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The mechanism of action of DD-peptidases: the role of tyrosine-159 in the *Streptomyces* R61 DD-peptidase

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Tyrosine-159 of the *Streptomyces* R61 penicillin-sensitive DD-peptidase was replaced by serine or phenylalanine. The second mutation yielded a very poorly active protein whose rate of penicillin binding was also drastically decreased, except for the reactions with nitrocefin and methicillin. The consequences of the first mutation were more surprising, since a large proportion of the thiolesterase activity was retained, together with the penicillin-binding capacity. Conversely, the peptidase properties was severely affected. In both cases, a drastic decrease in the transferase activity was observed. The results are compared with those obtained by mutation of the corresponding residue in the class A β-lactamase of *Streptomyces albus* G.

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, and the differences between the two types of enzyme can be summarized in a simple sentence: β-lactams inactivate PBPs, while β-lactamases inactivate β-lactams. Although this statement accounts for most enzyme–β-lactam interactions, it is an oversimplification since, for example, some β-lactams are known to also inactivate β-lactamases.

PBPs and β-lactamases appear to share some important properties. (1) As stated above, they are active-site-serine enzymes, with the exception of some Zn^{2+}-β-lactamases which will not be discussed here (Frère et al., 1976a; Waley, 1992). (2) Kinetically, their interaction with β-lactams can most often be represented by the simple, 3-step model shown below (Frère et al., 1975; Christensen et al., 1990).

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot C \xrightarrow{k_{+2}} E\text{-}C^* \xrightarrow{k_{+3}(H_2O)} E + P(s) \qquad \text{(model 1)}$$

where E, C, E·C, E-C* and P(s) are respectively the enzyme, the β-lactam, the non-covalent Henri–Michaelis complex, the acyl-enzyme and the reaction product(s), which are devoid of biological activity. (3) Some esters and thiolesters behave as substrates for both DD-peptidases and β-lactamases (Adam et al., 1990, 1991), although the latter enzymes fail to recognize the normal peptide substrates of the former. (4) X-ray diffraction data have indicated clear similarities in the three-dimensional structures of several β-lactamases and of the *Streptomyces* R61 extracellular DD-peptidase (Kelly et al., 1986; Samraoui et al., 1986), and sequence alignments have allowed the identification of several conserved elements (Joris et al., 1988), which are probably directly involved in the reaction mechanism (such as the active-site serine) or bordering the catalytic cavity.

Besides the specific interactions of the DD-peptidases with the D-alanyl-D-alanine-terminated peptides, the most striking difference between the two types of enzyme is the value of k_{+3}, which is generally quite high for β-lactamases (up to 4000 s^{-1}) and extremely low for DD-peptidases (10^{-3}–10^{-6} s^{-1}). This difference is responsible for the 'macroscopic' consequences described above.

One of the conserved elements is a Ser-Asp-Asn sequence situated on a loop near the active-site serine of class A β-lactamases (Joris et al., 1991). The corresponding triads are Tyr-Ala-Asn and Tyr-Ser-Asn in class C β-lactamases and in the R61 DD-peptidase respectively (Ghuysen, 1991). The roles of these three residues in the catalytic properties of class A β-lactamases have been extensively studied by site-directed mutagenesis (Jacob et al., 1990a,b), and the ionized group of the tyrosine residue of class C β-lactamases has been hypothesized to be the general base which activates the active serine by abstracting its proton (Oefner et al., 1990).

In this paper, we have assessed the importance of the corresponding tyrosine residue (Tyr-159) in the *Streptomyces* R61 DD-peptidase by site-directed mutagenesis. This enzyme is by far the most studied penicillin-sensitive DD-peptidase and the only one for which three-dimensional data are available.

KINETIC MODEL

The interaction between β-lactams and DD-peptidase is well represented by model 1 (Frère et al., 1975), with the first step in rapid equilibrium ($K = k_{-1}/k_{+1}$). When [C] $\ll K$, the rate of acylation is characterized by a second-order rate constant, k_{+2}/K, which is most often the parameter responsible for the sensitivity of the enzyme to the inactivator.

Model 1 also depicts the carboxypeptidation pathway where a first product, P1, is released in the k_{+2} step. With the best peptide substrate, $N^{\alpha}N^{\epsilon}$-bisacetyl-L-lysyl-D-alanyl-D-alanine (Ac_2KAA), k_{+2} is $\ll k_{+3}$ and thus $k_{cat.} = k_2$ and $K_m = K' = (k_{-1} + k_{+2})k_{-1}$ (Varetto et al., 1987); P1 is D-alanine. Conversely, with the thiolester benzoylglycylmercaptoacetate (C_6H_5-CO-NH-CH_2-CO-S-CH_2-COO^-), k_{+3} is $\ll k_{+2}$; thus $k_{cat.} = k_{+3}$ and $K_m = k_{+3}K'/k_{+2}$; P1 is mercaptoacetate (Jamin et al., 1991).

