Effect of Threonine-to-Methionine Substitution at Position 265 on Structure and Function of TEM-1 β -Lactamase

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Received 6 May 1994/Returned for modification 27 June 1994/Accepted 22 July 1994

TEM β -lactamase variants with the amino acid substitutions R164S, E104K, G238S, and E240K (ABL numbering) display increased activity toward extended-spectrum cephalosporins. The T265M substitution is frequently found to be associated with the above substitutions in extended-spectrum β -lactamases. However, the residue is located away from the active site in the three-dimensional structure and has been assumed to have no effect on catalysis. To examine the effect of the substitution on the structure and function of TEM β -lactamase we constructed the following mutants: G238S, T265M, T265M:G238S, and T265M:G238S:E240K. Each enzyme was purified to homogeneity and the kinetic parameters k_{cat} , K_m and k_{cat}/K_m were determined for cefotaxime, ceftazidime, cephaloridine, and ampicillin. The results indicate that the T265M mutation has little effect on hydrolysis. In addition, we used immunoblotting to show that the substitution has little or no effect on the in vivo steady-state levels of β -lactamase.

TEM β -lactamases are the most common plasmid-mediated β -lactamases (12). TEM-1 β -lactamase is considered a broad-spectrum β -lactamase because it can hydrolyze both penicillins and cephalosporins (3). However, it cannot efficiently hydrolyze extended-spectrum cephalosporins such as ceftazidime and cefotaxime. Derivatives of TEM β -lactamase that are able to hydrolyze extended-spectrum cephalosporins have been identified (11, 19, 20). These enzymes contain from one to three amino acid substitutions in the active site of the enzymes and have been termed extended-spectrum β -lactamases (4, 7). The active-site substitutions include E104K, R164S, G238S, and E240K (ABL numbering [1]). Other amino acid substitutions are often found to be associated with the active-site substitutions. For example, the T265M substitution has been identified in TEM-4, TEM-9, TEM-13, and TEM-14 (7, 11). The T265M substitution is located distant from the active site in the three-dimensional structure of the enzyme and thus is unlikely to exert a direct effect on substrate specificity (8). However, structural changes in the enzyme may indirectly affect the geometry of the active site and thus influence enzyme activity. To determine what influence, if any, the T265M substitution has on the structure and function of TEM-1 β -lactamase, the T265M substitution was constructed alone and in combination with other extended-spectrum substitutions, and the resultant enzymes were purified and characterized. Within the power of resolution of the experiments, the T265M was found to be neutral with respect to catalysis and enzyme expression.

MATERIALS AND METHODS

Materials. All enzymes except T7 DNA polymerase were purchased from New England Biolabs; T7 DNA polymerase was purchased from United States Biochemicals. The antibiotics ampicillin and cephaloridine were purchased from Sigma

Chemical Company. Ceftazidime was provided by Glaxo, and cefotaxime was provided by Hoechst-Roussel Pharmaceuticals Inc.

Plasmid pBG66 is the parent plasmid of all random library constructions (16). Plasmid pBG66 is a 4.8-kb derivative of pBR322 and pBR325 that contains the *bla*_{TEM-1} gene as well as the *cat* gene that encodes chloramphenicol acetyltransferase. The plasmid also contains the ColE1 and f1 origins of DNA replication.

Bacterial strains. *Escherichia coli* XL1-Blue was used in the study. The genotype is *endA1 gyrA96 hsdR17 lac recA1 relA1 supE44 thi-1* [F' *proAB, lacI^q lacZ Δ M15, Tn10(Tet^r)*] (Stratagene, Inc.).

Plasmid isolation and DNA sequencing. Single-stranded plasmid DNA was prepared for DNA sequencing as described previously (18). DNA sequencing was performed by the dideoxy-chain termination method. Custom-synthesized oligonucleotides were used to prime synthesis from defined sites within the *bla*_{TEM-1} gene. Plasmid DNA was prepared from *E. coli* by the alkaline lysis procedure (18).

