Catalytic mechanism of active-site serine β-lactamases: role of the conserved hydroxy group of the Lys-Thr(Ser)-Gly triad

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The role of the conserved hydroxy group of the Lys-Thr(Ser)-Gly [KT(S)G] triad has been studied for a class A and a class C β-lactamase by site-directed mutagenesis. Surprisingly, the disappearance of this functional group had little impact on the penicillinase activity of both enzymes. The cephalosporinase activity was much more affected for the class A S235A (Ser^{235}→Ala) and the class C T316V (Thr^{315}→Val) mutants, but the class C T316A mutant was less impaired. Studies were extended to β-lactams, where the carboxy group on C-3 of penicillins or C-4 of cephalosporins had been modified. The effects of the mutations were the same on these compounds as on the unmodified regular penicillins and cephalosporins. The results are compared with those obtained with a similar mutant (T299V) of the *Streptomyces* R61 DD-peptidase. With this enzyme the mutation also affected the interactions with penicillins and severely decreased the peptidase activity. The strict conservation of the hydroxy group on the second residue of the KT(S)G triad is thus much more easy to understand for the DD-peptidase and the penicillin-binding proteins than for β-lactamases, especially those of class C.

INTRODUCTION

In the active-site serine penicillin-recognizing enzymes whose third-dimensional structures are known, a wall of the catalytic cavity is formed by a piece of β-strand containing homologous triads of residues: K^{234}S(T)G in class A β-lactamases, K^{315}TG in class C β-lactamases and H^{298}TG in the *Streptomyces* R61 DD-peptidase [Herzberg and Moult (1991) and references cited therein]. Sequence alignments have shown that all the other known enzymes belonging to the serine β-lactamase–DD-peptidase–penicillin-binding protein (PBP) superfamily contained KTG, KSG or RSG triads in equivalent positions (Joris et al., 1991; Galleni et al., 1988a).

At the present time, two rules do apparently not suffer a single exception: the third residue is glycine and the second a hydroxylated residue, either serine or threonine. The conservation of the former is easily explained by considering that the presence of a side chain would, for steric reasons, severely interfere with the binding of the substrate. Conversely, the hydroxy group of the second residue appears to be well positioned to hydrogen-bond to the substrate, free carboxylate (Lamotte-Brasseur et al., 1991). Surprisingly, the T316V mutation in a class C β-lactamase did not appear to result in dramatic modifications of the catalytic properties of the enzyme (Tsukamoto et al., 1990). In parallel with the similar study of the equivalent T299 residue of the *Streptomyces* R61 DD-peptidase [the accompanying paper (Wilkin et al., 1994)], this contribution presents an analysis of the role of the Thr^{316} and Ser^{235} residues in a class C and a class A β-lactamase respectively. The interactions between β-lactams and all the studied enzymes obey the simple three-step pathway:

$$E+S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\cdot S \xrightarrow{k_{+2}} E\text{–}S^* \xrightarrow{k_{+3}} E+P$$

where E is the enzyme, S the β-lactam, E·S the Henri–Michaelis complex, E–S* the acyl-enzyme and P the product of hydrolysis. The characteristic steady-state constants are:

$$k_{cat.} = (k_{+2}\cdot k_{+3})/(k_{+2}+k_{+3})$$

and

$$K_m = (k_{+3}\cdot K')/(k_{+2}+k_{+3})$$

where

$$K' = (k_{-1}+k_{+2})/k_{+1}.$$

MATERIALS AND METHODS

Strains and plasmids

The strains of *Escherichia coli* K12 used in this work were SNO3 (ampA1, ampC8, pyrB, recA, rpsL) (Normark and Burman, 1977), FLO1 (recA derivative of *E. coli* MM294) (Lindberg and Normark, 1987) and TG1 (Δ(*lac-pro*), *SupE*, *thi*, *hsdD5*/*F'traD36*, *proA*$^+$*B*$^+$, *lacI*^{q}, *lacZ*Δ*M15*] for routine transformation and DNA preparation. *Salmonella typhimurium χ3000* (LT2-Z), a gift from Dr. R. Curtiss, III (Washington University, St. Louis, MO, U.S.A.) was used for production of the AmpC enzymes.

Bacteria were grown in Luria–Bertani broth or Terrific broth at 37 °C (Sambrook et al., 1989). When required, 20 μg/ml tetracycline was added. Solid media contained 1.5 % Difco Bacto agar.

Plasmid pAD25 (Figure 1) is a derivative of plasmid ptac11 (Amann et al., 1983): two complementary oligonucleotides (37 bases each) coding for the −35 promoter region of the tetracycline-resistance gene (Stüber and Bujart, 1981); corresponding

Abbreviations used: WT, wild-type; PBP, penicillin-binding protein; IPTG, isopropyl β-D-thiogalactopyranoside; Ac_2KAA, $N^{\alpha}N^{\epsilon}$-bisacetyl-L-lysyl-D-alanyl-D-alanine; DCC, deacetylcephalosporin C; Bp, benzylpenicillin; BpMe, benzylpenicillin methyl ester; the one-letter code for amino acids is used for mutants, e.g. S235A is Ser^{235}→Ala; EREF, enzyme rate enhancement factor.