In the presence of suitable aminated acceptors, the enzyme also catalyses the transfer of the Ac_2-L-lysyl-D-alanyl or benzoyl-

Abbreviations used: Ac_2KAA, $N^{\alpha}N^{\epsilon}$-bisacetyl-L-lysyl-D-alanyl-D-alanine; Ac_2KALa, $N^{\alpha}N^{\epsilon}$-bisacetyl-L-lysyl-D-alanyl-D-lactate; Ac_2KATl, $N^{\alpha}N^{\epsilon}$-bisacetyl-L-lysyl-D-alanyl-D-thiolactate; PBP, penicillin-binding protein; BPA, benzylpenicilloic acid.

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glycyl moieties of the donor substrates on to the acceptor amino group (transpeptidation). With the thiolester, alcohols can also serve as acceptors in transesterification reactions. When k_{+3} is $< k_{+2}$, the presence of an acceptor increases the rate of donor substrate disappearance by opening a new branch for the utilization of the acyl-enzyme, E-C*. However, a simple partition model is not sufficient to account for all of the experimental observations (Frère et al., 1973; Jamin et al., 1991).

MATERIALS AND METHODS

Chemicals

Enzymes for genetic engineering were purchased from Biolabs (Beverly, CA, U.S.A.) and Boehringer (Mannheim, Germany). [^{35}S]dATP (1000 Ci/mmol) was from Amersham; benzylpenicillin was from Rhône-Poulenc (Paris, France), [^{14}C]benzylpenicillin (53 Ci/mol) from Amersham, carbenicillin from Beecham Research Laboratories (Brentford, Middlesex, U.K.), ampicillin from Bristol Benelux (Brussels, Belgium), cephalosporin C from Elli Lilly and Co. (Indianapolis, IN, U.S.A.) and cefuroxime from Glaxo Group Research (Greenford, Middlesex, U.K.). All of the unlabelled β-lactam compounds were kindly given by the respective companies. Nitrocefin was purchased from Oxoid (Basingstoke, Hampshire, U.K.).

The tripeptides Ac_2KAA and $N^{\alpha}N^{\epsilon}$-bisacetyl-L-lysyl-D-alanyl-D-thiolactate (Ac_2KATl) were from UCB Bioproducts (Braine-l'Alleud, Belgium). $N^{\alpha}N^{\epsilon}$-Bisacetyl-L-lysyl-D-alanyl-D-lactate (Ac_2KALa) was a gift from Dr. R. F. Pratt (Middletown, CO, U.S.A.). The other substrates were synthesized in our laboratory. The structures of the various substrates are given in Table 1.

Rabbit anti-(R61 DD-peptidase) antiserum was from Gamma S.A. (Angleur, Belgium).

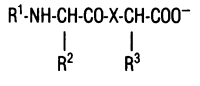
Oligonucleotides

Oligonucleotides were a gift of Dr. J. Brannigan, University of Sussex, Brighton, U.K. The crude oligonucleotides were purified with the help of an Oligonucleotide Purification Cartridge (Applied Biosystems).

The oligonucleotides used to introduce the mutations were the following: Tyr-159 → Phe, GGCCGCGCCTATTCATTCTCCAACACGAAC; Tyr-159 → Ser, GGCCGCGCCTATTCATCCTCCAACACGAAC; the modified bases are underlined (the wild-type codon was TAC).

Table 1 Structures of the substrates

The general formula is given by:



Substrate	R^1	R^2	R^3	X
Ac_2KAA	Ac_2L-Lys	CH_3 (D)	CH_3 (D)	NH
Ac_2KALa	Ac_2L-Lys	CH_3 (D)	CH_3 (D)	O
Ac_2KATl	Ac_2L-Lys	CH_3 (D)	CH_3 (D)	S
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_3 (DL)	H	S

Strains, plasmids and molecular biology techniques

Escherichia coli strain TG1 was used as the host for phage M13 and *Streptomyces lividans* strain TK24 (Hopwood et al., 1985) was used for enzyme expression and production.

The multicopy *Streptomyces* plasmid pDML115 was the expression vector. It carries the gene of the exocellular DD-peptidase of *Streptomyces* R61 (*dacR61*) and presents unique *Sph*I and *Pst*I sites (Duez et al., 1992).

The recombinant procedures were those described by Maniatis et al. (1982) and Hopwood et al. (1985), except that plasmid and M13 replicative form extractions were performed using the QIAGEN protocols and Tip-100 or Tip-20 columns (QIAGEN, Studio City, CA, U.S.A.). The *Streptomyces* plasmid extractions were performed as described by Hopwood et al. (1985), but the centrifugation step was replaced by a QIAGEN protocol. Elution of DNA fragments from agarose gels was carried out with the help of a Geneclean kit (Bio 101, La Jolla, CA, U.S.A.).

In order to introduce oligonucleotide-directed changes, the *Pst*I/*Sph*I fragment was subcloned in phage M13mp19 to provide single-stranded template DNA. The replacement of the tyrosine codon (TAC) by that for phenylalanine (TTC) or serine (TCC) was performed using the procedure of Taylor et al. (1985), with the help of an oligonucleotide-directed *in vitro* mutagenesis kit (Amersham International). The clones were screened by sequencing the complete fragment subcloned in M13. The dideoxy method was used with the T7 sequencing kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The mutated fragments were re-inserted into pDML115 to reconstitute the whole gene and the plasmids thus obtained were used to transform protoplasts of *Streptomyces lividans* TK24. The transformants were screened by immunodetection (Bio-Rad Immunoblot Assay kit). The absence of any other mutation was confirmed by resequencing the mutant DD-peptidase gene using the oligonucleotides priming at regular intervals of 150 bp.

