The mechanism of action of DD-peptidases: the role of Threonine-299 and -301 in the *Streptomyces* R61 DD-peptidase

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The side chains of residues Thr^{299} and Thr^{301} in the *Streptomyces* R61 DD-peptidase have been modified by site-directed mutagenesis. These amino acids are part of a β-strand which forms a wall of the active-site cavity. Thr^{299} corresponds to the second residue of the Lys-Thr(Ser)-Gly triad, highly conserved in active-site β-lactamases and penicillin-binding proteins (PBPs). Modification of Thr^{301} resulted only in minor alterations of the catalytic and penicillin-binding properties of the enzyme. No selective decrease of the rate of acylation was observed for any particular class of compounds. By contrast, the loss of the hydroxy group of the residue in position 299 yielded a seriously impaired enzyme. The rates of inactivation by penicillins were decreased 30–50-fold, whereas the reactions with cephalosporins were even more affected. The efficiency of hydrolysis against the peptide substrate was also seriously decreased. More surprisingly, the mutant was completely unable to catalyse transpeptidation reactions. The conservation of an hydroxylated residue in this position in PBPs is thus easily explained by these results.

INTRODUCTION

The membrane-bound penicillin-binding proteins (PBPs) control the biosynthesis of the bacterial-cell-wall peptidoglycan (Frère et al., 1992). *In vitro*, some of these proteins exhibit DD-carboxypeptidase and/or DD-transpeptidase activities. Together with the active-site-serine β-lactamases, they form a superfamily of penicillin-recognizing enzymes which share the following properties: (1) the three-step kinetic model shown below (Model 1) describes the interaction between the two types of enzymes and β-lactams (Frère and Joris, 1985; Christensen et al., 1990; Waley, 1992); (2) some esters or thiolesters behave as substrates for both types of enzymes (Govardhan and Pratt, 1987; Adam et al., 1990; Jamin et al., 1991) according to the same Model 1; (3) their three-dimensional structures exhibit striking similarities (Kelly et al., 1986; Samraoui et al., 1986; Oefner et al., 1990); (4) sequence alignments have allowed the identification of several conserved elements (Joris et al., 1988, 1991) which probably play important roles in the protein architecture or in the catalytic mechanism.

One of the conserved elements is the well-known Lys-Thr(Ser)-Gly sequence [KT(S)G], which is part of a β-sheet strand forming one of the active-site walls. In the *Streptomyces* R61 DD-peptidase (EC 3.4.16.4) this element is His^{298}-Thr^{299}-Gly^{300}-Thr^{301} and corresponds to Lys^{234}-Thr^{235}-Gly^{236}-Ala^{237} in the class A β-lactamases (Joris et al., 1988, 1991). Different studies have demonstrated the importance of this element in the class A β-lactamase catalysis: site-directed mutagenesis of the *Bacillus licheniformis* β-lactamase emphasized the role of Lys^{234} (Ellerby et al., 1990; Brannigan et al., 1991); molecular modelling of β-lactam antibiotics in the enzyme cavity of several class A enzymes (Lamotte-Brasseur et al., 1991, 1992; Moews et al., 1990; Strynadka et al., 1992) has shown that Thr^{235} might be involved in substrate binding via a hydrogen bond formed between the hydroxy group of the threonine and the carboxylate group of the β-lactam.

The residues which immediately follow the KT(S)G triad are not conserved among the penicillin-recognizing enzymes and do not play an essential role in the catalytic phenomena. However, they appear to significantly affect the substrate profile of class A β-lactamases. In 1976, Hall and Knowles, by applying a selective pressure on the TEM β-lactamase after chemical mutagenesis, observed a significant increase of the cephalosporinase/penicillinase ratio. The mutation was later identified as alanine-237→threonine ($Ala^{237}Thr$), and modifications of the same residue (Healey et al., 1989) resulted in alterations of the specificity profile of the enzyme. In the R61 DD-peptidase, the first residue of the corresponding HTG triad, His^{298}, has been replaced by Lys and Gln, and both mutations resulted in proteins seriously impaired in both their catalytic and penicillin-binding properties (Hadonou et al., 1992).

In the present study, the importance of the ubiquitous hydroxyl group of the second residue of the triad was assessed by replacing Thr^{299} by Val. In turn, Thr^{301}, which corresponds to Ala^{327} in the class A β-lactamases, was replaced by Ser and Ile in the hope of modifying the sensitivity of the DD-peptidase to penicillins or cephalosporins and of obtaining a better understanding of the secondary interactions which govern the relative efficiencies of these two families of β-lactams as inactivators of the DD-peptidases.

Kinetic models

Two kinetic models have been used to describe the different activities of the R61 DD-peptidase.

Model 1: carboxypeptidase activity and interaction with β-lactam inhibitors

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\cdot C \xrightarrow{k_{+2}} E\text{–}C^* \xrightarrow{k_{+3}} E + P(s) \qquad \text{(Model 1)}$$

where E, C, E·C, E–C* and P(s) are respectively the enzyme, the substrate of the β-lactam inactivator, the non-covalent Henri–Michaelis complex, and acylenzyme and the reaction products; k_{-1}, k_{+2} and k_{+3} are first-order rate constants and where $K' = (k_{-1}+k_{+2})/k_{+1}$.

