Site-directed Mutagenesis at the Active Site of Escherichia coli TEM-1 β-Lactamase

SUICIDE INHIBITOR-RESISTANT MUTANTS REVEAL THE ROLE OF ARGinine 244 AND METHIONINE 69 IN CATALYSIS

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Arginine 244 is a highly conserved residue in Class A β-lactamases, while methionine 69 is not. Informational suppression experiments show that replacement of M69 by a leucine, or that of R244 by most other amino acids leads to clavulanic acid-resistant phenotypes. The arginyl 244 side chain is tightly held in a network of interactions within the active site. Its replacement by a glutamine or a threonine perturbs the enzyme kinetics but to a smaller extent than would have been predicted if it were directly involved in substrate binding. Clavulanic acid and sulbactam still interact specifically with the mutant enzymes but are much less efficiently metabolized. Substitutions at position 244 also unveil interactions between the C6 substituent of substrates and the Arg^{122}/Glu^{104} region of the active site.

Methionine 69 is located in a region of strong structural constraints and presents an unusual conformation. Molecular dynamics simulation showed that its replacement by a leucine does not release the strain in this area and induces only minor structural changes. Accordingly, the kinetic behavior of the mutant is only marginally perturbed, except for suicide inhibitors. Both clavulanic acid and sulbactam are well degraded by the mutant enzyme, while irreversible inactivation is dramatically decreased. The contribution of both residues to catalysis is discussed in the light of the kinetic and structural data.

Increased and repeated use of β-lactams leads to their becoming ineffective, principally due to the onset and worldwide spread of enzymatic resistance via β-lactamase production by target organisms. To respond to this major challenge, the pharmaceutical industry adopted two strategies, the first one being the production of new β-lactams resistant to hydrolysis by β-lactamases (second and third generation cephalosporins). The second strategy consisted of combined use of a β-lactamase inhibitor with β-lactams (augmentin and timentin). Nevertheless, resistance against new antibiotics usually appears within a few years after their marketing (1), and recently hospital strains exhibiting resistance to ampicillin-clavulanate combination were isolated (2). In relation with this constant evolution of antibiotic resistance, understanding the catalytic mechanism and the inhibition pathway of β-lactamases becomes crucial for the design of improved β-lactams.

β-Lactamases are divided into four classes (A, B, C, and D) on the basis of their structures and substrate specificities (3, 4). Class A β-lactamases are the most commonly encountered. Sequence comparisons (4), recent structural information from crystallographic studies of different class A β-lactamases (5-8), modelling studies (9), and results from mutagenesis pointed out at least 4 residues which are believed to play a central role in catalysis: Ser^{70}, Lys^{72}, Glu^{106}, and Lys^{234} (10, 11). On the other hand, much less is known about the inhibition mechanism with site-directed inhibitors even though much attention has been devoted to inactivators involving β-elimination like clavulanic acid and penicillanic acid sulfone (sulbactam) (12). These two related compounds are believed to inactivate the TEM-1 β-lactamase as shown in Fig. 1 (13-16). They first react with the β-lactamase by ring scission to lead to an acy1 enzyme with the conserved serine 70. This intermediate may either hydrolyze, tautomerase to a more stable β-aminoacylate which is responsible for transient inhibition, or may produce permanent inactivation. It is suggested that this latter could result from a transamination reaction with an unknown active site residue (16, 17). However, with clavulanate, there are three distinct inactivation complexes (14), one being reversible with hydroxyamine treatment.

In order to better understand this mechanism, site-directed mutagenesis was performed at two positions which were presumed to interact in the inhibition pathway. Kinetics data were obtained from three purified variant enzymes (M69L, R244T, and R244Q) of the Escherichia coli TEM-1 β-lactamase, which, together with structural data, provided information on their respective role both in the catalytic and in the inhibition mechanisms of TEM-1 β-lactamase.

