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Research Article

The catalytic mechanism of DD-peptidases: unexpected importance of tyrosine 280 in the transpeptidation reaction catalysed by the *Streptomyces* R61 DD-peptidase

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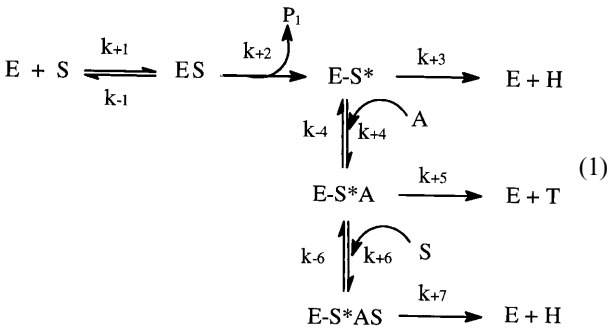
Abstract. The study of the interactions between the Tyr280Phe mutant of the *Streptomyces* R61 DD-peptidase, various substrates and β-lactam antibiotics shows that Tyr280 is involved not only in the formation of the acylenzyme with the peptide substrate and β-lactam antibiotics, but also and specifically in the catalysis of the transpeptidation reaction. Surprisingly, this residue does not belong to the conserved structural and functional elements which characterise the penicillin-recognising enzymes.

Key words. Penicillin-binding proteins; penicillin-recognizing enzymes; β-lactamase; site-directed mutagenesis; antibiotics; aminolysis.

For many years, the *Streptomyces* R61 DD-peptidase was utilised as a model for the study of penicillin-recognising enzymes [1–4]. Its three-dimensional structure is well documented [5], and it is the only penicillin-sensitive DD-peptidase whose transpeptidase properties have been studied in detail [6]. Site-directed mutagenesis experiments have indicated that Thr299, Tyr159, His298 and, to a lesser degree, Asn161 are directly involved in the transfer mechanism [7–10].

The Tyr280 residue is located at the entrance of the active site cavity with its hydroxyl oxygen in the close vicinity (3–5 Å) of His298-Nε, Tyr159-Oζ and Lys65-Nε, three of the conserved important catalytic residues. In this paper, we describe the physicochemical and kinetic properties of the Tyr280Phe mutant enzyme.

Kinetic model. All the data can be analysed on the basis of the following model [6].



(1)

The first, horizontal line accounts for the carboxypeptidase activity (S is the substrate and H, the hydrolysis products) and the inactivation by β-lactams (with S =

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β-lactam, k_{+3} is low and there is no P_1). In both cases, E-S* represents the acyl enzyme. The complete model describes the transpeptidation pathway where A, E-S*A, E-S*AS and T are respectively the acceptor substrate, the acyl enzyme-acceptor complex, the quaternary acyl enzyme-acceptor-donor complex and the transfer product, 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 and oligonucleotide. Chemicals and the various substrates were as previously described [7, 8, 10]. The oligonucleotide AGCACCCAGGGGTT$\underline{(A)}$CGGCCTCGGCCTG where the underlined and bracketed bases correspond to the mutant and wild-type bases was purchased from Eurogentec (Liège, Belgium) and purified as described [11].

Strains, plasmids and molecular biology techniques. *Escherichia coli* strain TG1 was used as the host for phage M13 as well as for enzyme expression and production. Plasmid pBK8 and its derivative pDML41 [12] both carry the gene of the *Streptomyces* R61 exocellular DD-peptidase (*dac*R61) and were used as expression vectors. These two plasmids differ only by the presence in pDML41 of a stop codon (TGA) introduced after the C-terminal residue of the mature protein (Ala350), which results in the presence of an additional DdeI restriction site in the dacR61 gene. This plasmid allows the expression of a correctly processed DD-peptidase in *E. coli* [12].

The recombinant procedures were those described before [8, 11]. In order to introduce oligonucleotide-directed changes, the 700-bp SphI/PstI fragment of pDML41 carrying the terminal half of the R61 gene was subcloned in phage M13mp18 to provide single-stranded template DNA. The mutation was introduced with the help of an oligonucleotide-directed in vitro mutagenesis kit (Amersham International). Screening of the clones and verification of the absence of additional unwanted mutations were achieved by sequencing the complete fragment subcloned in M13 using the dideoxy method with the T7 sequencing kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). The mutated SphI/PstI fragment carrying the stop codon was reinserted into pBK8 to reconstitute the whole gene, and the resulting plasmid pDML8 was used to transform *E. coli* TG1. The presence of the additional DdeI site allowed the selection of the transformants containing the mutated fragments.

