Kinetic analysis of an inhibitor-resistant variant of the OHIO-1 β-lactamase, an SHV-family class A enzyme

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The Met^{69} → Ile mutant of the OHIO-1 β-lactamase, an SHV-family enzyme, is resistant to inactivation by β-lactamase inhibitors. Analysis of purified Met^{69} → Ile enzyme reveals that its isoelectric point (pI 7.0) and CD spectrum are identical with those of the OHIO-1 enzyme. Levels of β-lactamase expression in *Escherichia coli* as determined by immunoblotting are similar for OHIO-1 and Met^{69} → Ile β-lactamase. The kinetic constants of the Met^{69} → Ile enzyme compared with OHIO-1 are smaller for benzylpenicillin ($K_m = 6\ \mu M$ compared with $17\ \mu M$; $k_{cat} = 234\ s^{-1}$ compared with $345\ s^{-1}$ respectively) and carbenicillin ($K_m = 3\ \mu M$ compared with $17\ \mu M$; $k_{cat} = 131\ s^{-1}$ compared with $320\ s^{-1}$ respectively). For the cephalosporins cephaloridine and 7-(thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethylaminophenylazo)-pyridinium-methyl]-3-cephem-4-carboxylic acid (PADAC), a similar pattern is also seen ($K_m = 38\ \mu M$ compared with $96\ \mu M$ and $6\ \mu M$ compared with $75\ \mu M$ respectively; $k_{cat} = 235\ s^{-1}$ compared with $1023\ s^{-1}$ and $9\ s^{-1}$ compared with $50\ s^{-1}$ respectively). Consistent with minimum inhibitory concentrations that show resistance to β-lactam β-lactamase inhibitors, the apparent K_i values, turnover numbers and partition ratios (k_{cat}/k_{inact}) for the mechanism-based inactivators clavulanate, sulbactam and tazobactam are increased. The inactivation rate constants (k_{inact}) are decreased. The difference in activation energy, a measurement of altered affinity for the wild-type and mutant enzymes leading to acylation of the active site, reveals small energy differences of less than 8.4 kJ/mol. In total, these results suggest that the Met → Ile substitution at position 69 in the OHIO-1 β-lactamase alters the active site, primarily affecting the interactions with β-lactamase inhibitors.

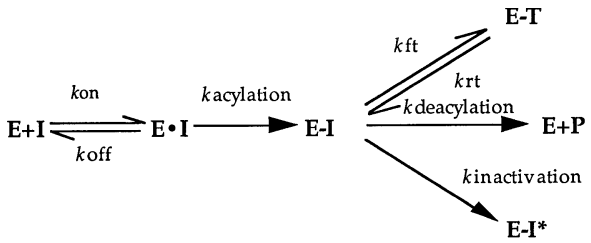
INTRODUCTION

The most common and important mechanism of bacterial resistance to β-lactam antibiotics is the production of β-lactamase enzymes. β-Lactamases are bacterial periplasmic enzymes that hydrolyse β-lactam antibiotics into inactive acids. Four classes of β-lactamases have been described. Classes A, C and D are serine hydrolytic enzymes; class B β-lactamases are zinc metalloenzymes [1,2]. So far more than 190 distinct enzymes have been recorded [3]. The diversity of these enzymes is matched only by their great catalytic efficiency. The rapid spread and evolution of these plasmid and chromosomally encoded enzymes have seriously threatened our antimicrobial arsenal [4].

To combat the resistance conferred by β-lactamase enzymes two strategies are employed. The first is to design or discover β-lactam antibiotics that are able to escape hydrolysis yet still are effective inactivators of penicillin-binding proteins (for example the penicillinase-resistant penicillins and oxyiminocephalosporins). The second is to couple a β-lactam antibiotic to a β-lactamase inhibitor. These inhibitors irreversibly inactivate class A β-lactamases. Ampicillin/sulbactam, amoxicillin/clavulanate, ticarcillin/clavulanate and piperacillin/tazobactam are marketed examples of this tactic.

Mechanistically, the β-lactamase inhibitor (I) first binds reversibly to the β-lactamase (Scheme 1). The enzyme is then acylated (E–I). The acyl intermediate has three potential fates: (1) hydrolysis of the active site ester resulting in the regeneration of active enzyme and hydrolysed inhibitor; (2) the formation of an irreversibly inactivated enzyme by secondary covalent modification; and (3) undergoing a reversible change that generates a transiently inhibited enzyme. This transiently inhibited enzyme is called a tautomer, and the process of generating a transiently inhibited enzyme is called tautomerization.

Point mutations in the TEM and SHV family of the class A β-



Scheme 1 Mechanism of activation of class A β-lactamase by inhibitors

Abbreviations: E, β-lactamase; I, inhibitor; E·I, Michaelis complex; E–I, acyl enzyme; E–T, tautomer; P, product; E–I*, irreversibly inactivated enzyme; *k*ft, forward tautomerization; *k*rt, reverse tautomerization.

