Mass Spectral Kinetic Study of Acylation and Deacylation during the Hydrolysis of Penicillins and Cefotaxime by β-Lactamase TEM-1 and the G238S Mutant†

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Received February 6, 1995; Revised Manuscript Received June 29, 1995⊗

ABSTRACT: The G238S substitution found in extended-spectrum natural mutants of TEM-1 β-lactamase induces a new capacity to hydrolyze cefotaxime and a large loss of activity against the good substrates of TEM-1. To understand this phenomenon at the molecular level, a method to determine the acylation and deacylation elementary rate constants has been developed by using electrospray mass spectrometry combined with UV spectrophotometry. The hydrolysis of penicillins and cefotaxime by TEM-1 and the G238S mutant shows that the behavior of penicillins and cefotaxime is very different. With both enzymes, the limiting step is deacylation for penicillin hydrolysis, but acylation for cefotaxime hydrolysis. Further analyses of the G238S mutant show that the loss of activity against penicillins is due to a large decrease in the deacylation rate and that the increase in catalytic efficiency against cefotaxime is the result of a better K_m and an increased acylation rate. These modifications of the elementary rate constants and the hydrolytic capacity in the G238S mutant could be linked to structural effects on the Ω-loop conformation in the active site.

The production of β-lactamases (EC 3.5.2.6) is the major mechanism used by Gram-negative bacteria to develop resistance to β-lactam antibiotics. TEM-1 and, to a lesser extent, SHV-1 are the most commonly encountered plasmid-mediated β-lactamases. They efficiently hydrolyze both penicillins and cephalosporins, but not third-generation cephalosporins. Nevertheless, the emergence of strains resistant to newer β-lactams occurs rapidly when these drugs are used intensively. Resistance to nonhydrolyzable third-generation cephalosporins, in particular to the oxyimino β-lactams ceftazidime and cefotaxime and to monobactams such as aztreonam, was described in Europe in 1983, 5 years after the introduction of this class of drugs (Knothe *et al.*, 1983). In some cases, resistance can be due to an overproduction of β-lactamase (Sougakoff *et al.*, 1989), but generally resistance is transferrable, is plasmid-mediated, and occurs simultaneously with resistance to some aminoglycosides and tetracyclines (Sirot *et al.*, 1987).

Several extended-spectrum β-lactamases have been characterized and sequenced. They mainly derive from SHV-1 and TEM-1 [or TEM-2, which differs from TEM-1 by the single substitution of a lysine for a glycine at position 39 (Q39K) without any alteration of the activity (Barthélémy *et al.*, 1985)]. Their primary sequences differ from those of the parent enzymes by 1−4 mutations in the vicinity of the active site. A few substitutions are found more frequently, alone or in combination: a lysine for a glutamic acid at position 104 (E104K); a serine or a histidine for an arginine at position 164 (R164S or R164H); a serine for a glycine at position 238 (G238S); a lysine for a glutamic acid at position 240 (E240K); and a methionine for a threonine at position 265 (T265M), according to Ambler's numbering (Ambler, 1980). The TEM-3, TEM-4, SHV-2, SHV-3, SHV-4, and SHV-5 enzymes are characterized by their capacity to efficiently hydrolyze cefotaxime. These enzymes, which all bear the G238S substitution, show a global loss of activity against substrates readily hydrolyzed by the parent enzyme, a better catalytic efficiency for cefotaxime hydrolysis, and a high sensitivity to inhibitors of β-lactamases (Paul *et al.*, 1988; Chanal *et al.*, 1988; Sougakoff *et al.*, 1988a,b).

Lenfant *et al.* (1990) have studied, by site-directed mutagenesis and informational suppression, positions 104 and 238 of the β-lactamase TEM-1. The lysine at position 104 is not crucial for the hydrolysis of third-generation cephalosporins and induces only a small improvement in the affinities for the substrates. Only three variants at position 238, obtained by site-directed mutagenesis (a serine, a threonine, and an alanine), keep a good level of activity. These substitutions lead to considerable loss of activity against the good substrates of TEM-1 β-lactamase, better substrate affinities, and cefotaxime hydrolysis. Venkatachalam *et al.* (1994) studied the effects of substitutions at positions 238 and 240 on the capacity to hydrolyze ceftazidime and cefotaxime. They have shown that the G238S substitution is sufficient to confer cefotaxime resistance. Moreover, the comparison of SHV-2 and SHV-1 enzymes, differing only by the G238S substitution (Huletsky *et al.*,

† This work was financially supported by the Centre National de la Recherche Scientifique, the Société de Secours des Amis des Sciences, the Région Midi-Pyrénées, and by Contract No. 930608 from the Institut National de la Santé et de la Recherche Médicale. The first two authors have made equal contributions to this work.

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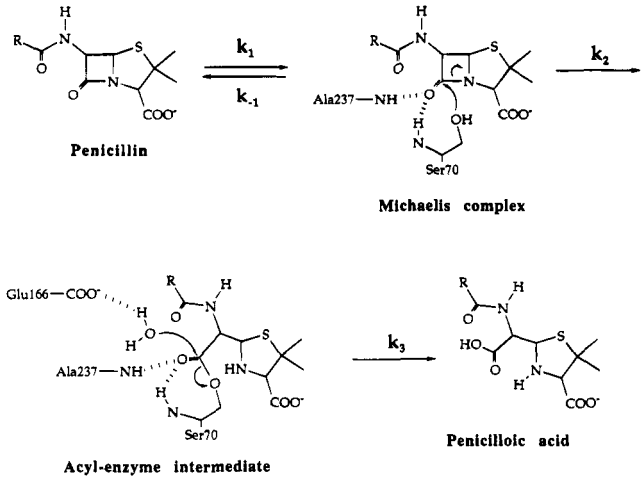
⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.