Production and purification of the mutant DD-peptidase. Two 400-ml precultures in LB medium [11] containing 12.5 mg/l of tetracycline were used to inoculate a 20-l BIOLAFITTE fermentor equipped with an antivortex shaking system containing 18 l of LB medium. The temperature was 37 °C, and the rate of aeration 2.5 l/min. After 5.5 h, the cells were harvested and lysed with lysozyme [12]. The periplasmic fraction was collected and dialysed against 10 mM Tris-HCl, pH 8.0, containing 50 μM EDTA. The mutant enzyme was purified as described previously [13].

Kinetics parameters, denaturation and stopped-flow experiments, HPLC and TLC procedures. The procedures for high-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC), the determination of the kinetics parameters and the denaturation and stopped-flow experiments were performed as described before [8, 10]. All experiments were done at 37 °C in 10 mM sodium phosphate buffer, pH 7.0, unless otherwise stated. Curve fitting was realized as before [6, 7], using equation 1 described in reference [7] for the analysis of the transpeptidation reactions.

Results

Physical properties and stability. After purification, about 7 mg of the Tyr280Phe mutant was obtained with a final recovery yield of 35%. Its absorption (230–320 nm), fluorescence emission (excitation at 280 nm) and far-ultraviolet (UV) circular dicroism (205–260 nm) spectra were similar to those of the wild-type enzyme. The half-life of the mutant at 60 °C in 10 mM phosphate buffer, pH 7.0, was 2.4 ± 0.2 min vs. 6.2 ± 0.6 min for the wild-type enzyme.

Carboxypeptidase activity of the Tyr280Phe enzyme. Table 1 compares the hydrolytic profiles of the tyrosine-modified and the wild-type enzymes. Fluorescence-quenching experiments indicated the formation of the acyl enzyme at $[S] > K_m$ with thiol esters S2a, S2c, S2d and S2Val (see legend of table 1), showing that for these substrates k_{+3} remained $< k_{+2}$, as observed with the wild-type enzyme. This was corroborated by the similar k_{cat} values obtained for substrates S2a and S2c, which form the same acyl enzyme. Fluorescence stopped-flow experiments performed at 10 °C yielded the values of k_{+2} and K' for the Tyr280Phe mutant and substrate S2a: wild-type, $k_{+2} = 151 \pm 6$ (s^{-1}), $K' = 8.5 \pm 0.8$ (mM) [6]; Tyr280Phe, $k_{+2} = 155 \pm 30$ (s^{-1}), $K' = 31 \pm 8$ (mM).

Transpeptidase activity of the Tyr280Phe enzyme. Figure 1 depicts the effects of increasing D-alanine concentrations on the k_{cat} values for the utilisation of thiol ester S2a by the wild-type and Tyr280Phe proteins. As observed with the wild-type enzyme, the k_{cat} curve fitted the empirical equation 1: $k_{cat} = (a + b\,[A])/(1 + c\,[A])$ with a slightly increased half-saturating D-alanine concentration ($[A]_{50} = 1/c$) for the mutant enzyme (140 mM vs. 50 mM for R61WT). Since K_m

Table 1. k_{cat}, K_m and k_{cat}/K_m values for the wild-type and Tyr280Phe enzymes.

Substrate	Tyr280Phe				R61WT			
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	M	k_{cat} (s^{-1})	K_m (mM)	$k_{cat}K_m$ (mM^{-1} s^{-1})	M
Ac_2KAA	3.1	50	0.06	B	55*	14	4	B
S1e	0.8	10.5	0.08	B	5†	0.9	5.5	A
S2a	5.8	0.32	18	A	5†	0.05	100	A
S2c	4	0.40	10	A	5†	0.05	100	A
S2d	64	0.70	91	A	70†	0.1	700	A
S2Val	3.1	1.60	1.9	A	4	0.5	8	A

Values for the wild-type are from references [1] (*) and [16] (†). The methods (M) used to calculate the steady-state parameters were A, complete time-course method [17], and B, initial-rate measurements. SD values did not exceed 15%. Ac_2KAA, N^{α},N^{ε}-diacetyl-L-lysyl-D-alanyl-D-alanine; S1e, hippuryl-DL-phenyllactate; S2a, hippuryl-thioglycolate; S2c, hippuryl-D-thiolactate; S2d, benzoyl-D-alanyl-D-thiolactate; S2Val, benzoyl-D-valyl-thioglycolate.

increased in a similar way (data not shown), the k_{cat}/K_m values did not significantly vary with [A], a behaviour similar to that of the wild-type enzyme.

