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 Thr²⁹⁹ and Thr³⁰¹ 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. Thr²⁹⁹ 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 Thr³⁰¹ 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 thioesters 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 His²⁹⁸-Thr²⁹⁹-Gly³⁰⁰-Thr³⁰¹ and corresponds to Lys²³⁴-Thr²³⁵-Gly²³⁶-Ala²³⁷ 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 Lys²³⁴ (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 Thr²³⁵ 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 (Ala²³⁷Thr), 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, His²⁹⁸, 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 Thr²⁹⁹ by Val. In turn, Thr³⁰¹, which corresponds to Ala³²⁷ 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

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

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 μ M enzyme solutions ($\lambda_{\text{excitation}} = 280$ nm; $\lambda_{\text{emission}} = 320$ nm) was continuously recorded.

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

Thus, the structures of the Thr³⁰¹Ile, Thr³⁰¹Ser and Thr²⁹⁹Val 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 $[S] \geq K_m$, 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 S1e, 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, Thr³⁰¹Ile and Thr³⁰¹Ser protein. As observed with the wild-type enzyme, the k_{cat} curve fitted the empirical equation:

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

The D-alanine concentrations $[A]$ corresponding to half-saturation ($[A]_{50}$) were about 100 and 590 mM ($[A]_{50} = 1/c$) for the Thr³⁰¹Ile and Thr³⁰¹Ser 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 $\delta k_{\text{cat}}/\delta[A]$ ratio or by the relative acceleration, $\delta k_{\text{cat}}/(\delta[A] \cdot k_{\text{cat}})$:

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

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

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

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

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

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

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 ·s ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ ·s ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ ·s ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ ·s ⁻¹)
Ac ₂ KAA	0.09	15	0.006	34.00	50	0.68	13.00	10	1.3	55*	13	4
S1e	ND	ND	< 1	0.41	13	0.30	1.12	0.630	1.8	5†	0.900	5.5
S2a	1.80	0.21	8.7	1.40	0.035	40	1.60	0.040	40	5†	0.050	100
S2c	2.00	0.11	18.7	2.00	0.030	65	1.80	0.025	72	5†	0.050	100
S2d	19.40	0.43	45.0	19.00	0.080	240	28.70	0.075	380	70	0.100	700
S2e	ND	ND	5.2	ND	ND	ND	ND	ND	ND	70	0.560	125
S2val	1.00	0.65	1.5	1.7	0.180	9.5	1.50	0.140	10.7	4	0.500	8

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 (○), Thr³⁰¹Ile (Δ) and Thr³⁰¹Ser (□) 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 $\pm$ S.D. ($n = 7$ or more). The k_{cat}/K_m values (not shown) were not significantly influenced.

With the Thr²⁹⁹Val 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 Thr³⁰¹Ile and Thr³⁰¹Ser enzymes, in

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

The donor substrate was 250 μ M S2a in 10 mM sodium phosphate, pH 7.0. (a) wild-type (○), Thr³⁰¹Ile (Δ) and Thr³⁰¹Ser (□) enzymes; (b) wild-type (○) and Thr²⁹⁹Val (◇) enzymes. The continuous curve are theoretical and were obtained by introducing the parameters of Table 8 into eqn. (6) of Jamin et al. (1993).

contrast with the Thr²⁹⁹Val 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_{-2}) parameters, and Table 6 details the individual values of k_{+2} and K' for carbenicillin and the three mutant enzymes.

For the Thr²⁹⁹Val 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 Thr²⁹⁹Val 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%.

Acceptor	Thr ²⁹⁹ Val			M	Wild-type			M
	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ · s ⁻¹)		k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ · s ⁻¹)	
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; ³re-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·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.