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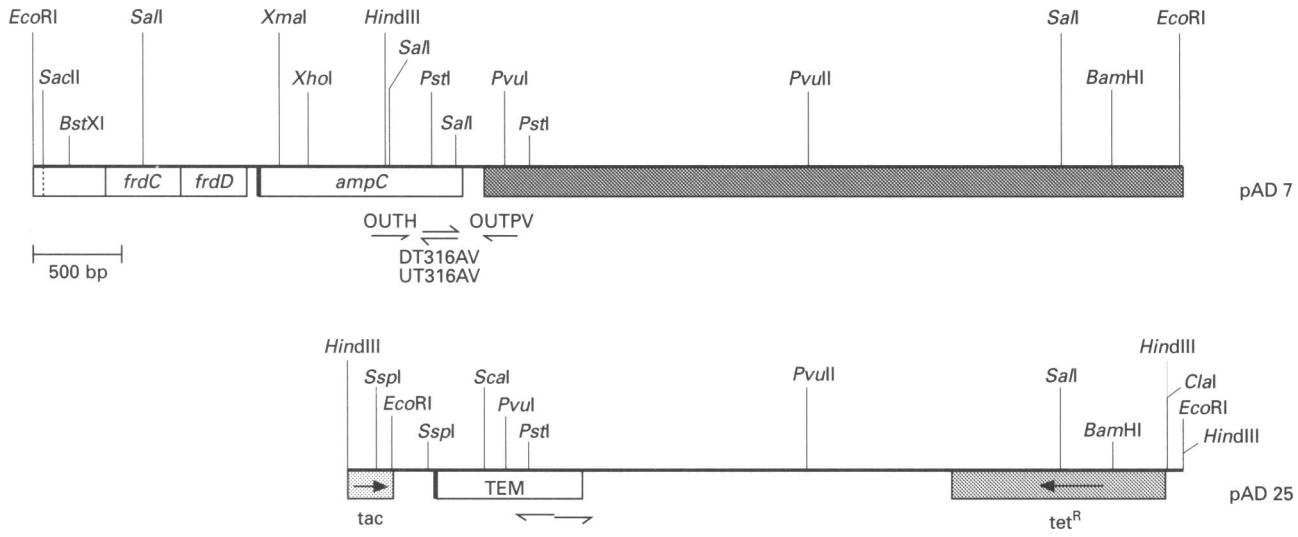

Figure 1 Linear restriction maps of plasmids pAD7 and pAD25

The shaded area in plasmid pAD25 represents part of pBR322 plasmid vector. The oligonucleotides used for the mutagenesis procedures are represented under the maps.

to the *Eco*RI/*Cla*I deletion made while constructing plasmid ptac11 were synthesized and ligated into the *Hin*dIII restriction site of ptac11 to restore the tetracyline resistance. Plasmid pAD25 exhibits thus both a selection marker and an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible TEM-1 β-lactamase expression. Plasmid pAD6 is a derivative of plasmid pNU35 (Grundström et al., 1980) from which a 1438 bp *Sac*II fragment has been deleted. The *ampC* gene encoded by this plasmid contains two up-promoter mutations which result in a high level of AmpC β-lactamase expression (Jaurin et al., 1981). A 2150 bp *Eco*RI/*Pvu*II fragment from pAD6 was cloned into the *Eco*RI/*Sca*I sites of pBR322, from which the unique *Hin*dIII restriction site had previously been deleted, to obtain plasmid pAD7 (Figure 1). This last plasmid was used for the subsequent mutagenesis procedure and the expression of wild-type (WT) and mutant AmpC enzymes.

Nucleic acid techniques

The plasmids were derivatives of pBR322 and were constructed by standard DNA-manipulation techniques (Sambrook et al., 1989). Sequencing was done by the dideoxy chain-termination method of Sanger et al. (1977) using the Sequenase DNA sequencing kit (United States Biochemical). T4 DNA ligase and kinase were purchased from Boehringer Mannheim G.m.b.H., restriction endonucleases were obtained from Gibco BRL, New England Biolabs or Boehringer and used in the buffer supplied by the manufacturer. Deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (10 mCi/ml) was purchased from Amersham International. Other dNTPs were from Boehringer. Oligonucleotides were purchased from the Protein Chemistry Laboratory at the Department of Biochemistry and Molecular Biophysics (Washington University), and from Eurogentec (Liège, Belgium).

Mutagenesis

For the AmpC β-lactamase, mutagenesis was performed by the overlap extension technique (Ho et al., 1988). PCR was done on a DNA thermal cycler (Perkin–Elmer/Cetus) with the Gene Amp DNA Amplification Reagent Kit (Cetus Corp.), the manufacturer's protocol for volumes and reagent concentrations being followed. The first two separate amplifications consisted of 25 cycles at 94 °C (60 s), 52 °C (60 s), 72° (140 s) in 100 μl of reaction mixtures with a DNA template of 2751 bp (*Xho*I/*Pvu*II fragment from pAD7) and two pairs of primers:

5′-TACATAAAG(T/C)AGGGGCGACCGG-3′	DT316AV
5′-GCTCCTTCGGTCCTCCGA-3′	OUTPV
5′-GTCGCCCCT(G/A)CTTTATGTACCC-3′	UT316AV
5′-CGCCTGGGGCGTTAGATG-3′	OUTH

designed to mutate the Thr codon (ACA) into the Ala (GCA) or Val (GTA) codons. The amplified DNA fragments resulting from this first set of reactions were chloroform-extracted, and portions of agarose-gel-purified products were used for the second amplification of 20 cycles at 94 °C (60 s), 50 °C (80 s), 72 °C (140 s). The PCR product was chloroform-extracted, digested with *Hin*dIII/*Pvu*I and the agarose-gel-purified 660 bp fragment containing the mutation was ligated into pAD7 and transformed in *E. coli* SNO3.

The S235A mutant was obtained through inverse PCR mutagenesis (Clackson et al., 1991) with the following oligonucleotides:

5′-TGATAAAG(T/C)TGGAGCCGGTGAGCG-3′

5′-GCAATAAACCAGCCAGCCGG-3′

the first one carrying the two mismatched bases designed to mutate the Ser codon (TCT) into either the Ala (GCT) or Val (GTT) codons. Only the former mutant was further purified and studied.