Production of the mutant DD-peptidases

For the expression of the mutant proteins, transformant *Streptomyces* colonies selected on thiostrepton-containing R2YE agar (Hopwood et al., 1985) were used to inoculate a 250 ml preculture in modified YEME medium (Erpicum et al., 1990). Thiostrepton (25 mg/l) was added to exert a selection pressure during the exponential growth phase, since the plasmid carried the resistance to this antibiotic. Baffled 1 litre Erlenmeyer flasks were used and incubations were at 28 °C with orbital agitation at 250 rev./min (New Brunswick Scientific incubator). After 3 days, a 4% inoculum was used to initiate the main culture, which was continued for 5 days under the same conditions.

Purification of the mutant DD-peptidases

This was performed as described by Hadonou et al. (1992). In summary, complete purification required three or four steps after the elimination of the mycelium by centrifugation: (1) adsorption at pH 4.0 on Amberlite CG-50 and desorption at pH 8.0; (2) chromatography on Q-Sepharose Fast Flow; (3) filtration on Sephadex G-75; (4) chromatography on a prepacked Hiload 26/10 Q-Sepharose Fast Flow column. The last step has not been described before. The buffer was 10 mM Tris/HCl containing 50 μM EDTA, pH 7.0, and a salt gradient up to 0.2 M NaCl was applied over 1.1 litres.

The enzyme was detected in the various fractions by SDS/PAGE using the wild-type *Streptomyces* R61 enzyme as a molecular mass standard.

Kinetic parameters

Substrates

Several substrates (see Table 1) could be directly and continuously monitored at 250 nm with the following absorbance variations: Sle ($\Delta\epsilon$ 540 $M^{-1}\cdot cm^{-1}$) S2a, S2c, S2d, S2e, S2-Val ($\Delta\epsilon$ −2200 $M^{-1}\cdot cm^{-1}$) and Ac_2KATl ($\Delta\epsilon$ −2000 $M^{-1}\cdot cm^{-1}$). Hydrolysis of thiolester substrates could also be monitored at 324 nm in the presence of 2 mM 4,4′-dithiodipyridine (Sigma Chemical Co., St. Louis, MO, U.S.A.), which reacts with the thiol groups released during the reaction ($\Delta\epsilon$ 20000 $M^{-1}\cdot cm^{-1}$) (Kelly et al., 1992).

Hydrolysis of the tripeptides Ac_2KAA and Ac_2KALa was monitored by estimating the release of D-alanine (D-amino acid oxidase procedure; Frère et al., 1975) or of hydrolysed product Ac_2KA (see h.p.l.c. procedure) respectively.

The steady-state parameters $k_{cat.}$, K_m and $k_{cat.}/K_m$ were determined either from complete time courses (method A; De Meester et al., 1987) or from initial rates using the Hanes linearization of the Henri–Michaelis equation (method B). This latter procedure was always utilized for the tripeptide and the depsipeptide.

β-Lactams

On the basis of model 1, β-lactams could be used as either inactivators or substrates. In the first case, the characteristic constants of the acylation (k_{+2}, K' or k_{+2}/K') and deacylation ($+k_3$) reactions were determined separately using one of the following methods. (1) The apparent first-order inactivation rate constant k_i was determined either by recording the decrease in protein fluorescence at 320 nm (method 1a) (excitation at 280 nm; Frère et al., 1975) or by the reporter substrate method (De Meester et al., 1987) with S2a or Ac_2KATl as reporter substrates (method 1b). When possible, the individual values of k_{+2} and K' were determined by fitting the measured k_i values to the equation:

$$k_i = k_{+2}[C]/(K' + [C])$$

with the help of the ENZFITTER program (Leatherbarrow, 1987). (2) k_{+2}/K' values could also be obtained by the competition method, with nitrocefin as reporter inactivator (Frère et al., 1992). Complete acylation of the enzyme by nitrocefin resulted in an increase in the solution absorbance at 482 nm (ΔA_0); when the enzyme was added to a mixture of nitrocefin (N) and a second β-lactam (C), this increase (ΔA_i) was smaller, since a proportion of the enzyme was acylated by the other antibiotic; it can be shown that:

$$\frac{\Delta A_0 - \Delta A_i}{\Delta A_i} = \frac{(k_{+2}/K')_C[C]}{(k_{+2}/K')_N[N]}$$

where $(k_{+2}/K')_C$ is the only unknown. This procedure could only be used if acylation reactions were completed over a time period much shorter than the half-lives of both acyl-enzymes. (3) The rate of deacylation (k_{+3}) was determined by monitoring the reactivation of the enzyme after elimination of the excess free β-lactam by addition of a small amount of *Bacillus licheniformis* β-lactamase. To determine the recovered activity, the tripeptide Ac_2KAA was used after inactivation by compounds exhibiting a low k_{+3} value (10^{-5}–10^{-6} s^{-1}; most cephalosporins), and the thiolester substrate S2a was also used after inactivation by compounds exhibiting a higher k_{+3} value (10^{-3}–10^{-4} s^{-1}; nitrocefin and penicillins). Identification of the degradation products of the [^{14}C]benzylpenicilloyl-enzyme adducts was performed by t.l.c. (Frère et al., 1976b).

