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Characterization of a New TEM-Derived β-Lactamase Produced in a *Serratia marcescens* Strain

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A natural TEM variant β-lactamase was isolated from an epidemic strain of *Serratia marcescens*. Nucleotide gene sequencing revealed multiple point mutations located in the 42-to-44 tripeptide and positions 145 to 146, 178, and 238. In addition, a glutamic acid 212 deletion was also found. The purified enzyme was studied from a kinetic point of view, revealing the highest catalytic efficiency (k_{cat}/K_m) values for ceftazidime and aztreonam compared with the TEM-1 prototype enzyme. The in vitro resistance correlated with kinetic parameters, and the enzyme also mediated resistance to some penicillins and an ampicillin-clavulanic acid combination. The mutational and kinetic changes are discussed in relation to the three-dimensional crystallographic structure of the wild-type TEM-1 enzyme.

TEM-type β-lactamases were first isolated 30 years ago, and these are now the most widespread plasmid-encoded serine β-lactamases among members of the family *Enterobacteriaceae*. The gene encoding the TEM-1 enzyme has undergone a very high selective pressure, because of a number of broad-spectrum β-lactam antibiotics which have been synthesized and introduced into clinical use since the 1980s. For this reason, the discovery of mutated enzymes produced by different clinical isolates and able to induce a particular pattern of resistance toward a large number of penicillins and cephalosporins is not surprising (7, 12, 16). Moreover, crystallographic studies carried out with TEM-1 and other class A β-lactamases (10, 11, 13, 17, 24, 30, 31, 41) have provided a large base of structural information useful in elucidating the behavior of mutated enzymes either naturally isolated or produced by site-directed mutagenesis. In particular, two major points can be underlined for the TEM family mutants: (i) a reduced catalytic efficiency toward molecules such as benzylpenicillin, ampicillin, and cephaloridine and (ii) a broadening of the substrate spectrum which includes the so-called normally stable β-lactams, such as ceftazidime, cefotaxime, and aztreonam.

The discovery of TEM- or SHV-derived mutants is not unexpected; however, it implies that the use of antibiotics thought to be suitable for treating frequently isolated bacterial strains, such as those of *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, etc., could become increasingly less successful.

Also, in Italy, recent observations point to the spread of extended-spectrum β-lactamases (ESβL) in the above-mentioned bacterial strains. In particular, in 1993 an epidemic spread of an *S. marcescens* strain which was resistant to ceftazidime, cefotaxime, and aztreonam was reported (33). This strain produced three different β-lactamases with pIs of 5.4, 5.5, and 8.4. The β-lactamases with pIs of 5.4 and 5.5 were plasmid-mediated enzymes, but only the β-lactamase at pI 5.5 hydrolyzes the oxyimino-cephalosporins and aztreonam. The β-lactamase with a pI of 8.4 was found to be an inducible chromosomal enzyme. However, no molecular data are currently available concerning the type of mutation or the kinetic characterization of the enzyme responsible for a similar resistance pattern.

The present study reports the characterization of the TEM-type β-lactamase produced by a strain of *S. marcescens*, named S5, from the above-mentioned epidemic spread. Kinetic data obtained from the purified enzyme, along with sequencing data and molecular modelling, indicated that a unique variant of TEM was produced by this strain.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. *E. coli* HB101 (37) was used as the host for recombinant plasmids. Cells were grown in Luria-Bertani medium (37) at 37°C under aerobic conditions. Plasmid pSM5 was a 54-kb plasmid from *S. marcescens* S5, which carried the gene encoding the ESβL produced by the above strain. pSM5 was extracted from *E. coli* K-12-J53-S5, which is a transconjugant harboring the plasmid (33), according to the method of Kado and Liu (19). The pBC-SK plasmid (Stratagene Corp., La Jolla, Calif.) was used as the genetic vector for cloning and expression of the ESβL-encoding gene in *E. coli*(pTEM-AQ).

Recombinant DNA methodology. Basic DNA recombination was performed according to the methods of Sambrook et al. (37). PCR to amplify the gene encoding the ESβL carried by plasmid pSM5 was performed in a 100-μl volume with, per reaction mixture, 2.5 U of *Taq* DNA polymerase (Boehringer, Mannheim, Germany), the reaction buffer provided by the *Taq* manufacturer (containing 1.5 mM $MgCl_2$), 200 μM each deoxynucleoside triphosphate, 0.5 μM each primer, and 2 ng of pSM5 as the template.

Amplification reactions were carried out as follows: denaturation at 94°C for 20 s, annealing at 52°C for 1 min, and extension at 72°C for 1 min, repeated for 30 cycles. A final DNA extension step at 72°C for 5 min was allowed at the end of the thermal cycling. DNA sequencing was performed according to the dideoxy-chain termination method (38) with a Sequenase 2.0 DNA sequencing kit (U.S. Biochemical, Amersham, United Kingdom). Direct sequencing of PCR amplimers was performed according to the manufacturer's instructions (2) on three PCR products derived from three independent PCRs with the original template. Nucleotide sequences were always determined for both strands.

Oligonucleotides. The oligonucleotides were designed on the basis of nucleotide sequences of TEM-derived ESβL as reported by Mabilat et al. (28). The

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sense oligonucleotide of 27 bp included a *Sac*I linker at the 5′ OH, and the antisense oligonucleotide of 26 bp had a *Kpn*I linker at the 5′ OH.