The amplification was performed with Vent DNA polymerase (Biolabs) in the buffer supplied by the manufacturer to which 10% formamide and 4 mM $MgSO_4$ (final concn.) were added; 30 cycles at 94 °C (60 s), 54 °C (60 s), 72 °C (290 s) were performed on 100 μl of reaction mixture. The PCR product was subsequently

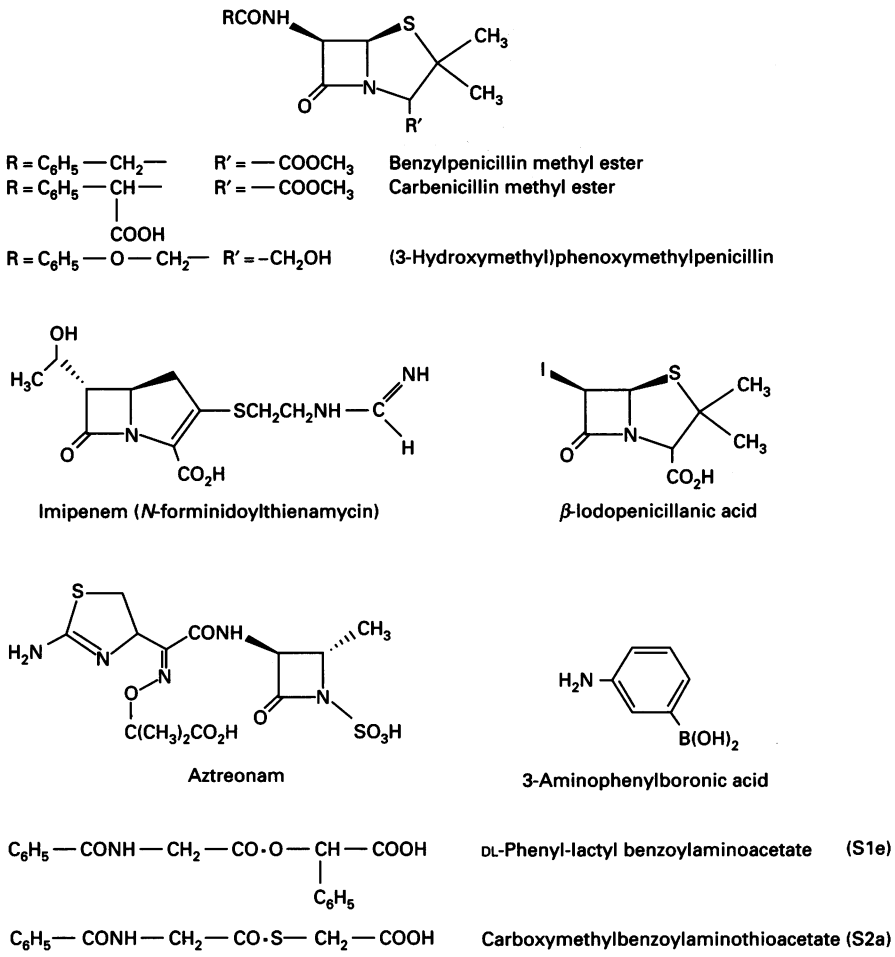

Figure 2 Structures of some of the studied compounds

treated as described by Imai et al. (1991) and transformed into *E. coli* TG1.

The mutagenesis reactions yielded three modified plasmids pNU643 (AmpC T316A), pNU644 (AmpC T316V) and pAD27 (TEM S235A). These were purified, and the modified genes sequenced to ensure that no other unwanted mutations were present.

Chemicals

Benzylpenicillin was from Rhône Poulenc (Paris, France), Phenoxymethylpenicillin was a gift of Dr. H. Vanderhaeghe and Dr. P. Claes (KUL, Leuven, Belgium). Ampicillin and aztreonam were from BristolMyers–Squibb S. A. (Brussels, Belgium). Deacetylcephalosporin C and its lactone, and carbenicillin and cefuroxime were from Glaxo Group Research (Greenford, Middx., U.K.); the (3-hydroxymethyl)phenoxymethylpenicillin was a gift from Dr M. I. Page (Huddersfield University, Huddersfield, U.K.).

Cefotaxime was from Hoechst–Roussel (Romainville, France), Imipenem and cefoxitin were from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.), cefazolin, cephalosporin C, cephalothin, cephalexin and cephaloridine were from Eli Lilly and Co. (Indianapolis, IN, U.S.A.), β-iodopenicillanic acid was from Pfizer Central Research (Sandwich, Kent, U.K.). All these compounds were kindly given by the respective companies. The β-lactam methyl esters were those described in Monnaie et al. (1994), the synthesis of the thioester depsipeptide (S2a) has been described by Adam et al. (1990). The structures that are not depicted by Matagne et al. (1990) and Varetto et al. (1991) are given in Figure 2. Nitrocefin was purchased from Oxoid (Basingstoke, Hants., U.K.). DL-phenyl-lactyl benzoylaminoacetate (Sle), 3-aminophenylboronic acid, IPTG, D-alanine, D-phenylalanine and tetracycline were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Production and purification of the mutant proteins

The *Salmonella* strains harbouring the plasmids coding for the WT or mutant AmpC β-lactamases were grown in 1-litre Erlenmeyer flasks each containing 500 ml of Terrific broth (Sambrook et al., 1989). The flasks were kept at 37 °C for 18 h with orbital shaking. The cells were collected by centrifugation, and the periplasmic contents of the cells were liberated by lysozyme treatment. The supernatant was then dialysed against 10 mM Tris/HCl buffer, pH 7.0, and placed on a 16/20 S-Sepharose Fast Flow column (Pharmacia) equilibrated in the same buffer. The enzymes were eluted by a gradient of 10–100 mM Tris/HCl, pH 7.0, over 300 ml. The active fractions were pooled. The final protein concentrations were 1–2 mg/ml.

The *E. coli* strains harbouring the plasmids coding for the TEM-1 enzyme and its S235A mutant were grown under similar conditions. When the A_{550} of the culture reached a value of 0.8, IPTG was added at a final concentration of 1 mM and the culture was continued for another 3 h. The periplasmic content was obtained as described above and the enzymes were purified as follows: the supernatant was dialysed against 5 mM cacodylate buffer, pH 6.5, and placed on a DEAE-cellulose column (4.6 cm × 30 cm) equilibrated with the same buffer. The enzymes were eluted by a linear NaCl gradient (500 mM final) over 1200 ml. The active fractions were pooled, dialysed against 5 mM cacodylate buffer, pH 6.5, and injected on to a Q-Sepharose Fast Flow column (2.6 cm × 30 cm) equilibrated with the same buffer. The enzymes were eluted under isocratic conditions. The active fractions were pooled and concentrated to 0.8–1.8 mg of protein/ml. The purity of all the enzyme preparations was verified by Coomassie Blue staining of SDS/PAGE gels, and found to be higher than 95%. The TEM mutant was also inactivated by β-iodopenicillanate, and the spectrum of the adduct exhibited an A_{325}/A_{280} ratio characteristic of pure enzyme (De Meester et al., 1986).