Abbreviations used: LB, Luria–Bertani; PADAC, 7-(thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethylaminophenylazo)pyridinium-methyl]-3-cephem-4-carboxylic acid; pIEF, preparative isoelectric focusing.

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lactamase are being described in the clinic and the laboratory that alter the ability of various inhibitors to inactivate the enzyme. Among the most common amino acid changes in TEM that confer resistance to inactivation by mechanism-based inhibitors are mutations at positions Met^{69}, Arg^{244} and Asn^{276} [5]. So far the only inhibitor-resistant β-lactamases that have been reported in the SHV family are the SHV-10 β-lactamase and laboratory isolates of the SHV-1, SHV-5 and OHIO-1 variety [6–10]. These include mutations at position 69 ($Met^{69} \rightarrow Ile$) and position 276 ($Asn^{276} \rightarrow Gly$) in the OHIO-1 β-lactamase, and positions 130 and 244 ($Ser^{130} \rightarrow Gly$, $Arg^{244} \rightarrow Ser$ and $Arg^{244} \rightarrow Cys$) in the SHV family. Although the TEM family of enzymes that possess resistance to β-lactamase inhibitors are the most prevalent, it is feared that with the extensive use of oral and intravenous β-lactam/β-lactamase inhibitor combinations there will also be an increase in the prevalence of resistant SHV enzymes found in the clinic. In this paper we describe in detail the kinetic interactions of a class A β-lactamase of the SHV family (the $Met^{69} \rightarrow Ile$ mutant of the OHIO-1 β-lactamase) that is resistant to inactivation by the mechanism-based inhibitors clavulanic acid, sulbactam and tazobactam.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli strain DH5α (*supE44*, Δ*lacU169*)(*Ø*80 *lacZ*ΔM15) hsdR17, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*) was employed in the experiments described. The OHIO-1 and $Met^{69} \rightarrow Ile$ β-lactamase genes were directionally subcloned into the phagemid vector pBC SK (−) (Stratagene, La Jolla, CA, U.S.A.), which encodes for chloramphenicol acetyltransferase (chloramphenicol resistance), and transformed into *E. coli* DH5α [7–9]. Plasmids were isolated with a Wizard Miniprep Kit (Promega Co., Madison, WI, U.S.A.). All bacteria were grown in Luria–Bertani (LB) broth and agar containing the appropriate antibiotic selection (20 μg/ml chloramphenicol or 100 μg/ml ampicillin and 20 μg/ml chloramphenicol). Experiments in our laboratory have shown that there is no effect of chloramphenicol on the level of expression of β-lactam resistance. Restriction enzymes were obtained from Promega (Madison, WI, U.S.A.) and United States Biochemical (Cleveland, OH, U.S.A.).

Chemicals

Benzylpenicillin, carbenicillin, ampicillin and cephaloridine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nitrocefin was purchased from Becton Dickinson Microbiological Systems (Cockeysville, MD, U.S.A.). 7-(Thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethylaminophenylazo)pyridiniummethyl]-3-cephem-4-carboxylic acid (PADAC) is a Calbiochem product (La Jolla, CA, U.S.A.). Clavulanate was kindly provided by Smith Kline Beecham Laboratories (Bristol, TN, U.S.A.). Tazobactam was provided by Lederle Laboratories (Pearl River, NY, U.S.A.). Sulbactam was a gift from Pfizer (New York, NY, U.S.A.). The chemical structures of these compounds are shown in Figure 1.

Phenotypic characterization

Minimum inhibitory concentrations were determined on LB agar plates with a Replicator (Chester, PA, U.S.A.) that delivered 10^4 colony-forming organisms per spot. After inoculation each plate was incubated for 18 h at 37 °C.

β-Lactamase purification

The OHIO-1 and $Met^{69} \rightarrow Ile$ β-lactamases were purified to homogeneity by preparative isoelectric focusing (pIEF). *E. coli* DH5α containing the OHIO-1 and $Met^{69} \rightarrow Ile$ β-lactamase genes was grown in LB broth containing 100 μg/ml ampicillin and 20 μg/ml chloramphenicol. β-Lactamase was liberated by stringent periplasmic fractionation with lysozyme by the method of Johnson and Hecht [11]. After liberation of the β-lactamase, crude lysates were clarified by passage through a Centricon filter. Samples were next concentrated with an Amicon concentrator with a 10 kDa cut-off.

pIEF was performed with a Multiphor II apparatus (Pharmacia, Piscataway, NJ, U.S.A.) with a pH gradient of 6–8. Ultradex gels were prepared with a concentrated protein sample in accordance with specifications outlined by the manufacturer. The pIEF gels were run overnight. β-Lactamase activity was identified by using finely cut strips of filter paper applied to the top of the gel matrix. The area where the β-lactamase was focused was identified by soaking strips of paper with nitrocefin and observing the colour change. Areas demonstrating β-lactamase activity were removed from the gel; the active enzyme was eluted from PEGG columns (Pharmacia, Piscataway, NJ, U.S.A.) with 20 μM potassium phosphate buffer, pH 7.4. Ampholines were removed by extensive dialysis in potassium phosphate buffer. Samples were again concentrated with Amicon filters (10 kDa cut-off) and purity was assessed by SDS/PAGE [15 % (w/v) gel]. Purified enzymes were stored at 4 °C until use. Specific activity was determined as μmol of nitrocefin hydrolysed per min divided by the total protein concentration.