Antibiotics. Piperacillin was from Cyanamid (Catania, Italy), carbenicillin and clavulanic acid were from SmithKline Beecham Research Laboratories (Brentford, United Kingdom), cefotaxime was from Hoechst AG (Frankfurt, Germany), aztreonam was from the Squibb Institute for Medical Research (Princeton, N.J.), 6-β-iodopenicillanic acid (6-β-IP) and sulbactam were from Pfizer Central Research (Sandwich, United Kingdom), and ceftazidime was from Glaxo (Verona, Italy). Benzylpenicillin, ampicillin, oxacillin, cephaloridine, cephalotin, and cefazolin were from Sigma Chemical Co. (St. Louis, Mo.), and nitrocefin was from Unipath (Milan, Italy).

Production and purification of TEM-AQ β-lactamase. An overnight culture of *E. coli* HB101(pTEMAQ) in Luria-Bertani broth was diluted 10-fold with 4 liters of the same medium containing chloramphenicol (30 μg/ml). Cells were grown for 18 h and harvested by centrifugation at 8,000 rpm for 10 min at 4°C, washed twice with 30 mM Tris-HCl buffer (pH 8.0), and lysed with lysozyme (2 mg/ml) for 1 h on ice, as previously reported by Lindström et al. (27). The lysate was centrifuged at 105,000 × *g* for 30 min, and the cleared supernatant containing the periplasmic extract was recovered and loaded onto a Sepharose-Q fast-flow column (2.0 by 20 cm; Pharmacia-LKB Biotechnology, Uppsala, Sweden) equilibrated with 30 mM Tris-HCl buffer, pH 8.0. The column was extensively washed to remove unbound proteins, and the β-lactamase was eluted with a linear gradient of NaCl (0 to 1 M) in the same buffer. The fractions containing β-lactamase activity were pooled, dialyzed overnight at 4°C against 25 mM Bis-Tris buffer (pH 7.0), and loaded onto a Mono P HR 5/20 column (Pharmacia), equilibrated with the same buffer. The proteins were eluted with 25 ml of 10-fold-diluted Polybuffer 74 in the pH range of 7 to 4.

Determination of β-lactamase activity. β-Lactamase activity was routinely measured spectrophotometrically following the hydrolysis of 100 μM nitrocefin as the substrate (λ = 482 nm; $\Delta\varepsilon_M^{482}$ = 15,000 $M^{-1} \cdot cm^{-1}$). One unit of β-lactamase activity was defined as the amount of the enzyme which hydrolyzes, 1 μmol of substrate per min in 50 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl at 30°C.

Protein determination. Protein content was determined by the method of Bradford (5) with bovine serum albumin as the standard.

Electrophoretic analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by following the method of Laemmli (23) with a fixed 12.5% (wt/vol) polyacrylamide gel in the presence of 0.1% SDS, with a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, Calif.). The β-lactamase M_r value was determined by comparison of its relative mobility with that of a standard protein mixture.

Gel isoelectric focusing was performed in 5% polyacrylamide gels containing ampholynes (pH range, 3.5 to 9.5) by using a Multiphor II apparatus (Pharmacia-LKB Biotechnology). The gels were focused at 4°C and 25 W for 180 min. The pI value was determined by focusing 5 μg of the purified enzyme.

In vitro susceptibility testing. The determination of MICs was performed by the conventional macrodilution broth procedure, as recommended by a National Committee for Clinical Laboratory Standards reference manual (32).

Determination of kinetic parameters. Kinetic experiments were performed following the hydrolysis of each substrate at 30°C in sodium phosphate buffer (pH 7.0), containing 0.2 M KCl to prevent enzyme instability. The data were collected with a Perkin-Elmer Lambda 2 spectrophotometer connected to a microcomputer. Each kinetic value is the mean of five different measurements; error was below 5%.

Inactivation rate constants for interaction with β-IP were determined by incubating 0.5 μM enzyme at 30°C with increasing inhibitor concentrations (0.5 to 5 μM). The rate of inactivation was measured by using 150 μM nitrocefin as the reporter substrate.

The inhibition constant for clavulanic acid was obtained by incubating 16 nM enzyme with 150 μM nitrocefin containing increasing concentrations of inhibitor (0.5 to 5 μM). Product accumulation was recorded for 1 min, before inactivation progress became detectable. The inhibition constant was calculated by the plot V_0/V_i versus I, yielding a line of slope $K_m/(K_m + S) \cdot K_i$. (V_0 and V_i are the initial rates of nitrocefin in the absence and presence of the inhibitor, respectively, I is the inhibitor concentration, and S is the concentration of nitrocefin). The inactivation rate constant for clavulanic acid was calculated as reported above for β-IP, by incubating 16 nM enzyme with 5 to 200 μM inhibitor.

Determination of N-terminal amino acid sequence. The N-terminal amino acid sequence of the mature form of TEM-AQ was characterized with the help of a gas phase Sequenator (Applied Biosystems, Foster City, Calif.).

Analysis of three-dimensional structure. The crystallographic structure of the parental TEM-1 β-lactamase (17), Protein Data Bank entry 1BTL, was examined on an Evans & Sutherland PS330 graphics system with FRODO (18). Figures were prepared with MOLSCRIPT (22).

Nucleotide sequence accession number. The nucleotide sequence data appear in the EMBL-GenBank-DDBJ databases under accession no. X97254.