Kinetic methods

The steady-state kinetic parameters ($k_{cat.}$, K_m, K_i, $k_{cat.}/K_m$) were determined by spectrophotometry as described before (Matagne et al., 1990; Dubus et al., 1993). Inactivation parameters (k_{+2}/K', k_{+2}, K') were determined using the reporter-substrate method (De Meester et al., 1987) with the help of either Uvikon 860 (Kontron Instruments) or Beckman DU-8 spectrophotometers interfaced to microcomputers in the case of slow reactions ($k_i \leqslant 0.05\ s^{-1}$, where k_i is the pseudo-first-order inactivation rate constant) or a Biologic SFM3 stopped-flow apparatus for faster reactions (Monnaie et al., 1992). The reporter substrate was either cefazolin or nitrocefin.

Re-activation phenomena (k_{+3}) were continuously monitored after diluting inactivated samples in reporter-substrate solutions (De Meester et al., 1987).

The values of the $k_{cat.}$ and K_m constants were deduced by fitting complete time courses to the integrated Henri–Michaelis equation (De Meester et al., 1987). Fitting the experimental data to the hyperbolic equation was performed with the help of the ENZFITTER program (Leatherbarrow, 1987) when initial rates were measured ($k_{cat.}$ and K_m for poor substrates exhibiting high K_m values) or for the determination of the individual k_{+2} and K' values from the concentration dependency of k_i.

Unless specifically mentioned otherwise, all the kinetic assays were performed at 30 °C in 10 mM Hepes/200 mM NaCl, pH 8.0, and 50 mM sodium phosphate buffer, pH 7.0, for the AmpC and TEM enzymes respectively.

The rate of thermal denaturation of the AmpC enzymes was determined by monitoring both the fluorescence at 370 nm ($\lambda_{excitation}$ 280 nm) and the enzyme activity in the presence of a reporter substrate (cefazolin) at a concentration well below its K_m value and under conditions where only a small proportion of this substrate was utilized. Denaturation induced an increase of the fluorescence intensity above 340 nm accompanied by a red shift. The loss of activity was irreversible, as verified in experiments where samples were diluted and assayed again at 30 °C.

For the TEM enzymes, the fluorescence at 340 nm ($\lambda_{excitation}$ 280 nm) was recorded as a function of increasing temperature (1.3 °C/min). Estimation of the transpeptidation products obtained with the thiolester and D-phenylalanine was done by h.p.l.c. on a Lichrospher 100 RP-18 column (Merck, Darmstadt, Germany) as described by Jamin et al. (1991).

RESULTS

Stability

Table 1 compares the thermostabilities of the three AmpC proteins. The two methods yielded similar results provided that the reporter substrate concentration was well below the K_m value. The loss of activity was irreversible in all cases.

Figure 3 shows the thermal-denaturation curves of the wild-type and mutant TEM enzymes as monitored by fluorescence. The melting temperature (T_m) values were 51.6 and 53.3 °C for the wild-type and S235A mutant proteins respectively. In both cases, after completion of the denaturation at 65 °C, samples were rapidly cooled to 30 °C, and it could be shown that the activity was fully recovered, indicating fully reversible phenomena. These results indicated that no major structural differences occurred between the WT and modified enzymes.

Kinetic properties: steady state

Five types of compounds were examined: (1) Unmodified penicillins and cephalosporins; (2) penicillins whose carboxylate group on C-3 had been esterified or replaced by a hydroxymethyl group, and a cephalosporin lactone in which the equivalent C-4 carboxylate had been esterified by the C-3′ hydroxy group

Table 1 Half-lives of the AmpC enzymes at 60 °C

The reporter substrate was 200 μM cefazolin (in 10 mM Hepes/200 mM NaCl, pH 8.2). K_m values are 1500, 7500 and > 2000 μM for the WT, T316A and T316V enzymes respectively. The enzyme concentrations ranged from 10–20 μg/ml in the fluorescence experiments and 0.04–2.4 μg/ml in the reporter substrate assays.

		Half-life (s)		
Method	Enzyme ...	Wild-type	T316A	T316V
Fluorescence		134 ± 4	45 ± 3	119 ± 4
Reporter substrate		139 ± 11	67 ± 10	108 ± 7

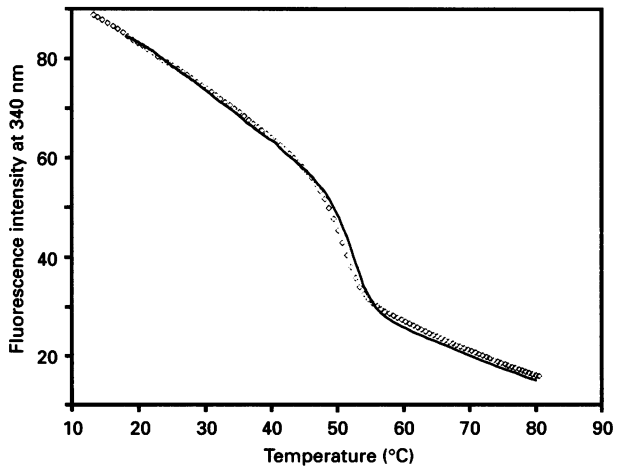

Figure 3 Thermal-denaturation curves of the WT (◇) and S235A (—) TEM enzymes

The enzymes (15 μg/ml) were in 50 mM sodium phosphate buffer, pH 7.0, and the temperature was increased at a rate of 1.3 °C/min. The fluorescence intensity (two readings/min; arbitrary units) was recorded at 340 nm.