MATERIALS AND METHODS

Chemicals—Antibiotics (Fig. 2) were a gift from their respective manufacturers: penicillin G (Rhône-Poulenc); amoxicillin, ticarcillin, clavulanic acid (Beecham); piperacillin (Lederle); cefoperazone, sulbactam (Pfizer); cephaloridine (Glaxo). Nitrocefin was purchased from Glaxo. Oligonucleotides were made as trityl derivatives on an Automated Biosystem 380B DNA synthesizer using the phosphoramidite chemistry and purified on Nensorb Prep columns as specified by the manufacturer (Du Pont).
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**FIG. 1.** Inactivation mechanism of class A β-lactamases by suicide inhibitors. For clavulanic acid, $X = O, R = \text{ethylidene}$; for sulbactam, $X = \text{SO}_2, R = \text{dimethyl}$.

**PENICILLINS**

- Penicillin G
- Amoxicillin
- Ticarcillin
- Piperacillin

**CEPHALOSPORINS**

- Cephalothin
- Ceferazone
- Cephaloridine

**INHIBITORS**

- Clavulanic acid
- Sulbactam

**FIG. 2.** β-Lactam antibiotics used in this study.

**Expression and Mutagenesis**—Bacterial strain *E. coli* XAC-1: F' lacZ, lacZ138am, proR/r/lac-pro, lacI, lacI, nalA rif argE, ara was used as expression host. The bla TEM-1 gene is expressed from the plasmid pCT1 previously described (18). To increase the expression of the revertant enzymes made in this study, the pct3 plasmid containing a stronger promoter was used. The method for informational suppression has been described (18): the amber mutated pCT1 plasmid is either expressed in XAC-1 strains carrying a pct2-sup plasmid or in strains each expressing a tRNA suppressor gene from their chromosome or their episome. Cells were grown in LB medium supplemented with 100 μg/ml ampicillin, 12 μg/ml tetracyclin, and 30 μg/ml chloramphenicol when a pct2 plasmid was present. Plasmid DNA was purified by the alkaline lysis method, and cloning techniques were based on Maniatis *et al.* (19). All enzymes for genetic engineering were obtained from Pharmacia LKB Biotechnology Inc. The preparation of competent *E. coli* cells and subsequent transformation with plasmid DNA were carried out according to Hanahan (20).

Mutants were made using the following oligonucleotides: M69am, 5′AAAAGTGCTCTACATTGGAAA3′; M69L, 5′AAAAGTGCTCA03′; R244am, 5′AATGATACCCTAAGACCCACG3′; R244Q, 5′AATGATACCCTGAGACCCACG3′; R244T, 5′AATGATACCCTAGACCCACG3′.

Purified oligonucleotides were phosphorylated as described previously (21). Site-directed mutagenesis was performed using the Eckstein method (22). The R244 and M69 ambers were selected for their sensitivity to ampicillin. Missense revertants were obtained by the same method starting from the amber mutated genes. The mutations were confirmed by sequencing using the Sanger dideoxy method (23) with the Pharmacia T7 polymerase kit.

**β-Lactamase Purification**—Cells were collected during exponential phase by centrifugation. β-Lactamase was extracted by osmotic shock as previously described (24) and then purified by preparative electrophoresis using the Multiphor II system (LKB) on a 5–8 pH gradient. The gel portion containing the β-lactamase was eluted with 20 mM Tris-HCl, pH 7.6, buffer. After extensive dialysis against the same buffer in order to eliminate the remaining ampholytes, the protein extract was chromatographed on a Pharmacia MonoQ anion-exchanger column. The protein was eluted with a gradient of NaCl in a 0.02
m Tris-HCl, pH 7.5, at a flow rate of 1 ml/min. Active fractions (detected using the nitrocefin assay in 50 mM sodium phosphate buffer, 37 °C, Δε = 20,500 M⁻¹ cm⁻¹(25) were eluted at 11% in NaCl gradient, collected, and concentrated on PD10 columns. The β-lactamase preparations were then found to be homogeneous as judged by analytical SDS-phastgel. Protein concentrations were determined at 595 nm according to the Bradford method (26) and confirmed by absorbance measurements at 280 nm with Δε = 18,200 M⁻¹ cm⁻¹ obtained with the wild-type enzyme.