RESULTS

Molecular cloning and sequencing of the gene encoding the ESβL carried by the *S. marcescens* plasmid pSM5. Several primers designed on the basis of consensus nucleotide sequences known to be flanking TEM-type β-lactamase-encoding genes (28) were used to attempt PCR amplification of the gene encoding the ESβL previously shown to be carried by the *S. marcescens* plasmid pSM5 (33). The PCR, performed on pSM5 extracted from a transconjugant *E. coli* strain, yielded an amplimer of 1,070 bp which was subjected to direct sequencing. The nucleotide sequence of the amplimer carried a 956-bp open reading frame potentially encoding a polypeptide of 319 amino acid residues which showed all the conserved structural elements typical of molecular class A β-lactamases and a high degree of sequence homology with TEM-1 β-lactamases. The N-terminal amino acid residue sequence (HPETLVKVKD. . .) determined by microsequencing is in complete agreement with that deduced from the gene sequence of the mature protein. Comparing amino acid residues of the TEM-AQ and TEM-1 enzymes, we found only eight residues mutated (Table 1). The fidelity of the sequence was confirmed by the full agreement in the sequences determined for every strand of three PCR products derived from three independent PCRs performed with the original template. The gene carried by pSM5, therefore, appeared to be a bla_{TEM} mutant which was named $bla_{TEM\text{-}AQ}$. Compared to TEM-1, the TEM-AQ enzyme showed the following mutations: A42S, R43T, V44S, P145S, K146Q, R178A, and G238R. A deletion of the glutamic acid residue at position 212 was also found. One or more of these mutations could affect the structure of the enzyme; this could result in a time-dependent instability of TEM-AQ enzyme, which loses about 70% of activity after 18 h of incubation at 30°C, with respect to TEM-1 β-lactamase (30% of activity decreased in the same conditions).

TABLE 1. Natural mutations in the TEM-AQ β-lactamase in respect to the TEM-1 prototype

β-Lactamase	Amino acid at position[a]:							
	42	43	44	145	146	178	212	238
TEM-AQ	S	T	S	S	Q	A	−[b]	R
TEM-1	A	R	V	P	K	R	E	G

[a] Numbering according to the work of Ambler et al. (1).
[b] −, amino acid deletion.

The PCR amplimer containing the $bla_{TEM\text{-}AQ}$ gene was also cloned in the plasmid vector pBC-SK, to obtain plasmid pTEMAQ. Confirmatory sequencing of the cloned copy of the $bla_{TEM\text{-}AQ}$ gene showed no differences with the sequence obtained with the PCR fragments.

Purification and biophysical characterization of the TEM-AQ enzyme. The TEM-AQ enzyme was purified from *E. coli* HB101(pTEMAQ) by two chromatographic steps which yielded the enzyme as more than 95% pure as evaluated by SDS-PAGE analysis.

The molecular mass and the isoelectric point calculated for the purified enzyme were 28,600 Da and 5.5, respectively. The molecular mass was determined on the basis of N-terminal sequence and is in good agreement with that determined by SDS-PAGE (28,000 Da).

Contribution of the TEM-AQ enzyme to the phenotype of resistance. In vitro susceptibility to various β-lactams was investigated for *E. coli* HB101(pTEMAQ) in comparison with HB101 recipient. Results of these experiments showed that production of the TEM-AQ enzyme was readily able to confer resistance toward ampicillin and piperacillin but not toward aztreonam, ceftazidime, or cefotaxime when conventional inocula (5×10^5 CFU) were used. With a larger inoculum (5 ×

TABLE 2. Pattern of resistance mediated by the TEM-AQ β-lactamase gene in *E. coli* HB101

Antibiotic	MIC (μg/ml) for *E. coli* strain (CFU):			
	pTEMAQ		HB101	
	5×10^7	5×10^5	5×10^7	5×10^5
Ampicillin	>1,000	>1,000	7.8	3.9
Carbenicillin	31.25	7.8	7.8	3.9
Cefoxitin	15.6	7.8	15.6	7.8
Aztreonam	>125	<0.48	<0.48	<0.48
Ceftazidime	7.8	<0.48	<0.48	<0.48
Piperacillin	>250	>250	1.9	1.9
Cefotaxime	0.96	<0.48	<0.48	<0.48
Cefuroxime	31.25	7.8	1.9	<0.48
Cefazolin	7.8	0.9	0.9	<0.48
Ampicillin-clavulanic acid[a]	>500	250	3.9	3.9

[a] 4-μg/ml fixed concentration of clavulanic acid.

10^7 CFU), a significant increase of the aztreonam and ceftazidime MICs was observed (Table 2).

Kinetic parameters and the effect of inhibitors on the TEM-AQ β-lactamase. Benzylpenicillin, ampicillin, nitrocefin, cephaloridine, and cephalotin were good substrates for the TEM-AQ enzyme as shown by k_{cat}/K_m values that generally differ by an order of magnitude or less from those reported for TEM-1 by Raquet et al. (36) (Table 3). In particular, the k_{cat} values for nitrocefin, cephaloridine, and cephalotin, ranging from 9 to 64 s^{-1}, account for the difference. The catalytic efficiency for oxacillin was 40-fold lower than the value for the TEM-1 enzyme. Surprisingly, aztreonam and ceftazidime were affected by TEM-AQ enzyme, with an exceptionally high catalytic efficiency, mainly for aztreonam. The K_m values for ceftazidime and aztreonam decreased 37- and 1,100-fold respectively, compared to TEM-1. The value of k_{cat}/K_m for cefotaxime was very similar to that reported for TEM-1 (36).