Table 2 Kinetic parameters of the penicillins with the WT and mutant AmpC enzymes

Substrate	AmpC... / Parameter...	WT $k_{cat.}$ (s^{-1})	WT K_m (μM)	WT $k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	T316A $k_{cat.}$ (s^{-1})	T316A K_m (μM)	T316A $k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	T316V $k_{cat.}$ (s^{-1})	T316V K_m (μM)	T316V $k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)
Benzylpenicillin		86±4	4.5±0.5	19500±2400	83±3	12.2±1.2	6800±700	39±4	10.1±2.3	3800±1000
Phenoxymethylpenicillin		43±4	6.9±1.5	6300±1500	38±3	16.8±2.4	2270±360	14±1	15±2	910±130
Ampicillin		3.00±0.07	3.07±0.04	980±30	2.5±0.1	3.73±0.06	670±30	0.82±0.02	2.6±0.1	320±10
Carbenicillin		(4.4±0.6)×10^{-3}	0.03†	135±3	(3.1±0.1)×10^{-3}	0.4†	7.4±0.5	(3.1±0.1)×10^{-3}	7.8†	0.4±0.02
Benzylpenicillin methyl ester		43±1	1.75±0.08	24000±1000	8.4±0.3	0.30±0.01	28000±2000	2.7±0.1	0.08±0.02	34000±8000
(3-Hydroxymethyl)-phenoxymethylpenicillin		11.0±0.2	2.25±0.80	4900±1700	4.8±0.6	2.9±1.0	2300±600	1.13±0.08	1.25±0.6	904±440
Carbenicillin methyl ester		(6±1)×10^{-3}	0.3†	23±1	(6±0.4)×10^{-3}	0.03†	176±10	(3.8±0.3)×10^{-3}	0.03†	110±10

* k_{+3} was derived from re-activation experiments.
† Calculated as $K_m = k_{+3} \cdot K'/k_{+2}$.

Table 3 Kinetic parameters of the cephalosporins with the WT and mutant AmpC enzymes

Substrate	AmpC... / Parameter...	WT $k_{cat.}$ (s^{-1})	WT K_m (μM)	WT $k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	T316A $k_{cat.}$ (s^{-1})	T316A K_m (μM)	T316A $k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	T316V $k_{cat.}$ (s^{-1})	T316V K_m (μM)	T316V $k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)
Nitrocefin		670±30	520±35	1290±100	645±36	658±60	980±100	36±2	1100±70	33±3
Cephalothin		300±15	42±2	7000±1000	175±6	930±40	188±4	46±2	2540±125	1.8±0.3
Cephalexin		38±3	4±0.4	9500±1700	15.6±1.4	105±7	150±20	6.5±0.3	600±40	10.8±0.8
Cephaloridine		188±13	190±10	990±90	100±8	1870±210	53±7	> 15	≫ 2000	8±0.5
Cefazolin		477±53	1510±180	320±50	90±8	7500±750	12±2	> 6	≫ 2000	3±0.3
Cephalosporin C		540±20	74±5	7300±600	216±12	360±70	600±100	94±6	1650±190	57±8
Deacetylcephalosporin C		364±7	50±4	7200±600	512±24	223±24	2300±200	152±5	340±14	450±10
Deacetylcephalosporin C lactone		113±4	18.6±1.4	6100±500	139±4	24.3±1.5	5700±390	4.0±0.5	6.1±0.3	660±90
Cefotaxime*		0.15±0.01	0.87±0.02	170±10	0.077±0.004	6.41±0.05	12±1	0.037±0.004	32.8±0.5	1.1±0.1
Cefuroxime*		0.050±0.001	0.087±0.007	575±50	0.032±0.001	0.52±0.02	61±3	0.025±0.001	3.0±0.1	8.3±0.4

* K_m was determined as a K_i in competition assays, $k_{cat.}/K_m = k_{cat.}/K_i$.

(Figure 2); (3) very poor substrates (aztreonam, imipenem), which behave as transient inactivators of the class C enzyme (Galleni and Frère, 1988); (4) with the class C enzyme, an irreversible inactivator (β-iodopenicillanate) and a reversible inhibitor (3-aminophenylboronic acid); and (5) linear depsipeptides, an ester and a thiolester whose structures are shown in Figure 2 and which can be hydrolysed by the WT enzymes and serve as donor substrates in the transfer reactions catalysed by the class C enzyme (this latter reaction is not significantly observed with the class A β-lactamases).

Tables 2 and 3 show the catalytic properties of the class C β-lactamase versus the unmodified and modified penicillins and cephalosporins. The most striking results are as follows.

(i) A nearly total absence of influence of the mutations on the regular and modified penicillins, with the sole exception of the $k_{cat.}/K_m$ values for carbenicillin. But conversely, the $k_{cat.}/K_m$ value of the carbenicillin methyl ester was larger with the modified than with the WT enzymes.

(ii) A much stronger effect of the mutations on the $k_{cat.}/K_m$ values for the cephalosporins, with the possible exceptions of deacetylcephalosporin C and nitrocefin. Again, the behaviour of the lactone is not significantly different from that of the parent compound. The T316A mutant consistently retained a higher proportion of the WT activity.

As shown in Table 4, the results obtained with the TEM WT and mutant enzymes indicated a somewhat similar pattern: a negligible influence of the mutations for penicillins, again with the possible exception of carbenicillin and a much more pronounced decrease of the acylation rate by cephalosporins. The disappearance of the free carboxylate on C-3 of penicillins results, however, in a dramatic decrease in the $k_{cat.}/K_m$ values (400- and 10000-fold), with both the WT and the modified protein.