Kinetics Parameters—The kinetic constants kₐ and Kᵣ for various substrates were determined at 37 °C by computerized microcideometry (27). Antibiotics used in this study are shown in Fig. 2. For irreversible inhibitors, i.e., clavulanic acid and sulbactam, the rate of substrate hydrolysis decreases with time. Kᵣ was determined from competition experiment with penicillin G. During the determination of the different inactivation parameters, no transient inhibition was observed in our conditions. Thus, the inhibition kinetics can be fitted to Scheme 1 (11) as follows.

\[
E + I \rightleftharpoons (E, I) \overset{k_{-1}}{\underset{k_1}{\rightleftharpoons}} E - I \\
K_{cat} \times V + P
\]

The rate constant of irreversible inactivation kₐ was determined by incubating the inhibitor in saturating concentration with the enzyme for various times. A large excess of benzylpenicillin was then added, and the remaining activity was monitored. The measure of the half-life time of inactivation then allowed to calculate the kₐ value (kᵣ = kₑ). The turnover numbers with clavulanic acid were also determined. After incubation of the enzyme and clavulanic acid in different ratio for 10 min, the remaining activity was measured. Turnover number was calculated from the extrapolated value for 100% inactivation from the plot of the remaining activity versus the I/E ratio. For sulbactam, a kᵣ (rate of hydrolysis) was measurable, and the turnover numbers were directly obtained by the ratio kₑ/KₛIE×P. For substrate hydrolysis, catalytic efficiency is defined as the ratio kₑ/KₛIE or kₑ/Kᵣ for inhibitor hydrolysis. An inhibition efficiency for irreversible inhibitors can be defined in the same way, as kₑ/Kᵣ. In the case of clavulanic acid where kₑ could not be measured, catalytic efficiency can be deduced from the turnover number and inhibition efficiency. One unit of β-lactamase activity is defined as the amount of enzyme hydrolyzing 1 μmol of substrate/min at pH 7 and 37 °C.

Preparation of Inactivated Enzyme and Hydroxylamine Reactivation—Preparation of inactivated enzyme was essentially as described (28) except that in order to obtain significant irreversible inhibition of our mutants, a 2000-fold molar excess of clavulanic acid over enzyme and a 7000-fold molar excess of sulbactam were used for incubations of 5 h at 30 °C. These values were derived from the turnover numbers of the antibiotics for mutant M69L. Inactivation of the enzymes was monitored after extensive dialysis of the same buffer, that is, 10 mM Tris, 0.1 M NaCl, pH 7.4. The inactivation, which was constant with time, results previously described (14), and by measurements of residual activity with nitrocefin.

Other Methods—Bacterial strains producing the different protein variants by informational suppression were assayed for growth in the presence of various antibiotics. Assays were based on the disc agar diffusion method as previously described (11, 18). Isoelectric points (pl) were determined by analytical isoelectric focusing; β-lactamases were visualized by the iodine procedure in agar gel containing benzylpenicillin as substrate (29).

RESULTS

Informational Suppression at Positions M69 and R244 of TEM-1 β-Lactamase—Amber mutations were introduced at codons 69 or 244 in the 6a gene by site-directed mutagenesis. The mutated plasmids were then introduced in the 14 available strains harboring an amber suppressor gene. Thus, with only one mutagenesis step, we generated 14 variants of the TEM-1 β-lactamase at either position. However, as discussed previously, the efficacy of the method depends both on the type of suppressor used and on the context of the amber codon to be suppressed (11, 18). Both suppression contexts at position 69 and 244 were poor, thus allowing only limited conclusions to be drawn. At position 69, only proline and arginine yielded totally inactive enzyme, all other substitutions resulting in active enzymes. Substitution by leucine gave strikingly small inhibition diameters with augmentin and timentin in antibiotic disc assays, indicating that the strain harboring the M69L variant exhibited increased resistance to clavulanic acid.

