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Implication of Ile-69 and Thr-182 Residues in Kinetic Characteristics of IRT-3 (TEM-32) β-Lactamase

SEDIGEH FARZANEH,[1] EL BACHIR CHAIBI,[1] JEAN PEDUZZI,[1] MICHEL BARTHELEMY,[1] ROGER LABIA,[1,2]* JESUS BLAZQUEZ,[3] AND FERNANDO BAQUERO[3]

URA 401 and UMR 175,[2] Centre National de la Recherche Scientifique,-MNHN, Quimper, France, and Hospital Ramon y Cajal, Madrid, Spain[3]

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The substitution of a methionine for an isoleucine at position 69 (Met69Ile), which causes inhibitor resistance to TEM-type β-lactamases (IRT-3 and IRT-I69), altered the positions of the Asn-170 and Glu-166 side chains as well as the position of the catalytic water molecule. A novel hydrogen bond between the hydroxyl of Thr-182 and the carbonyl of Glu-64 was expected to be responsible for the increase in the catalytic activity of the IST-T182 and IRT-3 enzymes compared with those of TEM-1 and IRT-I69, respectively.

Recently, a novel class of TEM-1-derived β-lactamases resistant to β-lactamase inhibitors has emerged, mostly in clinical isolates of *Escherichia coli*. The first published report of two such β-lactamases appeared in 1990 (4). The enzymes were named IRT-1 (TEM-31) and IRT-2 (TEM-30) (3, 26) for inhibitor-resistant TEM-derived β-lactamases. The TEM derivatives produced by clinical isolates (5, 9, 11, 19, 20, 22, 24, 26) or experimental mutants of genes specifying TEM-type (16, 23) or SHV-type (6) enzymes resistant to β-lactamase inhibitors have been reported, whereas other clavulanic acid-resistant TEM derivatives (10, 12, 17) and SHV derivatives (7, 8) were obtained by site-directed mutagenesis. Twelve IRT enzymes have been identified from clinical isolates (11, 20), and others are under study.

In 1993, Blazquez et al. (5) described IRT-3 (TEM-32), which differs from TEM-1 by two substitutions: Ile for Met at position ABL69 (standard numbering scheme for class A β-lactamases [1]) and Thr for Met at position ABL182. Both mutations were segregated by the construction of hybrid genes (5). The investigators numbered the two positions 67 and 180, according to the numbering of Sutcliffe (21).

According to a previously described method (2), we have purified to homogeneity the β-lactamases TEM-1, IRT-3 (TEM-32), and the two TEM derivatives constructed by Blazquez et al. (5): IRT-I69 (TEM-40) and the inhibitor-sensitive TEM IST-T182.

Kinetic constants were determined by computerized microacidimetry (15), at pH 7 and 37°C, in distilled water containing 85 mM NaCl. The standard deviations of the kinetic parameters (K_m and k_{cat}) were computed on the basis of the results of three or four independent experiments.

The k_{cat} values of the enzymes (Table 1) for benzylpenicillin showed that the two variants with substitutions of the Ile at position 69 (Ile-69) (IRT-I69 and IRT-3) were considerably less active than the parent TEM-1 or IST-T182. The catalytic activity (k_{cat}) of IRT-I69 for benzylpenicillin was 13% of that of the native TEM-1, whereas increases in catalytic activity of 28 and 33% were observed when Met-182 was replaced by a Thr in IRT-3 and IST-T182, respectively. However, IRT-3 and IRT-I69 presented K_m values for benzylpenicillin, amoxycillin, and piperacillin lower than those of TEM-1. Thus, the catalytic efficiencies (k_{cat}/K_m) were not significantly altered with the exception of that for ticarcillin. The presence of Thr-182 did not modify the K_m values for penicillins.

On the basis of 50% inhibitory concentrations (IC_{50}s) (Table 2), the β-lactamases IRT-3 and IRT-I69 were 150-fold more resistant to clavulanate, 15-fold more resistant to sulbactam, and 30-fold more resistant to tazobactam than the TEM-1 and IST-T182 β-lactamases. Thus, only mutation of a Met to an Ile at position 69 (Met69Ile) was involved in resistance to suicide inhibitors.

In order to investigate the role of the amino acid substitutions, the structures of IRT-I69, IRT-3, and IST-T182 were constructed by molecular modeling, starting with the crystal structure of the native *E. coli* TEM-1 β-lactamase (which is available from the Protein Data Bank, Brookhaven National Laboratory, Brookhaven, Conn.) at 0.18-nm resolution (13) as the entry 1BTL. For the molecular modeling of IRT-3, IRT-I69, and IST-182 β-lactamases, the side chains of Ile-69 and Thr-182 were placed according to the positions of Met-69 and Met-182 of the wild type, respectively. Energy was minimized locally and then all residues in a sphere with a 1.0-nm radius centered on α carbons 69 and 182 were selected. Minimization was carried out until the root-mean-square gradient was less than 1.0 kcal/nm. The models were energy minimized with the HyperChem software by using the Ambler force field (25).