Concerning the inhibitors, we studied the behavior of clavulanic acid and β-IP. The TEM-AQ β-lactamase inactivation by β-IP was complete, with an enzyme/inactivator ratio of 1:1. No enzyme reactivation was observed even after 2 h of incubation, thus indicating that no turnover occurred ($k_{+3} = 0$). The inactivation rate constants were computed according to the following scheme:

$$E + C \underset{}{\overset{K_s}{\rightleftharpoons}} EC \xrightarrow{k_{+2}} EC^* \xrightarrow{k_{+3}} EC_i$$

where EC* is the acyl enzyme and EC_i is the irreversible inactivated complex. An apparent first-order rate constant (k_{+2}) of 3.2×10^{-2} s^{-1} was measured, and a value of 1.97×10^4 M^{-1} s^{-1} was found for the k_{+2}/K ratio (9). On the basis of the k_{+2}/K ratio, the β-IP showed a 14-fold reduction with respect to the TEM-2 enzyme as computed by De Meester et al. (9). By using initial rate measurements of nitrocefin hydrolysis, a K_i value of 1.2 μM was estimated for clavulanic acid. The inactivation rate constant (k_i) was independent of the clavulanic acid concentration tested. Comparative analysis with TEM-1 enzyme showed similar behavior for the two β-lactamases. Sulbactam behaved like a good substrate for TEM-AQ, the hydrolysis obeyed Michaelis-Menten kinetics, and accumulation of enzyme inhibitor was not detected. The physiological efficiency of TEM-AQ for this sulfone compound was 16-fold higher than that of TEM-1.

DISCUSSION

A number of naturally occurring or laboratory mutants of TEM-1 or SHV-1 β-lactamases have been described recently, mutants which show an extension of the substrate spectrum, especially toward cefotaxime, ceftazidime, and monobactams, and a resistance to clavulanic acid inhibition, usually at the expense of their catalytic efficiency for penicillins (3, 4, 26). Based on the three-dimensional structure of several class A TEM-type enzymes, functional models have also been proposed for some of these ESβL to explain the relationships between amino acid variations and the modification of kinetic properties (8, 21, 25, 29, 34, 35, 40, 42, 43).

The enzyme described in this paper is a natural TEM derivative encountered in *S. marcescens* clinical isolates, which showed functional properties typical for ESβL yet with a peculiar set of mutations. Some of the mutations in the TEM-AQ enzyme occur at positions already known to be involved in altering the substrate spectrum of natural TEM derivatives. This is the case for mutations at positions 43 and 238. An R43S mutation which is active toward aztreonam, cefotaxime, and ceftazidime (6) has recently been reported for SHV-7. It should be noted that the TEM-AQ enzyme includes two additional mutations flanking position 43, A42S and V44S.

TABLE 3. Comparison of kinetic parameters between TEM-AQ and TEM-1 β-lactamases

Substrate	TEM-1[a]			TEM-AQ			$(k_{cat}/K_m)_{TEM\text{-}AQ}$
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	$(k_{cat}/K_m)_{TEM\text{-}1}$
Benzylpenicillin	25	1,600	6.4×10^4	19	165	8.7×10^3	0.13
Ampicillin	32	1,050	3.3×10^4	35	177	5.0×10^3	0.15
Oxacillin	3	60	2.0×10^4	10[b]	5[c]	5.0×10^2	0.02
Nitrocefin	55	930	1.7×10^4	120	64	5.3×10^2	0.03
Cephaloridine	680	1,500	2.2×10^3	412	43	1.0×10^2	0.04
Cephalotin	246	160	6.5×10^2	140	9	6.3×10	0.09
Cefazolin	513	200	3.9×10^2	120	39	3.2×10^2	0.8
Cephalosporin C	400	36	0.9×10^2	560	178	3.2×10^2	3.5
Cefotaxime	6,000	9	1.5	190	0.3	1.6	1
Ceftazidime	4,280	0.3	7.0×10^{-2}	115	0.4	3.0	43
Aztreonam	1,430	1.0	0.7	1.3	0.13	1.0×10^2	143
Cefuroxime	1,000	6.0	6.0	920	6.1	6.6	1
Sulbactam	1[b]	0.06[c]	0.06×10^3	1.9[b]	1.7[c]	1.0×10^3	16

[a] Data from reference 36.
[b] As K_i with 150 μM nitrocefin as reporter substrate.
[c] k_{cat} was calculated by direct hydrolysis. For more details, see Materials and Methods.