To determine the precise pI of the purified enzymes, analytical isoelectric focusing was performed using pH gradients of 3–11 and 6–8 using the Bio-Rad apparatus (Bio-Rad, Hercules, CA, U.S.A.). β-Lactamase activity was detected by nitrocefin staining, by the method of Vecoli et al. [12]; the homogeneity of the sample was further assessed by staining with Coomassie Blue. The first 20 residues of the purified wild-type enzyme were sequenced at the Molecular Biology Core Facility at Case Western Reserve University School of Medicine (Cleveland, OH, U.S.A.).

Immunoblotting

Steady-state expression levels of OHIO-1 and $Met^{69} \rightarrow Ile$ β-lactamases were determined by immunoblotting, with a modification of the method developed by Petrosino and Palzkill [13]. *E. coli* and DH5α cells containing the wild-type and $Met^{69} \rightarrow Ile$ β-lactamases were grown in LB broth containing ampicillin and chloramphenicol. Cell pellets were suspended in Tris/HCl buffer, pH 7.4, and treated with lysozyme and EDTA. Crude lysates were tested for their ability to hydrolyse nitrocefin. Equal amounts of crude lysate (25 μg of protein) were resolved on SDS/PAGE gels containing 15 % (w/v) polyacrylamide. Proteins were next transferred to nitrocellulose membranes by electroblotting. The blot was probed by an anti-TEM polyclonal antibody kindly provided by T. Palzkill. We have repeatedly shown in our laboratory that this antibody cross-reacts with SHV-1 type enzymes. Bound antibodies were revealed by using anti-rabbit antibodies labelled with horseradish peroxidase and with the Amersham Western Blot Kit.

Kinetic analysis

Protein concentration was determined by absorbance measurements at 280 nM ($\Delta\epsilon$ 33400 $M^{-1} \cdot cm^{-1}$) [14]. A uniform concentration of enzyme was used for determining hydrolytic activity

