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Point mutations of two arginine residues in the *Streptomyces* R61 DD-peptidase

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Incubation of the exocellular DD-carboxypeptidase/transpeptidase of *Streptomyces* R61 with phenylglyoxal resulted in a time-dependent decrease in the enzyme activity. This inactivation was demonstrated to be due to modification of the Arg-99 side chain. In consequence, the role of that residue was investigated by site-directed mutagenesis. Mutation of Arg-99 into leucine appeared to be highly detrimental to enzyme stability, reflecting a determining structural role for this residue. The conserved Arg-103 residue was also substituted by using site-directed mutagenesis. The modification to a serine residue yielded a stable enzyme, the catalytic properties of which were similar to those of the wild-type enzyme. Thus Arg-103, although strictly conserved or replaced by a lysine residue in most of the active-site penicillin-recognizing proteins, did not appear to fulfil any essential role in either the enzyme activity or structure.

INTRODUCTION

The exocellular DD-carboxypeptidase/transpeptidase of *Streptomyces* R61 serves as a model for the membrane-bound DD-peptidases involved in bacterial cell-wall biosynthesis (Frère & Joris, 1985). It is inactivated by β-lactam compounds in a reaction involving acylation of the Ser-62 residue (Frère *et al.*, 1976; Joris *et al.*, 1987).

Active-site serine DD-peptidases and β-lactamases form a large family of penicillin-recognizing proteins, and studies of the functional role of the active-site residues have until recently been restricted to the latter enzymes. Although some mutations that influence penicillin binding or the physiological activity of various penicillin-binding proteins have been described (Dowson *et al.*, 1989; Laible *et al.*, 1989; Brannigan *et al.*, 1990; Schuster *et al.*, 1990; Lujan *et al.*, 1991), no detailed analysis of the kinetic properties of the mutants has been reported. In the R61 DD-peptidase, the role of the two tryptophan residues, Trp-233 and Trp-271, has been probed by chemical modification and site-directed mutagenesis (Bourguignon-Bellefroid *et al.*, 1992) and that of His-298 by the latter technique (Hadonou *et al.*, 1992), but the residues that assist the active Ser-62 residue in catalysis and in the interaction with β-lactams remain unknown.

In the present study, we have investigated the importance of arginine residues in this enzyme. Indeed, it has been generally assumed that a positively charged group of the enzyme is involved in charge-pairing with the *C*-terminal carboxylate of the substrate or the carboxylate on C-3 of penicillins (or C-4 of cephalosporins). Such a positively charged group might be important for the recognition of substrates and inactivators by the DD-peptidases and the β-lactamases (Varetto *et al.*, 1987, 1991; Laws & Page, 1989). The first residue of the 'HTG' motif (box VII; Joris *et al.*, 1988) has been considered as a likely candidate for charge-pairing (Kelly *et al.*, 1989), but the pH-dependence of both hydrolysis of the substrates and inactivation by penicillins seems to indicate the importance of a group of p*K* close to or larger than 9 (Varetto *et al.*, 1987). The only phenomenon exhibiting a strong pH-dependence between 6 and 8 is the transpeptidation reaction (Frère *et al.*, 1973). The lysine residue of the S*XXK motif, where S* is the active-site serine, is strictly conserved in all active-site-serine penicillin-recognizing proteins. Many results obtained with β-lactamases indicate that substitutions affecting this residue result in a very important loss of activity (Madgwick & Waley, 1987; Tsukamoto *et al.*, 1990), although it does not seem to be involved in an interaction with the penicillin carboxylate (Moews *et al.*, 1990; Herzberg, 1991). Chemical-modification experiments (Georgopapadakou *et al.*, 1981) have been interpreted by assuming that an arginine residue was present in the active site of the *Streptomyces* R61 DD-peptidase. However, a reaction with one or more lysine residues could also explain these results (Means & Feeney, 1964). The R61 DD-peptidase contains 14 arginine and seven lysine residues (Duez *et al.*, 1987). In the present investigation we have examined the effect of phenylglyoxal on the enzyme activity and penicillin-binding properties. An apparently essential arginine residue was identified and its role further investigated by site-directed mutagenesis. Another arginine residue, formerly characterized as being conserved in most penicillin-recognizing enzymes, was also modified by the latter method.