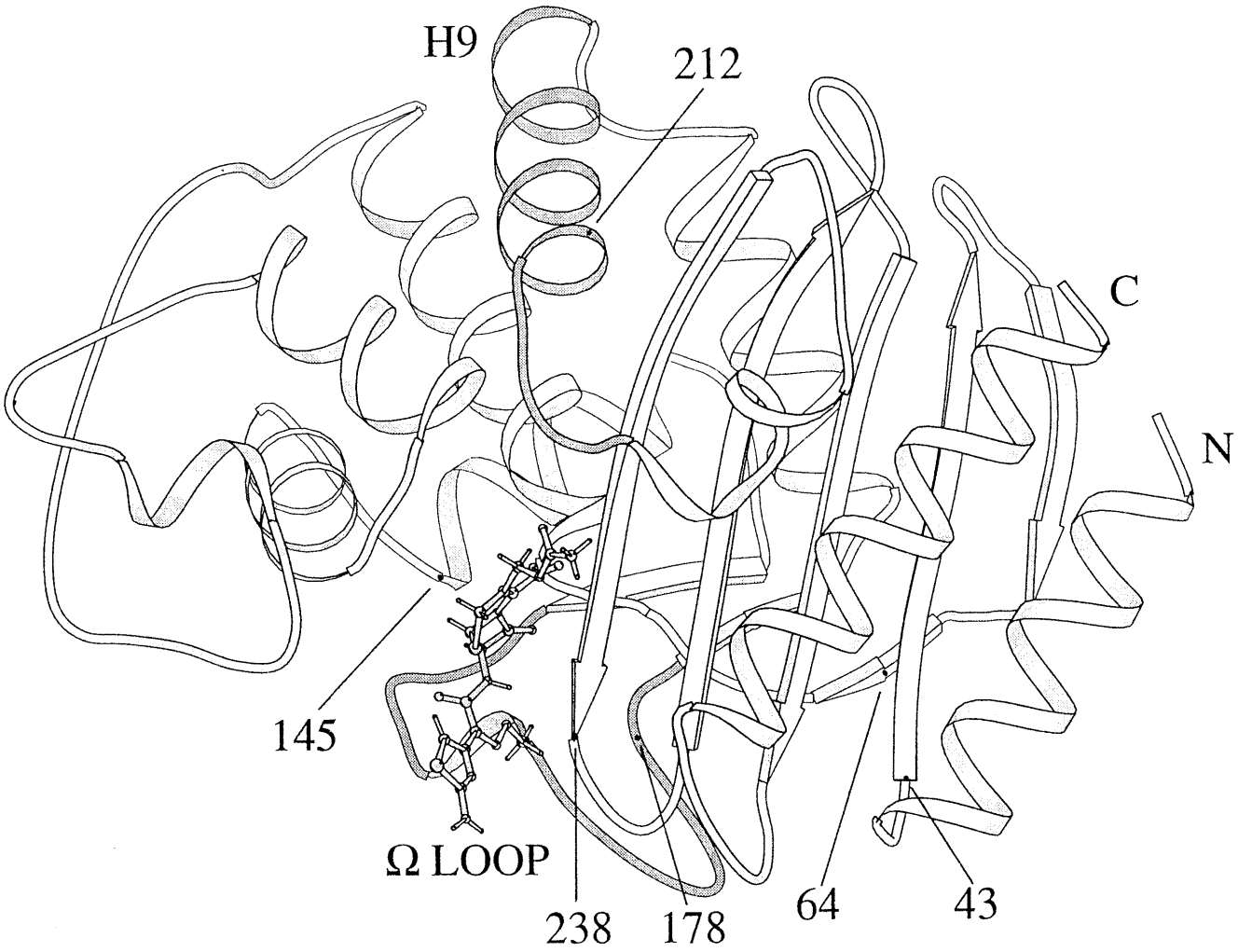

FIG. 1. Folding of TEM-1 β-lactamase (17) from Protein Data Bank 1BTL drawn with MOLSCRIPT (22). Numbers indicate amino acid positions found mutated in TEM-AQ. Cefotaxime is shown in the binding site.

Figure 1 is a global view of the folding of the 264-residue TEM-1 parental β-lactamase (11, 17, 41), showing the points of mutation discussed below in more detail.

A-R-V to S-T-S at positions 42 to 44. Introduction of threonine at position 43 and hydrophilic side chains at positions 42 and 44 is unique among TEM mutants (20). In TEM-1, as in all class A β-lactamases, the 42-to-44 tripeptide is part of the B1 β strand (Fig. 2). In wild-type β-lactamases, the side chains of residues 42 and 44 are always hydrophobic and lie on the front side of the β sheet and behind the N- and C-terminal α helices. In TEM-1, R43 is hydrogen bonded to E64 (2.95 Å) and, more weakly, to the backbone carbonyl group of residue 65. This bonding may be important in stabilizing the 64-to-69 polypeptide strand running behind the β sheet to the reactive S70. The more important mutation in this tripeptide is therefore likely to be at position 43, where the much shorter threonine side chain may not be able to reach the hydrogen bonding groups on the strand.

P-K to S-Q at position 145 to 146. The segment 145 to 146 is at the beginning of helix H6 and touches the omega loop, which forms the base of the binding site (Fig. 3). The side chain of K146 projects into solvent, and its alteration to glutamine probably has little consequence. P145 is part of a hydrophobic shell of amino acids (F72, L139, L148, W165, and L169) surrounding L162 on the omega loop. Mutation of the proline to a polar serine could modify the hydrophobic stabilization of L162, changing slightly the position of the omega loop and the catalytic E166.

R to A at position 178. In TEM-1, R178 is located in the omega loop but has no contact with β-lactam substrates. Its exposed side chain donates two hydrogen bonds to D176 (Fig. 3). The R178-D176 ion pair linkage is near a similar linkage between R164 and D179 at the neck of the loop. All amino acids found at position 178 in the class A β-lactamases (1) are polar and capable of hydrogen bonding; thus, the occurrence in TEM-AQ of a hydrophobic alanine at 178 is quite unusual. Loss of the ion pair linkage to D176 might change the shape or flexibility of the omega loop. Coupled with this R-to-A mutation may be the additional mutation at position 238 on β-strand B3, discussed below.