The results obtained with the very poor substrates and β-iodopenicillanate are presented in Table 4 for the TEM enzymes and aztreonam and in Table 5 for the class C enzyme. The mutations did not seem to significantly affect the k_{cat} (TEM) or k_{+3} (= $k_{cat.}$, AmpC) values, but significantly decreased the k_{+2}/K' or $k_{cat.}/K_m$ values. With the acyclic ester and thiolester, the $k_{cat.}/K_m$ values were only significantly decreased with the class C mutants and the ester (Table 6). In the other cases, the decreases did not exceed a factor of 20. Transpeptidation assays were performed with the AmpC WT and mutant proteins. With 0.5 mM thioester and 20 mM D-phenylalanine, the transpepti-

Table 4 Kinetic parameters for the WT and mutant TEM enzymes

	TEM-1 ...	WT			S235A		
Substrate	Parameter ...	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)
Benzylpenicillin		1420±80	25±3	57000±8000	413±43	23±4	18000±4000
Phenoxymethylpenicillin		410±70	6±1.4	69000±10000	123±16	5.5±1.3	22000±4000
Ampicillin		880±10	76±3	11600±500	600±11	93±6	6430±430
Carbenicillin		110±10	9±1.6	12000±2500	33±1.4	67±5	490±50
Benzylpenicillin methyl ester		> 9	> 1600	5.6±0.1	> 18	> 2000	9.1±0.2
(3-Hydroxymethyl)-phenoxymethylpenicillin		285±30	1800±250	160±30	345±50	3290±580	105±25
Nitrocefin		850±70	46±10	18480±4660	> 175	> 1000	195±4
Cephalothin		95±10	166±15	570±100	14±1	6640±600	2.1±0.2
Cephalexin		15±1	1190±120	13±2	> 0.5	> 3000	0.149±0.002
Cephaloridine		1500±150	670±100	2240±400	33±4	4600±700	7.1±1.4
Cefazolin		140±8	380±20	370±35	> 3.7	> 2500	1.50±0.05
Cephalosporin C		28±2	414±14	67±5	3.1±0.5	11700±2000	0.26±0.06
Deacetylcephalosporin C		11±1	530±80	21±3	0.48±0.06	7700±1200	0.062±0.012
Cefotaxime		> 1.5	> 1000	1.5±0.1	> 0.0056	> 1000	0.0056±0.0009
Cefuroxime		6±1	1050±150	5.7±1.3	> 0.014	> 1000	0.0144±0.0004
Aztreonam		1±0.1	1500±200	0.67±0.11	> 0.192	> 3000	0.0064±0.0004

Table 5 Kinetic parameters of poor substrates and inactivator for the WT and mutant AmpC enzymes

	Aztreonam		Imipenem		β-Iodopenicillanate
AmpC	k_{+2}/K' ($mM^{-1} \cdot s^{-1}$)	k_{+3} (s^{-1})	k_{+2}/K' ($mM^{-1} \cdot s^{-1}$)	k_{+3} (s^{-1})	k_{+2}/K' ($mM^{-1} \cdot s^{-1}$)
WT	410±10	$(2.4 \pm 0.2) \times 10^{-3}$	9.3±0.2	$(5 \pm 2) \times 10^{-3}$	0.194±0.002
T316A	77±4	$(1.4 \pm 0.1) \times 10^{-3}$	0.11±0.01	$(4 \pm 1) \times 10^{-3}$	0.0083±0.0001
T316V	104±5	$(2.6 \pm 0.1) \times 10^{-3}$	0.034±0.002	$(3 \pm 1) \times 10^{-3}$	0.0028±0.0003

Table 6 Kinetic parameters for the acyclic depsipeptides with the WT and mutant enzymes

	Substrate ...	Sle (ester)			S2a (thiolester)		
Enzyme		$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)
AmpC							
WT		30±1	1320±100	22.7±0.3	> 8	> 2500	2.9±0.1
T316A		> 0.4	> 2000	0.19±0.01	> 1.75	> 2500	0.70±0.07
T316V		–	–	0.02	> 0.6	> 2500	0.24±0.04
TEM-1							
WT		0.10±0.01	1920±250	0.052±0.008	20±2	3800±500	5.2±0.8
S235A		> 0.008	> 2500	0.004	> 0.75	> 2500	0.3±0.01

dation/hydrolysis ratios were 3.5 with the WT and 0.06 with both mutants, thus demonstrating a nearly complete loss of the transpeptidation properties of the mutants. It should be remembered that the WT TEM enzyme is unable to catalyse this type of reaction. Finally, with the AmpC enzyme, the mutations did not significantly modify the K_i values for 3-aminophenylboronate: 9.2 μM with the WT enzyme, and 16.6 and 17.2 μM with the T316A and T316V mutants respectively.

Kinetic properties: pre-steady-state parameters

The k_{+3} and k_{+3} values for the good substrates of class C enzymes

Table 7 Individual values of the catalytic parameters for the WT and mutant AmpC enzymes

	AmpC... Wild-type	T316A	T316V
Carbenicillin			
k_{+2} (s^{-1})	25 ± 1	3.25 ± 0.1	0.93 ± 0.04
K' (mM)	0.24 ± 0.02	0.45 ± 0.03	4.0 ± 0.3
k_{+2}/K' ($mM^{-1} \cdot s^{-1}$)	110 ± 13*	7.3 ± 0.7*	0.23 ± 0.03*
	135 ± 3†	7.4 ± 0.5†	0.40 ± 0.02†
k_{+3} (s^{-1})	$(4.4 \pm 0.6) \times 10^{-3}$‡	$(3.1 \pm 0.1) \times 10^{-3}$‡	$(3.1 \pm 0.1) \times 10^{-3}$‡
Cefotaxime			
k_{+2} (s^{-1})	> 130	36 ± 2	> 15
K' (mM)	> 1	2.5 ± 0.2	> 4.5
k_{+2}/K' ($mM^{-1} \cdot s^{-1}$)	150 ± 2*	14 ± 2*	3.3 ± 0.3*
$k_{cat.}/K_i$ ($mM^{-1} \cdot s^{-1}$)	170 ± 10	12 ± 1	1.1 ± 0.1
$k_{+3} = k_{cat.}$ (s^{-1})	0.15 ± 0.01	0.077 ± 0.004	0.037 ± 0.004

* Calculated from the individual values.
† Measured directly at low carbenicillin ($\ll K'$) concentrations.
‡ Measured by re-activation (see Table 2).