Concerning the Met69Ile mutation, the α carbons at resi-

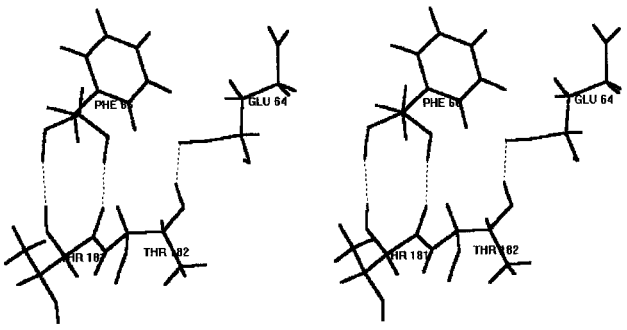

FIG. 1. Stereo view of the environment of Thr-182 in the IST-T182 molecular model. A novel hydrogen bond links the hydroxyl of Thr-182 and the carbonyl backbone amide of Glu-64, in addition to the two hydrogen bonds between Thr-181 and Phe-66.

* Corresponding author. Mailing address: UMR 175 CNRS-MNHN, 6 rue de l'Université, 29000 Quimper, France. Phone: (33) 2 98 90 80 35. Fax: (33) 2 98 90 80 48.

TABLE 2. IC_{50}s of β-lactamase inhibitors for β-lactamase activity[a]

Inhibitor	IC_{50} (μM)			
	TEM-1	IST-T182	IRT-I69	IRT-3
Clavulanate	0.08	0.1	13	12
Sulbactam	10	11	150	160
Tazobactam	0.16	0.15	5	5

[a] Data are from reference 6.

dues 69 to 72 were shifted by 0.042, 0.028, 0.021, and 0.027 nm, respectively. The α carbon at Asn-170 was shifted by 0.01 nm, but the α carbons of the other residues were not affected (<0.01 nm). However, the position of the side chain amide of Asn-170 was altered (shift of 0.057 nm for the oxygen of the side-chain amide), as were the hydroxyl of Ser-70, the carboxylate of Glu-166, and the catalytic water molecule (a shift of 0.030 nm for its oxygen). The ethyl moiety (γ1 and δ1 carbons) of Ile-69 was roughly in the position of carbon γ and sulfur δ of Met-69. Then, the Ile-69 methyl (γ2 carbon) produced steric interactions with the side chain of Asn-170. The catalytic water molecule displacement should be responsible for the decrease in the k_{cat} and K_m values for the IRT-I69 and IRT-3 β-lactamases and resistance to site-directed inhibitors.

Molecular modeling of IST-T182 suggests that the hydroxyl of Thr-182 forms a hydrogen bond with the carbonyl of the amide bond of Glu-64 (Fig. 1). That adds an extra hydrogen bond to the three others that already link together two domains of the β-lactamase: the first one located between the S2 strands and helix H2 (residues 60 to 69) and the second one just before helix H8 (residues 180 to 182); two hydrogen bonds link the NH and CO amides of Thr-181 and Phe-66; another one is formed between the hydroxyl of Thr-180 and a hydrogen of the guanidinium of Arg-65. Therefore, the presence of Thr-182 probably strengthens the dense hydrogen bond network that stabilizes the active site. Knox (14) suggested that the required structural change at position 69 is probably facilitated by the introduction of a branched β carbon at position 182.

It is noteworthy that with the sequences of 28 class A β-lactamases recently aligned (18), TEM-1 was the sole protein exhibiting a Met at position 182, a position that generally holds a hydrogen bond forming residues such as Thr for 18 β-lactamases, Ser for 8 β-lactamases, and Cys for the remaining enzyme.

In conclusion, the Met69Ile substitution, which induced inhibitor resistance in TEM-1, is associated with a considerable decrease in k_{cat} for most β-lactam substrates. In IRT-3, the Met182Thr substitution partially restores this catalytic activity.

TABLE 1. Kinetic data for TEM-1 β-lactamase and mutants IST-T182, IRT-169, and IRT-3[a]

Antibiotic	TEM-1			IST-T182			IRT-I69			IRT-3		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Benzylpenicillin	1,440	29	49.6	1,920	26	73.8	180	7	26	230	9	25
Amoxicillin	1,080	55	19.6	1,830	46	39.8	275	24	11.4	390	30	13
Piperacillin	1,300	55	23.6	1,900	50	38	230	18	13	235	15	15.7
Ticarcillin	160	12	13.5	310	10	31	90	170	0.5	110	80	1.4
Cephalothin	290	520	0.5	500	260	1.9	40	320	0.1	65	340	0.2
Cephaloridine	1,800	800	2.2	2,300	480	4.8	200	420	0.5	310	750	0.4

[a] Coefficients of variation were ±5% for k_{cat}, ±10% for a K_m of ≤100 μM, and 20% for a K_m of >100 μM.

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