0006-2960/95/0434-11660$09.00/0

Scheme 1: Simplified Proposed Mechanism of Penicillin Hydrolysis by Class A β-Lactamases



1993; Lee *et al.*, 1991), and the finding of this substitution in a mutant of OHIO-1 β-lactamase resistant to oxyimino β-lactams (Schlaes & Currie-McCumber, 1992) clearly indicate that this substitution is responsible for the new hydrolysis capacity of extended-spectrum β-lactamases derived from TEM-1 (or TEM-2) and SHV-1 and for the reduced enzymatic activity of these variants toward penicillins and first- and second-generation cephalosporins. The Voisse mutant (Lenfant *et al.*, 1988) bearing the G238S substitution on the TEM-2 amino acid sequence was used to study the effect of the G238S substitution.

The general mechanism of action of class A β-lactamases may be decomposed into three elementary reactions (Scheme 1) as follows: (i) formation of the reversible, noncovalent Michaelis complex; (ii) conversion of the Michaelis complex into a covalent acyl-enzyme intermediate; and (iii) hydrolysis of the acyl-enzyme to produce penicilloic acid (the product of the reaction) and the active enzyme. At the molecular level, serine 70 (according to Ambler's numbering) is responsible for the opening of the β-lactam ring, which leads to the formation of the covalent acyl-enzyme (Strynadka *et al.*, 1992). Deacylation of this complex is then catalyzed by glutamic acid 166 (Delaire *et al.*, 1991; Adachi *et al.*, 1991; Escobar *et al.*, 1991) through the activation of a water molecule (Scheme 1).

To understand the global effect of the G238S substitution on the hydrolysis of β-lactams, it is necessary to measure the effect of the substitution on each step of the catalytic mechanism (Scheme 1). A method to determine the elementary rate constants of hydrolysis was developed that was based on electrospray mass spectrometry (ESMS)[1] and UV spectrophotometry. The effect of this amino acid mutation on acylation and deacylation steps was studied with both penicillins and cefotaxime.

MATERIALS AND METHODS

Production, Extraction, and Purification of β-Lactamases. *Escherichia coli* strain XAC-1 (Normanly *et al.*, 1986) was used to produce β-lactamases. This strain was transformed by the plasmid pAra 15, a derivative of pAra 14 (Cagnon *et al.*, 1991) expressing the *bla* gene for the production of the wild-type TEM-1, and by the plasmid T28C2+ (Lenfant *et al.*, 1988) for the production of the G238S variant of TEM-2. The preparation of competent cells and transformation with plasmid DNA were performed according to Hanahan (1985).

Bacterial cells were grown at 30 °C on Luria broth supplemented with 0.5% $MgSO_4$, 0.2% glycerol, and 100 μg/mL ampicillin (Sigma Chemical Co., St. Louis, MO). Cells were harvested by centrifugation during the exponential phase (A_{600} = 2) at 5000*g* for 10 min and washed with 200 mL of 200 mM Tris-HCl (pH 8). The cell pellet was resuspended to A_{600} = 60 with 200 mM Tris-HCl (pH 8); 1 vol of 1 M sucrose in 200 mM Tris-HCl (pH 8), 0.1 vol of 100 mM EDTA (pH 7.6), and 30 mg of lysozyme were added. The suspension was incubated for 20 min at 4 °C, and 0.2 vol of 0.5 M $MgCl_2$ was added. The periplasmic fluid was collected by centrifugation at 18000*g* over 20 min and dialyzed against 200 mM Tris-HCl (pH 8), to eliminate sucrose.

This extract was then concentrated and desalted by ultrafiltration using an Amicon system 8200 (Amicon GmbH, Witten, Germany) with a PM10 membrane. The final volume was approximately 5 mL. This suspension was then purified by preparative electrofocusing using the Multiphor II system (Pharmacia, Uppsala, Sweden) on a 4−6.5 pH gradient. The gel portion containing the β-lactamase was eluted with 20 mM Tris-HCl (pH 7.5) and dialyzed extensively against the same buffer. The protein extract was chromatographed on a Pharmacia Mono Q HR5/5 anion exchange column. The proteins were eluted with a 0−0.2 M linear NaCl gradient in 20 mM Tris-HCl (pH 7.5) buffer at a flow rate of 0.5 mL/min. Active fractions detected with nitrocefin (Oxoid, Basingstoke, Hampshire, U.K.) were found to be homogeneous, as judged by analytical SDS Phastgel (Pharmacia) and silver staining. The pure enzyme TEM-1 was further dialyzed against 10 mM ammonium bicarbonate (pH 7) for mass spectrometric analyses. Protein concentration was determined according to the Bradford method (1976), given that the molecular mass of TEM-1 is 28 933 Da and that of the G238S is 28 938 Da, as determined from the protein sequences.

Kinetic Parameters. The Michaelis−Menten constants k_{cat} and K_m were determined at 2.5 and 37 °C, in 10 mM ammonium bicarbonate (pH 7). The kinetic measurements were carried out spectrophotometrically. The changes in absorbance due to the opening of the β-lactam ring of cefotaxime (a gift from Roussel-Uclaf, Paris), cloxacillin (a gift from Smith Kline Beecham Laboratories, Nanterre, France), oxacillin (Sigma), and penicillin G (Rhône-Poulenc, Paris) were monitored with a UVIKON 930 (Kontron Instruments) at 280, 263, 260, and 227 nm, respectively. The corresponding values of $\Delta\epsilon$, calculated according to Samuni (1975), were 2120, −116, −248, and 982 M^{-1} cm^{-1}, respectively. The steady state kinetic parameters, V_m and K_m, were determined by nonlinear regression analysis of the progress curve of the reaction at various antibiotic concentrations ([S]) with the program ≪Minim ≫. The k_{cat} value was determined by calculating V_m/[E], where [E] is the enzyme concentration.