MATERIALS AND METHODS

Enzymes, chemicals and antiserum

The enzymes used in the recombinant DNA techniques were from Bethesda Research Laboratory (Gaithersburg, MD, U.S.A.), New England Biolabs (Beverly, MA, U.S.A.), Boerhinger (Mannheim, Germany) and Amersham International, Amersham, Bucks., U.K. The wild-type R61 enzyme was produced by the *Streptomyces lividans* TK24 strain containing plasmid pDML115 (Hadonou *et al.*, 1992) and purified as described previously (Fossati *et al.*, 1978). The class A β-lactamase of *Bacillus licheniformis* 749/C was purified according to the method of Matagne *et al.* (1990).

[γ-[^{35}S]Thio]dATP (1350 Ci/mmol) was from NEN (Boston, MA, U.S.A.), and [7-^{14}C]phenylglyoxal (25 nCi/nmol) and [^{14}C]benzylpenicillin (54 nCi/nmol) were from Amersham International; benzylpenicillin was from Rhône–Poulenc (Paris, France), ampicillin was from Bristol Benelux (Brussels, Belgium),

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carbenicillin was from Beecham Research Laboratories (Brentford, Middx., U.K.), cefuroxime was from Glaxo Group Research (Greenford, Middx., U.K.) and cefoxitin was from Merck, Sharp and Dohme (Rahway, NJ, U.S.A.). All the unlabelled β-lactams were kindly given by the respective companies. Nitrocefin was from Oxoid (Basingstoke, Hampshire, U.K.). Phenylglyoxal and cyclohexane-1,2-dione were from Janssen Chimica (Beerse, Belgium); the tripeptide Ac_2-L-Lys-D-Ala-D-Ala was from UCB Bioproducts (Braine-l'Alleud, Belgium). The poor substrate Ac-L-Ala-D-Glu-Gly was a gift from Dr. H. R. Perkins (University of Liverpool, Liverpool, U.K.). The thioester substrate (C_6H_5–CO–NH–CH_2–CO–S–CH_2–CO_2^-) was synthesized in our laboratory (Adam *et al.*, 1990). Rabbit anti-(R61 DD-peptidase) serum was from Gamma S. A. (Angleur, Belgium).

Modification procedure

Modification experiments with phenylglyoxal and cyclohexane-1,2-dione were carried out in 10 mM-sodium borate buffer, pH 8, and 20 mM-boric acid, pH 9, respectively. Samples were withdrawn and diluted at least 40-fold, and the residual enzymic activity was determined.

Labelling experiments

For the binding of [^{14}C]phenylglyoxal, reaction mixtures contained 1 mM-[^{14}C]phenylglyoxal (5 nCi/nmol) and 38 μg of pure enzyme (1 nmol) in a total volume of 25 μl of 10 mM-sodium borate, pH 8. Binding was determined by measuring the amount of radioactivity associated with the protein after separation from the excess reagent by t.l.c. (solvent: butanol/water/acetic acid/ethanol, 10:4:3:3, by vol.). Parallel determination of the residual hydrolytic activity allowed the computation of the number of inactivator molecules bound per mol of inactivated enzyme. Another sample was incubated with 5 nmol of benzylpenicillin before the treatment with phenylglyoxal.

[^{14}C]Benzylpenicillin-binding assays were performed at 37 °C for 10 min. Assay mixtures containing 38 μg of pure enzyme 80%-inactivated by 25 nmol of phenylglyoxal were centrifuged on Centricon-10 (molecular-mass cut-off 10 kDa) to eliminate the excess of phenylglyoxal and incubated with 2 nmol of [^{14}C]benzylpenicillin (40 μM, final concentration). The labelled protein was isolated by t.l.c. before liquid-scintillation counting.