When β-lactams were used as substrates (method 4), the time courses exhibited a burst, followed by steady-state linear hydrolysis. Analysis of the burst yielded a k value $\{= k_{+3} + [k_{+2}[C]/(K' + [C])\}$ and the steady-state rate was equal to $k_{+3}[E_0]$, assuming that k_{+3} was much smaller than k_i $\{k_{+2}[C]/K' + [C])\}$, a condition that was verified in all cases. When k_i varied linearly with [C], only k_{+2}/K' could be computed. This procedure thus allowed a rapid determination of the acylation and deacylation parameters. The time courses were recorded at 482 nm (nitrocefin), 260 nm (other cephalosporins) or 230 nm (penicillins).

All kinetic experiments were performed at 37 °C (unless otherwise stated) with a Uvikon 860 or Hewlett Packard 8452A spectrophotometer coupled to microcomputers via RS232 interfaces, or with a Perkin–Elmer MPF44 spectrofluorimeter.

Reactions between substrate S2a and mutant DD-peptidases under pre-steady-state conditions were monitored with a Biologic SFM3 stopped-flow apparatus. As shown before (Jamin et al., 1991) the fluorescence of the acyl-enzyme intermediate was lower than that of the free enzyme, and quenching of the fluorescence emission at 20 °C was recorded through a 310–490 nm band filter with excitation at 290 nm.

Denaturation experiments

Denaturation of the *Streptomyces* R61 DD-peptidase induced a shift in the fluorescence emission maximum from 320 nm to 340 nm (excitation 280 nm) (Nieto et al., 1973). The quenching of fluorescence at 320 nm obeyed first-order kinetics, and the denaturation first-order rate constant (k_d) was computed with the help of the following equation:

$$\ln(F_t - F_\infty) = \ln(F_0 - F_\infty) - k_d t$$

where F_0, F_t, F_∞ were the fluorescences at times zero, t and infinity respectively.

Two sets of denaturation conditions were used to characterize the stability of the mutant enzyme: 10 mM sodium phosphate buffer, pH 7.0, at 60 °C, or 6 M urea in the same buffer at 56 °C.

H.p.l.c. procedures

Chromatography experiments were performed on a Nucleosil 5-C_{18} (Machery–Nagel) column (ET250/8/4) coupled to a Merck h.p.l.c. apparatus. Separation of Ac_2KALa and its hydrolysis product, Ac_2KA, was performed under isocratic conditions using 10 mM sodium acetate/acetic acid buffer, pH 3.0, containing 3% acetonitrile. The retention times were 8.6 and 5 min for the substrate and the product respectively. For transpeptidation reactions with S2a, the conditions were as described by Jamin et al. (1991). Quantification was achieved by integration of the peak areas using pure Ac_2KALa, Ac_2KA, hippuric acid or S2a as standards.

RESULTS

Mutagenesis, production and purification

The mutagenesis procedures yielded two modified plasmids, pDML35 (Tyr-159 → Phe) and pDML36 (Tyr-159 → Ser). The plasmids were purified and the modified genes were sequenced; no additional mutations were detected.

Enzyme production was performed in the optimized YEME medium (Erpicum et al., 1990). Maximum production was obtained after 5 days of growth, as observed for the wild-type protein. This yielded 120 and 45 mg of modified enzyme per litre for the Tyr-159 → Phe and Tyr-159 → Ser proteins respectively.

The purifications are summarized in Table 2. Step 4 allowed the purification of two enzyme populations of similar M_r (37000–38000), but which were eluted at different ionic strengths.

Table 2 Purification of the Tyr-159 mutant proteins

Culture volumes were 2.25 litres for both mutant enzymes. S.D.s were ⩽ 10% of means.

Enzymes...	Tyr-159 → Phe			Tyr-159 → Ser		
Step	Total protein (mg)	Total enzyme (mg)	Purity (%)	Total protein (mg)	Total enzyme (mg)	Purity (%)
1	6400	300	4.7	2600	100	3.8
2	460	200	43	275	100	36
3	200	150	75	100	100	> 95
4	100	100	> 95	–	–	–

Table 3 $k_{cat.}$, K_m and $k_{cat.}/K_m$ values for the wild-type and Tyr-159-substituted mutant enzymes

Values for the wild-type enzyme are from *Frère and Joris (1985), †Varetto et al. (1987) or ‡Adam et al. (1990). M is the method used to calculate the steady-state parameters: A, complete time course method (De Meester et al., 1987); B, linearization of the Henri–Michaelis equation (see the Materials and methods section). S.D.s did not exceed 15% of the means. ND, not determined.