Determination of Acylation and Deacylation Rate Constants by Mass Spectrometry. All analyses were performed using a TRIO 2000 quadrupole mass spectrometer (VG Biotech, Altrincham, U.K.) equipped with an electrospray

[1] Abbreviations: PG, penicillin G; OXA, oxacillin; CLOX, cloxacillin; CTX, cefotaxime; ESMS, electrospray mass spectrometry.

Table 1: Kinetic Data for TEM-1 and Mutant G238S[a]

		TEM-1			G238S−Q39K			
	T (°C)	k_{cat} (s^{-1})	K_m (μM)	eff (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	eff (M^{-1} s^{-1})	%eff
PG	2.5	ND	ND	ND	15.3 ± 0.8	7 ± 0.9	2.2×10^6	ND
	37	1170 ± 90	62 ± 14	1.9×10^7	47.6 ± 0.8	11 ± 2	4.3×10^6	23
OXA	2.5	27.8 ± 1.1	25 ± 8	1.1×10^6	<0.1			
	37	41 ± 5	130 ● 42	3.2×10^5	0.7 ± 0.03	12 ± 5	5.8×10^4	18.1
CLOX	2.5	5.8 ± 0.8	72 ± 19	8.0×10^4	<0.1			
	37	9.9 ± 0.5	104 ± 24	9.5×10^4	1.43 ± 0.24	69 ± 12	2.1×10^4	22.1
CTX	2.5	<0.1			0.51 ± 0.04	92 ± 19	5.5×10^3	
	37	2.5 ± 0.9	1684 ± 690	1.5×10^3	20.3 ± 10.8	188 ± 92	1.1×10^5	7297

[a] ND, not determined; eff, catalytic efficiency defined as k_{cat}/K_m; %eff, relative efficiency of the mutant enzyme from the wild-type TEM-1.

source. Enzymatic reactions were carried out at saturating substrate concentration, at 2.5 or 37 °C, by mixing the enzyme solution (10 mM ammonium bicarbonate, pH 7) with an aqueous solution of antibiotic and quenched after various times by acidification with formic acid to pH 2.5. For acyl-enzyme quantitation analyses, oxacillin was used to form the acyl-enzyme with TEM-1 (reaction for 10 s at 2.5 °C). The reaction was quenched with acetonitrile (half the reaction volume) and formic acid to pH 2.5, vortex mixed, and centrifuged. An aliquot of TEM-1 was then added to the reaction mixture. Reaction mixtures were directly introduced into the electrospray source via a 10 μL injection loop at 10 μL/min using a 50:48:2 (v:v:v) mixture of acetonitrile/water/formic acid delivered by a syringe pump (140 A solvent delivery system, Applied Biosystems, Foster City, CA). Mass spectra were acquired in multichannel analyzer mode at a scan rate of 15 s/scan.

The determination of k_2 and k_3 is based on eqs 1 and 2, where k_{cat} is the catalytic rate constant of the reaction determined by UV spectrophotometry. The acyl-enzyme concentration at the steady state of the reaction is determined by the initial enzyme concentration and the evaluation of the ratio of free enzyme to acyl-enzyme in solution by electrospray mass spectrometry. The variation in product concentration with time is given by UV absorbance monitoring (even though the product formation could be followed by mass spectrometry, a direct correlation between absolute peak intensity and product concentration could not be simply established).

$$\text{d[penicilloic acid]}/\text{d}t = k_3[\text{acyl-enzyme}] \quad (1)$$

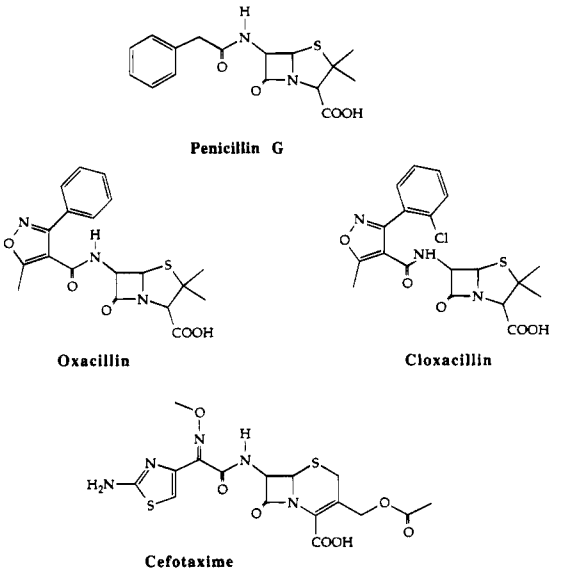
$$k_2 = \frac{k_3 k_{cat}}{k_3 - k_{cat}} \quad (2)$$

RESULTS

To better understand the effect of the G238S substitution, the determination of the acylation and deacylation rate constants k_2 and k_3 for TEM-1 and the mutant is necessary. It was achieved by quantitative evaluation of the acyl-enzyme.

Kinetic analyses by ESMS were carried out by quenching the enzymatic reaction with formic acid to pH 2.5 at various times and immediately injecting the sample into the mass spectrometer. Samples to which an equal volume of denaturing solvent (acetonitrile) was added to the enzyme reaction mixture gave identical spectra; this shows that the observed species were covalent complexes of the enzyme with the β-lactam, most likely the acyl-enzyme intermediate.

Scheme 2: Structures of Antibiotics Used



Hydrolysis of Penicillins by Wild-Type TEM-1. In order to trap and detect the acyl-enzyme intermediate, substrates with good affinity and a sufficiently slow rate of hydrolysis at a given temperature (2.5 or 37 °C) were selected. Oxacillin and cloxacillin, whose kinetic parameters are given in Table 1, fulfilled these requirements at 2.5 °C (compared to an excellent substrate, such as penicillin G). These two penicillin substrates have similar structures, cloxacillin being a chlorinated analogue of oxacillin (Scheme 2). Oxacillin and cloxacillin were characterized by using ESMS before enzymatic hydrolyses. Their spectra in the *m/z* range from 100 to 500 (data not shown) presented singly charged ions corresponding to the protonated molecules and to complementary fragment ions resulting from the cleavage of their β-lactam ring. The spectra of the corresponding penicilloic acids exhibited peaks 18 mass units above those of the protonated substrates and peaks of the decarboxylated products. The identification of these species in the mass range used to detect the antibiotics (*m/z* 100−500) provided a way to follow the course of the reaction. Similarly, at a higher mass range (*m/z* 900−1300), the different forms of the enzyme can be observed and identified.