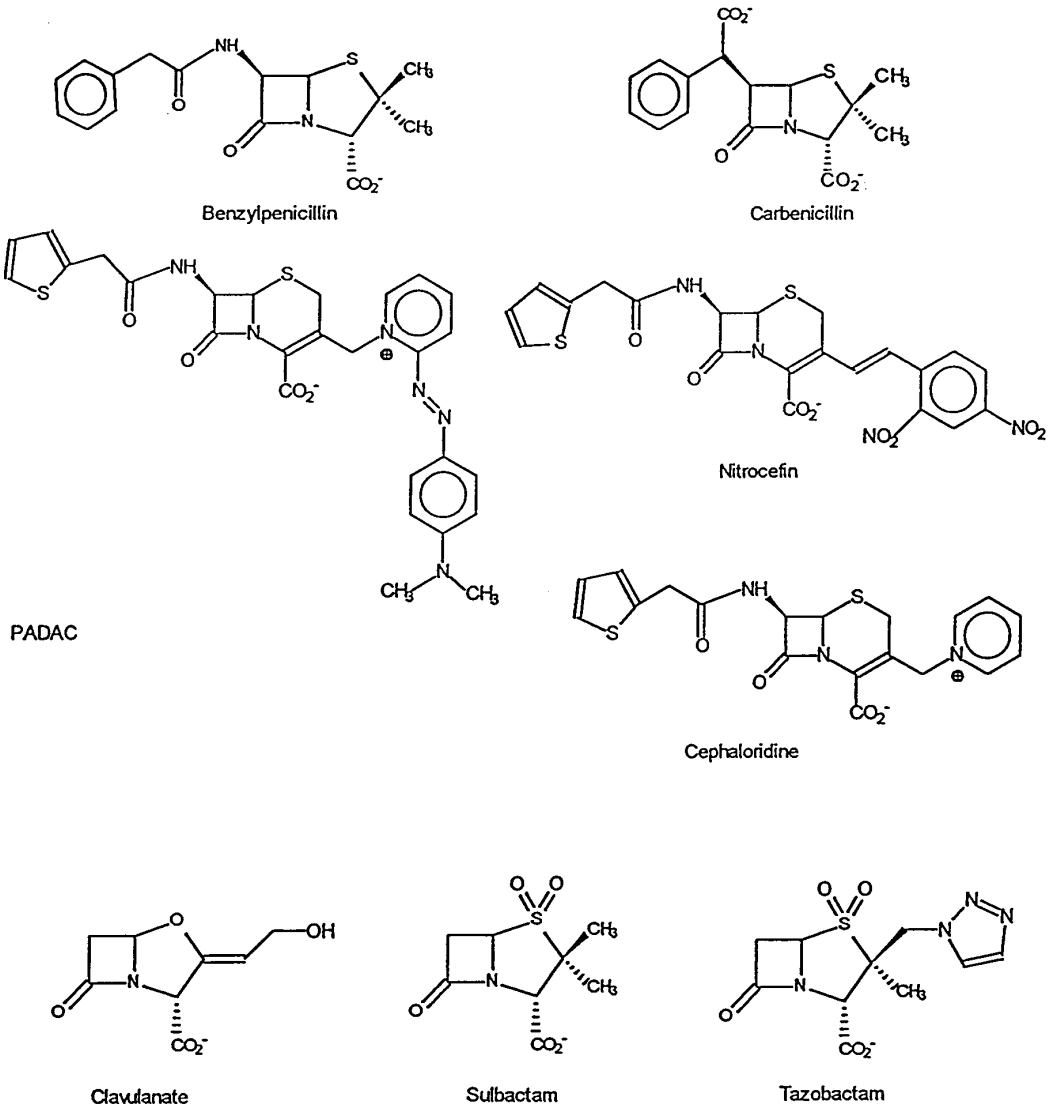

Figure 1 Chemical structures of β-lactam antibiotics used in this study

(typically 1.2 nM β-lactamase). β-Lactamase activity was monitored with a spectrophotometer at 25 °C in 20 mM phosphate buffer, pH 7.4.

Hydrolysis of the β-lactam substrates was performed at 25 °C in a HP 8453 UV–visible spectrophotometer in a cell with a 1 cm path length. The spectrophotometric assays of β-lactamase activity were performed in 20 mM phosphate buffer, pH 7.4. The molar absorption coefficients used were as follows: benzylpenicillin, $\Delta\epsilon_{240}$ 529 $M^{-1}\cdot cm^{-1}$; carbenicillin, $\Delta\epsilon_{240}$ 400 $M^{-1}\cdot cm^{-1}$; nitrocefin, $\Delta\epsilon_{482}$ 17400 $M^{-1}\cdot cm^{-1}$; cephaloridine, $\Delta\epsilon_{267}$ 1000 $M^{-1}\cdot cm^{-1}$; PADAC, $\Delta\epsilon_{466}$ 9590 $M^{-1}\cdot cm^{-1}$; sulbactam, $\Delta\epsilon_{236}$1780 $M^{-1}\cdot cm^{-1}$.

Initial rates (v) were determined from the first 5–10 % of the reactions at various substrate concentrations. The data were fitted to the Michaelis–Menten equation by a non-linear least-squares algorithm with the program ENZFITTER (Sigma). Each determination of V_{max} and the K_m possessed an S.E.M. of approx. 10 %.

For the determination of the dissociation equilibrium constant, K_i, the enzymes (1 nM) and stock concentrations of clavulanate, sulbactam and tazobactam were preincubated for 10 min at room temperature before the addition of a uniform concentration of indicator substrate (100 μM nitrocefin), which served as a competitor. All inhibition experiments were performed in 20 μM phosphate buffer, pH 7.4. Standard plots of $1/v$ against [I] (Dixon plots) were prepared and the apparent K_i was calculated.

To measure the turnover number (t_n) of the enzyme for the inhibitors, each inhibitor and enzyme were incubated at various concentrations for 10 min and the residual activity was measured. The turnover value was deduced from the extrapolated value for 100 % inactivation from the plot of the remaining activity against the ratio of [I] to [E] [15].

The partition ratios (k_{cat}/k_{inact}) for the inhibitors with wild-type and mutant enzymes were determined by the titration method, as described by Imtiaz et al. [16]. Various ratios of inhibitor and enzyme concentrations were mixed and incubated overnight at 4 °C (at least 18 h). The enzyme activity remaining was assayed by monitoring the rate of hydrolysis of 100 μM nitrocefin. Control samples containing enzymes without the inactivator were similarly tested. Residual activity was then

assayed and a plot was made of the activity remaining against the inhibitor-to-enzyme ratio.

The maximal inactivation rate constant, k_{inact}, was obtained as the absolute value of the slope of $\ln v$ against time (t). A large excess of nitrocefin was added and the remaining activity (v) was measured. For clavulanate and tazobactam, k_{cat} could not be measured directly. For sulbactam a k_{cat} (rate of hydrolysis) was measured directly ($\Delta\epsilon_{236}$ 1780 $M^{-1} \cdot cm^{-1}$).

