactamases together, we propose that, in the monozinc form, the metal ion is alternatively located in both sites. The substitution of the histidine side chain modifies the affinity for zinc of the catalytic site. It could be possible that, in the presence of substrate, the zinc ion occupies the so-called second binding site at some stage during catalysis. Therefore, its integrity is needed to yield a fully active monozinc enzyme. The elimination of His^{121} decreases the affinity of the second binding site and may favor an enzyme species where the zinc is mainly located in the first binding site. This phenomenon yields an enzyme with reduced catalytic efficiencies compared with the wild type FEZ-1. The addition of zinc ion allows the formation of a dizinc species and the restoration of a very active enzyme.

**Substitution of Residues Involved in FEZ-1 Substrate Specificity**

**Effects of the Y156A Substitution**—Only one major difference was observed between the Y156A mutant and the wild type FEZ-1 (Table II). A 10–100-fold decrease of k_{cat}/K_m for imipenem was noted depending on the zinc concentration. In contrast to that of the WT, the catalytic efficiency of the Y156A mutant toward imipenem was not significantly modified by increasing the metal ion concentration up to 100 μM. Crystallographic structures of metallo-β-lactamases highlight a flexible amino acid chain that extends over the active site. Studies of the CcrA (21, 22) and IMP-1 (14) enzymes show that this loop “clamps down” on inhibitors upon binding. A similar behavior is thus expected in the presence of the substrate. This phenomenon
enon may participate in determining the catalytic efficiency of the \(\beta\)-lactamases. The crystal structure of the L1 \(\beta\)-lactamase shows that there is a large loop that extends over the active site, and modeling studies have predicted that Phe\(^{156}\) (for L1) and Tyr\(^{156}\) (for FEZ-1) can make significant contacts with large substituents at the C-6 or C-7 positions in penicillins or cephalosporins, respectively. In L1, the F156A mutation yields a \(\beta\)-lactamase with a catalytic efficiency similar to that of the wild type (32). In consequence, the experimental data do not support the conclusions of the modeling studies (18).

**Kinetic Properties of the S221A Mutant**—The substitution of Ser\(^{221}\) in alanine resulted in a modification of the FEZ-1 activity spectrum (Table II). Although somewhat decreased, the Activities against cephalosporins and penicillins were well conserved. The \(K_m\) values for these antibiotics remained similar to those calculated for the wild type enzyme. The \(k_{cat}\) values were generally 2–10-fold lower than for the wild type enzyme. In contrast, the activity of S221A was poor against all tested carbapenems (imipenem, biapenem, and meropenem). The \(k_{cat}/K_m\) values were 10–100-fold lower than those of the wild type enzyme. The decrease of the catalytic efficiency versus meropenem was due to a 100-fold decrease of the \(k_{cat}\) value.
Ser^{221} is thus not essential for the hydrolysis of cephalosporins and penicillins but plays a more significant role during the hydrolysis of carbapenems. The docking of the penam (for penicillins), the cephem (for cephalosporins), and the carbapenem moieties, respectively, in the active site of the wild type enzyme indicated that the carboxylate group at position C-3 (penicillins and carbapenems) and position C4 (cephalosporins) may interact with the side chain of Ser^{221}. In the S221A FEZ-1 mutant, the interaction is not possible and should thus be marked by a clear increase of $K_m$. This phenomenon was observed in the presence of high zinc ion concentrations in the case of penicillin and cephalosporins. In the case of carbapenems, the absence of the side chain of Ser^{221} results in the loss of a strong interaction either with the hydroxyl moiety of the serine side chain or with

![MALDI-TOF/TOF MS spectrum of the tryptic peptides of FEZ-1 Y228A (a) and FEZ-1 Y228A incubated with cefuroxime (b). The peptide containing the free cysteine Cys^{200} (m/z 927.48) was found in the active and inactivated enzymes. The peaks at m/z of 959.379 and 893.011 indicate the peptides with an extra sulfur atom or after loss of H_2S, respectively.](image)

![Possible mechanism of inactivation of Y228A by cefuroxime.](image)
the second water molecule in the active site, which would be necessary in order to have an efficient hydrolysis of the β-lactam.

Kinetic Properties of the Asn225 Mutant—It has been proposed that the catalytic mechanism of class B β-lactamases involves the hydroxide bridging the two zinc ions, which can serve as the attacking nucleophile on the carbonyl carbon of the β-lactam ring (4). Asn225 and Zn-1 are likely to form an oxygen hole that stabilizes the putative tetrahedral intermediate and to contribute to the FEZ-1 catalytic properties. The behavior of N225A was not strongly modified versus penicillins (Table II). The catalytic efficiency of the mutant against third generation cephalosporins was even increased compared with the wild type enzyme. The major impact of the mutation was observed on the catalytic properties against carbapenems.