Abbreviation used: PBP(s), penicillin-binding protein(s).

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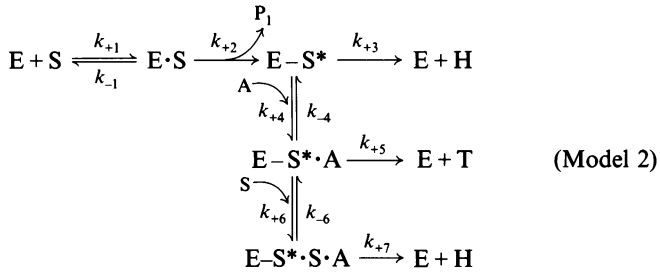
Model 2: transpeptidase activity (Jamin et al., 1993)



(Model 2)

where E, S, A, E·S, E–S*, P_1, H and T are respectively the enzyme, the donor substrate, the acceptor substrate, the Henri–Michaelis complex, the acylenzyme, the leaving group, the hydrolysis and transfer products and where $K' = (k_{-1}+k_{+2})/k_{+1}$, $\alpha = (k_{-4}+k_{+5})/k_{+4}$ and $\beta = (k_{-6}+k_{+7})/k_{+6}$.

MATERIALS AND METHODS

Chemicals were of the same origin as in the previous papers (Wilkin et al., 1993a,b) and Table 1 summarizes the structures of the various substrates.

Oligonucleotides were a gift of SmithKline Beecham, Brockham Park, Surrey, U.K. The oligonucleotides utilized to introduce the mutations were purified as described by Maniatis et al. (1989). They had the following sequences:

Thr[301]Ile, CACACGGGCATCGTGCAGGGC
C

Thr[301]Ser, CACACGGGCAGCGTGCAGGGC
C

Thr[299]Val,
GTGTACGGCCACGTGGGCACCGTGCAGGGC
AC

where, at the level of the mutations, the lower nucleotides are those of the wild-type gene.

Recombinant DNA procedures were as described by Wilkin et al. (1993a). The construction of plasmids pDML42 (Thr[301]Ile), pDML43 (Thr[301]Ser) and pDML39 (Thr[299]Val) by site-directed mutagenesis was as described by Hadonou et al. (1991).

The production of the mutant DD-peptidases was done as previously. Two different culture media were used: the YEME medium (Wilkin et al., 1993a) and the TSB medium (Wilkin et al., 1993b). The Thr[301]Ile and Thr[301]Ser mutants were produced in the first medium and the Thr[299]Val mutant in the second.

Purification, determinations of kinetic parameters, denaturation and h.p.l.c. experiments were done exactly as described by Varetto et al. (1987) and Wilkin et al. (1993a). All experiments were performed at 37 °C in 10 mM sodium phosphate buffer, pH 7.0, unless otherwise stated.

Curve fitting was realized with the help of two software packages. The ENZFITTER package allows data analysis by non-linear regression (Leatherbarrow, 1987). The SIMFIT software package which was kindly given by Dr. H. Holzhütter (Institute of Biochemistry, Humbolt University, Berlin, Germany), allows simulations and data analysis using models based upon algebraic and/or differential equations (Holzhütter and Colosimo, 1990). The equations used for fitting were those described by Jamin et al. (1993). An additional simplification could be made, since the substrate concentration could be considered as much smaller than K', so that eqn. (5) of Jamin et al. (1993) became:

$$\frac{V_o}{E_o} = \frac{f'(k_{+2}/K')[S]}{f' + g'(k_{+2}/K')[S]\,g'} \quad (1)$$

where:

$f' = k_{+3}\alpha\beta + k_{+5}\beta[A] + k_{+7}[A][S]$

$g' = \alpha\beta + \beta[A] + [A][S]$

Fitting was used to calculate the kinetics parameters in the transpeptidation pathway and was performed on three sets of data: the variations of initial rates with donor and acceptor concentrations and of transfer/hydrolysis ratios (T/H) with the acceptor concentration.

Table 1 Structures of the substrates

A and K are D-alanine and L-lysine respectively; Ac is acetyl.

R–NH–CH(R′)–CO–X–CH(R″)–COO^{-1}

Substrate	R	R′	R″	X
Ac_2KAA	Ac_2K	CH_3(D)	CH_3(D)	NH
S1e	C_6H_5–CO	H	C_6H_5–CH_2	O
S2a	C_6H_5–CO	H	H	S
S2c	C_6H_5–CO	H	CH_3(D)	S
S2d	C_6H_5–CO	CH_3(D)	H	S
S2e	C_6H_5–CO	CH_3(D)	CH_3(DL)	S
S2Val	C_6H_5–CO	$(CH_3)_2$–CH(DL)	H	S

RESULTS

Mutagenesis, production and purification

The three modified plasmids, pDML42 (Thr[301]Ile), pDML43 (Thr[301]Ser) and pDML39 (Thr[299]Val) were purified. That no additional, unwanted mutations had been introduced was verified as follows: firstly, the modified gene fragment was sequenced in M13 immediately after the site-directed-mutagenesis procedure and, secondly, after reinsertion in the *Streptomyces* plasmid, the same fragment was again subcloned in M13 and the presence of the mutation confirmed by sequencing.