Analysis of a TEM-1/oxacillin mixture, at the beginning of the reaction, showed a single molecular species corresponding to the acyl-enzyme complex (measured molecular mass of 29 333 ± 3 Da) (Figure 1a). The difference between the molecular mass of this complex and that of TEM-1 (28 933 Da) was 400 ± 3 Da, which is in excellent agreement with the molecular mass of oxacillin (401 Da). At longer

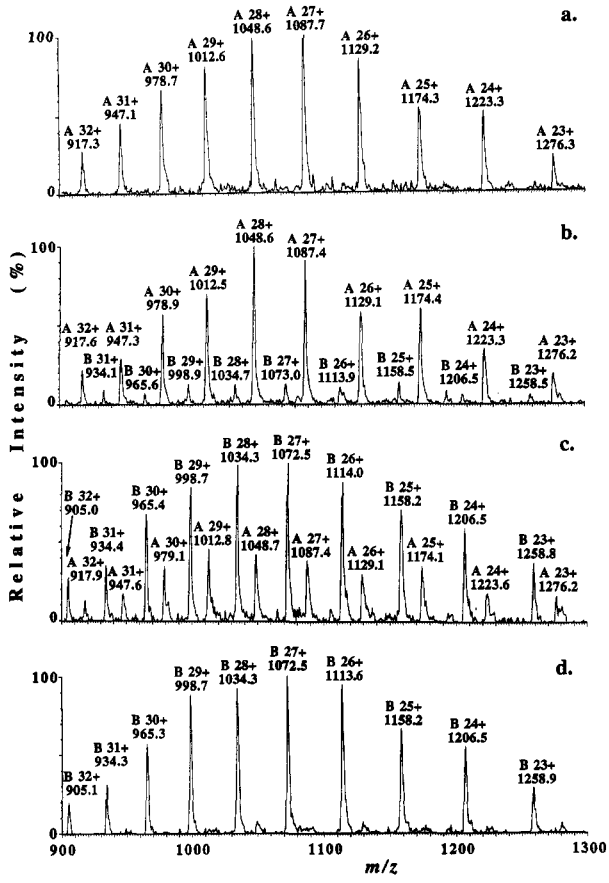


FIGURE 1: Electrospray mass spectra, m/z 900−1300, of the reaction mixture of TEM-1 (20 μL, 10 μM) and oxacillin (3 μL, 10 mM) (1:150 enzyme to substrate molar ratio, 2.5 °C, pH 7) after reaction times of (a) 10, (b) 15, (c) 20, and (d) 30 s. Reactions were stopped by acidification to pH 2.5 with formic acid. Component A designates the TEM-1/oxacillin complex with a measured molecular mass of 29 333 ± 3 Da, and component B designates TEM-1 with a measured molecular mass of 28 932 ± 3 Da. The number of charges is indicated after the component labels for each multicharged ion peak.

reaction times, two species with molecular masses of 29 333 ± 3 and 28 932 ± 3 Da were detected, corresponding to the acyl-enzyme and free TEM-1, respectively (Figure 1b,c). Analyses at even longer reaction times only revealed the presence of the free enzyme (Figure 1d). Figure 2a shows that the acyl-enzyme complex is the unique enzymatic form during the time course of the steady state of oxacillin hydrolysis. Corresponding analysis in the mass range of the antibiotic (data not shown) showed that the time at which the substrate is fully transformed correlates with the rapid disappearance of the acyl-enzyme complex to give the free enzyme.

Direct correlation between ESMS responses and concentration is allowed because of similar ionization behavior of the free and acylated enzymes. Indeed, no shift in the charge state distribution is observed upon acylation. Thus, multiple analyses of an equimolar mixture of TEM-1 and acylated TEM-1 gave spectra displaying two envelopes of identical charge state distribution and similar intensities (data not shown). The signal intensity corresponding to the acyl-enzyme was calculated to be 53 ± 3% of that of TEM-1. Another series of analyses showed that the detection limit of one of the two enzymatic species in solution was around 2%. Below this value, no minor signal was detected, and between 2 and 10%, a minor signal was detected but precise

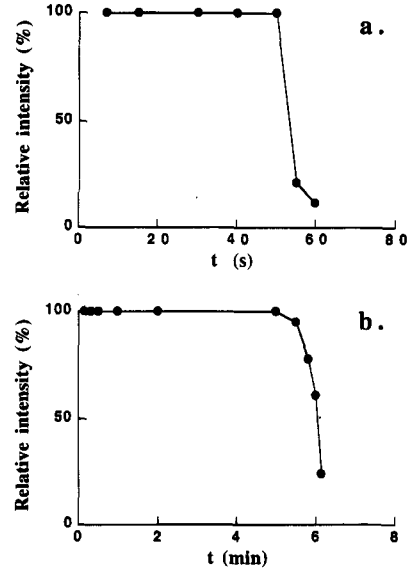


FIGURE 2: Plots of the evolution versus time of the (a) TEM-1/oxacillin complex for a 1:300 molar ratio and (b) TEM-1/cloxacillin complex for a 1:300 molar ratio [reaction mixture of TEM-1 (20 μL, 10 μM) and oxacillin or cloxacillin (3 μL, 10 mM)]. The reaction was stopped by acidification to pH 2.5 with formic acid. The percentage of acyl-enzyme complex has been defined as 100E-S/(E-S + E), where E-S and E are the peak heights of the complex and of the enzyme, respectively, after deconvolution of the raw data signal.

quantitation was difficult due to the low relative intensity of the signal. Thus, when only the acyl-enzyme is detected, it can reasonably be assumed that it represents at least 98% of the enzyme in solution.