	Tyr-159 → Phe				Tyr-159 → Ser				Wild type			
Substrates	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	M	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	M	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	M
Ac_2KAA	0.006	13000	0.5	B	0.02	13000	1.3	B	55*	14000	4000	B
Ac_2KALa	ND	ND	1.4	B	8	45000	180	B	32†	40000	800	B
Ac_2KATLa	ND	ND	ND		30	4000	9000	B	72	8000	9000	B
S1e	ND	ND	< 1	B	0.02	1400	14	A	5‡	900	5500	A
S2a	0.008	70	100	B	0.3	5	60000	A	5‡	50	100000	A
S2c	ND	ND	ND		0.22	2	114000	A	5‡	50	100000	A
S2d	0.05	50	1000	B	9	50	200000	A	70	100	700000	A
S2e	ND	ND	ND		11	200	52000	A	70	560	80000	A
S2Val	ND	ND	ND		0.46	100	4500	A	4	500	8000	A

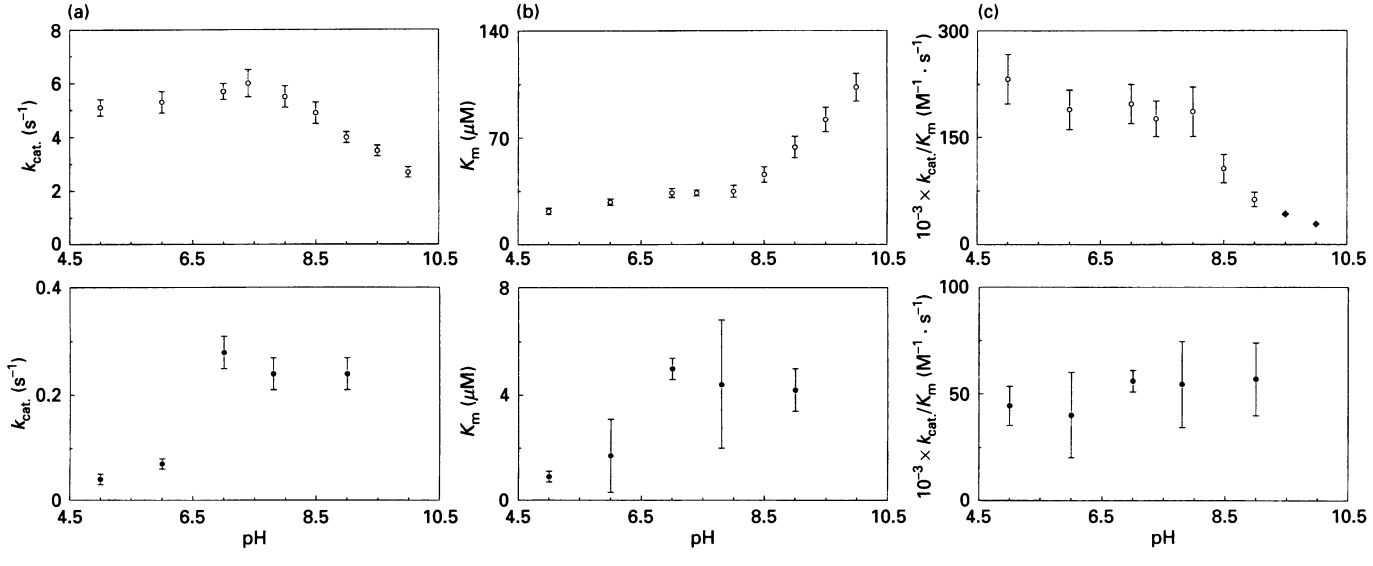


Figure 1 pH-dependence of the kinetic parameters for the hydrolysis of substrate S2a by the wild type (○) and Tyr-159 → Ser (●) enzymes: (a) $k_{cat.}$ values, (b) K_m values, (c) $k_{cat.}/K_m$ values

The values were obtained by analysing complete time-courses. Reactions were performed at 37 °C in the following buffers: pH 5.0, 10 mM sodium cacodylate/HCl; pH 6.0, 13 mM sodium cacodylate/HCl; pH 7.0, 10 mM sodium phosphate; pH 8.0, 7 mM sodium phosphate; pH 9.0, 6 mM potassium borate/NaOH. The ionic strength was adjusted to the same value throughout by the addition of NaCl. The pH profile of the wild-type enzyme is from Varetto (1991). Error bars represent the S.D.s. ($n = 6$ or more).

Table 4 Influence of the acceptor concentration on the $k_{cat.}$, K_m and $k_{cat.}/K_m$ values for the wild-type and mutant enzymes

S2a was the donor substrate. M is the method used to calculate the steady-state parameters: A, complete-time course method (De Meester et al., 1987); B, linearization of the Michaelis equation. S.D.s did not exceed 15% of the means. ND, not determined.

	Tyr-159 → Phe				Tyr-159 → Ser				Wild type			
Acceptor	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	M	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	M	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	M
None	0.008	70	100	B	0.30	5.0	60000	A	5.0	50	100000	A
D-Alanine												
5.7 mM	ND	ND	ND		0.29	5.0	58000	A	17.0	170	100000	A
15 mM	0.011	100	110	B	0.26	5.2	50000	A	29.0	290	100000	A
45 mM	ND	ND	ND		0.25	4.4	57000	A	61.0	610	100000	A
55 mM	0.013	83	150	B	ND	ND	ND		67.0	670	100000	A
Gly-Gly												
20 mM	0.006	40	150	B	0.25	6.0	42000	A	9	90	100000	A

Table 5 Transpeptidation/hydrolysis ratios for the wild-type and mutant enzymes

Transpeptidation was with S2a as donor substrate (250 μM) and D-alanine as acceptor substrate.