Maximum productions were obtained after 120 and 72 h of growth in the YEMET medium (Thr[301]Ile and Thr[301]Ser) and in the TSB medium (Thr[299]Val) respectively, as observed for the wild-type enzyme in both cases. The yields were 100 mg/l for the Thr[301]Ile and Thr[301]Ser mutants, 6 mg/l for the Thr[299]Val mutant. The production of the Thr[299]Val enzyme was thus relatively low when compared with that of the wild-type enzyme under the same conditions (usually about 30–40 mg/l).

Purification of the three modified proteins were performed as described by Wilkin et al. (1993a). The final yields were 80% for the Thr[301]Ile and Thr[301]Ser and 66% for the Thr[299]Val mutants respectively. The purity of the mutant proteins was evaluated as higher than 95% by SDS/PAGE.

Physical properties and stability

Absorption (230–320 nm) and fluorescence emission (excitation at 280 nm) spectra of the three modified proteins were superimposable on those of the wild-type enzyme. Moreover, the mutations did not result in major modifications of the protein stabilities (Table 2).

Table 2 Half-lives of the wild-type and mutant enzymes

The quenching of fluorescence emission of 0.2 μM enzyme solutions ($\lambda_{excitation}$ = 280 nm; $\lambda_{emission}$ = 320 nm) was continuously recorded.

Enzyme	Half-lives (min)	
	60 °C	56 °C + 6 M urea
$Thr^{299}Val$	8.0 ± 0.1	6.1 ± 0.1
$Thr^{301}Ile$	6.6 ± 0.5	6.2 ± 0.4
$Thr^{301}Ser$	8.2 ± 0.6	4.8 ± 0.2
Wild-type	6.2 ± 0.6	8.6 ± 0.9

Thus, the structures of the $Thr^{301}Ile$, $Thr^{301}Ser$ and $Thr^{299}Val$ proteins were assumed to be similar to that of the wild-type enzyme.

Kinetic properties

Carboxypeptidase activity

Table 3 shows the hydrolytic profiles of the three modified enzymes. In all cases, fluorescence-quenching experiments allowed us to reveal the acyl-enzyme at $[S] \geqslant K_m$, with substrates S2a, S2d and S2val showing that the hydrolysis of those substrates remained k_3-limiting ($k_3 < k_2$), as found with the wild-type enzyme. Moreover, this assumption was corroborated by the similar $k_{cat.}$ values for substrates Sle, S2a and S2c (Table 1), which form the same acyl-enzyme.

Transpeptidase activity

Figure 1 compares the effects of increasing D-alanine concentrations on the $k_{cat.}$ and K_m values of the wild-type, $Thr^{301}Ile$ and $Thr^{301}Ser$ protein. As observed with the wild-type enzyme, the $k_{cat.}$ curve fitted the empirical equation:

$$k_{cat.} = \frac{a + b[A]}{1 + c[A]}$$

The D-alanine concentrations [A] corresponding to half-saturation ($[A]_{50}$) were about 100 and 590 mM ($[A]_{50} = 1/c$) for the $Thr^{301}Ile$ and $Thr^{301}Ser$ enzymes respectively, while the wild-type value was 50 mM. Since K_m increased according to a similar equation, the $k_{cat.}/K_m$ was not significantly modified, as found for the wild-type enzyme. Thus the behaviour of the mutants seemed to be similar to that of the wild-type enzyme if one excepts the increase of the $[A]_{50}$ values. At low acceptor concentrations, $k_{cat.}$ increased linearly with the acceptor concentrations, and the quality of an acceptor could be characterized by the $\delta k_{cat.}/\delta[A]$ ratio or by the relative acceleration, $\delta k_{cat.}/(\delta[A] \cdot k_{cat.})$:

$Thr^{301}Ile$: $\delta k_{cat.}/\delta[A] = 640 \pm 100$ ($M^{-1} \cdot s^{-1}$)

$\delta k_{cat.}/(\delta[A] \cdot k_{cat.}) = 460 \pm 80$ (M^{-1})

$Thr^{301}Ser$: $\delta k_{cat.}/\delta[A] = 860 \pm 90$ ($M^{-1} \cdot s^{-1}$)

$\delta k_{cat.}/(\delta[A] \cdot k_{cat.}) = 540 \pm 60$ (M^{-1})

Wild-type: $\delta k_{cat.}/\delta[A] = 3340 \pm 350$ ($M^{-1} \cdot s^{-1}$)

$\delta k_{cat.}/(\delta[A] \cdot k_{cat.}) = 670 \pm 70$ (M^{-1}) (Jamin et al. 1991)

Table 3 $k_{cat.}$, K_m and $k_{cat.}/K_m$ values for the wild-type and the mutant enzymes

Values for the wild-type are from Frère and Joris (1985) (*) or Adam et al. (1990) (†); methods used to calculate the steady-state parameter (M) were: A, complete time-course method (De Meester et al., 1987) and B, initial-rate measurements. ND, not determined; S.D. values did not exceed 15%.