Deletion at position 212. In TEM-1, the side chain of E212 is exposed to solvent on the surface of helix H9 at the top of the enzyme (Fig. 1 and 4), 10 Å distant from the β-lactam binding site. Its deletion in TEM-AQ is unique to the family of known TEM mutants. Shortening helix H9 by one residue could change the position of a C-terminal loop of the polypeptide

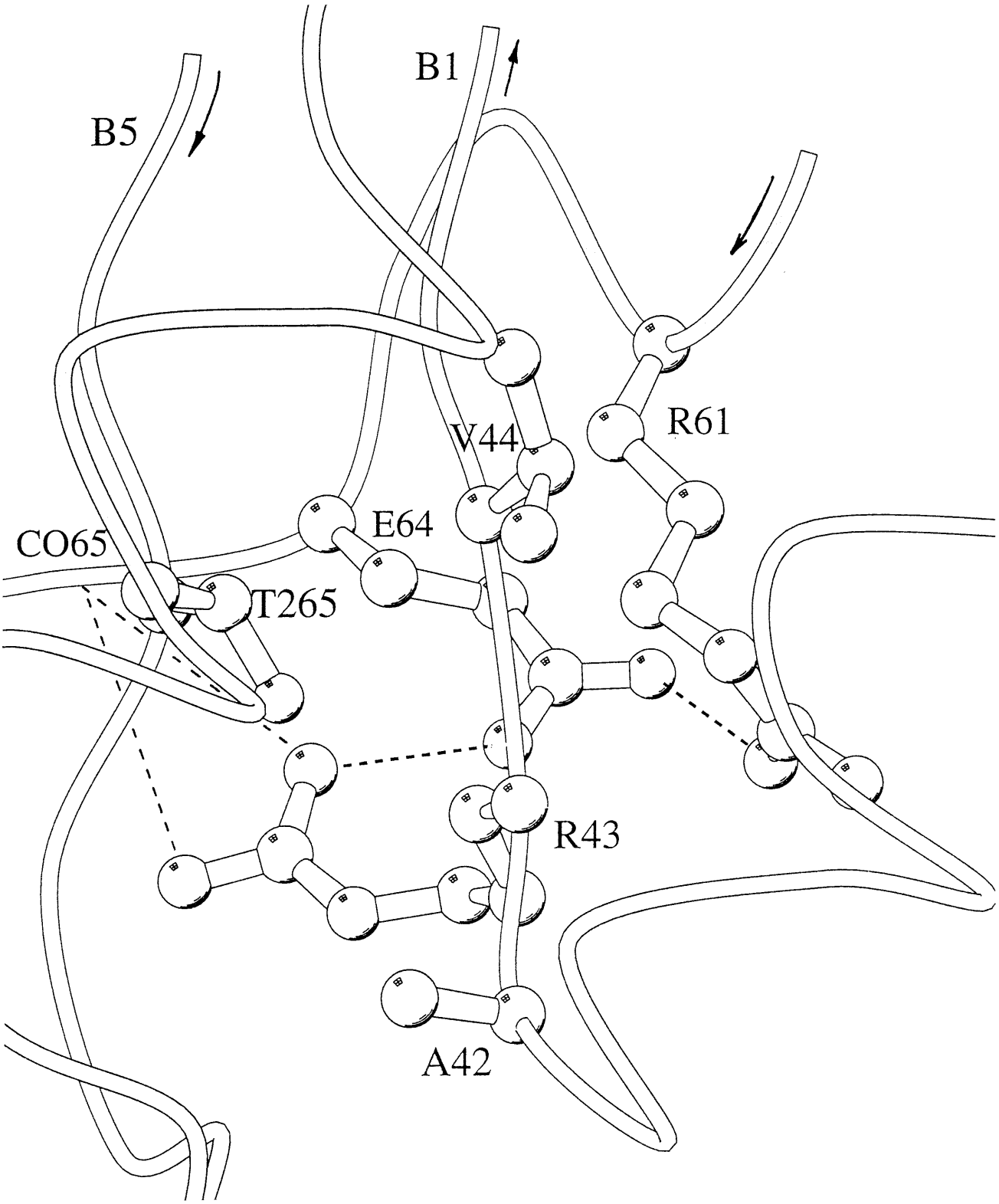

FIG. 2. Hydrogen bonding of R43 in TEM-1. The view direction corresponds to that in Fig. 1.