CD measurements

CD spectra were recorded on a Jasco Model J-600 spectropolarimeter. The protein concentration was 6.9 μM for both the wild-type and mutant enzymes in 20 μM potassium phosphate, pH 7.4. We used quartz cuvettes with a 1 cm path length.

RESULTS

Minimum inhibitory concentration

To evaluate the phenotypic effect of the Met^{69} → Ile mutation in the OHIO-1 β-lactamase enzyme, the wild-type and Met^{69} → Ile β-lactamase genes were directionally subcloned into the phagemid vector pBC SK(−). This construct was transformed into a uniform genetic background, *E. coli* DH5α. The wild-type and the Met^{69} → Ile-bearing strains were tested against ampicillin, ampicillin/clavulanate and ampicillin/sulbactam. The results are summarized in Table 1. The Met^{69} → Ile β-lactamase expressed in *E. coli* DH5α was more resistant than the wild-type enzyme to β-lactam/β-lactamase inhibitor combinations [7,8].

Table 1 Minimum inhibitory concentration for *E. coli* DH5α with and without phagemid vector pBC SK(−)

OHIO-1 and Met^{69} → Ile are isogenic strains bearing the OHIO-1 and Met^{69} → Ile β-lactamases.

	Minimum inhibitory concentration (μg/ml)		
	Ampicillin	Ampicillin/clavulanate	Ampicillin/sulbactam
DH5α without pBC SK(−)	2	⩽ 2/0	⩽ 2/0
DH5α with pBC SK(−)	2	⩽ 2/0	⩽ 2/0
OHIO-1	4096	8/4	128/64
Met^{69} → Ile	2048	32/16	256/128

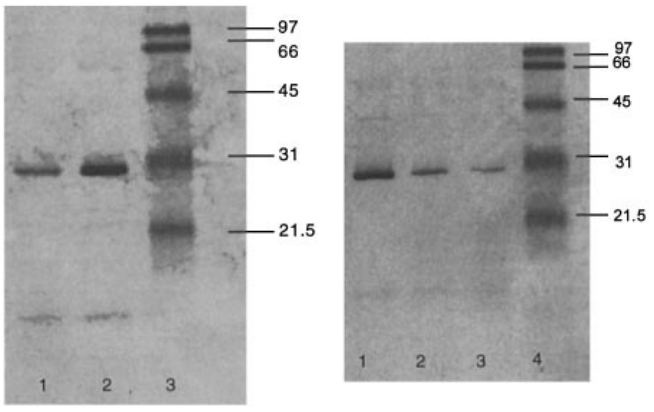

Figure 2 Purified protein preparations of the OHIO-1 and Met^{69} → Ile β-lactamases resolved by SDS/PAGE [15% (w/v) gel]

Left panel: lanes 1 and 2, purified OHIO-1 β-lactamase from different pIEF gels; lane 3, molecular mass markers (identified in kDa at the right). Right panel: lanes 1–3, purified Met^{69} → Ile β-lactamase from different pIEF gels; lane 4, molecular mass markers (identified in kDa at the right).

Figure 3 Immunoblots of crude lysates of OHIO-1 and Met69 → Ile β-lactamases resolved by SDS/PAGE [15% (w/v) gel]

Left lane, OHIO-1 β-lactamase; right lane, Met^{69} → Ile β-lactamase.

Purification and characterization of the OHIO-1 and Met69 → Ile β-lactamases

The OHIO-1 and Met^{69} → Ile β-lactamase were purified to homogeneity (Figure 2). The specific activity of samples prepared in this manner increased 140–150-fold. The typical yield of enzyme by our purification method was 20%. N-terminal amino acid sequencing revealed that the mature protein began at ABL residue Ser^{26} [16a]. Isoelectric focusing experiments demonstrated that the pI values for OHIO-1 and Met^{69} → Ile β-lactamases were both 7.0. The far-UV CD spectra of the OHIO-1 and Met^{69} → Ile mutant β-lactamases at pH 7.4 were nearly superimposable.

Immunoblotting

β-Lactamase expression levels *in vivo* were examined by immunoblotting; crude lysates were screened. Identical amounts of protein were resolved on a SDS/PAGE gel. Immunoblots with anti-TEM polyclonal antibodies revealed that the expression levels of OHIO-1 and Met^{69} → Ile β-lactamases were similar (Figure 3).

Kinetic analysis

The K_m values for benzylpenicillin, carbenicillin, PADAC and cephaloridine were lower with the Met^{69} → Ile β-lactamase than with OHIO-1 β-lactamase (Table 2). Similarly, the k_{cat} values for the Met^{69} → Ile mutant were lower for all substrates. The catalytic efficiencies, defined as k_{cat}/K_m, were larger for some substrates (penicillin, carbenicillin and PADAC) but smaller for others (cephaloridine and nitrocefin). Nitrocefin was the only β-lactam substrate that demonstrated a slightly larger K_m.