At position 244, all substitutions lead to enzymes retaining some activity, but the main result is that many variants lead to reduced inhibition diameters with augmentin, meaning that these variant enzymes also display an altered behavior toward clavulanic acid. This result suggests that arginine 244 plays a specific role in the inactivation process. In order to obtain more detailed results on substrate and inhibitor interactions, a number of revertants were made for both positions, and the corresponding enzymes were purified and submitted to kinetic analysis.

Mutant Enzymes R244T and R244Q—Table I shows kinetic parameters both for the R244Q and R244T variants and the wild-type β-lactamase toward a wide range of substrates: amino-, carboxy-, and ureidopenicillins and first and second generation cephalosporins. Replacement of arginine 244 by a threonine or glutamine leads to mutant enzymes exhibiting decreased affinity toward all substrates. However this decrease is greater for penicillins. All kₑ values are also decreased but more significantly for cephalosporins than for penicillins. This leads to a large reduction of catalytic efficiency (kₑ/Kᵣ) of the mutant enzymes: 0.15-5% of wild type efficiency for R244T and 0.3-14% efficiency for R244Q. As these reductions in efficiency affect all the substrates, there is no major change in substrate profile. However, we can note the large decrease in the value of the activity rate constant for ticarcillin which therefore presents an original behavior compared to all other penicillins, and more generally, a lower efficiency of the mutant enzymes toward substrates bearing a thiazole ring on their side chain (ticarcillin, cephalothin, and cefaloridin).

The mutations at position 244 perturb the interactions with suicide inhibitors in a more dramatic way (Table II). The Kᵣ values increase a 100- to 300-fold, while kₑ values drop over a 1000-fold for clavulanic acid and 2- to 4-fold for sulbactam. Therefore, catalytic efficiencies (kₑ/Kᵣ) for sulbactam drop 6000-fold (for R244T) or 2000-fold (for R244Q), while irreversible inhibition efficiencies (kₑ/Kᵣ) drop “only” 500- and 700-fold, respectively. For clavulanic acid, catalytic efficiencies and turnover numbers cannot be estimated, while irreversible inhibition efficiency drop over 10,000-fold. Nevertheless, Kᵣ values are still in the micromolar range, and interactions can still be considered specific. In fact, all the inactivation process is altered, and both molecules behave more like competitive inhibitors than suicide inhibitors toward the R244Q or R244T mutants.

However, 50% irreversible inhibition of both mutant proteins can still be obtained by incubating the enzymes with large excess of clavulanic acid for long periods of time (5-h incubation with a 2000-fold molar excess of antibiotic), whereas the wild type enzyme is rapidly and totally inhibited in the same conditions. Subsequent reactivation of the enzymes can be achieved by hydroxylamine treatment as described by Charnas et al. (28), but TEM-1 only recovers 20% of its activity through this treatment, while the R244T and R244Q mutants recover over 90% of their activity. This can be interpreted as a quantitative change in the respective proportions of the three irreversible inactivation complexes as described by Charnas and Knowles (14), although such a change is not obvious from the isoelectric focusing experiments on the inactivated enzymes (not shown).

The pl value is 5.15 for both R244T and R244Q enzymes. kₑ and Kᵣ profiles as functions of pH were also obtained (not shown), but no substantial changes were observed, raising
the question of the role of the guanidinium of residue 244 in catalysis.

**Mutant Enzyme M69L**—Kinetic parameters for the M69L mutant are shown in Table III. Except for ticarcillin, all $K_{cat}$ values are slightly lower than for the wild type enzyme, whereas most affinity constants increased to a small extent. Catalytic efficiencies ($k_{cat}/K_m$) are therefore decreased by a ratio ranging from 2 for cefoperazone, to 8 for ticarcillin. These changes can be considered as minor and the substrate profile of the mutant enzyme is not significantly modified compared to TEM-1 β-lactamase.