Substrate	$Thr^{299}Val$				$Thr^{301}Ile$				$Thr^{301}Ser$				Wild-type			
	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	M	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	M	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	M	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	M
Ac_2KAA	0.09	15	0.006	B	34.00	50	0.68	B	13.00	10	1.3	B	55*	13	4	B
Sle	ND	ND	< 1	B	0.41	13	0.30	B	1.12	0.630	1.8	B	5†	0.900	5.5	A
S2a	1.80	0.21	8.7	A	1.40	0.035	40	A	1.60	0.040	40	A	5†	0.050	100	A
S2c	2.00	0.11	18.7	A	2.00	0.030	65	A	1.80	0.025	72	A	5†	0.050	100	A
S2d	19.40	0.43	45.0	A	19.00	0.080	240	A	28.70	0.075	380	A	70	0.100	700	A
S2e	ND	ND	5.2	A	ND	ND	ND		ND	ND	ND		70	0.560	125	A
S2val	1.00	0.65	1.5	B	1.7	0.180	9.5	A	1.50	0.140	10.7	A	4	0.500	8	A

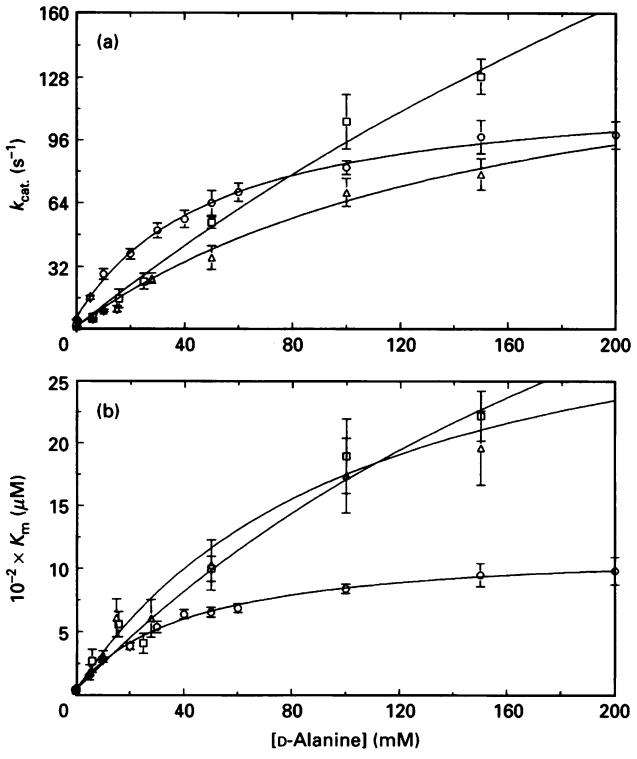


Figure 1 Influence of the D-alanine concentration on the $k_{cat.}$ and K_m values for the hydrolysis of S2a by the wild-type (○), Thr^{301}Ile (□) and Thr^{301}Ser (□) enzymes

(**a**) $k_{cat.}$ values; (**b**) K_m values. Initial-rate measurements or the complete-time-course method (De Meester et al., 1987) were used at 37 °C in 10 mM sodium buffer, pH 7.0. Values for the wild-type enzyme are from Jamin et al. (1991). Results are means ± S.D. ($n = 7$ or more). The $k_{cat.}/K_m$ values (not shown) were not significantly influenced.

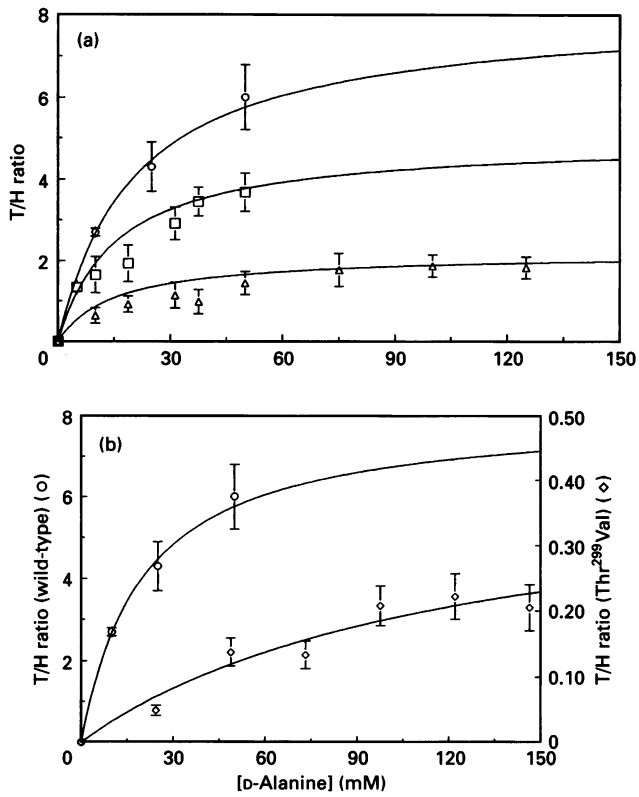


Figure 2 Variation of the T/H ratios with increasing concentrations of D-alanine (37 °C)

The donor substrate was 250 μM S2a in 10 mM sodium phosphate, pH 7.0. (**a**) wild-type (○), Thr^{301}Ile (△) and Thr^{301}Ser (□) enzymes; (**b**) wild-type (○) and Thr^{299}Val (◇) enzymes. The continuous curve are theoretical and were obtained by introducing the parameters of Table 8 into eqn. (6) of Jamin et al. (1993).