[D-Alanine] (mM)	Transpeptidation/hydrolysis ratio		
	Tyr-159 → Phe	Tyr-159 → Ser	Wild type
5	0.01 ± 0.005	–	–
10	0.16 ± 0.02	–	2.7 ± 0.1
15	0.13 ± 0.02	–	–
20	0.21 ± 0.03	–	–
25	–	–	4.3 ± 0.6
50	0.19 ± 0.02	0.07 ± 0.02	6.0 ± 0.8
100	0.25 ± 0.04	0.20 ± 0.05	–
150	–	0.33 ± 0.07	–
200	–	0.40 ± 0.08	–

The first (70% of the total) was eluted with 73 mM NaCl, and the second (30%) with 85 mM NaCl. Both variants hydrolysed substrate S2a with apparently identical kinetic parameters within the limits of experimental error, and were thus considered to be kinetically equivalent. This phenomenon, which was observed previously with the wild-type enzyme and other mutants (Bourguignon-Bellefroid et al., 1992), is under current investigation.

The purity of the mutant proteins was verified by SDS/PAGE and evaluated to be greater than 95%.

Stability and physical properties

Absorption spectra of the modified proteins between 230 and 320 nm were superimposable on that of the wild-type enzyme. The fluorescence emission spectrum of the Tyr-159 → Phe mutant was also identical to that of the wild-type enzyme but, with the Tyr-159 → Ser protein, a small blue shift of the maximum (317 nm instead of 319 nm) was observed, indicating a slight modification of the fluorophore environment. No major change in stability was observed in the mutant proteins. The half-lives were 6.2 ± 0.6 (wild type), 6.0 ± 0.6 (Tyr-159 → Phe) and 3.4 ± 0.3 (Tyr-159 → Ser) min at 60 °C in buffer, and 8.6 ± 0.9 (wild type), 2.3 ± 0.2 (Tyr-159 → Phe) and 2.3 ± 0.2 (Tyr-159 → Ser) min at 56 °C in the presence of 6 M urea.

Kinetic properties

Carboxypeptidase and esterase activities

Table 3 compares the substrate profiles of the wild-type and mutant enzymes. Stopped-flow experiments, in which the accumulation of acyl-enzyme was monitored by recording the rate of fluorescence decrease at various substrate concentrations, allowed the calculation of the individual k_{+2} and K' values at 20 °C: Tyr-159 → Phe, $k_{+2} = 2.6 \pm 0.5\ s^{-1}$, $K' = 17 \pm 3$ mM; Tyr-159 → Ser, $k_{+2} = 300 \pm 50\ s^{-1}$, $K' = 14 \pm 2$ mM; wild type, $k_{+2} = 200 \pm 50\ s^{-1}$, $K' = 5 \pm 0.5$ mM (Jamin et al., 1991).

These results showed that, with substrate S2a, deacylation remained rate-limiting ($k_{+3} < k_{+2}$) for the mutants, as found for the wild-type enzyme. The same situation prevailed with substrates S2c, S2d and S2e, for which fluorescence quenching experiments permitted the visualization of the accumulation of the acyl-enzyme at [S] > K_m.

The pH-dependence of the kinetic parameters was only studied in the most favourable case, i.e. the Tyr-159 → Ser–S2a interactions. The results are presented in Figure 1.

Transpeptidase activity

The influence of two acceptors, D-alanine and glycyl-glycine, on the kinetic parameters for the utilization of substrate S2a is shown in Table 4. The most striking observation was that, in sharp contrast to the situation with the wild-type enzyme, the presence of an acceptor failed to increase the $k_{cat.}$ and K_m values for the mutants, indicating that the transpeptidation pathway was grossly impaired. Accordingly, only a small proportion of transpeptidation product could be detected by h.p.l.c. (Table 5).

Interactions with β-lactam antibiotics

The results presented in Table 6, show drastic decreases in all of the acylation rates for the Tyr-159 → Phe mutant compared with the wild-type enzyme. With the Tyr-159 → Ser mutant, the k_{+2}/K' value was not significantly decreased. In fact, they were even increased in several cases. For carbenicillin as substrate, individual k_{+2} and K' values were determined. They were $0.50 \pm 0.08\ s^{-1}$ and 0.18 ± 0.03 mM respectively, not very different from those observed with the wild-type enzyme ($0.10\ s^{-1}$ and 0.11 mM). The mutations did not seem to affect the k_{+3} values. However, at pH 7.0 the rate-limiting step in the degradation of the benzylpenicilloyl–wild-type-enzyme adduct is the hydrolysis of the C_5–C_6 bond of the antibiotic moiety, rapidly followed by

Table 6 k_{+2}/K' and k_{+3} values for several antibiotics with the wild-type and mutant enzymes

Determinations of the k_{+2}/K' and k_{+3} values were performed as described in the Materials and methods section. Numbers in parentheses refer to the various procedures. S.D.s did not exceed 15%; ND, not determined.