In contrast, major changes are observed concerning the behavior of the mutant protein toward irreversible inactivators (Table IV). First, we note a decrease in affinity for both clavulanic acid and sulbactam. Indeed, $K_v$ values increase 24- and 33-fold, which is much more than the $K_m$ increase for substrates, thus indicating a preferential decrease of affinity for inhibitors. In parallel, inactivation rate constants $k_{inact}$ also decrease 5-fold for sulbactam and 20-fold for clavulanic acid. Another important feature of the M69L mutant is its enhanced turnover number and catalytic rate constant $k_{cat}$ for sulbactam. Opposite variations of $k_{cat}$ and $k_{inact}$ in fact account for a decrease of irreversible inactivation rate constant $k_v$ and an increase of the desactivation rate constant $k_d$ (Fig. 3). All this translates into catalytic efficiencies decreasing by one order of magnitude for both inhibitors, while inactivation efficiencies decrease by two orders of magnitude, thus indicating that both clavulanic acid and sulbactam are still good substrates for the enzyme, but much less potent inhibitors (Table IV). In the inactivation-hydroxylamine reactivation experiments, total inactivation of both M69L and the wild type enzyme was obtained with a 2000-fold molar excess of hydroxylamine, as opposed to only 20% for wild type TEM-1.

$k_{cat}$ and $K_m$ profiles as functions of pH were similar for both the wild type and the mutant enzymes thus indicating no direct role of this residue in catalysis (not shown). This is not surprising within the context of the x-ray structure of the wild type enzyme (5).
**DISCUSSION**

In the course of systematic replacements of residues in the active site region of TEM-1 β-lactamase by informational suppression, several mutants obtained at positions 69 and 244 led to clavulanic acid resistance phenotypes.

In the wild-type enzyme, arginine 244 is positioned at the beginning of strand α4, on the opposite side of the catalytic site compared to Ser^{22}, Lys^{23}, and Glu^{106}. As judged from x-ray structure (5), the mobility of Arg^{244} side chain is restricted by Leu^{220}, Met^{272}, Asn^{276}, and Arg^{275} side chains. The last 3 residues belong to the C-terminal α helix, and their side chains complete, together with Arg^{244}, one side of the substrate binding area. The side chain of Arg^{244} is tightly held in a network of interactions involving a sulfate group, water molecules and protein residues including Gly^{236} (Fig. 3). Thus, from its location and chemical type, Arg^{244} must play a role in maintaining the active site integrity.

Most substitutions at position 244 resulted in clavulanate-resistant phenotypes, although these enzymes were clearly less active than the wild type. Such results are consistent with several TEM-1 β-lactamase mutants reported recently, either isolated from blood culture (R244C) or obtained by mutagenesis (R244C, R244S), all exhibiting resistance to clavulanate (2, 30). This suggests that the presence of the arginine residue at position 244 is of crucial importance for the inactivation process.

All the $K_{m}$ values are greatly increased for mutants R244T and R244Q, while they exhibit decreased rates of hydrolysis for all the substrates tested. The present results clearly illustrate that arginine 244 plays a role in the catalytic apparatus of the TEM-1 enzyme. Such conclusions are consistent with the fact that this residue is well conserved in class A β-lactamases except for Streptomyces albus. Substitution of arginine for a threonine or a glutamine modifies the electronic features of the cavity. However, when the penicillin substrate is modeled into the active site, in a suitable position for acylation of the carbonyl group of the β-lactam ring by Ser^{22}, there seems to be no direct interactions between Arg^{244} guanidinium group and the carboxylic acid substituent at C3 of the substrate. The thiazolidin ring occupies the sulfate binding site found in the wild-type native crystal structure. A small concerted movement of Asn^{276} and Arg^{244} side chains is sufficient to position the thiazolidin ring and Arg^{244} side chain at reasonable van der Waals distance. From these observations, it appears that Arg^{244} does not act directly as a required ionic partner for substrate binding, an hypothesis in agreement with the kinetic data. Indeed, mutations on residues essential for substrate binding such as Lys^{236} led to drastic decreases of both $k_{cat}$ and $K_{m}$ values (11), whereas mutations of Arg^{244} did not. We would suggest that the role of this arginine residue could rather be to destabilize the enzyme-product complex and thus to be responsible for the high turnover of the enzyme. Moreover, this hypothesis seems to be confirmed by the lower $K_{m}$ values exhibited by the mutant enzymes R244T and R244Q.