Oligonucleotides

Oligodeoxynucleotides were obtained from Eurogentec (Liège, Belgium). The crude oligonucleotides were purified by electrophoresis on a 20% (w/v) polyacrylamide gel containing 8 M-urea and desalted by using a spun column (Maniatis *et al.*, 1982). The oligonucleotides used to introduce the mutations were the following: R99L, TGCTGCCCGACGACCTGATCACC GTGCGTC; R103S, TCACCGTGAGTCAGGTGAT.

Strains, plasmids and growth conditions

The *Escherichia coli* TG1 strain was used as a host for bacteriophage M13, and the *S. lividans* TK24 strain (Hopwood *et al.*, 1985) was used for enzyme expression and production. The *Streptomyces* plasmid pIJ702 (Katz *et al.*, 1983) was modified in order to remove its *Sph*I and *Pst*I sites. Plasmid pDML110, as prepared by Duez *et al.* (1987), was used as a source of the DD-peptidase gene. The expression vector was pDML115 (Hadonou *et al.*, 1992). Growth conditions for the expression of the mutant proteins were as described in Jacob *et al.* (1990).

DNA recombinant techniques

The procedures used were those described in Maniatis *et al.* (1982) and Hopwood *et al.* (1985). Site-directed mutagenesis was performed using the 'Oligonucleotide-directed *in vitro* mutagenesis' kit from Amersham. Nucleotide sequencing was carried out by the bacteriophage M13 dideoxy chain-termination method using the USB Sequenase kit.

Mutagenesis, expression and purification

These were as described in Bourguignon-Bellefroid *et al.* (1992). The concentration of the pure enzymes (> 90%) was determined by u.v.-absorbance measurements by using a molar absorption coefficient at 280 nm of 38000 $M^{-1} \cdot cm^{-1}$.

Instrumentation

Isoelectric focusing was performed on a Pharmacia Phast system apparatus in the pH range 3–10. T.l.c. was performed on Polygram Sil-G plates (Macherey and Nagel, Düren, Germany). The solvent was butanol/water/acetic acid/ethanol (10:4:3:3, by vol). High-voltage electrophoresis was carried out on Whatman 3MM paper for 45 min in pyridine/acetic acid/water (25:1:225, by vol), pH 6.5, in a Gilson model DW electrophorator (60V/cm). Radioactive peptides were localized by liquid-scintillation counting. The [^{14}C]phenylglyoxal-labelled Arg-99-containing peptide was purified on a C_{18} reverse-phase PEP-RPC HR 5/5 chromatography column connected to an f.p.l.c. system (Pharmacia, Uppsala, Sweden). Solvent A was 0.1% (v/v) trifluoroacetic acid and solvent B was 70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Elution was performed with a linear gradient (27–40% of solvent B) over 11 ml at a flow rate of 0.5 ml/min. The peptide was sequenced with the help of an Applied Biosystem gas-phase Sequenator (Hewick *et al.*, 1981). Amino acid analysis was performed with a Pico-Tag analyser (Waters, Millipore).

Fractionation of the *Streptomyces* cells

Three fractions were separated: the 'lysozyme-releasable' fraction, the membranes and the cytoplasm; fractionation was performed as described by Leyh-Bouille *et al.* (1977).

Determination of kinetic parameters

Absorbance measurements were performed with a Uvikon 860 spectrophotometer coupled to a microcomputer via an RS232 interface. Hydrolysis of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala was monitored by estimating the amount of released D-alanine with the help of the D-amino acid oxidase procedure (Frère *et al.*, 1976). The $k_{cat.}/K_m$ values were determined by using first-order analyses of the time-courses at $[S] < K_m$ and on the basis of Hanes plots. Hydrolysis and aminolysis of the thioester substrate were monitored spectrophotometrically at 250 nm by using an absorption coefficient variation value of 2200 $M^{-1} \cdot cm^{-1}$. Separate values for $k_{cat.}$ and K_m were computed from complete time courses as described by De Meester *et al.* (1987).