With the Thr^{299}Val enzyme, the presence of the acceptor failed to increase the rate of donor hydrolysis (Table 4), indicating that the transpeptidation was not faster than the hydrolysis in the absence of acceptor.

Figures 2(a) and 2(b) show the transpeptidation/hydrolysis ratios (T/H ratios) measured with the three mutants under steady-state conditions (less than 10% utilization of the donor substrate). As observed with the wild-type enzyme, these data indicated a clear saturation effect. The T/H ratios were not strongly affected for the Thr^{301}Ile and Thr^{301}Ser enzymes, in contrast with the Thr^{299}Val mutant, for which the T/H ratios remained lower than 5% of those observed with the wild-type.

Interaction with β-lactams

Table 5 gives an overview of the acylation (k_{+2}/K') and deacylation (k_{+3}) parameters, and Table 6 details the individual values of k_{+2} and K' for carbenicillin and the three mutant enzymes.

For the Thr^{299}Val enzyme, the values of k_{+2}/K' decreased

Table 4 Influence of acceptor concentrations on the $k_{cat.}$, K_m and $k_{cat.}/K_m$ values for the wild-type and Thr^{299}Val enzymes with S2a as donor substrate

Methods used to calculate the steady-state parameters (M) were: A, complete-time-course method (De Meester et al., 1987); B, initial-rate measurements. S.D. values did not exceed 15%.

	Thr^{299}Val				Wild-type			
Acceptor	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	M	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	M
None	1.8	0.21	8.7	A	5.0	0.05	100	A
D-Alanine								
10 mM	1.8	0.19	9.3	B	28.1	0.28	100	A
50 mM	1.9	0.18	10	A	63.8	0.65	97.4	A
150 mM	2.1	0.19	11	A	97.0	0.95	102	A

Table 5 k_{+2}/K' and k_{+3} values for several antibiotics with the wild-type and mutant enzymes

Abbreviation: Benzylp-Me, benzylpenicillin methylester. Notes: *value from Varetto et al. (1991). The numbers refer to the various procedures described in Wilkin et al. (1993a): [1a]fluorescence quenching; [1b]reporter substrate method; [3]re-activation of the isolated acylenzyme; [1c]sampling procedure (Varetto et al., 1987): apparent first-order rate constants of 2.7×10^{-4} (s^{-1}) and 9.4×10^{-5} (s^{-1}) were obtained for 2.4 mM cephalosporin C and 1.6 mM cefuroxime respectively; these values would translate into 0.1 and 0.05 $M^{-1} \cdot s^{-1}$ k_{+2}/K' values, but at these high antibiotics concentrations the cephalosporin concentrations might no longer be much smaller than K'. S.D. values did not exceed 15%; ND, not determined.

	Thr[299]Val		Thr[301]Ile		Thr[301]Ser		Wild-type	
Antibiotic	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)	k_{+3} (s^{-1})	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)	k_{+3} (s^{-1})	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)	k_{+3} (s^{-1})	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)	k_{+3} (s^{-1})
Benzylpenicillin	[1a]600	[3]5.4×10^{-4}	[1a]460	[3]1.5×10^{-4}	[1a]4000	[3]3.5×10^{-4}	[1a]18000	[3]1.4×10^{-4}
Carbenicillin	[1a]26	[3]5.5×10^{-4}	[1a]100	[3]1.8×10^{-4}	[1a]360	[3]3.5×10^{-4}	[1a]830	[3]1.4×10^{-4}
Ampicillin	[1a]2	[3]2.7×10^{-4}	[1a]35	[3]2.0×10^{-4}	[1a]111	[3]3.5×10^{-4}	[1a]111	[3]1.4×10^{-4}
Benzylp-Me	[1a]1	ND	ND	ND	ND	ND	[1a]24	[3]1.0×10^{-5}*
Nitrocefin	[1b]30	[3]2.5×10^{-3}	[1a]1140	[3]6.0×10^{-4}	[1a]680	[3]3.6×10^{-4}	[1a]4100	[3]3.0×10^{-4}
Cephalosporin C	[1c] < 1	[3]9.0×10^{-6}	[1a]300	[3]1.5×10^{-6}	[1a]850	ND	[1a]1500	[3]1.0×10^{-6}
Cefuroxime	[1c] < 1	[3]2.2×10^{-5}	[1a]130	[3]1.2×10^{-6}	[1a]430	ND	[1a]350	[3] $< 4 \times 10^{-6}$

Table 6 Individual k_{+2} and K' values for carbenicillin and the wild-type and mutant enzymes

The fluorescence quenching results from method 1a in Wilkin et al. (1993a) were analysed with the help of ENZFITTER program.

	Carbenicillin	
Enzymes	k_{+2} (s^{-1})	K' (μM)
Thr[299]Val	0.09 ± 0.01	3250 ± 1000
Thr[301]Ile	0.33 ± 0.04	3000 ± 470
Thr[301]Ser	0.84 ± 0.22	3700 ± 1000
Wild-type*	0.09 ± 0.01	110 ± 20

* Frère and Joris (1985).