Our results rule out the hypothesis of Jacob-Dubuisson et al. (31) who postulate that residue R220 in S. albus and R244 in TEM-1 β-lactamases play the same role by participating in the proper positioning of the antibiotic, forming together with Lys^{236} and the dipole of the α2 helix a large positive field on that side of the cavity, which is necessary for the carboxylate group in C3 or C4 of β-lactams. In contrast with our present findings with TEM-1 mutants R244T and R244Q, in vivo data concerning the β-lactamase of S. albus show that this enzyme, carrying an Asn residue at position 244, is also less efficiently inhibited by clavulanic acid (32) but exhibits a much higher turnover number than other class A β-lactamases. Therefore, there seem to be several very significant differences between the S. albus and TEM-1 enzymes.

Analysis of Table III indicates that all compounds bearing a thiazole ring as the C6 substituent (Fig. 2) are very poor substrates for the Arg^{244} mutants. Modeling penicillin substrates binding onto the protein shows that the C6 substituent is oriented toward the solvent side of the catalytic cavity (Fig. 4) and that the carboxylic group and the sulfur atom of the thiazole ring can be brought in the vicinity of Asn^{276} and Glu^{104} side chains, providing additional interactions between protein and substrate in this case. These interactions would be expressed among the penicillins substrates as a better $K_{m}$ and a reduced $k_{cat}$, because the product might behave as a weak inhibitor (see the wild-type kinetic values for ticarcillin in Table III). When Arg^{244} is replaced by glutamine or threonine, the repulsive effect mentioned above, which controls in part the turnover number, is canceled allowing the interactions between the protein and the substituent at C6 to become kinetically significant and observable. These interactions reduce the turnover number, and this is indeed expressed by a significant reduction of $k_{cat}$ for these substrates when compared to penicillin G; the $k_{cat}$ ratio between penicillin and ticarcillin is 10 for the wild-type enzyme and 50 and 200 for R244T and R244Q, respectively.

Concerning inhibition by site-directed inhibitors, kinetic parameters determination has clearly shown that the resistance arises by the modification of both affinity for the inhibitors and inactivation constants, with $k_{cat}$ being largely decreased for both inhibitors. Moreover, the results obtained with penicillanic acid sulfone show that the hydrolysis of the inhibitor is also strikingly affected. The two inhibitors thus behave more like competitive inhibitors of the mutant enzymes than irreversible inactivators. Is such a behavior only

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**Fig. 3. Environment of arginine 244 side chain (thick line) in the wild type x-ray structure.** The solvent molecules shown in this diagram display high occupancy values after refinement at 2Å resolution. Dotted lines connect atoms found at hydrogen bond distance.
Replacement of Arginine 244 and Methionine 69 in TEM-1 β-Lactamase

**FIG. 4.** Stereo view of the protein residues surrounding the thiazole ring of ticarcillin when the substrate (*thick lines*) is modeled into the binding site.

**FIG. 5.** Stereo view around residue 69: M69 (*thick lines*), L69 (*thin lines*). Leucine 69 has been modeled from the x-ray structure of the wild-type enzyme and both structures (wild type and L69 model) were subjected to 5-ps molecular dynamics simulation (see text).

due to structural modifications caused by the replacement of the charged arginine, as it is proposed for substrates, or to a more direct role of this residue in the inhibition mechanism? Moews et al. (33) proposed that Arg^244 in *Bacillus licheniformis* 749/C β-lactamase is a candidate for inactivation with sulfone or clavulanate by transimination, this residue being closer to the double bond at C2 than Lys^25 or Lys^244. However, when considering structural data of TEM-1 β-lactamase (5), the side chain of Arg^244 is obviously too far from the reactive carbon atom to play this postulated role in this enzyme. Moreover, inactivation experiments and kinetic results clearly show that the irreversible inactivation still occurs with the R244 variants, albeit at a much reduced rate. We can conclude that Arg^244 is not a target for these inhibitors.