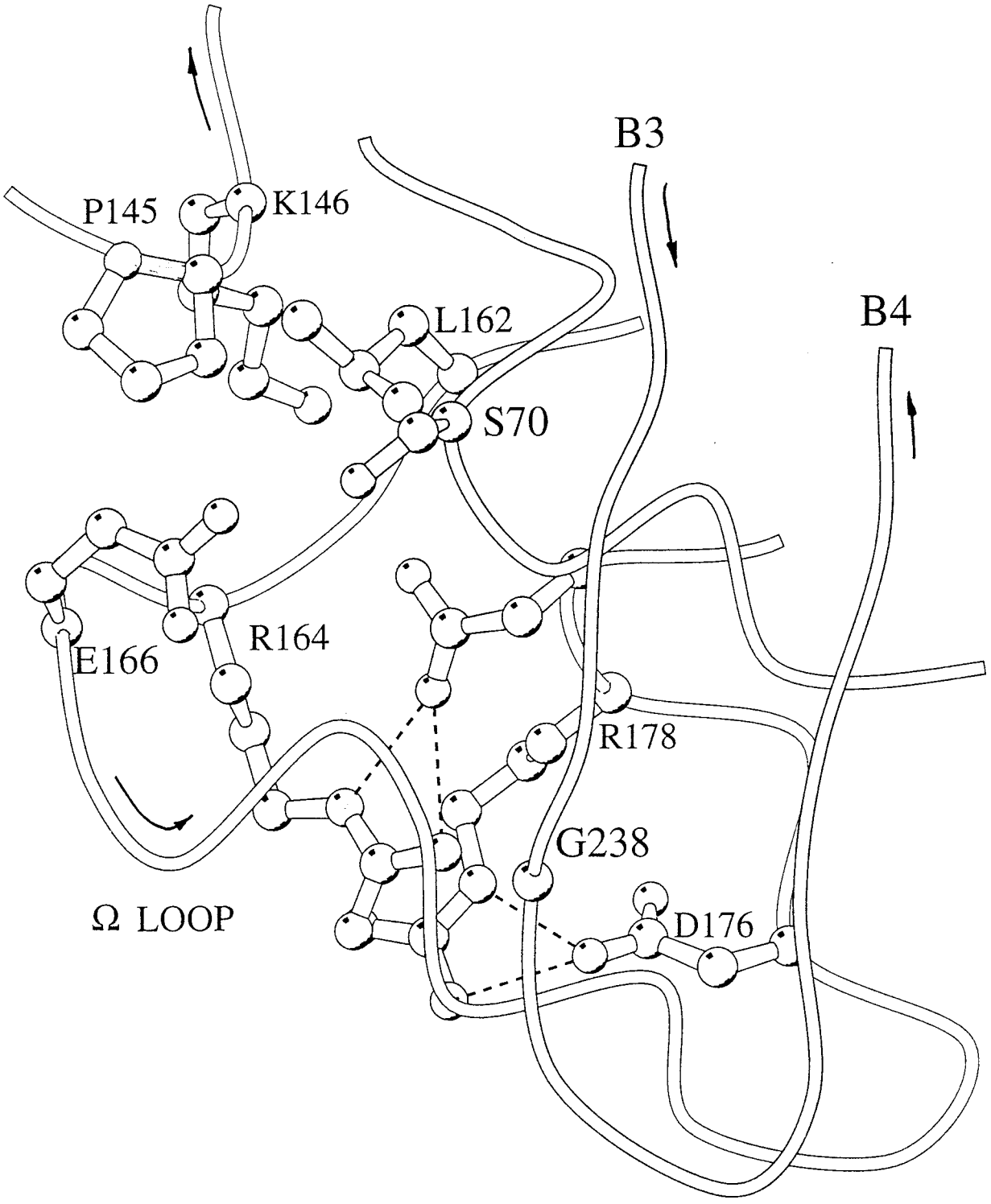

FIG. 3. The β-lactam binding site of TEM-1, showing the D176-R178 ion pair linkage and the position of G238. View direction is as in Fig. 2.

(213 to 220) extending over the top of the binding site. Any loop movement may have consequences for the binding of cephalosporins with large C3 substituents, as such substituents can easily make contact with this loop (20).

The loop movement may also affect binding and reactivity with clavulanic acid, based on an inhibition mechanism according to the work of Imtiaz et al. (15). In their mechanism, one step of the inhibition reaction relied upon activation of a bound water molecule by R244 (Fig. 4). In the crystal structures of all class A β-lactamases, this water molecule is seen to be hydrogen bonded to the backbone carbonyl group of V216. Clearly, displacement of V216 by deletion of the residue at 212