Inactivation experiments were performed by mixing enzyme, reporter thioester substrate and inactivator and analysing the time course of thioester hydrolysis as described in De Meester *et al.* (1987). The deacylation rate constant (k_3) was determined as described in Frère *et al.* (1974): after complete inactivation of the enzyme and elimination of the excess of free β-lactam by addition of a small amount of the *Enterobacter cloacae* P99 β-lactamase, recovery of the enzyme activity was monitored with the tripeptide Ac_2-L-Lys-D-Ala-D-Ala.

All incubations were performed at 37 °C in 10 mM-sodium phosphate buffer, pH 7.

RESULTS

Inactivation by phenylglyoxal

Incubation of the DD-peptidase with phenylglyoxal resulted in progressive loss of the enzyme hydrolytic activity as assayed on

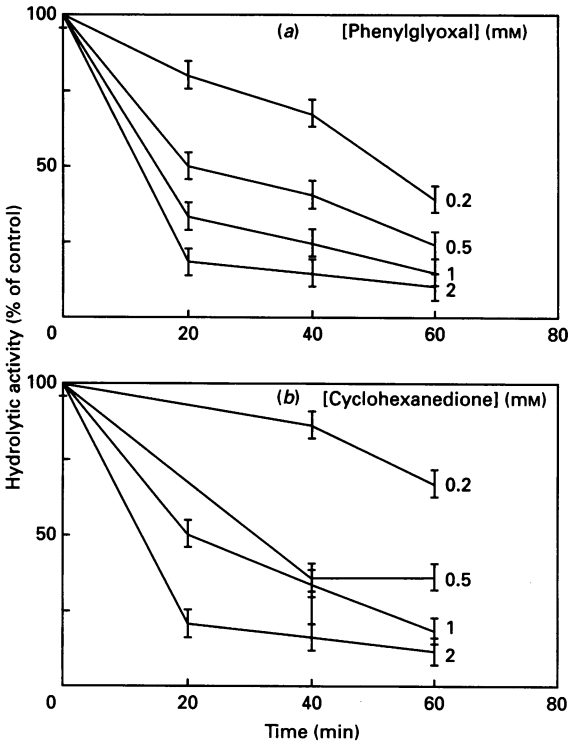

Fig. 1. Inactivation of the R61 enzyme by phenylglyoxal and cyclohexane-1,2-dione

Residual hydrolytic activity after incubation of the enzyme (25 μM) with (*a*) phenylglyoxal at the concentrations indicated at 37 °C in 10 mM-sodium borate buffer, pH 8 and (*b*) cyclohexane-1,2-dione at the concentrations indicated at 30 °C in 20 mM-boric acid, pH 9. Activities are expressed as a percentage of that of an unmodified control.

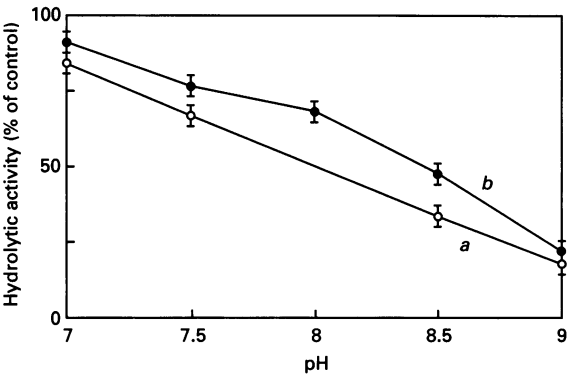

Fig. 2. Effect of pH on inactivation by phenylglyoxal and cyclohexane-1,2-dione

Residual hydrolytic activity after a 60 min incubation of the R61 enzyme (25 μM) with (curve *a*) 0.5 mM-phenylglyoxal at 37 °C and (curve *b*) 2 mM-cyclohexane-1,2-dione at 30 °C at the pH values indicated. Activities are expressed as in Fig. 1. The following buffers were used: pH 7.0 and 7.5, 50 mM-sodium phosphate; pH 8.0, 10 mM-sodium borate; pH 8.5, 10 mM-Hepes + 25-μM EDTA; pH 9.0, 20 mM-boric acid.