With meropenem, the decrease of the kcat/Km versus penicillins and cephalosporins was catalyzed with conserved efficiency after the elimination of the S221A and N225A mutants underline the fact that the hydrolysis of carbapenems requires the WT active site, whereas that of penicillins and cephalosporins can be catalyzed with conserved efficiency after the elimination of the serine and asparagine side chains at positions 221 and 225, respectively.

Kinetic Properties of the Y228A Mutant—Tyr228 does not play a major role in the hydrolysis of penicillins and carbapenems (Table II). The main impact of the mutation was the large increases of the Km values, which underline a less efficient interaction between the enzyme and its substrate. The steady state kinetic parameters for cefuroxime, cefotaxime, nitrocefin, and cephalothin were estimated by measuring the initial rates of hydrolysis. Km values for the WT and Y228A were similar and little affected by the presence of zinc ions. By contrast, we noted a large increase of the kcat/Km parameters. For the WT, the hydrolysis of cefuroxime was not modified in the presence of a large excess of zinc ions, whereas the catalytic constants for cefotaxime and nitrocefin increased by factors of 5 and 16, respectively. Nevertheless, a time-dependent inactivation of the Y228A mutant was observed. In the presence of increasing concentrations of cefuroxime, the rate of enzyme inactivation was dependent on the initial substrate concentration. For a concentration higher than 500 μM, the limit value of the pseudofirst-order rate constant of inactivation was $1.6 \times 10^{-2} \text{ s}^{-1}$.

In order to determine whether the inactivation was due to the substrate or the cephalosporin hydrolysis products, the Y228A mutant was incubated in the presence of the hydrolysis product of cefuroxime. It behaved as competitive inhibition ($K_i = 140 \mu\text{M}$). In addition, prolonged incubation induced the inactivation of the enzyme, and an apparent $k_i$ value of $2.7 \times 10^{-3} \text{ s}^{-1}$ was obtained, which was not dependent on the tested hydrolysis product concentrations. These data suggested that the inactivation event is more efficient when the hydrolysis product is generated in the active site of Y228A. The influence of the size of the C7 lateral chain on the formation of inactive enzyme was studied. 7-ACA was similarly hydrolyzed by Y228A and the wild type enzyme (Table II). In addition, no inactivation of the mutant was observed. The difference between cefuroxime and 7-ACA is the presence of a bulky C7 lateral chain (Scheme 1). These data suggest that this lateral chain is essential to the inactivation of the Y228A mutant.

### Table III

| Parameter | Value |
|-----------|-------|
| a | 44.76 Å |
| b | 77.43 Å |
| c | 78.50 Å |
| β | 101.70° |

* Data collection obtained on an x-ray rotating anode generator Nonius FR-591 coupled to a MarResearch Image Plate at the Laboratory of Macromolecular Crystallography (Grenoble, France).

* Last resolution shell: 2.12 to 2.01 Å.

* $R_{sym} = \sum |I_j| - \langle I_j \rangle / \sum |I_j|$, where $I_j$ is the intensity for reflection $j$, and $\langle I_j \rangle$ is the mean intensity.

* $R_{working} = \sum |F_{o} - F_{c}| / \sum |F_{c}|$, calculated with the working set.

* $R_{free}$ was similarly calculated with 9.5% of the data excluded from the calculation of $R_{working}$. 

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**FIG. 6. Native and Y228A mutant active sites.** Superposition of the crystallographic structures of wild-type FEZ-1 (cyan coil) and the Y228A mutant (white coil, Ala225 marked in red). The active site residues and the mutated residues described in this work are labeled. Ser227 in native FEZ-1 is shown in its double conformation. This figure was generated using BOBSCRIPT (35).
To determine whether the interaction between the hydrolysis product of cephalosporins and the Y228A mutant can lead to a covalent intermediate, ESI-MS was used to monitor the mass increment of the enzyme. The mass spectrum of Y228A is shown in Fig. 2a. Two masses were obtained. The mass of 29,358 Da corresponds to the theoretical mass of the Y228A, and the mass of 29,227 Da corresponds to the loss of the N-terminal methionine. After reaction with cefuroxime, mass shifts to 29,739 and to 29,608 Da are observed (Fig. 2b). For the WT enzyme, only a small fraction undergoes the mass shift (Fig. 2c). This mass increase of 381 Da corresponds well with the mass of hydrolyzed cefuroxime after departure of the 3′ leaving group -NH₂COOH. Such as inactivation mechanism is in good agreement with the reported inactivation of Aeromonas hydrophila metallo-β-lactamase CphA by cefotaxime (33). In the latter case, a disulfide bridge is possibly formed between the only cysteine of CphA and the dihydrothiazine sulfur atom of cefotaxime or between the cysteine and the exomethylene group of the hydrolyzed cefotaxime.