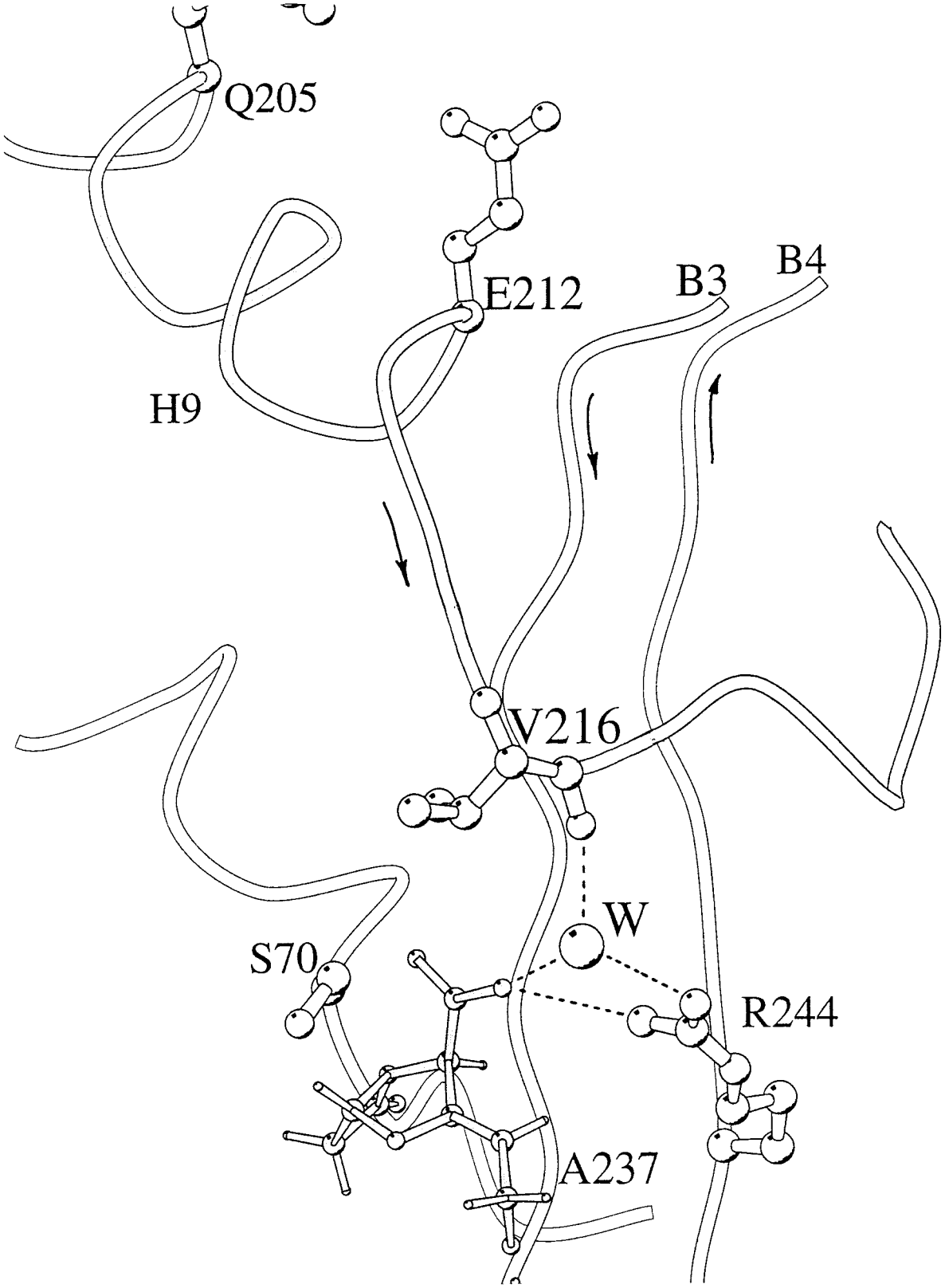

FIG. 4. The position of deleted E212 on the H9 helix above the β-lactam binding site. Modelled (15) hydrogen bonding of clavulanic acid, R244, water (W), and the backbone CO group of V216 is shown.

will alter this hydrogen bonding pattern and possibly change the course of the inhibition by clavulanic acid.

G-to-R mutation at 238. Perhaps the most important change occurring in TEM-AQ is the G-to-R mutation on β-strand B3. Five TEM mutants have serine here, and valine is found in laboratory variants of the related SHV enzyme (20, 39). The presence of a large side chain at this position could be responsible for an outward displacement of the B3 β strand, causing an expansion of the binding site and better accessibility of cephalosporins to the backbone groups of residue 238 (14). A side chain as long as that of arginine, and one that is positively charged as well, is very exceptional. If the side chain is fully extended behind the β sheet, it is rather buried in the enzyme above the omega loop. Presumably, the buried positive charge must be neutralized for structural stability. D176 could fulfill this role, but only if it rotates in the direction of R238. Removal of the ion pair linkage of D176 to R178 is necessary to allow the rotation. This scenario therefore provides an explanation for the radical R-to-A mutation at 178 and suggests that it is directly correlated with the equally radical G-to-R mutation at 238.

In order to elucidate the effect of these mutations, the TEM-AQ β-lactamase has been studied regarding its kinetic profile, and the kinetic parameters calculated with some representative β-lactams have been compared to those previously reported for the parental TEM-1 enzyme by Raquet et al. (36). On the basis of the data here reported, we find that TEM-AQ β-lactamase shows a lower catalytic efficiency with respect to TEM-1 enzyme, when benzylpenicillin, ampicillin, nitrocefin, cephaloridine, and cephalotin are considered; cefazolin and cefotaxime are hydrolyzed with k_{cat}/K_m ratios of the same order of magnitude as those reported for TEM-1. Moreover, TEM-AQ β-lactamase hydrolyzes ceftazidime and aztreonam with catalytic efficiencies 43- and 143-fold higher than those of TEM-1 β-lactamase, respectively. It is very important to emphasize that the improvement in the performance of the mutant is due to an increase in the affinity of the enzyme for ceftazidime and aztreonam; in fact, the K_m value for ceftazidime is 37-fold lower with respect to that calculated with TEM-1 enzyme, whereas an 1,100- and a 30-fold-lower K_m value is calculated for aztreonam and cefotaxime, respectively.

The TEM-AQ enzyme was quite resistant to the serine β-lactamase inhibitors clavulanic acid and sulbactam and sensitive to β-iodopenicillanate. TEM-2–β-IP interaction is thought to involve a turnover, whereas TEM-AQ does not. Clavulanic acid at a fixed concentration of 4 μg was not able to restore efficiently the MICs of ampicillin in both inocula used.

Evolutionary pressure to broaden the spectrum of activity of an enzyme which shows a catalytic efficiency close to the diffusion limit has once more generated a mutated enzyme which is not able to maintain the same values in the presence of a classical β-lactam such as benzylpenicillin, whereas the mutant shows an extremely high k_{cat}/K_m ratio value for aztreonam.

The data reported in the present paper allow us to propose the following tentative conclusions: (i) A unique TEM β-lactamase, produced during an epidemic infective episode by *S. marcescens* S5 in an Italian hospital, has been characterized. (ii) This new TEM-type enzyme is able to inactivate ceftazidime and aztreonam by increasing its affinity, thus conferring increased resistance to these two β-lactam molecules. (iii) The observed mutations can also be involved in moderate resistance to the inhibitory effect of clavulanic acid.

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