Informational suppression at position 69 revealed the leucine revertant as an acid clavulanic-resistant mutant. Analysis of the three-dimensional structure of the enzyme (5) suggests that residue 69 is a structurally important residue. Its side chain, buried and part of the hydrophobic interface between the two protein domains, is facing the pro-L hydrogens of the Cα of glycines 236 and 245 which belong to strands β3 and β4 (Fig. 5). These glycine residues are required for steric reasons. G236 is a strikingly conserved residue in class A β-lactamases from various sources, whereas G245 is not. However, when another residue is found at position 245, a compensatory mutation is found at position 69. In the crystal structure of the enzyme, M69 is in a high energy conformation, with (φ, ψ) dihedral angles outside the "allowed" regions of the Ramachandran plot. Docking experiments in the refined structure show that M69 is not directly involved in substrate binding, which is confirmed by the fact that many substitutions at that position yielded enzymes almost as active as the wild type. Among the 14 substitutions tested, only M69L resulted in an enzyme less sensitive to clavulanate.

Kinetic data show that, in fact, leucine in position 69 barely affects the catalytic constants of the enzyme toward all the substrates tested. Moreover, two other revertants at this position (M69G and M69Q) were also tested but neither led to significant changes of the different kinetic parameters (data not shown). These results thus indicate that methionine 69 does not play a key role in the binding of substrates nor their hydrolysis. Such conclusions are not surprising as comparison of class A β-lactamase sequences (34) at this position shows that this residue is not conserved.

However, if the behavior of M69L is almost the same as that of the wild-type enzyme with a wide range of substrates, this is not true concerning the irreversible inhibitors tested. All kinetics values are perturbed: the mutation leads to a greatly decreased affinity for both inhibitors, to a decreased efficiency of inactivation but to a much less decreased effi-
ciency for hydrolysis. This results in a shift in the inhibition pathway, favoring hydrolysis over irreversible inhibition.

In fact, the behavior of inhibitors toward M69L mutation stresses the fact that important properties should be associated to this high-conformational energy residue. They could be related either to its proximity to essential Ser59, to its location at the domain interface, or to folding aspects. In view of our mutagenesis results, such conformational strain would be related to catalytic requirements rather than to requirements for protein folding. Such conclusions were also reported by Herzberg (7) concerning *Staphylococcus aureus* PC1 β-lactamase. The mutation of methionine to leucine is readily accommodated in the wild-type x-ray structure. Molecular dynamics simulations have been performed using the GROMOS software on the M69L mutant and on the wild type structure as a reference. After 5-ps simulation, no major changes have been observed in both cases (Fig. 5), and interestingly, there was no tendency to release the strain on the (ϕ, ψ) values of residue 69. This indicates that the overall structure constraints dominate in this area, although the atomic compacity is not optimal in this region, both proteins displaying cavities located slightly differently. We suggest that the substitution of Met59 by a leucine residue leads to a modified active site where good substrates can still be well fitted, whereas small molecules such as clavulanic acid and sulbactam do not orient in the same way and consequently cannot undergo inactivation as with the wild-type enzyme.

In conclusion, resistance to inhibitors by TEM-1 β-lactamase can occur in different ways: M69L and R244 mutants both lead to resistance, the former by hydrolyzing the inhibitor and the latter by interacting competitively with it. Moreover, the mechanism of inactivation appears to be readily perturbed by structural changes as exemplified by differences between PC1 and TEM-1 β-lactamases despite their relatedness (5, 7). Residue(s) uniquely required for the irreversible inactivation are still to be found. It is somewhat surprising that the only irreversible inhibitor-resistant mutants that have been uncovered, either as natural isolates or by random or directed mutagenesis, display mutations at residues that are not directly involved in the inactivation mechanism. This seems to indicate that the primary target(s) of these inhibitors is a residue that is essential for enzyme activity and cannot therefore be easily changed. One would then expect to find it among the active site residues strongly conserved throughout the class A enzymes. Such potential candidates are in limited number, if indeed transamination is the mechanism by which inactivation occurs. Mutagenesis at such positions is currently under way. The study of this complex inactivation mechanism will bring more information on the exquisitely sophisticated mechanism of β-lactam hydrolysis by β-lactamases.

REFERENCES