the synthetic tripeptide Ac_2-L-Lys-D-Ala-D-Ala. The time-course of inactivation by four concentrations of phenylglyoxal is shown in Fig. 1(*a*). Inactivation was not reversed by overnight dialysis (4 °C) against 10 mM-potassium phosphate buffer, pH 7, followed

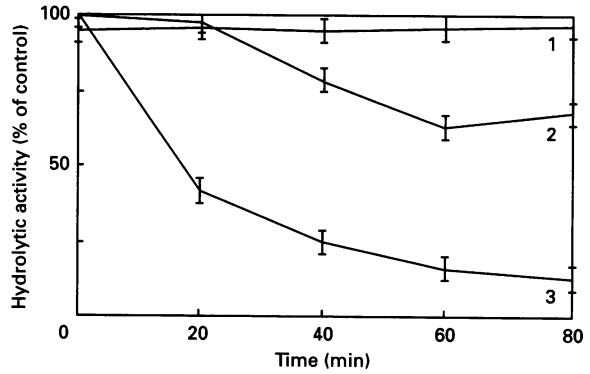

Fig. 3. Protection of the R61 enzyme against phenylglyoxal inactivation by the Ac-L-Ala-D-Glu-Gly peptide

All incubations were carried out at 37 °C. The R61 enzyme was preincubated with the 20 mM-peptide for 15 min before 0.5 mM-phenylglyoxal was added. Curve *1*, R61 + peptide; curve *2*, R61 + peptide + phenylglyoxal; curve *3*, R61 + phenylglyoxal. Activities are expressed as in Fig. 1.

by a 17 h incubation at 37 °C. Inactivation rate increased with increasing pH, control activity remaining virtually unchanged in the pH range examined (7.0–9.0) (Fig. 2, curve *a*).

When the enzyme was inactivated by a 10-fold stoichiometric excess of benzylpenicillin before phenylglyoxal treatment, a complete recovery of the enzyme activity was observed after addition of the β-lactamase and dilution. The decay of the inactive acyl-enzyme occurred with a half-life similar to that recorded in the absence of phenylglyoxal treatment. In conditions similar to those of Fig. 1(*a*), the activity loss caused by 2 mM-phenylglyoxal decreased from 80 to 40% in 60 min when the experiment was performed in the presence of 20 mM-Ac-L-Ala-D-Glu-Gly, a very poor substrate for which a K_i value of about 15 mM was measured by competition with the usual substrate Ac_2-L-Lys-D-Ala-D-Ala (Fig. 3). Protection against inactivation was thus afforded if benzylpenicillin or the poor substrate Ac-L-Ala-D-Glu-Gly occupied the enzyme active site, suggesting the presence of a reactive arginine or lysine residue in the active site. [^{14}C]Benzylpenicillin binding was also inhibited by phenylglyoxal. After a 10 min incubation with [^{14}C]benzylpenicillin, the phenylglyoxal-treated 80%-inactivated enzyme was found to bind only 54% of the labelled benzylpenicillin bound by the same amount of intact enzyme. This showed that the rate of binding of penicillin to the modified enzyme was drastically decreased, since, with the intact enzyme, the same level of labelling should be reached in about 1 s.

Similar inactivation time courses (Fig. 1*b*), pH-dependence (Fig. 2, curve *b*) and protection by benzylpenicillin (results not shown) were also obtained with cyclohexane-1,2-dione.

Stoichiometry of inactivation of the DD-peptidase by phenylglyoxal

Incubation of 1 nmol of enzyme with 25 nmol of [^{14}C]phenylglyoxal for 20 min (residual hydrolytic activity about 20%) followed by t.l.c. resulted in incorporation of radioactivity into the protein peak corresponding to 3.4 ± 0.34 nmol of [^{14}C]phenylglyoxal/nmol of inactivated enzyme. If the modification reaction was performed when the active site was protected by benzylpenicillin, only 2.5 ± 0.5 nmol of [^{14}C[phenylglyoxal was incorporated per nmol of enzyme. This indicated that several enzyme residues were modified and confirmed that the presence of the penicilloyl residue in the active site apparently prevented the modification of one of them.