Site-directed mutagenesis. The T265M mutation was constructed by oligonucleotide-directed mutagenesis by the method of Kunkel et al. (9). In the following oligonucleotide, the underlined A residue results in the conversion of codon 265 from ACG-Thr to ATG-Met: 5'-GACTCCCCGTCATG TAGATAAC-3'.

The G238S and G238S:E240K mutants were constructed by cassette mutagenesis by using the plasmid containing the L238-241 excision linker insert (16). Digestion of this plasmid with the restriction endonuclease *Bsp*M1 created a deletion in the *bla*_{TEM-1} gene with the sticky ends shown below.

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234 235 236          241 242 243 244
5'-AAA TCT GG      T GGG TCT CGC-3'
3'-TTT AGT CCT CGG      AGA GCG-5'
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Double-stranded DNA fragments were constructed from complementary oligonucleotides and were ligated into the pBG66 plasmid containing the deletion shown above to create the appropriate site-directed mutations. The sequences are shown

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below, with the oligonucleotide-generated fragments given in italics and the nucleotide substitution resulting in the mutation given in boldface:

G238S

234 235 236 237 238 240 241 242 243 244
5'-AAA TCT GGA GCC **AGT** GAG CGT GGG TCT CGC-3'
3'-TTT AGT CCT CGG **TCA** CTC GCA CCC AGA GCG-5'

G238S:E240K

234 235 236 237 238 240 241 242 243 244
5'-AAA TCT GGA GCC **AGT** **AAG** CGT GGG TCT CGC-3'
3'-TTT AGT CCT CGG **TCA** **TTC** GCA CCC AGA GCG-5'

The G238S:T265M and G238S:E240K:T265M mutants were constructed by introducing the T265M mutation into the already constructed G238S and G238S:E240K mutants by site-directed mutagenesis (9).

Antibiotic susceptibilities. MICs were determined by broth microdilution by the reference procedure of the National Committee for Clinical Laboratory Standards (13). Briefly, twofold dilutions of each antibiotic were made in cation-supplemented Mueller-Hinton broth. The ranges of antibiotic concentrations tested for ampicillin were 0.031 to 8192 $\mu\text{g/ml}$, those for cephaloridine were 0.062 to 640 $\mu\text{g/ml}$, those for cefotaxime were 0.25 to 64 $\mu\text{g/ml}$, and those for ceftazidime were 0.031 to 64 $\mu\text{g/ml}$. The inoculum was prepared from overnight cultures grown on tryptic soy agar supplemented with 5% sheep erythrocytes. The final concentration of inoculum in each well was 5×10^5 CFU/ml. Microtiter trays were incubated at 37°C for 18 to 24 h. The trays were examined visually, and the lowest concentration of antimicrobial agent that inhibited visual growth was recorded as the MIC.

Immunoblots of β -lactamases. Steady-state levels of β -lactamase were examined by immunoblotting exactly as described previously (17).

β -lactamase purification. TEM-1 β -lactamase and the mutant β -lactamases were purified to $\geq 90\%$ homogeneity. Cultures containing the wild-type and mutant β -lactamases were grown overnight at 37°C in 10 ml of Luria-Bertani (LB) broth supplemented with 12.5 μg of chloramphenicol per ml to select for the presence of the plasmid. A total of 2 ml of the overnight cultures was then used to inoculate 2.5 liters of LB plus 12.5 μg of chloramphenicol per ml, and the cultures were grown at 37°C for 6 h so that the culture was in the late log phase. β -lactamase and other periplasmic proteins were isolated by osmotic shock (15). The wild-type and mutant enzymes were purified by using an anti- β -lactamase antibody affinity column. The affinity matrix was prepared by coupling anti- β -lactamase antibody to protein A beads as described by Harlow and Lane (6). For this procedure, the periplasmic extract was bound to the affinity beads in $1 \times$ phosphate-buffered saline (PBS) overnight at 4°C with gentle agitation. The beads were then put in a column and washed with several bed volumes of $1 \times$ PBS. β -lactamase was eluted from the column with 100 mM sodium acetate (pH 4.0)–500 mM NaCl. The fractions were neutralized to pH 7 with 2 M Tris (pH 9). The purity of the preparation was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10) (see Fig. 1).