30–50-fold with penicillins and more than three orders of magnitude for cephalosporins. For carbenicillin, the decrease could be attributed to an increased K' value.

The Thr[301] mutants were much less affected and the properties of the Thr[301]Ser protein were not very different from those of the wild-type. Surprisingly, the individual values of k_{+2} and K' for carbenicillin were increased by similar factors, which explained the small variations of the resulting k_{+2}/K' values.

The k_{+3} values for the Thr[301] mutant enzymes did not seem to be affected. Conversely, for the Thr[299]Val mutant, there was a 10-fold increase in nitrocefin, cefuroxime and cephalosporin C.

At pH 7.0, the Thr[299]Val enzyme also exhibited a significant increase of the penicilloic acid/phenylacetylglycine ratio during the re-activation step: 0.6 ± 0.1 versus < 0.1 for the wild-type.

DISCUSSION

The physical properties of the three modified proteins, including their thermal stabilities, were not significantly different from those of the WT enzyme, which indicated the absence of important structural alterations. Accordingly, the mutants retained, to various degrees, some of the catalytic and/or penicillin binding properties of the original enzyme.

Thr[301]

As explained in the Introduction, and on the basis of the results obtained with the TEM β-lactamase, one might have expected selective alterations of the sensitivity of the DD-peptidase to penicillins and cephalosporins after replacement of the Thr[301] residue by Ile (elimination of a possible H-bonding function) or by Ser (a smaller residue conserving the H-bonding function). In contrast with these expectations, the modifications did not result in dramatic changes of the protein properties. The Thr[301]Ser mutation barely affected the rates of substrate utilization and of acylation by pencillins, the largest factor (4.5) being observed with benzylpenicillin. The Thr[301]Ile mutant was somewhat more affected, up to 40-fold, again with benzylpenicillin. Unexpectedly, the decrease of $k_{cat.}/K_m$ value for the tripeptide substrate was less important (6-fold), so that this protein could be considered as a DD-peptidase exhibiting increased benzylpencillin resistance. This

Table 7 Kinetic parameters for the concomitant hydrolysis and aminolysis (D-alanine) of substrate S2a catalysed by the R61 DD-peptidase at 37 °C

The k_2/K' and k_{+3} values were those directly measured in the absence of acceptor. The other parameters were obtained by fitting initial rates (except for the Thr[299]Val enzyme) and T/H ratios on eqn. (1) of the Materials and methods section and eqn. (6) of Jamin et al. (1993). S.D. values are the averages of the S.D. values obtained after each fitting experiment.

Constants	Thr[301]Ile	Thr[301]Ser	Thr[299]Val	Wild-type
k_{+2}/K' ($mM^{-1} \cdot s^{-1}$)	40 ± 6	44 ± 7	8.7 ± 1.5	100 ± 10
k_{+3} (s^{-1})	1.37 ± 0.09	1.64 ± 0.15	1.8 ± 0.2	5 ± 0.5
k_{+5} (s^{-1})	45 ± 15	190 ± 21	2.3 ± 0.2	200 ± 50
α (mM)	250 ± 26	370 ± 100	364 ± 36	100 ± 16
k_{+7} (s^{-1})	73.4 ± 15	180 ± 20	21.5 ± 2.1	84 ± 2
β (mM)	0.82 ± 0.20	1.26 ± 0.12	0.96 ± 0.10	0.82 ± 0.01
S.D....	0.06	0.034	0.019	0.002

Table 8 Comparison of the kinetic parameters of DD-transpeptidase mutants

The T/H values were obtained with 250 μM S2a and 10 mM D-alanine as donor and acceptor substrates respectively. Values in square brackets were computed from $k_{cat.}$ increases; the real T/H values might thus be somewhat lower. All the kinetic parameters are taken from Hadonou et al. (1992) and Wilkin et al. (1993a,b). Abbreviations used: Pen G, penicillin G; Ceph C, cephalosporin C.

	Substrates					Inhibitors	
	Peptide	Thiolester				Pen G	Ceph C
Enzyme	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)	T/H	$\delta k_{cat.}/\delta$ [A] ($M^{-1} \cdot s^{-1}$)	$\delta k_{cat.}/k_{cat.} \delta$ [A] (M^{-1})	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)
Wild-type	4000	100000	2.70	3400	670	18000	1500
$His^{298}Gln$	25	10000	[0.20]	70	50	120	26
$His^{298}Lys$	37	44000	[0.60]	200	210	240	120
$Thr^{299}Val$	6	8700	0.04	0	0	600	< 1
$Thr^{301}Ile$	680	40000	0.70	800	600	460	300
$Thr^{301}Ser$	1300	40000	1.70	1000	600	4000	850
$Tyr^{159}Ser$	1.3	60000	0.02	0	0	10000	540
$Tyr^{159}Phe$	0.5	100	0.16	0	0	55	14
$Asn^{161}Ser$	30	38000	0.40	340	150	14000	500
$Asn^{161}Ala$	10	3200	0.60	1140	100	230	35

conclusion could, however, not be extended to the other tested β-lactams, for which the rates of acylation only decreased 3–8-fold. If one excepts the benzylpenicillin–$Thr^{301}Ile$ interaction, no modification of the penicillin-versus-cephalosporins pattern could be detected.