The enzymatic reaction of TEM-1 with cloxacillin was conducted similarly to that with oxacillin. Figure 3a,b shows the raw data spectra in the m/z ranges of the enzyme and the antibiotic, respectively. The mass calculated for the acyl-enzyme complex (Figure 3a) is 29 370 ± 3 Da, corresponding to the addition of one molecule of cloxacillin (molecular mass = 435.5 Da) to one molecule of the enzyme. Figure 3b shows the simultaneous presence of cloxacillin (substrate) and hydrolyzed cloxacillin (product) in the reaction mixture. The plots of the evolution of the TEM-1/cloxacillin complex with time (Figure 2b) show that, as for oxacillin, the acyl-enzyme is the only species observed during the steady state of the reaction. It lasts for a longer time than that with oxacillin, in agreement with the lower k_{cat} value measured for cloxacillin (Table 1). Moreover, the almost complete disappearance of the substrate from the reaction mixture matches the decomposition of the enzyme−substrate complex (data not shown).

Under our experimental conditions, with both oxacillin and cloxacillin, the acyl-enzyme concentration during the steady state of the reaction was equal to the initial TEM-1 concentration. Calculation and comparison of k_{cat}, k_3, and k_2 rate constant values (Table 2) indicate that, for both substrates, deacylation is the limiting step of the reaction. k_3 and k_{cat} values are similar, and the acylation rate constant (k_2) values are about 3−4 times larger than the k_3 values.

Hydrolysis of Penicillins by G238S Mutant. The G238S substitution resulted in a significant loss of activity toward penicillins. The catalytic efficiencies obtained with penicillin G, cloxacillin, and oxacillin were 17−23% of those of TEM-1 (Table 1). The hydrolysis of penicillin G (Scheme

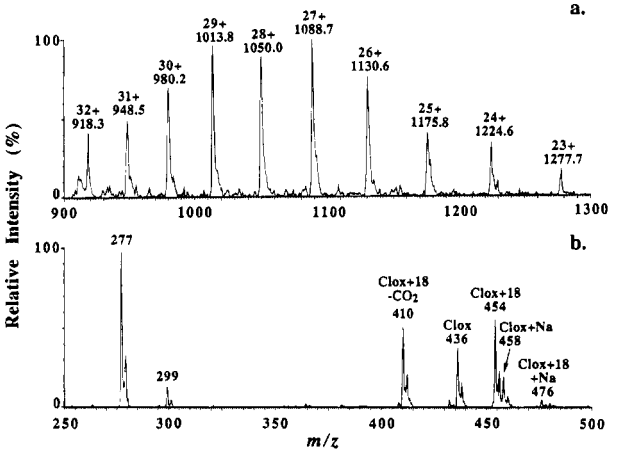


FIGURE 3: Electrospray mass spectra of the reaction mixture of TEM-1 (20 μL, 10 μM) and cloxacillin (3 μL, 20 mM) (1:300 enzyme to substrate molar ratio) at 2.5 °C and pH 7 after 1 min of reaction: (a) *m/z* 900−1300; (b) *m/z* 250−500. The measured molecular mass from the top spectrum is 29 370 ± 3 Da, which corresponds to the TEM-1/cloxacillin complex. In the bottom spectrum, peaks labeled Clox and Clox+18 correspond to protonated cloxacillin (substrate) and protonated hydrolyzed cloxacillin (product), respectively. Corresponding sodium adduct peaks are labeled Clox+Na and Clox+18+Na. The peaks found two *m/z* units higher than the labeled peaks are due to the ^{37}Cl isotope of chlorinated compounds. For the sake of clarity, the most abundant ^{35}Cl isotope is the only one labeled and referred to when necessary. The peak at *m/z* 277 corresponds to a fragment ion resulting from the cleavage of the β-lactam ring of cloxacillin, and the peak at *m/z* 410, labeled Clox+18-CO_2, results from decarboxylation (loss of 44 Da) of hydrolyzed cloxacillin. Corresponding sodium adducts are found 22 mass units higher.

Table 2: Kinetic Elementary Rate Constants for TEM-1 and G238S Mutant

	TEM-1				G238S			
	T (°C)	k_2 (s^{-1})	k_3 (s^{-1})	k_{cat} (s^{-1})	T (°C)	k_2 (s^{-1})	k_3 (s^{-1})	k_{cat} (s^{-1})
PG	20[a]	2800[a]	1500[a]	980[a]	2.5	≫16	16	15.3
OXA	2.5	104	38	27.8	37	≫0.7	0.7	0.69
CLOX	2.5	21	8	5.8	2.5	ND[c]	ND	ND
CTX	37		2.5[b]	≫2.5	2.5	37	20[b]	≫20	20.3

[a] Determined by Christensen *et al.* (1990). [b] Estimated from the k_{cat} value and eq 2, given that the k_3 value is very large. [c] ND, not determined.

2), which was too rapid with the wild-type TEM-1 even at 2.5 °C, could now be studied by ESMS. For the purpose of comparison with the wild-type enzyme, the substrates used for the study of the mutant enzyme were oxacillin and penicillin G.