Enzyme kinetics. The kinetics of TEM-1 β -lactamase and the mutant derivatives were determined with ampicillin, cephaloridine, cefotaxime, and ceftazidime. The following extinction coefficients were used: ampicillin, 235 nm, $\epsilon = 2,160 \text{ M}^{-1} \text{ cm}^{-1}$, $\Delta\epsilon = 900 \text{ M}^{-1} \text{ cm}^{-1}$; cephaloridine, 260 nm, $\epsilon = 13,570 \text{ M}^{-1} \text{ cm}^{-1}$, $\Delta\epsilon = 10,125 \text{ M}^{-1} \text{ cm}^{-1}$; cefotaxime, 264 nm, $\epsilon = 16,893 \text{ M}^{-1} \text{ cm}^{-1}$, $\Delta\epsilon = 6,900 \text{ M}^{-1} \text{ cm}^{-1}$; ceftazidime, 260 nm,

TABLE 1. MICs for β -lactamase mutants

Mutant	MIC ($\mu\text{g/ml}$)			
	Ampicillin	Cephaloridine	Cefotaxime	Ceftazidime
Wild type	1,024	128	0.03	0.125
T265M	2,048	256	0.06	0.25
G238S	512	64	0.25	0.5
G238S:T265M	512	128	0.5	1.0
G238S:E240K	1,024	64	1.0	16
G238S:E240K:T265M	1,024	256	2.0	16

$\epsilon = 18,630 \text{ M}^{-1} \text{ cm}^{-1}$, $\Delta\epsilon = 9,390 \text{ M}^{-1} \text{ cm}^{-1}$. The hydrolysis of the substrates was monitored spectrophotometrically at 30°C in 0.05 M phosphate buffer (pH 7.0) on a Beckman model DU 640 spectrophotometer. Hydrolysis of the cephalosporin substrates was monitored in a 1-mm-path-length cuvette, while ampicillin hydrolysis was monitored in a 10-mm-path-length cuvette. The kinetic parameters V_{max} and K_m were determined by (i) initial velocity kinetic analysis by fitting to the equation of a rectangular hyperbola by using unweighted nonlinear least squares (2) or (ii) by using complete progress curves with starting concentrations of at least three times the K_m . The progress curves were made by determining the instantaneous rate at many points in the reaction by taking the derivative of the substrate concentration at many points in the reaction course. These data were then fitted to the Michaelis-Menten equation. To ensure that product inhibition was not occurring, initial rates were determined at four substrate concentrations chosen to bracket the K_m value determined by the progress curves. The k_{cat}/K_m values for ceftazidime and cefotaxime for the enzymes when k_{cat} and K_m could not be determined independently were estimated by using the equation $v = [k_{\text{cat}}/K_m] [E] [S]$ when $[S] \ll K_m$, where $[E]$ is enzyme and $[S]$ is substrate (5). The value reported is based on velocity measurements at 10, 25, and 50 μM substrate concentrations.

RESULTS

Effect of T265M on β -lactam resistance. To determine the effect of the T265M mutation on TEM-1 β -lactamase structure and function the mutation was introduced into the wild-type *bla*_{TEM-1} gene. Since the T265M mutation is often found to be associated with amino acid substitutions in extended-spectrum

FIG. 1. SDS-PAGE of purified β -lactamases. The enzymes were stained with Coomassie blue and are labelled at the top. M, prestained low-molecular-weight marker (Bio-Rad). WT, wild type.