The kinetic impact of the Met^{69} → Ile substitution against irreversible inhibitors was quite pronounced (Table 3). The dissociation equilibrium constant, K_i, for each inhibitor with the wild-type and mutant enzymes was increased significantly. These

Table 2 Kinetic parameters of OHIO-1 and Met^{69} → Ile β-lactamases

For all results the S.E.M. was ±10%.

Substrate	Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	$\Delta G^{\ddagger}_{app}$ (kJ/mol)
Benzylpenicillin	OHIO-1	17	345	20	
	Met^{69} → Ile	6	234	39 ↑	−1.7
Carbenicillin	OHIO-1	17	320	19	
	Met^{69} → Ile	3	131	44 ↑	−2.1
PADAC	OHIO-1	75	50	0.6	
	Met^{69} → Ile	6	9	1.5 ↑	−2.1
Cefaloridine	OHIO-1	96	1023	10	+1.3
	Met^{69} → Ile	38	235	6 ↓	+1.3
Nitrocefin	OHIO-1	11	115	9	
	Met^{69} → Ile	22	13	0.6 ↓	+6.7

Table 3 Kinetic parameters for inhibition

Inhibitor	Enzyme	K_i (μM)	k_{inact} (s^{-1})	Turnover number	k_{cat}/k_{inact}
Clavulanate	OHIO-1	0.4	0.01	80	8
	Met^{69} → Ile	10	0.002	2000	193
Sulbactam	OHIO-1	17	0.001	40000	422
	Met^{69} → Ile	68	0.0004	200000	5174
Tazobactam	OHIO-1	0.30	0.01	400	4
	Met^{69} → Ile	18	0.0016	2000	586

experiments were done with a concentration of inhibitor 1000-fold greater than that of the enzyme.

The turnover number, the number of molecules of inhibitor required to inactivate the Met^{69} → Ile β-lactamase, was greater for each of the inhibitors than for the wild-type enzyme (Table 3). The most striking proportional increase in turnover number activity was for clavulanate (25-fold). The turnover numbers for sulbactam for the wild-type OHIO-1 β-lactamase and the Met^{69} → Ile β-lactamase were both markedly greater.

The partition ratio, k_{cat}/k_{inact}, for each inhibitor was also larger in the mutant (Table 3). In comparison with the wild-type enzyme, the Met^{69} → Ile enzyme possessed a partition ratio 25-fold greater for clavulanate, 5-fold greater for sulbactam and more than 100-fold greater for tazobactam. The measured k_{cat} for sulbactam was 2.0 s^{-1} for the Met^{69} → Ile enzyme, and 0.4 s^{-1} for the OHIO-1 β-lactamase.

DISCUSSION

The minimum inhibitory concentration results show that the Met → Ile substitution at position 69 of the OHIO-1 β-lactamase primarily affects the β-lactam/β-lactamase inhibitor combination. In normally susceptible *E. coli* DH5α, the Met^{69} → Ile β-lactamase-expressing mutants are resistant to all the mechanism-based inhibitors (Table 1).

The Ile substitution did not seem to change the isoelectric point or CD spectrum of the β-lactamase. The nearly superimposable CD spectra suggests that the Ile substitution at position 69 did not induce a major change in the secondary and tertiary structural elements of the Met^{69} → Ile enzyme. This suggests that the observed kinetic differences are not due to major changes in the structure of the mutant β-lactamase. As determined by immunoblotting, resistance to inhibitors does not seem to be a consequence of increased levels of expression or changes in stability of the β-lactamase enzymes.

Our analysis of substrate hydrolysis reveals that there is a major effect of the Ile substitution on the catalytic behaviour of the enzyme. For each of the substrates tested except nitrocefin, there was a decrease in K_m. Similarly, the k_{cat} values were decreased for all the β-lactams tested. Although k_{cat}/K_m (catalytic efficiency) was increased for some substrates (penicillin, carbenicillin and PADAC) and decreased for others (cephaloridine and nitrocefin), the overall trend was that the Ile-substituted enzyme turned over substrate more slowly than does the wild-type enzyme. This difference is not unique to our mutant enzyme. Farzaneh et al. [17] also showed that for the TEM variant of Met^{69} → Ile, the k_{cat} values are uniformly decreased. Although we were not able to test it directly, this consistent decrease in both k_{cat} and K_m suggests that the deacylation step might be the rate-determining step. This is in accordance with the electrospray MS findings of Saves et al. [18] that show that deacylation is the rate-determining step, k_{cat}, for penicillin by the TEM-1 enzyme.