The hydrolysis of oxacillin was followed as described for TEM-1, at both 2.5 and 37 °C. The analysis of the reaction mixture shows that the unique form of the enzyme at steady state is the acyl-enzyme complex with a molecular mass of 29 339 ± 4 Da (28 935 + 401 Da) (Figure 4a). In the mass range of the antibiotic, peaks corresponding to oxacillin and hydrolyzed oxacillin (18 mass units above) are observed (Figure 4b). At 2.5 °C, after a 10 min reaction time, the substrate is still present, while in the reaction between wild-type TEM-1 and oxacillin, all of the substrate is consumed within 3 min. This is in line with the loss of catalytic efficiency for oxacillin due to the G238S substitution. The concentration of the acyl-enzyme at the steady state of the reaction is equal to the initial enzyme concentration, and the

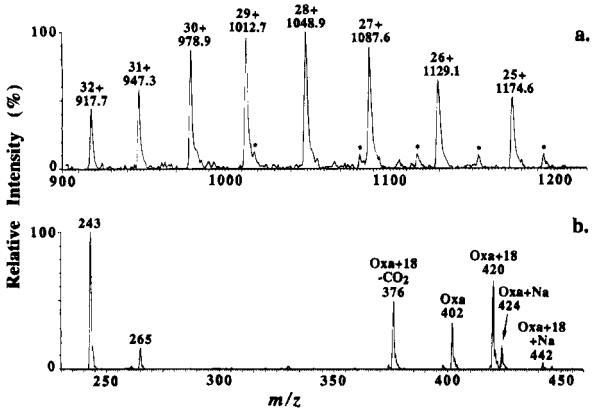


FIGURE 4: Electrospray mass spectra of the reaction mixture of the G238S variant of TEM-2 β-lactamase (10 μL, 6 μM) and oxacillin (4 μL, 10 mM) (1:500 enzyme to substrate molar ratio) at 2.5 °C and pH 7 after 5.5 min of reaction: (a) *m/z* 900−1220; (b) *m/z* 230−460. The calculated mass of the acyl-enzyme complex from the top spectrum is 29 339 ± 4 Da. Asterisks indicate contamination by a heavier protein that coeluted with the β-lactamase. In the bottom spectrum, peaks labeled Oxa and Oxa+18 correspond to protonated oxacillin (substrate) and protonated hydrolyzed oxacillin (product), respectively. The peak at *m/z* 243 corresponds to a fragment ion resulting from the cleavage of the β-lactam ring of oxacillin, and the peak at *m/z* 376, labeled Oxa+18-CO_2, results from decarboxylation (loss of 44 Da) of hydrolyzed oxacillin. Corresponding sodium adducts are found 22 mass units higher.

k_3 determined value at 37 °C is 0.7 s^{-1}, which is identical to the k_{cat} value (Table 2) measured from steady state enzyme kinetics. It expresses the fact that the deacylation step is the rate-limiting step for the hydrolytic reaction of oxacillin.

The hydrolysis of penicillin G with the G238S mutant was studied in the same way. The reaction was performed at 2.5 °C because of the rapidity of hydrolysis at 37 °C (Table 1). The acyl-enzyme complex was observed with a molecular mass of 29 270 ± 3 Da, exactly corresponding to the addition of one molecule of penicillin G (334 Da) to the G238S β-lactamase (28 935 Da). It was the unique enzymatic form at steady state, and the k_3 and k_{cat} values were almost identical (16 s^{-1}).

For the two penicillin substrates, the difference between k_{cat} and k_3 is too small to compute reliable values of k_2. All experimental values, however, show that the loss of activity against penicillins upon the G238S mutation is due to a considerable decrease in the deacylation rate.

Hydrolysis of Cefotaxime by Wild-Type TEM-1. Cefotaxime (Scheme 2) was characterized by ESMS. Its spectrum displays a peak corresponding to the singly charged, protonated antibiotic. When cefotaxime is hydrolyzed, a characteristic peak appears corresponding to a fragment ion of the protonated cephalosporoic acid, resulting from the loss of acetic acid (data not shown).

In spite of the high K_m value of cefotaxime for TEM-1 compared to other substrates, the k_{cat} value is adequate to follow the reaction at 37 °C (Table 1). At all reaction times, even at the beginning of the reaction, the free enzyme is the only observed species. Nevertheless, the reaction product is formed (data not shown). This means that the acyl-enzyme intermediate does not accumulate, indicating that the deacylation step occurs faster than the acylation step. Even though the k_3 value could not be determined under these conditions, it can be assumed that its value is significantly larger than that of k_2.

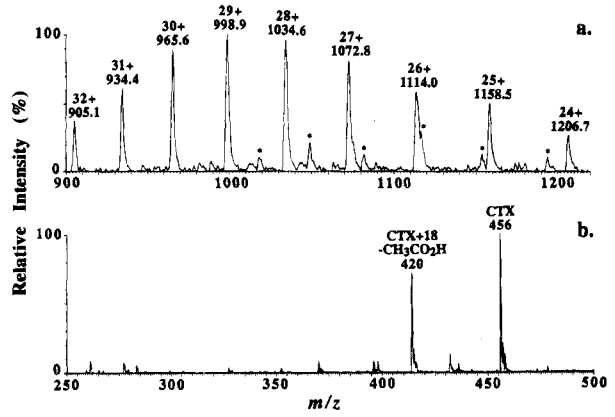


FIGURE 5: Electrospray mass spectra of the reaction mixture of the G238S variant of TEM-2 β-lactamase (10 μL, 6 μM) and cefotaxime (2 μL, 20 mM) (1:500 enzyme to substrate molar ratio) at 2.5 °C and pH 7 after 3 min of reaction: (a) *m/z* 900−1300; (b) *m/z* 100−500. From the top spectrum, the measured molecular mass is 28 938 ± 4 Da, which corresponds to the free G238S variant. Asterisks indicate a higher molecular mass contaminant. In the bottom spectrum, peaks labeled CTX and CTX+18-CH_3COOH correspond to protonated cefotaxime (substrate) and a fragment ion resulting from the loss of acetic acid (loss of 60 Da) from hydrolyzed cefotaxime, respectively.