TABLE 2. Kinetic parameters of wild-type and mutant β -lactamases for ampicillin

β -Lactamase	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	Relative ^a
Wild-type T265:G238:E240	$1,450 \pm 57$	71 ± 13	2.04×10^7	1.0
T265M	$1,329 \pm 71$	87 ± 12	1.53×10^7	0.75
G238S	23 ± 1	21 ± 3	1.10×10^6	0.05
G238S:T265M	55 ± 2	37 ± 6	1.49×10^6	0.07
G238S:E240K	40 ± 2	31 ± 5	1.29×10^6	0.06
G238S:E240K:T265M	55 ± 7	50 ± 10	1.10×10^6	0.05

^a Ratio of k_{cat}/K_m of the mutant relative to that of the wild type.

β -lactamases, it is possible that the T265M mutation exerts an effect only when it is present in combination with other extended-spectrum mutations. Therefore, we also constructed T265M mutations in combination with the common G238S substitution and the G238S:E240K double mutant. To assess the effects of these mutations on antibiotic resistance, the MICs of ampicillin, cephaloridine, cefotaxime, and ceftazidime were determined (Table 1). The MIC data indicate that the T265M mutation does not significantly alter the resistance profile of TEM-1 β -lactamase. In addition, comparison of the G238S and G238S:T265M mutants and the G238S:E240K and G238S:E240K:T265M mutants shows that the combination of the T265M mutation with other extended-spectrum mutations does not significantly alter the antibiotic resistance profile. Note that although the T265M mutation appears to cause small increases in the MICs of several antibiotics, repeated MIC determinations routinely gave twofold fluctuations in the MICs so that the small effects seen were within the error of the experiment.

Enzyme kinetics. Since the MIC is a discontinuous measurement, it is possible that changes in the substrate specificity of the enzyme caused by the T265M mutation may not be detected by the MIC determination. Small changes in catalytic efficiency may be important for the selection of mutant enzymes during the strong selective pressure of antibiotic therapy. Therefore, wild-type TEM-1 β -lactamase as well as each of the mutant enzymes listed in Table 1 were purified and the kinetic parameters for ampicillin, cephaloridine, ceftazidime, and cefotaxime hydrolysis were determined. The level of purity of the enzyme preparations was evaluated by SDS-PAGE to be $\geq 90\%$ for each of the enzymes (Fig. 1).

The results of the kinetics experiments are shown in Tables 2 through 5. It is apparent from these data that the T265M mutation alone has no significant effect on either k_{cat} or K_m for any of the substrates tested. Similarly, the addition of the T265M mutation to the extended-spectrum G238S and G238S:

TABLE 4. Kinetic parameters of wild-type and mutant β -lactamases for ceftazidime

β -Lactamase	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	Relative ^a
Wild-type T265:G238:E240	NM ^b	NM	21.40	1.0
T265M	NM	NM	28.30	1.3
G238S	0.9 ± 0.1	532 ± 119	1.69×10^3	79
G238S:T265M	0.3 ± 0.1	587 ± 80	5.11×10^2	24
G238S:E240K	23.2 ± 2	406 ± 22	5.71×10^4	2,668
G238S:E240K:T265M	12 ± 1	641 ± 50	1.87×10^4	873

^a Ratio of k_{cat}/K_m of the mutant relative to that of the wild type.^b NM, not measurable.

E240K β -lactamases does not significantly affect the kinetic parameters of these enzymes for any of the substrates tested (Tables 2 through 5). A possible exception is the effect of the T265M mutation on the G238S and G238S:E240K enzymes in which a two- to threefold decrease in ceftazidime hydrolysis was observed (Table 4). A detailed description of the kinetic effects of the G238S and G238S:E240K amino acid substitutions on β -lactam catalysis has been described elsewhere (22). The data are included here for comparison with those for the T265M mutant derivatives.