Previous experiments have shown the transpeptidation properties to be highly sensitive to most structural modifications (Hadonou et al., 1992; Wilkin et al., 1993a,b).

The detailed analysis of the data (Table 7), however, indicated some interesting features with both mutants, the α values were somewhat increased and thus contributed to decreasing the T/H ratios, a trend which was reinforced by the lower k_{+5} value of the $Thr^{301}Ile$ mutant. Interestingly, the k_{+7} value for the $Thr^{301}Ser$ mutant was slightly increased, which explained the higher $k_{cat.}$ values observed with this protein at large acceptor concentrations, a somewhat paradoxical result, similar to that obtained with the $Asn^{161}Ser$ mutant with which an increased relative efficiency of the 'vertical' hydrolysis pathway (Model 2) was also observed (Wilkin et al., 1993b).

Finally, the significant increase (30-fold) of the K' value for carbenicillin was rather surprising and contrasted with the situation with the peptide substrate, for which K_m ($= K'$, rate-limiting acylation) increased only 4-fold with $Thr^{301}Ile$ and not at all for $Thr^{301}Ser$.

In consequence, it can be concluded that the side chain of residue 301 only plays a minor role in the interactions with the peptide substrates and β-lactam inactivators of the enzyme. As proposed by Kelly et al. (1989), the functions of this residue mainly involve its backbone amide NH and CO groups respectively in contributing to the oxyanion hole and in forming a hydrogen bond with the amide NH of the side chain on C-6 of penicillins or C-7 of cephalosporins, and these interactions should not be significantly altered by the mutations performed here.

Thr^{299}

In contrast with the results discussed above, the $Thr^{299}Val$ mutation resulted in marked modifications of the enzymic properties. Modelling of the Henri–Michaelis complexes formed upon binding penicillins in the active sites of various class A β-lactamases indicated that the hydroxy group of the corresponding Thr^{235} or Ser^{235} residue could hydrogen-bond to the substrate carboxylate, contributing to its proper positioning in the enzyme cavity (Moews et al., 1990; Lamotte-Brasseur et al., 1991; Strynadka et al., 1992). The validity of this hypothesis was tested by replacing Thr^{299} by a Val residue.

This resulted in a dramatic decrease (700-fold) of the $k_{cat.}/K_m$ value for the peptide substrate and in a strong impairment of the esterase activity. The thiolesterase activity was, conversely, much less affected, but a similar situation appears to prevail with many other mutants of the R61 DD-peptidase, including, interestingly, those in which the neighbouring His^{298} residue has been replaced by Gln or Lys.

Even when the Thr^{299} and His^{298} mutants retained an important fraction of their hydrolytic capacities (against the thiolester S2a), the transpeptidase activities were nearly totally abolished. Residue 298 had been hypothesized to play a pivotal role in the transpeptidation reaction (Hadonou et al., 1992), but this activity was even more affected by the $Thr^{299}Val$ mutation. As shown in Table 7, the loss of transpeptidase activity could be attributed to a dramatic (100-fold) decrease of the k_{+5} value with the $Thr^{299}Val$ mutant.

Several residues have now been identified as playing a significant role in the transpeptidation reaction (Table 8) and, among these, Tyr^{159} and Thr^{299} appear to be the most essential. Strikingly, the impairment of the transpeptidation efficiency appears to closely parallel that of the hydrolysis of the peptide substrate, and these two phenomena relate to different parts of the reaction pathway. Indeed, in the hydrolysis of the peptide, acylation is rate-limiting, and what occurs after formation of the acyl-enzyme has little influence on the rate of the reaction, while the transpeptidation pathway only contains intermediates derived from the acyl-enzyme. Thus Thr^{299} and Tyr^{159} are deeply involved in both stages of the reactions when the natural substrates are transformed.

Modification of the Thr^{299} side chain significantly decreased the rates of acylation by penicillins (30–50-fold), and, interestingly, the reaction with cephalosporins became exceedingly slow. Only nitrocefin retained a non-negligible inactivating potency, but the behaviour of this compound has often been found to be rather different from that of more classical cephems. The modified enzyme thus became practically

resistant to cefuroxime and cephalosporin C, a situation reminiscent of that observed with the peptide substrate. It was somewhat surprising to note that the decreased k_{+2}/K' values for carbenicillin and the tripeptide were due to an increased K' and decreased k_{+2} ($= k_{cat.}$) respectively, a result which might indicate that it is somewhat artificial to try to individualize the two constants which determine the rate of acylation, but which could also reflect distinct behaviours of the substrate and inactivators in the enzyme catalytic site.

The behaviour of the benzylpenicillin methyl ester was more surprising. Indeed, when compared with benzylpenicillin, its efficiency was decreased by similar factors with the wild-type (800-fold) and the mutant (600-fold) enzymes. The disappearance of the Thr^{299} hydroxy group thus similarly decreases the efficiencies of both benzylpenicillin and its ester.

These results and others presented here will be further discussed in the companion paper (Dubus et al., 1994), which analyses the consequences of mutations of the corresponding residues in a class A and a class C β-lactamase.