For the wild-type peptidase, at low acceptor concentrations, the k_{cat} values increase linearly with the acceptor concentration, and the quality of an acceptor is characterised by the acceleration factor $\Delta k_{cat}/\Delta[A]$ or by the relative acceleration $\Delta k_{cat}/(\Delta[A]k_{cat})$ [3]. When compared with that of the wild-type enzyme, the acceptor profile of the mutant depicted in table 2 was sharply modified, with a strong decrease of the acceleration factors of the best acceptors. Two compounds, D-aspartate and D-lactate exhibited 'negative' acceleration factors, thus behaving as inhibitory acceptors. A loss of stereospecificity was also recorded, since a low but significant acceleration was observed with L-alanine.

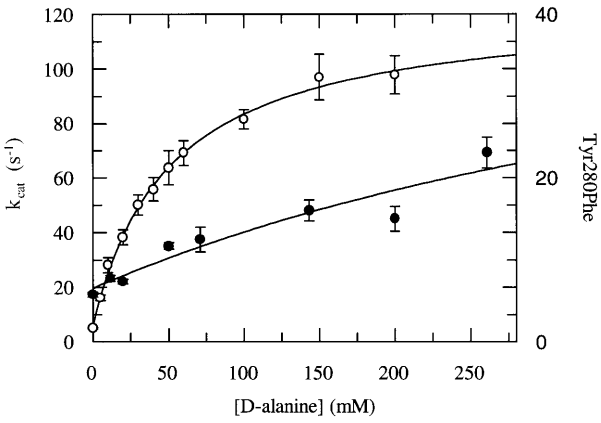

Figure 1. Influence of the D-alanine concentration on the k_{cat} values for the hydrolysis of S2a by the wild-type (○) and Tyr280Phe (●) enzymes. Initial rates were measured or the complete time courses monitored [17] at 37 °C in 10 mM sodium phosphate buffer, pH 7.0. Values for the wild-type are from [3]. Results are means ± SD ($n = 7$ or more). The continuous curves were fitted according to the empirical eq. (1) described in the Results section.

Measurement by HPLC of the quantities of transpeptidated and hydrolysed products liberated during the transpeptidation reactions allowed determination of transpeptidation/hydrolysis (T/H) ratios. Table 2 shows the T/H ratios observed with the Tyr280Phe and wild-type enzymes after complete utilisation of substrate S2a in the presence of L-alanine, D-aspartate and D-lactate, and figure 2 depicts the influence of increasing D-alanine concentrations on the Tyr280Phe T/H ratio measured under steady-state conditions (less than 10% utilization of the donor substrate). A clear saturation effect with a slightly decreased limit T/H value (3.6) and an increased half-saturating D-alanine concentration $^{T/H}[A]_{50}$ (130 mM) when compared with the wild-type values (7.8 and 20 mM, respectively) were observed.

Interaction of the Tyr280Phe enzyme with β-lactams. Table 3 presents an overview of the acylation (k_{+2}/K') and deacylation (k_{+3}) parameters for the Tyr280Phe and wild-type enzymes with several β-lactam antibiotics. For carbenicillin, the individual values of k_{+2} and K' were determined by fluorescence quenching, indicating that the decrease of the k_{+2}/K' value was mainly due to a sharp increase of K' (2.50 ± 0.33 vs. 0.11 ± 0.02 mM), whereas k_{+2} exhibited a moderate increase (0.20 ± 0.02 vs. 0.09 ± 0.01 s^{-1}). The values of k_{+2}/K' for the mutant decreased 10–110-fold with the penicillins and less than 10-fold with the cephalosporins except nitrocefin, for which the value was increased 3-fold. By contrast, the k_{+3} values were not modified except again with nitrocefin, for which a 10-fold increase over the wild-type value was observed. Finally, at pH 7, the benzylpenicilloyl-mutant adduct was fragmented, yielding a nearly quantitative formation of phenylacetylglycine, as observed with the wild-type enzyme (penicilloic acid/phenylacetylglycine ratio <0.1) as determined by TLC [14].