Steady-state β -lactamase levels. Since it is possible that the T265M mutation may exert an effect on the expression levels of β -lactamase rather than on catalytic efficiency, the in vivo expression levels of wild-type TEM-1 β -lactamase and each of the mutants was determined by immunoblotting (Fig. 2). Total protein from the *E. coli* strain that produced the mutant β -lactamases was screened with anti-TEM-1 β -lactamase polyclonal antibodies. The results indicate that the levels of expression of the wild-type, T265M, and G238S enzymes were similar. However, when the T265M mutation was combined with the G238S and G238S:E240K extended-spectrum enzymes there appeared to be small reductions in the expression levels. Thus, rather than providing a stabilizing effect, the T265M mutation may actually destabilize the extended-spectrum enzymes to a small extent.

DISCUSSION

The results of the present study indicate that the T265M substitution has little measurable effect on either the wild-type or the G238S and G238S:E240K extended-spectrum enzymes. A possible exception is the effect of the T265M substitution on the G238S and G238S:E240K enzymes, in which it appears to decrease ceftazidime hydrolysis two- to threefold. This result is consistent with previous observations that the TEM-9 β -lacta-

TABLE 3. Kinetic parameters of wild-type and mutant β -lactamases for cephaloridine

β -Lactamase	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	Relative ^a
Wild-type T265:G238:E240	962 ± 67	704 ± 65	1.37×10^6	1.0
T265M	483 ± 20	596 ± 46	8.10×10^5	0.6
G238S	82 ± 3	102 ± 13	8.04×10^5	0.6
G238S:T265M	91 ± 6	120 ± 21	7.58×10^5	0.6
G238S:E240K	25 ± 1	61 ± 12	4.10×10^5	0.3
G238S:E240K:T265M	16 ± 1	33 ± 4	4.84×10^5	0.4

^a Ratio of k_{cat}/K_m of the mutant relative to that of the wild type.TABLE 5. Kinetic parameters of wild-type and mutant β -lactamases for cefotaxime

β -Lactamase	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	Relative ^a
Wild-type T265:G238:E240	NM ^b	NM	3.90×10^3	1.0
T265M	NM	NM	4.74×10^3	1.2
G238S	56 ± 1	314 ± 36	1.78×10^5	46
G238S:T265M	55 ± 5	350 ± 57	1.57×10^5	40
G238S:E240K	47 ± 3	100 ± 7	4.68×10^5	120
G238S:E240K:T265M	37 ± 1	73 ± 3	5.07×10^5	130

^a Ratio of k_{cat}/K_m of the mutant relative to that of the wild type.^b NM, not measurable.

FIG. 2. Immunoblot of TEM-1 β -lactamase and mutant derivatives. The blot was treated with anti-TEM-1 β -lactamase antibodies. Lanes: +, wild-type; 1, T265M; 2, G238S; 3, G238S:T265M; 4, G238S:E240K; 5, G238S:E240K:T265M.

mase exhibits decreased ceftazidime hydrolysis relative to that of the TEM-26 β -lactamase (14, 21). Since the enzymes differ only in the T265M substitution being present in the TEM-9 enzyme but not in the TEM-26 enzyme, this observation suggests that the T265M substitution results in decreased ceftazidime hydrolysis when it is present in combination with other extended-spectrum mutations (14, 21).

An interesting question is why, if the T265M substitution confers no selective advantage, has it been found in extended-spectrum β -lactamases? One explanation is that the *bla*_{TEM} gene encoding the T265M mutation is a neutral allele that arose by genetic drift. If the mutation is neutral, the fact that it is found in association with extended-spectrum β -lactamases suggests that the mutation is prevalent among bacteria containing a *bla*_{TEM} gene. An alternate explanation is that the T265M mutation may provide some selective advantage that we were not able to measure in the present study. This possibility is very difficult to rule out since we do not know how small an effect constitutes a selective advantage. A third possibility is based on the observation that multiple TEM β -lactamase variants can exist within the same bacterial strain (11). If high rates of recombination between *bla*_{TEM} genes occurred in these strains, it is feasible that the T265M mutation could become associated with extended-spectrum mutations in *bla*_{TEM} genes even in the absence of selection.

ACKNOWLEDGMENT

This work was supported by NIH grant AI32956 to T.P.

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