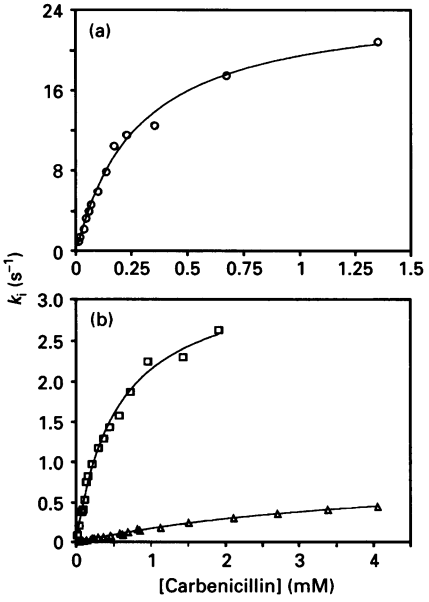

Figure 4 Influence of the carbenicillin concentration on the rate of acylation of the WT (a), T316A (□) and T316V (△) (b) mutant AmpC enzymes

The values obtained by non-linear regression are shown in Table 7.

are generally very high (Galleni and Frère, 1988; Galleni et al., 1988b). When the $k_{cat.}$ values are greater than 100 s^{-1}, the steady state is usually established within the mixing dead time of the stopped-flow apparatus. In consequence, accumulation of the acyl-enzyme could only be directly observed with some poor substrates (Monnaie et al., 1992) of the *Enterobacter cloacae* 908R β-lactamase and, even in that case, the k_{+2} and K' values were often so high that only the k_{+2}/K' ratio could be directly determined in stopped-flow experiments.

In the present study, carbenicillin and cefotaxime were selected for a detailed analysis on the basis of apparently suitable $k_{cat.}$ and $k_{cat.}/K_m$ values with the *E. coli* AmpC enzyme and its mutants. However, with cefotaxime, only minimum k_{+2} and K' values could be determined for the WT enzyme. The results presented in Table 7 and Figure 4 indicate that the mutations did not affect K' more significantly than k_{+2}.

DISCUSSION

In the present study the role of hydroxy group of the second residue of the KT(S)G triad has been analysed in detail.

With both a class A and a class C β-lactamase, the disappearance of this hydrogen-bonding group has much more pronounced effects on the cephalosporinase than on the penicillinase activity. These results are in good agreement with those of Imtiaz et al. (1993), who studied the same S235A mutant of the TEM-1 enzyme, but at variance with those of Tsukamoto et al. (1990), who did not record an influence of a similar mutation on the cephalosporinase activity of another class C enzyme. These observations raise an interesting question. Indeed, class A β-lactamases exhibit a very small number of strictly conserved functional groups or residues, and this hydroxy group is one of them. Why would it be so generally conserved if it were useless? As proposed by Imtiaz et al. (1993), the only rational answer seems to be that the 'natural' selective pressure (as opposed to the artificial one recently exerted by the clinical utilization of β-lactam antibiotics) which was applied on β-lactamases during the long period since these enzymes diverged from the putative ancestral gene was mainly due to cephalosporins and not to penicillins. This might easily be understood for bacteria which produce cephalosporins, such as members of the Actinomycetales family, or which compete with these for the same ecological niches, but it is much more difficult to rationalize for other species, such as *E. coli*, which usually live in a very different environment.

In contrast with the situation with the β-lactamases, the T299V mutation in the DD-peptidase significantly decreased the rate of acylation by penicillins, but again the acylation efficiencies of cephalosporins were more affected. In consequence, the three enzymes exhibited a consistent behaviour: the acylation parameter (k_{+2}/K', corresponding to $k_{cat.}/K_m$ for the β-lactamase) was always significantly more affected with cephalosporins than with penicillins (Table 8). This observation might be correlated

Table 8 Comparison of the WT over mutant ratio of the $k_{cat.}/K_m$ (or k_{+2}/K') values for the β-lactamases (class A and C) and the *Streptomyces* R61 DD-peptidase

	Enzyme...	R61 DD-peptidase	*E. coli* AmpC		*E. coli* TEM-1
Substrate	Mutant...	T299V	T316A	T316V	S235A
Ac_2KAA		600*	Not a substrate for WT and mutant enzymes		
S1e		> 50	110	500	13
S2a		12†	5	15	17
Benzylpenicillin		30	2.9	5.1	3
Ampicillin		50	1.5	3	2
Carbenicillin		30‡	18§	340	25
Benzylpenicillin methyl ester		20	0.9	0.7	0.6
Nitrocefin		135	1.3	40	100
Cephalosporin C		> 1500	12	120	260
Cefuroxime		> 1600	10	75	400
Deacetylcephalosporin C		nd‖	3	16	nd
Other cephalosporins		nd	20–60	100–3500	90–300

* Only due to k_{+2}; K_m is unchanged.
† $k_{cat.} = k_{+3}$; the factor of 12 results from factors of 3 and 4 on $k_{cat.}$ and K_m respectively.
‡ Increase of K'.
§ Decrease of k_{+2}.
‖ Not determined.

with the results obtained by Juteau et al. (1992), who docked ampicillin and cephalexin in the active site of another class A β-lactamase and showed that the latter antibiotic minimized significantly 'higher' in the active site (i.e. probably closer to the Ser^{235} side chain) than the former.

To complement the site-directed-mutagenesis approach, the consequences of the modification of the substrate carboxylate group were also analysed. Indeed, the presence of such a negatively charged group on C-3 of penicillins and C-4 of cephalosporins appears to be a prerequisite for their antimicrobial activity, and it has been hypothesized to interact with the hydroxy group of the second residue of the KT(S)G motif studied here (Lamotte-Brasseur et al., 1991; Juteau et al., 1992). It is not easy to propose clear-cut conclusions from the numerous results presented here, and the fact that the intermediate complexes are too unstable to allow a direct observation by X-ray crystallography or n.m.r. represents a major difficulty in attempting to clarify the situation. Indeed, some of the results might appear contradictory, and the peptidase and β-lactamases of both classes do not always behave as a homogeneous group of enzymes.

The T299V mutation yielded a DD-peptidase nearly inactive towards the tripeptide, an analogue of the substrate of bacterial transpeptidases. This was probably due to a decrease in k_{+2}, which contrasted with the major effect on K' observed with carbenicillin, and these results underline again the distinct behaviours of substrates and penicillins when they interact with the DD-peptidases. The mutant also became unable to catalyse the transacylation reactions, which are essential to the physiological role of these enzymes. Interestingly, the T316A and T316V mutant class C β-lactamases suffered a similar impairment of their transacylation properties, suggesting that the corresponding residues in the two enzymes participate in this reaction. In contrast to the situation with β-lactamases, the strict conservation of the Thr residue of the KTG motif in all DD-peptidases and PBPs is thus easy to understand, since this side chain appears to be essential to both the acylation by the donor peptide and the transfer of the acyl moiety on to the acceptor, pivotal reactions in the formation of the peptidoglycan cross-bridges.