A significant difference in inactivation was seen when the β-lactamase inhibitors were tested against the wild-type and mutant OHIO-1 enzymes. For each inhibitor, the dissociation equilibrium constant, K_i, was increased. This increase was determined with a concentration of inhibitor in 1000-fold excess. For clavulanate and tazobactam the increases were 25-fold and 60-fold respectively. For sulbactam the dissociation equilibrium constant for the wild-type enzyme was already increased relative to that for clavulanate and tazobactam; the increase over wild-type for sulbactam with the Met^{69} → Ile enzyme was 4-fold.

β-Lactamase inhibitors are mechanism-based inactivators. Hence they are recognized as a substrate by the enzyme [16]. The turnover numbers of each β-lactamase inhibitor were uniformly increased. The inactivation rate constant, k_{inact}, was decreased for clavulanate, sulbactam and tazobactam.

The partition ratio, the ratio of the amount of inhibitor that is hydrolysed to the amount that inactivates the enzyme, was also increased in Met^{69} → Ile compared with OHIO-1. From k_{cat}/k_{inact}, the Met^{69} → Ile mutant enzyme hydrolyses sulbactam > tazobactam > clavulanate. The wild-type enzyme hydrolyses sulbactam > clavulanate > tazobactam. The directly measured k_{cat} for sulbactam with the mutant enzyme is 5-fold that for the wild-type (2.0 s^{-1} compared with 0.4 s^{-1}). The decreased efficiency of inactivation and increased efficiency of hydrolysis of β-lactam inhibitors indicate that there is a shift in the inhibition pathway. The k_{cat}/k_{inact} of 8 for the OHIO-1 enzyme is significantly lower than the k_{cat}/k_{inact} of 160 for TEM-1 [19]. However, it is in

accordance with the observation [6,15] that the IC_{50} of clavulanate for SHV enzymes is lower than for TEM enzymes.

A unique feature of SHV-family enzymes is their intrinsic resistance to sulbactam. Under the conditions of our experiments we found that the sulbactam turnover numbers were 40000 and 200000 for wild-type and inhibitor-resistant enzymes respectively. Others also have found that turnover numbers are elevated for sulbactam [15]. These experiments suggest that the significant differences in the interaction of sulbactam with TEM and SHV enzymes might be related to differences in tertiary structure and to each agent's mechanism of inhibition.

The contribution of the $Met^{69} \rightarrow Ile$ substitution to the overall catalytic efficiency can be calculated for each substrate from the results in Table 2. This was done with the equation:

$$\Delta G^{\ddagger} = -RT\ln (k_{cat}/K_m)_{Met69 \rightarrow Ile}/(k_{cat}/K_m)_{OHIO\text{-}1}$$

which calculates the difference in binding energy, $\Delta G^{\ddagger}$, between the wild-type and mutant enzymes in going from the free enzyme plus substrate to the transition state. For penicillin, carbenicillin and PADAC, the $Met^{69} \rightarrow Ile$ mutation decreases the free energy barrier by approx. 2.1 kJ/mol. For nitrocefin and cephaloridine $\Delta G^{\ddagger}$ increases by 1.3 and 6.7 kJ/mol. In magnitude these changes are relatively small (Table 2) and are similar to $\Delta G^{\ddagger}$ values that we calculated for the $Met^{69} \rightarrow Ile$ mutant and the $Met^{69} \rightarrow Leu$ mutant of TEM-1 [17,20].

On the basis of the modelling and energy minimization of the crystal structure of the TEM-1 β-lactamase, Farzaneh et al. [17] proposed that the $Met^{69} \rightarrow Ile$ substitution in TEM results in a 0.3–0.4 Å shift in the α carbons from residues 69 to 72. Similar shifts also occur in the side chain amide of Asn^{170} (0.6 Å), the hydroxy group of Ser^{70}, the carboxylic group of Glu^{166} and the oxygen atom of the catalytic water molecule. These authors contend that this shifting causes a steric interaction between the side chains of Asn^{170} and Ile^{69}. They also state that a small displacement (0.3 Å) causes the catalytic water molecule to be displaced, resulting in a decrease in k_{cat} and K_m, and resistance to inhibitors, particularly when deacylation is the rate-determining step. In their analysis of the $Met^{69} \rightarrow Ile$ mutant, Chiabi et al. have suggested that both hydrophobicity and steric constraints are responsible for the altered activity [21].

The crystal structure of the OHIO-1 or an SHV β-lactamase has not yet been determined. To understand the implications of our findings, we previously constructed a three-dimensional model of the OHIO-1 β-lactamase [8,9].