ESI-MS of the trypsin-digested enzyme and of the enzyme-CEFuroxime complex reveals a doubly charged peptide (m/z 654.7) present only in the complex (Fig. 3). The presence of this 1307.4-Da peptide confirms that the Gly199-Lys206 peptide (m/z 927.5), containing the free cysteine Cys200, binds a cefuroxime fragment of 381 Da. The residue to which the covalent bond is formed could be identified by MALDI-TOF/TOF MS. The Gly199-Lys206 peptide (m/z 927.5) is observed in both the native enzyme and the complex-derived peptide map (Fig. 4). Two additional peaks at m/z 959.5 and m/z 893.1, however, are present for the complex (Fig. 4b). This 32-Da mass increase and 34-Da mass decrease correspond to the addition of a sulfur atom and the loss of HS, respectively. It thus appears that the cefuroxime is linked to the peptide by a disulfide bond. Due to the high laser intensity of the MALDI source, the 1307.4-Da peptide (Gly199-Lys206 plus 381-Da cefuroxime fragment) can break in three different positions around the disulfide bond. The 927.5-Da peptide is obtained by the cleavage of the disulfide bond itself (X-CH₂-S-). The 893.1-Da peptide is due to the Gly199-Lys206 peptide without the cysteine sulfur atom (X-CH₂). Finally, the 959.5-Da peptide corresponds to the peptide containing the disulfide group but not the cefuroxime moiety (X-CH₂-S-S-). A similar cleavage of disulfide bridges in MALDI-MS has recently been reported (34). After derivatization of Cys200 with iodoacetamide, cefuroxime was no longer able to form a complex with Y228A, showing that the cefuroxime fragment resulting from the departure is indeed bound to this residue (result not shown).

Based on these data, the kinetic scheme of inactivation can be described by a branched pathway (Fig. 5). The hydrolysis of the β-lactam ring proceeds via the formation of noncovalent complexes. The EP complex obtained by the interaction between the enzyme and the hydrolysis product of cephalosporins can be transformed in an EP’ complex, resulting from the departure of a C-3′ leaving group and the appearance of an exomethylene moiety. EP’ can evolve into three different species. The first corresponds to the separation of the free enzyme and the P’ product. The second species is a covalent intermediate obtained by the addition of the free thiol group of Cys200 onto the exomethylene group. This reaction gives a stable thioether and was observed with the A. hydrophila enzyme but does not seem to occur here. Finally, the third possible dead end intermediate can be obtained by the rupture of the C-6–S-1 bond of the six-membered ring. The reaction yields a free sulfur group that will react with the Cys200 side chain to form a stable disulfide bond. Although the opening of the thiazolidine ring seems unlikely, we cannot exclude this possibility based on the analysis of the MS results described above.

Active Site of FEZ-1: Native Form and Y228A Mutant—X-ray data collection and structure refinement of Y228A FEZ-1 mutant are shown in Table III. Except in the vicinity of the mutated Tyr215 residue, there are no significant differences between the three-dimensional structures of the wild-type and the mutant enzyme. The active sites of both the wild-type and Y228A mutant enzyme are depicted in Fig. 6. The root mean square deviation between the two main chain atoms (residues 36–311) is 0.18 Å. The main difference between the two structures is at His121, which rotates 94° around the C-γ-C-β bond. This movement affects the neighboring residues Phe154 and Tyr156.

We propose that the replacement of the tyrosine side chain by a methyl group, which increases the space in the vicinity of the active site, changes the position of the antibiotic in the catalytic pocket. After hydrolysis of cephalosporins, this space allows a direct interaction between Cys200 and the cephem ring, yielding the formation of a covalent and inactive complex. Unfortunately, up to now, we were not able to solve the structure of the Y228A-cefuroxime complex.

CONCLUSIONS

The His121 is essential for the production of a dizinc form of FEZ-1 at low zinc concentration. The monozinc enzyme is active and stable. The addition of zinc ion allowed the production of a dizinc enzyme as active as the wild type FEZ-1. All of our data indicate that the main function of His121 is to interact with zinc ions. Our studies confirmed that Tyr156 does not play an important role in the subclass B3 β-lactamases. Substitutions of Ser221 and Asn225 modify the activity spectrum of the enzyme. In both cases, the catalytic efficiency of the two mutants against carbapenems decreases. These two residues are involved in the correct positioning of the carbapenem in the catalytic pocket. Finally, we could demonstrate that Tyr228 is important in the processing of bulky cephalosporins. The Y228A mutant is inactivated by the hydrolysis product of cephalosporin. Our studies describe the mechanism of inactivation of a subclass B3 enzyme by a β-lactam antibiotic.

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Probing the Specificity of the Subclass B3 FEZ-1 Metallo-β-lactamase by Site-directed Mutagenesis
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