	Tyr-159 → Phe		Tyr-159 → 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})
Benzylpenicillin	55 (2)	2.1×10^{-4} (3)	10000 (1b)	3.0×10^{-4} (3)	14000 (1a)	1.4×10^{-4} (3)
Carbenicillin	< 5 (2)	ND	2600 (1a)	3.3×10^{-4} (3)	800 (1a)	1.4×10^{-4} (3)
Ampicillin	< 5 (2)	ND	740 (1a)	5.9×10^{-4} (3)	110 (1a)	1.4×10^{-4} (3)
Methicillin	10 (1a)	ND	ND	ND	10 (1a)	ND
Nitrocefin	4500 (4)	4.6×10^{-4} (3)	5000 (1a)	4.3×10^{-4} (3)	4100 (1a)	3.0×10^{-4} (3)
Cephalosporin C	14 (2)	ND	540 (1a)	5.0×10^{-6} (3)	1500 (1a)	1.0×10^{-6} (3)
Cefuroxime	< 5 (2)	ND	740 (1a)	6.6×10^{-6} (3)	350 (1a)	4.0×10^{-6} (3)

the hydrolysis of the phenylacetylglycyl-enzyme thus formed (Frère et al., 1976b). Surprisingly, under the same conditions, the degradation of the adducts formed with the mutants mainly yielded benzylpenicilloic acid (BPA). The BPA/phenylacetylglycine ratios were 50:1 and 10:1 with the Tyr-159 → Phe and Tyr-159 → Ser mutants respectively.

DISCUSSION

At present, high-resolution refined structures are only available for three class A and one class C β-lactamases. Unfortunately, the X-ray data on the *Streptomyces* R61 DD-peptidase are still rather incomplete. In class A enzymes, the catalytic cavity is limited on one side by the Lys-234-Thr(Ser)-Gly motif, corresponding to similar Lys-Thr-Gly and His-298-Thr-Gly sequences in the class C β-lactamases and the *Streptomyces* R61 DD-peptidase respectively. The highly conserved Ser-130-Asp-Asn loop forms another boundary of the class A enzyme active site, and site-directed mutagenesis studies have demonstrated the important role of these three residues in the stability and catalytic properties of these enzymes. The corresponding loop is found in the class C (Tyr-180-Ala-Asn triad) and R61 (Tyr-159-Ser-Asn triad) enzymes. If the other conserved elements [the active-site serines and the Lys(His)-Thr-Gly triads] are superimposed, the hydroxy group of Ser-130 (class A β-lactamases), Tyr-180 (class C β-lactamases) and Tyr-159 (R61 DD-peptidase) are found in very similar positions. In consequence, this group could be expected to take an active part in the catalytic and penicillin-binding properties of the DD-peptidase, a hypothesis that was fully confirmed by the present study, although with some rather surprising observations. By using site-directed mutagenesis techniques, the Tyr-159 residue was replaced by either Phe or Ser. The first mutation eliminated the hydroxy group while it was conserved in the second mutation, probably 0.35–0.40 nm further away from the active-site-serine oxygen.

The stabilities of the modified proteins were not severely decreased (never more than 3.7-fold), whereas the Ser-130 → Ala and Ser-130 → Gly mutants of *Streptomyces albus* G β-lactamase were 36- and 16-fold more unstable than the wild-type enzyme (Jacob et al., 1990b). Thus no gross modifications of the protein structures were suspected, although the small blue shift in the fluorescence emission maximum of the Tyr-159 → Ser mutant suggested a slightly more hydrophobic environment for the tryptophane residues responsible for enzyme fluorescence. The fluorescence emission was quenched upon formation of the acyl-enzyme with substrates and β-lactam inactivators, a situation similar to that found with the wild-type enzyme.

The rates of substrate utilization and penicillin inactivation of the Tyr-159 → Phe mutant were drastically decreased, except for the reactions with nitrocefin and methicillin. The particular behaviour of these compounds was reminiscent of the situation with the Ser-130 → Ala and Ser-130 → Gly mutants of the *S. albus* G enzyme, which hydrolysed these two substrates significantly more efficiently than most other β-lactams (Jacob et al., 1990b). In the interaction with the thiolester substrate S2a, which could be studied in more detail, the acylation and deacylation rate constants were severely impaired, by 100- and 1000-fold respectively. The loss of catalytic efficiency was even more striking with the peptide substrate, for which the $k_{cat.}$ value, probably corresponding to k_{+2}, was only 0.01% of that of the wild type. Conversely, although the Tyr-159 → Ser mutant also became a very poor peptidase, its esterase activity was somewhat better, and the thiolesterase and penicillin-binding properties were nearly identical to those of the wild type (and sometimes better). With substrate S2a and carbenicillin, for which all of the individual constants were measured, the values were very close to those observed with the wild-type enzyme.