Residue 69 is located in a structurally conserved area of the enzyme. A Met or Ile residue at this position has no direct interaction with β-lactam substrates as they enter the active site (Figure 3). The substitution of Ile, with its branched side chain, having the β-methyl group position behind the B3 and B4 β-strands and somewhat within the oxyanion pocket, is predicted to affect the interaction of inhibitors with this enzyme. The 2-fold greater hydrophobicity and steric bulk of the side chain of Ile might perturb the shape and character of the oxyanion pocket that binds the carbonyl group of the β-lactam ring. In addition, a change in the position of the guanidinium group of Arg^{244} on B4 is expected. The effects of these changes are a decrease in the catalysis of other β-lactams and an increase in the catalytic rate constant for the hydrolysis of clavulanate, sulbactam and tazobactam. In other words, because clavulanate, sulbactam and tazobactam do not have large substituents at the C-6 position, the catalysis of the β-lactamase inhibitors is increased. These compounds might be better able to enter a more constrained active site. Hence there is an increase in k_{cat}/k_{inact}. In contrast, the size and character of the large R2 group at the C-6 position of the other β-lactams decrease their turnover. The superimposable CD spectra and the small changes calculated in the apparent binding energy to the transition state are consistent with this model.

On the basis of the investigations of Farzaneh et al. [17], Delaire et al. [20], Chiabi et al. [21] and the present study, the kinetic impact of a mutation at position 69 seems to be similar in both the TEM and OHIO-1 β-lactamases. However, it is possible that important structural differences between TEM and OHIO-1 β-lactamases do exist. The catalytic differences between mutations at position 69 and those at position 244 suggest that the two sites affect substrate hydrolysis and confer resistance to inhibition by very different mechanisms.

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REFERENCES

1 Ambler, R. P. (1980) Philos. Trans. R. Soc. London B **289**, 321–331
2 Joris, B., Ghuysen, J. M., Dine, G., Renard, A., Dideberg, O., Charlier, P., Frere, J. M., Kelly, J. A., Moews, P. C. and Knox, J. R. (1988) Biochem. J. **250**, 313–324
3 Bush, K., Jacoby, G. A. and Medeiros, A. A. (1995) Antimicrob. Agents Chemother. **39**, 1211–1233
4 Medeiros, A. A. (1997) Clin. Infect. Dis. **24** (suppl. 1), S19–S45
5 Knox, J. R. (1995) Antimicrob. Agents Chemother. **39**, 2593–2600
6 Prinarakis, E. E., Mirriagon, V., Tzelepi, E., Gazouli, M. and Tzouvelekis, L. S. (1997) Antimicrob. Agents Chemother. **41**, 838–840
7 Bonomo, R. A., Currie-McCumber, C. and Shlaes, D. M. (1992) FEMS Microbiol. Lett. **92**, 79–82
8 Bonomo, R. A., Dawes, C. G., Knox, J. R. and Shlaes, D. M. (1995) Biochim. Biophys. Acta **1247**, 113–120
9 Bonomo, R. A., Dawes, C. G., Knox, J. R. and Shlaes, D. M. (1995) Biochim. Biophys. Acta **1247**, 121–125
10 Giakkoupi, P., Tzelepi, E., Legakis, N. J. and Tzouvelekis, L. S. (1998) FEMS Microbiol. Lett. **160**, 49–54
11 Johnson, B. H. and Hecht, M. H. (1994) Biotechnology **12**, 1357–1361
12 Vecoli, C., Prevost, F. E., Ververis, J. J., Medeiros, A. A. and O'Leary, G. P. (1983) Antimicrob. Agents Chemother. **24**, 186–189
13 Petrosino, J. F. and Palzkill, T. (1996) J. Bacteriol. **178**, 1821–1830
14 Edelhoch, H. (1967) Biochemistry **6**, 1948–1954
15 Bush, K., Macalintal, C., Rasmussen, B. A., Lee, V. J. and Yang, Y. (1993) Antimicrob. Agents Chemother. **37**, 851–858
16 Imtiaz, U., Billings, E., Knox, J. R., Manavathu, E. V., Lerner, S. A. and Mobashery, S. (1993) J. Am. Chem. Soc. **115**, 4435–4442
16a Ambler, R. P., Coulson, A. F. W., Frère, J.-M., Ghuysen, J.-M., Joris, B., Forsman, M., Levesque, R. C., Tiraby, G. and Waley, S. G. (1990) Biochem. J. **276**, 269–272
17 Farzaneh, A., Charlie, E., Peduzzi, J., Barthelemy, M., Labia, R., Blazquez, J. and Baquero, F. (1996) Antimicrob. Agents Chemother. **40**, 2434–2436
18 Saves, I., Burlet-Schlitz, O., Maveyraud, L., Samama, J.-P., Promé, J.-C. and Masson, J.-M. (1995) Biochemistry **34**, 11660–11667
19 Imtiaz, U., Billings, E., Knox, J. R. and Mobashery, S. (1994) Biochemistry **33**, 5728–5738
20 Delaire, M., Labia, R., Sammama, J.-P. and Masson, J.-M. (1992) J. Biol. Chem. **267**, 20600–20606
21 Chiabi, E. B., Peduzzi, J., Farzaneh, S., Barthelemy, M., Sirot, D. and Labia, R. (1998) Biochim. Biophys. Acta **1382**, 38–46

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