Conclusion

The relatively importance of the two threonine residues of the β3-strand is very different in the R61 DD-peptidase. The side chain of residue Thr^{301} plays a minor role and its modification only results in subtle alterations of the specificity pattern of the enzyme. By contrast, that of Thr^{299} is a major participant in several important events in the catalytic cycle.

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REFERENCES

Adam, M., Damblon, C., Plaitin, B., Christiaens, L. and Frère, J.-M. (1990) Biochem. J. **270**, 525–529

Brannigan, J., Matagne, A., Jacob, F., Damblon, C., Joris, B., Klein, D., Spratt, B. G. and Frère, J.-M. (1991) Biochem. J. **278**, 673–678

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Christensen, H., Martin, M. T. and Waley, S. G. (1990) Biochem. J. **266**, 853–861

De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourguignon, C., Frère, J. M. and Waley, S. G. (1987) Biochem. Pharmacol. **36**, 2393–2403

Dubus, A., Wilkin, J.-M., Raquet, X., Normark, S. and Frère, J.-M. (1994) Biochem. J. **300**, 485–494

Ellerby, L. M., Escobar, W A., Fink, A L., Mitchinson, C. and Wells, J. A. (1990) Biochemistry **29**, 5797–5806

Frère, J.-M. and Joris, B. (1985) CRC. Crit. Rev. Microbiol. **1**, 299–396

Frère, J.-M., Nguyen-Distèche, M., Coyette, J. and Joris, B. (1992) in The Chemistry of Beta-Lactams (Page, M. I., ed.), pp. 148–197, Chapman and Hall, Andover

Govardhan, C. and Pratt, R. (1987) Biochemistry **26**, 3385–3395

Hadonou, A. M., Jamin, M., Adam, M., Joris, B., Dusart, J., Ghuysen, J.-M. and Frère, J.-M. (1992) Biochem. J. **282**, 495–500

Hall, A. and Knowles, J. R. (1976) Nature (London) **264**, 803–804

Healey, W. J., Labgold, M. R. and Richards, J. H. (1989) Prot. Struct. Funct. Gen. **6**, 275–283

Holzhütter, H. G. and Colosimo, A. (1990) CABIOS **6**, 23–28

Jamin, M., Adam, M., Damblon, C., Christiaens, L. and Frère, J.-M. (1991) Biochem. J. **280**, 499–506

Jamin, M., Wilkin, J.-M. and Frère, J.-M. (1993) Biochemistry **32**, 7278–7285

Joris, B., Ghuysen, J.-M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frère, J.-M., Kelly, J., Boyington, J., Moews, P. and Knox, J. (1988) Biochem. J. **250**, 313–324

Joris, B., Ledent, P., Dideberg, O., Fonze, E., Lamotte-Brasseur, J., Kelly, J. A., Ghuysen, J.-M. and Frère, J.-M. (1991) Antimicrob. Agents Chemother. **35**, 2294–2301

Kelly, J. A., Dideberg, O., Charlier, P., Wéry, J., Libert, M., Moews, P., Knox, J., Duez, C., Fraipont, C., Joris, B., Dusart, J., Frère, J.-M. and Ghuysen, J.-M. (1986) Science **231**, 1429–1431

Kelly, J. A., Knox, J. R., Zhao, H., Frère, J.-M. and Ghuysen, J.-M. (1989) J. Mol. Biol. **209**, 281–295

Lamotte-Brasseur, J., Dive, G., Dideberg, O., Charlier, P., Frère, J.-M. and Ghuysen, J.-M. (1991) Biochem. J. **279**, 213–221

Lamotte-Brasseur, J., Jacob-Dubuisson, F., Dive, G., Frère, J.-M. and Ghuysen, J.-M. (1992) Biochem. J. **282**, 189–195

Leatherbarrow, R. J. (1987) ENZFITTER, Elsevier Biosoft, Cambridge

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, NY

Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P. and Frère, J.-M. (1990) Prot. Struct. Funct. Genet. **7**, 156–171

Oefner, C., D'Arcy, A., Daly, J. J., Gubernator, A., Charnas, R. L., Heinze, I., Hubschwerlen, C. and Winkler, F. K. (1990) Nature (London) **343**, 284–288

Samraoui, B., Sutton, B., Todd, R., Atrymiuk, P., Waley, S. G. and Phillips, D. (1986) Nature (London) **320**, 378–380

Strynadka, N. C. J., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betzel, C., Sutoh, K. and James, M. N. J. (1992) Nature (London) **359**, 700–705

Varetto, L., Frère, J.-M., Nguyen-Distèche, M., Ghuysen, J.-M. and Houssier, C. (1987) Eur. J. Biochem. **162**, 525–531

Varetto, L., De Meester, F., Monnaie, D., Marchand-Brynaert, J., Dive, G., Jacob, F. and Frère, J.-M. (1991) Biochem. J. **278**, 801–807

Waley, S. G. (1992) in The Chemistry of β-Lactams (Page, M. I., ed.), pp. 198–228, Chapman and Hall, Glasgow

Wilkin, J.-M., Jamin, M., Damblon, C., Zhao, G. H., Joris, B., Duez, C. and Frère, J.-M. (1993a) Biochem. J. **291**, 537–544

Wilkin, J.-M., Jamin, M., Joris, B. and Frère, J.-M. (1993b) Biochem. J. **293**, 195–201