In the β-lactamases, the mutations consistently decreased the $k_{cat.}/K_m$ values for the cephalosporins more significantly than the $k_{cat.}$ values. For the class C enzyme, deacylation is generally rate-limiting, and it can thus be concluded that k_{+3} was only a little or not at all affected when water was the attacking nucleophile. Interestingly, the largest decreases in the acylation rates were observed with some of the poorest substrates of the WT enzyme (imipenem and the ester S1e) and with the rather inefficient inactivator, β-iodopenicillanate. The unchanged K_i values observed in the interactions between the AmpC mutants and the boronic acid suggested that the decrease of $k_{cat.}/K_m$ was mainly due to increased K' values but the k_{+2} value for carbenicillin was somewhat affected by the mutation (Table 7).

It was also surprising that the T316A substitution yielded a consistently more active enzyme than the more conservative T316V mutation. This could be due to unfavourable interactions with the more hydrophobic Val side chain, as suggested by the increased K' value for the T316V mutant with carbenicillin (Table 7).

The behaviours of the TEM-1 class A enzyme and of its S235A mutant are even more difficult to interpret since the relative values of k_{+2} and k_{+3} are not known for their interactions with cephalosporins. An increase of K' or a decrease of k_{+2} might both account for the present observations.

The results obtained with the modified substrates can be most significantly analysed by making use of the 'Enzyme Rate Enhancement Factor' (EREF) introduced by Laws and Page (1989), which represents the ratio of the $k_{cat.}/K_m$ value of the enzymic reaction to the second-order rate constant, k_{OH}, observed for the alkaline hydrolysis of the β-lactam, which reflects the intrinsic susceptibility of the compound to a nucleophilic attack. In agreement with previous results (Laws and Page, 1989; Varetto et al., 1991; Laws et al., 1993), and despite the fact that the negative electrostatic potential maps for the benzylpenicillin

Table 9 EREF values and ratios for modified and unmodified substrates

	Mutant or WT	Benzylpenicillin (Bp)	BpMe*	BP / BpMe	Deacetylcephalosporin C (DCC)	DCC lactone	DCC / DCC lactone
Peptidase R61	WT	140000	11	12700	25000	10	2500
	T299V	4600	0.5	9200	–	–	–
Class A	TEM-1: WT	440×10^6	2600	170000	230000	–	–
	S235A	140×10^6	4300	33000	700	–	–
	Bacillus licheniformis WT†	500×10^6	1000	500000	500000	160000	3
	B. cereus WT	280×10^6‡	2800‡	100000	(67000)§	(28000)§	(2.5)
	S. albus WT†	22×10^6	400	55000	120000	2000	60
	S. albus R220L‖	0.38×10^6	3500	110	550	3900	0.14
Class C	*E. coli* K12 WT	150×10^6	11×10^6	14	80×10^6	0.32×10^6	250
	T316A	52×10^6	13×10^6	4.1	25×10^6	0.30×10^6	80
	T316V	29×10^6	16×10^6	1.8	5×10^6	0.035×10^6	140
	Enterobacter cloacae 908R WT†	250×10^6	5.4×10^6	46	100×10^6	0.13×10^6	770

* Me = methyl ester.
† Varetto et al. (1991); Varetto (1991).
‡ Laws and Page (1989).
§ Results for benzyldeacetyl cephalosporanic acid and its lactone (Laws et al., 1993).
‖ Jacob-Dubuisson et al. (1991).
–, Not done.

methyl ester and for the deacetylcephalosporin C lactone were not very different from those of benzylpenicillin and deacetylcephalosporin C (Varetto et al., 1991), the disappearance of the substrate free carboxylate often, but not always, seriously decreased their EREF values (Table 9). For the peptidase, the acylating potencies of the modified substrates were consistently and very significantly impaired when compared with those of the parent compounds bearing the net negative charge. For the class A enzymes, the decrease was extremely large in the case of the methyl ester, but nearly negligible for the lactone. Conversely, for the class C enzymes, the methyl ester and the other modified penicillins were nearly as efficient as the parent compounds, but the lactonization appeared to result in a somewhat larger impairment, although not as dramatic as that observed for the class A enzymes and the methyl ester. Again, these results probably underline subtle differences in the interactions between the two classes of β-lactamases and their substrates.

However, the experiments performed with the modified proteins and the modified substrates yielded an unexpected, but consistent, result. The EREF values for the modified substrates were never less significantly affected by the mutations than those of the parent compounds, an observation which appears to rule out any important interaction between the substrates' carboxylate and the hydroxy group of the second residue of the triad. In fact, the only mutation which was found to selectively affect the EREF values of the modified substrates when compared with those of the parent compounds was the R220L substitution in the *Streptomyces albus* G class A β-lactamase (Table 9; Jacob-Dubuisson et al., 1991). This result suggested a major direct or indirect interaction between the positive Arg guanidinium group, which is not far from the KTG triad, and the substrate negative charge, but the interpretation which had been formerly proposed, i.e. an influence of the Arg side chain on the orientation of the Thr hydroxy group, resulting in an optimal interaction of the latter with the substrate carboxylate, appears to be ruled out by the present results, unless the respective roles of Thr^{235} in the *S. albus* G and Ser^{235} in the TEM-1 enzymes are significantly different, a supposition which does not presently appear to be very realistic.

Further work seems necessary before coherent catalytic mechanisms can be proposed for this group of very important enzymes. It is also evident that site-directed-mutagenesis experiments do not always supply clear-cut answers when multi-step catalytic pathways are involved and that conclusions relying on the analysis of the behaviour of one mutant towards a limited number of substrates can lead to a very incomplete picture of a complex phenomenon.

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