Hydrolysis of Cefotaxime by G238S Mutant. The value of the catalytic constant for hydrolysis (k_{cat}) indicates that the suitable temperature at which to study the reaction is 2.5 °C (Table 1). The reaction mixture was analyzed at different times. In all cases, the acyl-enzyme complex was not detected and only the free enzyme was observed (Figure 5a). Nevertheless, the substrate disappeared and the product was formed (Figure 5b).

For hydrolysis of cefotaxime, the G238S mutant and the wild-type TEM-1 behave similarly, *i.e.*, the acylation step limits the overall reaction rate. Comparison of the results obtained for the hydrolysis of cefotaxime and penicillins by both the wild-type TEM-1 and the G238S mutant reveals that their behavior is very different in terms of the rate-limiting step, the deacylation step being more rapid than the acylation step in the case of cefotaxime.

DISCUSSION

ESMS is a new method that has been used mainly to determine molecular masses of biomolecules, such as proteins. This soft ionization technique allows the formation of gas phase macromolecular multicharged ions directly from solution via ion evaporation (Covey *et al.*, 1988; Smith *et al.*, 1990; Fenn *et al.*, 1989). The high charge state obtained in this way allows the detection of these protein ions in an *m/z* range amenable to conventional quadrupole analyzers. This technique has recently been applied to the detection of covalent enzyme−substrate (Stevenson *et al.*, 1992; Aplin *et al.*, 1990; Leung *et al.*, 1994) and enzyme−inhibitor complexes (Ménard *et al.*, 1991; Rahill & Pratt, 1994). For β-lactamases, the covalent acyl-enzyme intermediate in the mechanism of hydrolysis of β-lactams can be observed by using ESMS (Aplin *et al.*, 1990). However, to determine the acylation and deacylation rate constants k_2 and k_3, quantitative evaluation of the reaction intermediates is required. Different indirect methods have been described to determine k_3, using either a reporter substrate (Fisher *et al.*, 1980; Galleni *et al.*, 1988; Monnaie *et al.*, 1992), quenched-flow experiments in viscous medium (Christensen *et al.*, 1990), or penamaldate precipitation of the acyl-enzyme (Martin & Waley, 1988). Thanks to the ability of ESMS to detect the enzyme and its covalent complex with the substrates during the enzymatic reaction, the proportion of acyl-enzyme complex and free enzyme at the steady state could be determined, thus leading to the quantitation of the acyl-enzyme complex and direct determination of the deacylation constant (see Materials and Methods). The conditions for applying this method are that the overall reaction should be sufficiently slow (relatively low k_{cat} values) and that the deacylation rate should be slower than the acylation rate to enable the detection of acyl-enzyme species. The kinetic properties of the G238S mutant allowed us to use ESMS to study the effect of this substitution on the acylation and deacylation steps of β-lactam hydrolysis. Although quantitative rate constants could not always be determined, the rate-limiting step between acylation and deacylation could be identified, providing a better understanding of the catalytic events of this mutant.

The kinetic results for penicillin hydrolysis by β-lactamase TEM-1 show that the deacylation rate k_3 is 3−4 times smaller than the acylation rate constant k_2, indicating that the former is rate limiting. This is consistent with Christensen's results (1990), where the values of k_2 and k_3 measured by quenched-flow experiments with penicillin G were similar, k_2 being larger than k_3 (Table 2). The k_{cat} for cefotaxime is 5−500 times lower than those of penicillins, but the acylation step is now limiting the reaction ($k_2 \ll k_3$) (Table 2). These results are consistent with the fact that β-lactamase TEM-1 is a penicillinase. It was shown from the TEM-1 X-ray structure at 1.8 Å resolution (Jelsch *et al.*, 1993) that the active site, which is at the interface of the two domains of the protein, is more closed compared to that of the homologous PC1 enzyme. The large number of hydrogen bond interactions are not in favor of large conformational changes, and it was reported in the acyl-enzyme structure of the E166N mutant enzyme (Strydnadka *et al.*, 1992) that structural differences occurred upon substrate binding. The chemical structure of cefotaxime, a third-generation cephalosporin, differs from those of penicillins (Scheme 2) by the presence of a large substituent on the β-lactam ring that likely impairs cephalosporin access to the substrate-binding site. The few cefotaxime molecules that could reach the active site lead to acyl-enzyme complexes, which are very rapidly deacylated to give the free enzyme and hydrolyzed cefotaxime. The small k_2 value likely expresses the inappropriate shape of the wild-type active site to covalently bind cefotaxime.

The efficiency of penicillin hydrolysis by the G238S mutant is 17−23% of that of TEM-1, and the calculated k_3 values are almost equal to the k_{cat} values. The very small difference between k_3 and k_{cat} does not allow the determination of k_2, but it is clear from the observation of the acyl-enzyme by ESMS that the deacylation step is limiting the reaction with penicillin substrates. From the results in Table 2, it is also apparent that the decrease in the k_{cat} values is mainly, or totally, due to a decrease in the deacylation rate constant k_3, which is reduced by 98% for oxacillin. Modeling of the G238S substitution on the three-dimensional structure of β-lactamase TEM-1 suggests that the hydroxyl group of the serine might be too close to the asparagine 170 main chain atoms (Figure 6). Release of the steric constraints seems most easily achieved through movement of the Ω-loop