Table 2. Acceleration factors ($\Delta k_{cat}/\Delta[A]$) and relative accelerations ($\Delta k_{cat}/\Delta[A]\ k_{cat}$) for the wild-type and Tyr280Phe enzymes.

Acceptor	Tyr280Phe		R61WT [6]	
	Acceleration factor ($M^{-1}\ s^{-1}$)	Relative acceleration (M^{-1})	Acceleration factor ($M^{-1}\ s^{-1}$)	Relative acceleration (M^{-1})
D-Phenylalanine	360	60	15500	3100
D-Leucine	50	9	9700	1940
D-Histidine	280	48	9500	1900
D-Asparagine	190	33	5600	1120
D-Alanine	110	20	3340	670
L-Alanine	5 (0.5)	0.9	<5 (0.18)	-
D-Aspartate	−20 (0)	−4	<5 (0.6)	-
Meso-A_2pm	70	11	6540	1300
D-Lactate	−5 (1.2)	−0.9	1800 (14.1)	360
Glycylglycine	140	24	490	100

The donor substrate was S2a in all cases. Meso-A_2pm, mesodiaminopimelic acid. The complete time-course method [17] was used to calculate the k_{cat} values. Negative values indicate a decrease in the rate of donor hydrolysis. Values between parentheses give the T/H ratios measured after complete utilisation of 250 μM S2a in the presence of 200 mM L-alanine or D-lactate or 75 mM D-aspartate at 37 °C in 100 mM sodium phosphate buffer, pH 7.0. SD values did not exceed 15%.

Discussion

The physical properties of the Tyr280Phe protein, including its thermal stability, were not significantly different from those of the wild-type enzyme, indicating the absence of important structural alterations. Variations of the mutant kinetic parameters can thus be discussed as resulting from a direct effect on catalysis rather than on structural characteristics.

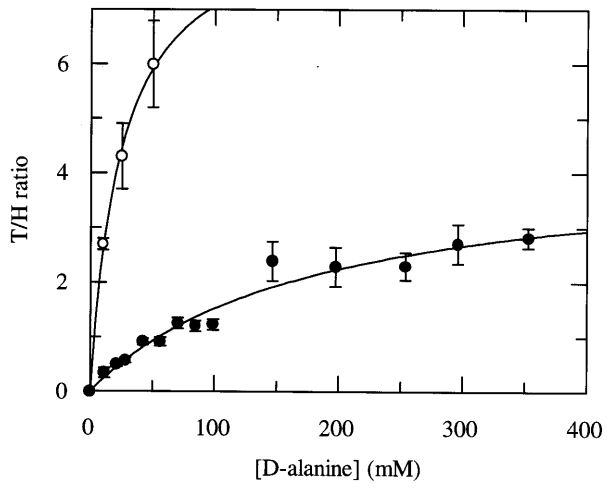

Figure 2. Influence of the D-alanine concentration on the T/H ratios for the hydrolysis of S2a by the wild-type (○) and Tyr280Phe (●) enzymes. The donor substrate was 250 μM S2a in 10 mM sodium phosphate buffer, pH 7.0. (37 °C). The continuous curves are theoretical and were obtained by introducing the parameters of table 4 into eq. (6) of ref. [6].

DD-Carboxypeptidase profile and interaction with β-lactam antibiotics. The mutation did not induce strong modifications of the thiolesterase activity as shown by the k_{cat}/K_m values with substrates S2a, S2c and S2Val. In addition, with these substrates, for which $k_{cat} = k_{+3}$, the k_{cat} values were similar to those of the wild-type which implied that the deacylation rate was little or not modified. By contrast, the k_{cat}/K_m ($=k_{+2}/K'$) values for ester S1e and peptide Ac_2KAA both decreased 70-fold. In the latter case, the alteration resulted mainly from the decrease of the k_{cat} value (18-fold), while the K_m value was less strongly increased (3.5-fold). If we assume that acylation remains rate limiting with this substrate as observed with the wild-type enzyme ($k_{cat} = k_{+2}$ and $K_m = K'$), these results indicate that catalysis of the acylation reaction (k_{+2}) is more affected by the mutation than the binding of the substrate (K').