Table 1. Separation of the [^{14}C]phenylglyoxal-labelled peptides by high-voltage electrophoresis at pH 6.5

Mobilities are relative to lysine = +1.00 (+ and − indicate migration towards cathode and anode respectively). The radioactivity found in the various peptides essentially accounted for that present in the initial sample before proteolytic digestion (about 100%). Abbreviation: PenG, benzylpenicillin.

Peptide	Mobility at pH 6.5	R61 + [^{14}C]phenylglyoxal		R61 + PenG + [^{14}C]phenylglyoxal	
		(c.p.m.)	(%)	(c.p.m.)	(%)
1	+1.00	2100±70	2.6±0.08	1300±50	2.8±0.10
2	+0.77	900±40	1.1±0.05	400±30	0.9±0.10
3	+2.51	3500±80	4.4±0.10	2000±60	4.3±0.10
4	+0.11	36800±270	45.9±0.30	14700±170	31.7±0.40
5	+0.00	27000±230	33.7±0.30	17400±190	37.6±0.40
6	−0.26	4500±100	5.6±0.10	5500±100	11.9±0.20
7	−0.80	5300±100	6.6±0.10	5000±100	10.8±0.20
		80100±900	100±1.00	46300±700	100±1.50

Identification of the amino acid residue involved in the inactivation reaction

The enzyme was inactivated to 80% by [^{14}C]phenylglyoxal and the complex thus formed was filtered on Sephadex G-25 in 50 mM-NH_4HCO_3 and freeze-dried. The labelled enzyme was digested with pepsin [2% (w/w) with respect to the DD-peptidase] in 5% (v/v) formic acid at 37 °C for 4 h and then with trypsin [2% (w/w)] in 25 mM-NH_4HCO_3 at 37 °C for 4 h. The digest was then submitted to electrophoresis at pH 6.5, giving seven radioactive peaks (Table 1). The radioactivity of peak 4 decreased when the enzyme was protected by benzylpenicillin before reaction with phenylglyoxal suggesting that it contained the 'active-site' peptide. Peptide 4 was eluted from the electrophoretogram with 1% (v/v) formic acid and purified by three successive identical chromatographies on the reverse-phase column. Its amino acid sequence was determined and corresponded to that of the Thr-89–Arg-99 segment of the DD-peptidase (Thr^{89}-Tyr-Leu-Pro-Gly-Leu-Leu-Pro-Asp-Asp-Arg^{99}). In parallel to this, a similar analysis of Pronase digests [Pronase (10%, w/w) in 10 mM-phosphate buffer, pH 7, for 2 h at 37 °C] of the modified and subsequently heat-denatured DD-peptidase was performed. The 'active-site' labelled protein fragment isolated under those conditions contained only an arginine residue, as revealed by amino acid analysis. These results indicated that Arg-99 was the residue involved in the inactivation reaction by phenylglyoxal. Its role, together with that of the conserved Arg-103 residue, was further investigated by site-directed mutagenesis: Arg-99 was replaced by leucine and Arg-103 by serine.

Production of the mutant DD-peptidases

The leucine and serine residues were chosen to replace the arginine residues in order to eliminate the positive charge associated with the arginine side chain.

After mutagenesis and sequencing, the *PstI–SphI* fragments (0.5 kb) coding for the *N*-terminal part of the mutant DD-peptidases were cloned into the previously described *Streptomyces* plasmid (Hadonou *et al.*, 1992) to replace the equivalent wild-type fragment. After transformation of the *S. lividans* TK24 protoplasts, the recombinant plasmids pDML22 (R103S) and pDML32 (R99L) were purified, and the DNA fragments coding for the DD-peptidases were released by restriction, recloned into bacteriophage M13 and resequenced in the region of the mutation. This procedure was designed to avoid the occurrence of a wild-type gene in the plasmid preparations. The presence of the mutations in pDML22 and pDML32 was thus confirmed. Mutant DD-peptidases were then produced in 5-litre 7-day cultures and purified as described by Bourguignon-Bellefroid *et al.* (1992). The R103S enzyme preparation was more than 90% pure as estimated by SDS/PAGE.