Antibiotic	Thr ²⁹⁹ Val		Thr ³⁰¹ Ile		Thr ³⁰¹ Ser		Wild-type	
	k_{+2}/K' (M ⁻¹ ·s ⁻¹)	k_{+3} (s ⁻¹)	k_{+2}/K' (M ⁻¹ ·s ⁻¹)	k_{+3} (s ⁻¹)	k_{+2}/K' (M ⁻¹ ·s ⁻¹)	k_{+3} (s ⁻¹)	k_{+2}/K' (M ⁻¹ ·s ⁻¹)	k_{+3} (s ⁻¹)
Benzylpenicillin	^{1a} 600	3.54×10^{-4}	^{1a} 460	3.15×10^{-4}	^{1a} 4000	3.5×10^{-4}	^{1a} 18000	3.14×10^{-4}
Carbenicillin	^{1a} 26	3.5×10^{-4}	^{1a} 100	3.18×10^{-4}	^{1a} 360	3.5×10^{-4}	^{1a} 830	3.14×10^{-4}
Ampicillin	^{1a} 2	3.27×10^{-4}	^{1a} 35	3.20×10^{-4}	^{1a} 111	3.5×10^{-4}	^{1a} 111	3.14×10^{-4}
Benzylp-Me	^{1a} 1	ND	ND	ND	ND	ND	^{1a} 24	3.10×10^{-5} *
Nitrocef	^{1b} 30	3.25×10^{-3}	^{1a} 1140	3.60×10^{-4}	^{1a} 680	3.6×10^{-4}	^{1a} 4100	3.0×10^{-4}
Cephalosporin C	^{1c} < 1	3.90×10^{-6}	^{1a} 300	3.15×10^{-6}	^{1a} 850	ND	^{1a} 1500	3.10×10^{-6}
Cefuroxime	^{1c} < 1	3.22×10^{-5}	^{1a} 130	3.12×10^{-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.

Enzymes	Carbenicillin	
	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 Thr³⁰¹ mutants were much less affected and the properties of the Thr³⁰¹Ser 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 Thr³⁰¹ mutant enzymes did not seem to be affected. Conversely, for the Thr²⁹⁹Val mutant, there was a 10-fold increase in nitrocef, cefuroxime and cephalosporin C.

At pH 7.0, the Thr²⁹⁹Val enzyme also exhibited a significant increase of the penicilloic acid/phenylacetylglutamine 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.

Thr³⁰¹

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 Thr³⁰¹ 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 Thr³⁰¹Ser mutation barely affected the rates of substrate utilization and of acylation by penicillins, the largest factor (4.5) being observed with benzylpenicillin. The Thr³⁰¹Ile 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 benzylpenicillin 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 Thr²⁹⁹Val 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 ·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 μ M 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.

Enzyme	Substrates					Inhibitors	
	Peptide		T/H	Thiolester		Pen G	Ceph C
	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)		$\delta k_{cat}/\delta [A]$ ($M^{-1} \cdot s^{-1}$)	$\delta k_{cat}/k_{cat} \delta [A]$ (M^{-1})	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)
Wild-type	4000	100 000	2.70	3400	670	18 000	1500
His ²⁹⁸ Gln	25	10 000	[0.20]	70	50	120	26
His ²⁹⁸ Lys	37	44 000	[0.60]	200	210	240	120
Thr ²⁹⁹ Val	6	8700	0.04	0	0	600	< 1
Thr ³⁰¹ Ile	680	40 000	0.70	800	600	460	300
Thr ³⁰¹ Ser	1300	40 000	1.70	1000	600	4000	850
Tyr ¹⁵⁹ Ser	1.3	60 000	0.02	0	0	10 000	540
Tyr ¹⁵⁹ Phe	0.5	100	0.16	0	0	55	14
Asn ¹⁶¹ Ser	30	38 000	0.40	340	150	14 000	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–Thr³⁰¹Ile 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 Thr³⁰¹Ile mutant. Interestingly, the k_{+7} value for the Thr³⁰¹Ser 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 Asn¹⁶¹Ser 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 Thr³⁰¹Ile and not at all for Thr³⁰¹Ser.

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.

Thr²⁹⁹

In contrast with the results discussed above, the Thr²⁹⁹Val 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

Thr²³⁵ or Ser²³⁵ 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 Thr²⁹⁹ 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 His²⁹⁸ residue has been replaced by Gln or Lys.

Even when the Thr²⁹⁹ and His²⁹⁸ 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 Thr²⁹⁹Val 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 Thr²⁹⁹Val mutant.

Several residues have now been identified as playing a significant role in the transpeptidation reaction (Table 8) and, among these, Tyr¹⁵⁹ and Thr²⁹⁹ 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 Thr²⁹⁹ and Tyr¹⁵⁹ are deeply involved in both stages of the reactions when the natural substrates are transformed.

Modification of the Thr²⁹⁹ 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 cepheps. 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} ($=k_{\text{cat}}$) 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 Thr²⁹⁹ 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 β 3-strand is very different in the R61 DD-peptidase. The side chain of residue Thr³⁰¹ plays a minor role and its modification only results in subtle alterations of the specificity pattern of the enzyme. By contrast, that of Thr²⁹⁹ is a major participant in several important events in the catalytic cycle.

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