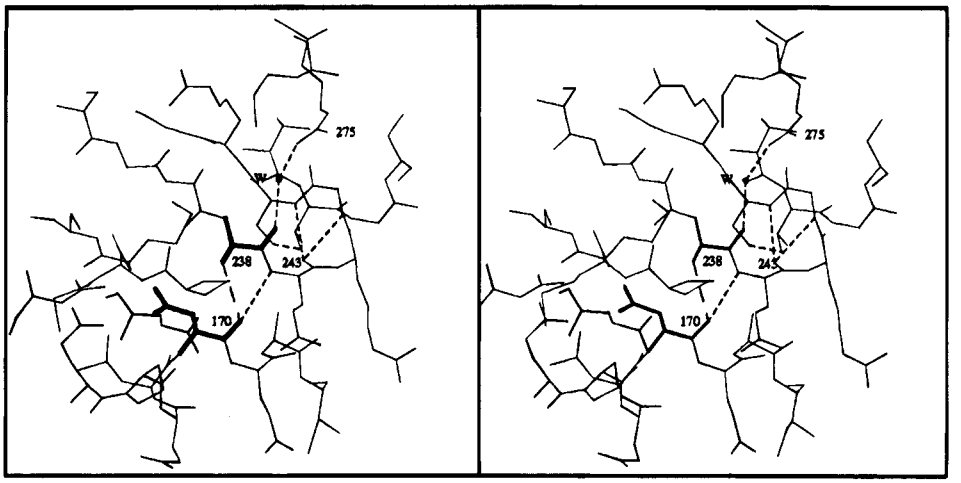


FIGURE 6: Stereoview of the G238S mutation modeled on the 1.8 Å resolution TEM-1 structure. Short-dashed lines indicate the hydrogen bond interactions. Ser 243 OG interacts with Thr 266 OG1 from strand S5. The large-dashed line represents the 3.2 Å distance between the CB atom of residue 238 and the main chain oxygen atom of residue 170.

(residues 161−179) that forms one edge of the substrate-binding site and carries the deacylating glutamic acid 166. The proper orientation of this essential residue is provided by the *cis* peptide bond of the proline 167, and its side chain is oriented by a hydrogen bond with the asparagine 170 side chain and by van der Waals contacts with the buried leucine 169 (Jelsch *et al.*, 1993). Movement of the Ω-loop should affect the position of the glutamic acid 166 side chain, resulting in a less efficient deacylation reaction of the acyl-enzyme complex, which would explain the decreased k_{cat} values observed for hydrolysis of penicillin substrates.

In the case of cefotaxime hydrolysis by the G238S mutant enzyme, the 73-fold increase in the catalytic efficiency is the result of both a better K_m for the β-lactam antibiotic and a higher k_{cat} value. As with the wild-type TEM-1 β-lactamase, no covalent acyl-enzyme complex but hydrolyzed cefotaxime is detected by ESMS, indicating that the acylation step is limiting the overall catalytic reaction. The 8-fold increase in the k_{cat} value for the mutant enzyme compared to the wild-type enzyme, in which acylation is the rate-limiting step, must then result from an increase in the k_2 value, which still remains much lower than the k_3 value. Two hypotheses so far have been proposed to explain the kinetic properties of the G238S mutant. The first one, proposed by Labia *et al.* (1988) and Barthélémy *et al.* (1988), suggests a direct interaction between the hydroxyl group of serine 238 and the oxime nitrogen of cefotaxime resulting in a new hydrogen bond, which would overcome the steric hindrance and allows the binding of cefotaxime and, hence, its hydrolysis. Lenfant *et al.* (1990) have shown that this hypothesis is unlikely because the G238A substitution also leads to an extended-spectrum β-lactamase, and because hydrolysis of cefotaxime occurs even though the formation of a hydrogen bond cannot be postulated. The analysis of the wild-type X-ray structure and the molecular modeling clearly show that the serine at position 238 is not oriented so as to form an hydrogen bond with cefotaxime in the active site. The second hypothesis has been proposed by Huletsky *et al.* (1993). It is based on a modeling study of the SHV-2 enzyme from the refined crystal structure of the sequence-homologous *Bacillus licheniformis* 749/C β-lactamase. They proposed that the substitution of glycine 238 on the S3 strand (residues 230−238) by a serine induces a movement of the C-terminal edge of this strand away from the active site by about 1−2 Å, allowing the cefotaxime to enter the catalytic pocket and be hydrolyzed more efficiently. Modeling of the G238S substitution on the three-dimensional structure of TEM-1 indicates that the CB carbon atom is 3.2 Å from the asparagine 170. This distance is smaller than a normal van der Waals contact, and this steric effect could be released through a slight movement of either one of these residues.

The number of hydrogen bonds found in the 238−243 region (Figure 6) suggests important conformational and positional constraints that are not in favor of movement of the C-terminal edge of strand S3. On the contrary, a single hydrogen bond is exchanged between O170 and N240 main chain atoms, and it seems that the substitution would more easily induce a displacement of part of the Ω-loop. The conformational constraints in this loop provided by the salt bridge R164−E171, *cis*-P167, and the hydrogen bond exchanged between N170 and E166 side chains might explain that a slight displacement of the main chain atoms of residue 170 could affect the position of E166. This would explain the decreased k_{cat} values observed for penicillin substrate hydrolysis and the less efficient deacylation reaction in that case. Such structural changes would result in better access of cefotaxime to the active site and, thus, a more efficient acylation of cephalosporin. One can hypothesize that both the acylation and deacylation steps are affected by this change of the active site conformation for both cefotaxime and penicillins. The hypothesis that a slight displacement of the Ω-loop could, in some way, be responsible for the hydrolysis of cefotaxime is in line with the kinetic results of Sowek *et al.* (1991) and Delaire *et al.* (1991) on mutants of Ω-loop residues. Arginine 164 and glutamic acid 166 of the β-lactamase TEM-1 were substituted by a serine and a tyrosine, respectively. In both cases, a better K_m for cephalosporins was measured and an extension of the substrate spectrum to the cephalosporins was observed. These data and our structural interpretation of the G238S catalytic properties based on the X-ray structure of the TEM-1 enzyme suggest that there might be a link between the hydrolysis of cephalosporins and structural changes in this area.

ACKNOWLEDGMENT

We are indebted to Arlette Savagnac for skillful technical assistance. We thank Neil Johnson for reading the manuscript, Marie-Hélène Remy for helpful discussions, and Lionel Mourey for efficient help in computer modeling.

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BI950273K