Interestingly, the mutation similarly modified the reaction parameters with the antibiotics, and the ester and peptide substrates. With the former, the disappearance of the Tyr280 hydroxyl group did not alter the deacylation rates (k_{+3}) but resulted in a decrease of the acylation rates (k_{+2}/K'), the penicillins being more affected than the cephalosporins. With carbenicillin, this variation was due to a 25-fold increase of the K' value, while the k_{+2} value was barely affected, a behaviour in contrast to that of the peptide substrate. This difference between the peptide Ac_2KAA and carbenicillin has been observed before with the Asn161Ala, Thr299Val, Thr301Ile and Thr301Ser mutants [8, 10]. It shows that with the *Streptomyces* R61 DD-peptidase, the acylation requirements are different for the two molecules.

These data surprisingly demonstrate that the Tyr280 residue is clearly involved in the mechanism of acyl enzyme formation with both substrates and β-lactams,

Table 3. k_{+2}/K' and k_{+3} values with β-lactam antibiotics for the wild-type and Tyr280Phe enzymes.

Antiobiotic	Tyr280Phe		R61WT	
	k_{-2}/K' (M^{-1} s^{-1})	k_{+3} (s^{-1})	k_{+2}/K' (M^{-1} s^{-1})	k_{+3} (s^{-1})
Benzylpenicillin	1000	82×10^{-6}	18000	140×10^{-6}
Carbenicillin	70	100×10^{-6}	830	140×10^{-6}
Ampicillin	1	72×10^{-6}	110	140×10^{-6}
Nitrocefin	15000	3.3×10^{-3}	4100	0.3×10^{-3}
Cephalosporin C	160	1.6×10^{-6}	1500	1.0×10^{-6}
Cefuroxime	150	2.5×10^{-6}	350	$<4.0 \times 10^{-6}$

k_{+2}/K' and k_{+3} values were measured according to methods 1a (quenching of fluorescence) and 3 (reactivation of the isolated acylenzyme) as described previously [8]. SD values did not exceed 15%.

and at both levels of the process: binding of the ligand and acylation.

Transpeptidase profile. The influence of the acceptor concentrations on k_{cat} and T/H indicated that the Tyr280Phe mutant obeyed a catalytic mechanism similar to that of the wild-type enzyme with somewhat increased $[A]_{50}$ (2.8-fold) and $^{T/H}[A]_{50}$ (6.5-fold) values, and a slightly decreased maximum T/H ratio (2-fold). So with D-alanine as acceptor, all the data could be fitted to the model proposed for the wild-type enzyme, and table 4 summarises the individual transpeptidation parameters thus calculated. The largest effect is an almost 10-fold decrease of the k_{+5} value, the first-order rate constant characterising the decay of the ES*A complex into free enzyme and transpeptidation product, a factor which is directly reflected in the lower T/H ratios shown in figure 2.

This conclusion also applies to the other acceptors, as indicated by the alterations of the profile of mutant acceleration factors. Based on the kinetic model, $\Delta k_{cat}/\Delta[A]$ varies with the substrate concentrations according to eq. (2):

$$\frac{\Delta k_{cat}}{\Delta[A]} = \frac{k_{+5}}{\alpha} + \frac{k_{+7}[A][S]}{\alpha\beta} \quad (2)$$

Table 4. Kinetic parameters for the concomitant hydrolysis and aminolysis (D-alanine) of substrate S2a catalysed by the wild-type and Tyr280Phe enzymes at 37 °C.

Constants	Tyr280Phe	R61WT
k_{+2}/K' (mM^{-1} s^{-1})	18 ± 2.0	100 ± 10
k_{+3} (s^{-1})	5.0 ± 0.5	5.0 ± 0.5
k_{+5} (s^{-1})	25 ± 6	200 ± 50
α (mM)	175 ± 30	100 ± 16
k_{+7} (s^{-1})	33 ± 6	84 ± 2
β (mM)	1.2 ± 0.2	0.82 ± 0.01

The k_{+2}/K' and k_{+3} values were those directly measured in the absence of acceptor. The other parameters were obtained by fitting two sets of experimental data – initial rates and T/H ratios as a function of the concentration of D-alanine – on eqs. (1) of ref. [7] and (6) of ref. [6].