The behaviour of the three substrates containing the Ac_2-L-Lys-D-Ala acylating moiety was particularly striking. The rates of acylation of the Tyr-159 → Ser mutant were respectively 130- and 7000-fold larger with the lactate and thiolactate derivatives than with the peptide, thus directly reflecting the 'quality' of the leaving group, in contrast to the properties of the wild-type enzyme. These results thus suggested a role for the Tyr-159 hydroxy group in the protonation of the substrate's leaving group similar to that proposed for the hydroxy group of Ser-130 in the mechanism of action of the *S. albus* G β-lactamase (Lamotte-Brasseur et al., 1991). Indeed, the pK_a values increase from 7.0 with the thiolactate to 13–14 with the lactate and to $\gg$ 14 with alanine. Although a similar explanation seems to hold for the other thiolesters, for which acylation of the Tyr-159 → Ser mutant appeared to be unimpaired, it does however consitute an oversimplification, since the acylation of the Tyr-159 → Phe mutant by thiolesters was severely impaired (about 1000-fold). Moreover, with thiolesters containing the benzoyl group, the Tyr-159 side-chain also appeared to be involved in deacylation, since the k_{+3} value decreased by one and three orders of magnitude for the Ser and Phe mutants respectively. In the absence of detailed structural information, it seems somewhat dangerous to assume that the serine hydroxy group can replace that of the tyrosine, even with a decreased efficiency. In fact, the ability of the Tyr-159 → Ser mutant to perform some, but not all, of the functions of the wild-type enzyme remains a complete surprise. Similarly, acylation of the Tyr-159 → Ser mutant by β-lactams

was unimpaired and was sometimes even faster than with the wild-type enzyme, contrasting again with the dramatic loss of penicillin-binding capacity of the Tyr-159 → Phe mutant. Thus by an unknown mechanism such as a structural adjustment or the involvement of an additional water molecule, the serine mutant could somehow compensate for the modified position of the hydroxy group. However, this compensation was only efficient with the thiolester substrates and β-lactam compounds, indicating more stringent structural requirements for the hydrolysis of peptide substrates. It could be hypothesized that the thiolester substrates better mimicked the behaviour of β-lactams than the peptide substrates, but this was somewhat in contradiction with the results obtained with other mutants (His-298 → Lys and His-298 → Gln), for which the mutation affected acylation by the β-lactams 4–20-fold more strongly than that by the thiolester S2a (Hadonou et al., 1992).

These results are also reminiscent of those obtained with the Lys-213 → Arg mutant of the *E. coli* PBP5, which seemed to lose its peptidase activity completely while retaining its penicillin-binding properties (Malhotra and Nicholas, 1992).

The most striking result, however, was the near-complete loss of transpeptidation activity exhibited by both mutants, as demonstrated by the failure of good acceptors to increase $k_{cat.}$ values for the thiolester substrate and by the extremely low transpeptidation/hydrolysis ratios recorded, even at high D-alanine concentrations. Similar, although less drastic, decreases in transpeptidation efficiency were also reported for His-298 mutants. By analogy with known class A β-lactamase structures, and on the basis of the available X-ray data, an interaction between Tyr-159 and His-298 could be suggested. These two side-chains must somehow be involved in the interaction with the acceptor substrate, and might co-operate in the catalysis of the transfer step. Indeed, modifications of these residues resulted in both cases in a disproportionate decrease in transferase activity compared with hydrolysis. For instance, with the Tyr-159 → Ser mutant and substrate S2a, the rate of hydrolysis and aminolysis (by 200 mM D-alanine) were decreased 17- and 2500-fold respectively.

The pH-dependence of the $k_{cat.}$ and K_m values of the Tyr-159 → Ser mutant was seemingly characterized by the appearance of a new pK of around 6–6.5. This might correspond to the shifting to higher pH values of a pK which is below 4.5 with the wild-type enzyme, and thus outside the pH range which can be easily studied since the enzyme becomes unstable at low pH. The invariance of the $k_{cat.}/K_m$ ratio in the same pH range suggested a specific influence on k_{+3}. Conversely, a pK of about 8.5, which seemed to influence the acylation of the wild-type enzyme by S2a, was not found with the mutant, and it was thus very tempting to attribute this pK to the tyrosine side-chain. It is, however, quite dangerous to accept such a simple explanation, since the attribution of pK values to specific side-chains in an enzyme active site appears to remain a difficult problem.

Finally, the nature of the benzylpenicillin degradation products revealed another rather subtle function of the Tyr-159 side-chain. With the wild-type enzyme at pH 7.0, the major product (> 90%) was phenylacetylglycine, formed by a rate-limiting hydrolysis of the C_5–C_6 bond of the antibiotic moiety, followed by a rapid decay of the phenylacetylglycyl-enzyme thus obtained. In more basic conditions, benzylpenicilloate was also obtained, probably by a direct attack by OH^- ions. With the mutants at neutral pH, direct hydrolysis became predominant, and it could be calculated that the efficiency of this reaction increased more than 20-fold while that of the rupture of the C_5–C_6 bond decreased 5–20-fold. Thus the mutations seemed not only to allow a direct access of water molecules or OH^- ions to the acyl-enzyme ester bond, but also to impair the more complex degradation pathway in which Tyr-159 might thus play a determining role.

In conclusion, although several observations remain surprising, it is clear that the Tyr-159 side-chain contributes in an important way to the carboxypeptidase and transpeptidase activities of the *Streptomyces* R61 DD-peptidase. Its influence on the thiolesterase and penicillin-binding properties is more complicated to assess in the light of the very different behaviour of the two mutants Tyr-159 → Ser and Tyr-159 → Phe. These results underline the dangers of forming hypotheses on the basis of the properties of one single mutant and interactions with only one substrate and one β-lactam.

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