At low donor and acceptor substrate concentrations – i.e. the conditions in which $\Delta k_{cat}/\Delta[A]$ is measured – the second term of the equation can be neglected, and the acceleration factors thus reflect the k_{+5}/α ratios, which quantify the efficiency of the transfer reaction. As shown by table 3, the acceleration factors of the best acceptors ($\Delta k_{cat}/\Delta[A] > 3000$ M^{-1} s^{-1}) decreased 30–90-fold, indicating a proportional decrease of the k_{+5}/α values. The effect of the mutation on the acceleration factors of the poorer acceptors ($\Delta k_{cat}/\Delta[A] <$ 3000 M^{-1} s^{-1}) was even more surprising. D-Aspartate and D-lactate presented negative acceleration factors. On the basis of the kinetic model, this would indicate that with these compounds, the value of k_{+5} is lower than those of k_{+3} and k_{+7}, which would immobilise the enzyme in a somewhat less productive intermediate. This assumption is supported by the 14-fold decrease of the T/H value measured with D-lactate and by the complete absence of transpeptidation product with D-aspartate ($k_{+5} = 0$). A very striking and unexpected result was a significant loss of stereospecificity for the transpeptidation reaction. Indeed, when D- and L-alanine were compared, the D/L ratio of the acceleration factors decreased from more than 650 to 22. Accordingly, the T/H value recorded with the L isomer increased almost threefold compared with that of the D isomer.

All these data underline an important and unexpected role of Tyr280 in the transpeptidation reaction mechanism. As discussed above with D-alanine, its major contribution appears to be at the level of the k_{+5} step, but it might also participate in determining the stereospecificity, and the disappearance of the hydroxyl group might allow the binding of the wrong (i.e. L) stereoisomer of alanine. Interestingly, the compound for which the lesser decrease was recorded was the nonchiral glycylglycine dipeptide.

Structural and mechanistic considerations. The Tyr280 residue is well situated to participate in the transpeptidation mechanism. Its hydroxyl group lies in close vicinity (5.1 Å) of the Tyr159-Oζ, at about 8 Å from the Thr299 and Ser62 hydroxyl group oxygens and within hydrogen-bonding distance (2.71 Å) from the His298-Nδ. The His298-Tyr280 pair might then act as a single entity. This hypothesis is supported by the fact that replacement of His298 by Lys or Gln resulted in enzymes exhibiting kinetic characteristics similar to those of the Tyr280Phe enzyme. Indeed, with both His298 mutants no important modifications of the thiolesterase activity were observed; the acylation constants (k_{+2}/K') were impaired 120–180-fold with substrate Ac_2KAA and 75–160-fold with benzylpenicillin, while they were less affected with cephalosporin C (15–60-fold); and their transpeptidase activity was altered, as evidenced by 4- and 10-fold decreases of the D-leucine acceleration factors for the Lys and Gln mutations, respectively [9].

We propose that the disappearance of the hydrogen-bonded Tyr280-His298 pair is responsible for the altered acylation and transfer properties of the Tyr280Phe mutant enzyme. The mutation would result in a disorientation of both side chains, which in turn would impair the catalytic mechanism.

One particularly original property of the Tyr280Phe enzyme was that L-alanine became an acceptor for the mutant. The Tyr280 OH group thus seems to influence the acceptor stereospecificity and could be related to the acceptor binding site. Tyr280 is located at the junction of two enzyme cavities. The first cavity corresponds to the part of the active site where the donor substrate is thought to bind antiparallel to the β3 strand carrying the His298-Thr-Gly300 conserved element with its carboxylate oriented towards the conserved basic residues (Lys65 and His298) and within hydrogen-bonding distance from the Thr299-0γ [15]. The second cavity is bordered by the Leu83, Arg103 → His108, Ala155 → Tyr157, Gln264 → Thr273 and Gly279 → Gln303 residues and connects the solvent outer shell to the first cavity. It presents a volume of approximately 3000 Å^3 (10 Å × 15 Å × 20 Å). These properties correspond to the requirements for an acceptor-binding site, and we propose that this second cleft actually is the acceptor-binding site.

In conclusion, the Tyr280 OH group is involved (i) in the formation of the acyl enzyme with ester and peptide substrates and with β-lactams and (ii) in the transpeptidation mechanism at the level of the transfer process and in the configuration of the acceptor-binding site. This might result from a hydrogen bond formed with the histidine 298 side chain. For the first time, a residue that does not belong to one of the three conserved elements is found to be of great importance for the R61 DD-peptidase catalytic mechanism, especially the transpeptidation pathway.

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