R99L mutant DD-peptidase

No enzymic activity was detected in the culture supernatant. Attempts to detect an inactive protein were made by using SDS/PAGE, with the wild-type enzyme as an M_r standard, followed by Western blotting for immunological localization. All these tests remained negative. Cells were then fractionated and all fractions submitted to SDS/PAGE and Western blotting. Only a very weak response was obtained in the 'lysozyme-releasable', membrane and cytoplasmic fractions, corresponding to a production lower than 0.05 mg of recombinant protein/l of culture. No hydrolytic activity could be detected with the good substrate Ac_2-L-Lys-D-Ala-D-Ala, which showed that the immunological method detected a protein with less than 0.1% activity of the wild-type. It thus appeared that the enzyme was either underexpressed or that its rate of folding was significantly decreased, resulting in a much faster proteolysis of the nascent protein, and that its secretion was also impaired. This underexpression could not be attributed to the choice of an infrequent codon for leucine, since CTG is the most often used in the *Streptomyces* R61 DD-peptidase gene (Duez *et al.*, 1987). The modified enzyme could not be produced and purified.

R103S mutant DD-peptidase

The mutation R103S yielded an enzyme the stability of which at 37 °C did not differ significantly from that of the wild-type (inactivation $t_{\frac{1}{2}} > 1500$ min for both enzymes).

Isoelectrofocusing revealed the presence of two bands for both wild-type and mutant enzyme preparations, which, however, behaved homogeneously on SDS/PAGE. The pI values were 4.2 and 4.5 for the wild-type and 4.1 and 4.4 for the R103S enzymes, thus confirming the more acidic character of the mutant. For the wild-type enzyme, the two forms have been separated by ion-exchange chromatography and exhibit identical enzymic and penicillin-binding properties (M. Jamin & J.-M. Frère, unpublished work). The structural basis for the presence of two active protein variants is presently under investigation. It is interesting to note that such a phenomenon had not been previously observed when the enzyme was produced by the R61 strain itself (Frère *et al.*, 1973).

The catalytic properties of the mutant DD-peptidase with the

Table 2. Kinetic parameters for the interaction of the wild-type and R103S enzymes with substrates and β-lactams

The kinetic parameters for the hydrolytic + transpeptidase activity were determined in the presence of 290 μM-thioester substrate and 10 mM-D-alanine. Unless otherwise stated S.D. values are within 20%.

		Wild-type	R103S
Hydrolytic activity (Ac_2-L-Lys-D-Ala-D-Ala)			
$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)		3900*	2250
Esterase activity (thioester)			
$k_{cat.}$	(s^{-1})	5±0.3	5.3±0.6
K_m	(μM)	41±4	49.5±4
$k_{cat.}/K_m$	($M^{-1} \cdot s^{-1}$)	120000±19000	107000±20000
Esterase + transpeptidase activity (thioester-D-Ala)			
$k_{cat.}$	(s^{-1})	47±13	32.5±4.5
K_m	(μM)	308±60	385±60
$k_{cat.}/K_m$	($M^{-1} \cdot s^{-1}$)	153000±70000	84000±24000
Interaction with β-lactams†			
Benzylpenicillin	k_2/K	14000	14170
	k_3	1.4×10^{-4}*	1.1×10^{-4}
Carbenicillin	k_2/K	830*	600
	k_3	1.4×10^{-4}*	1.3×10^{-5}
Ampicillin	k_2/K	110*	100
	k_3	1.4×10^{-4}*	0.9×10^{-4}
Nitrocefin	k_2/K	4100	4140
	k_3	3×10^{-4}*	2×10^{-4}
Cefuroxime	k_2/K	1400	520
	k_3	4×10^{-6}*	5.3×10^{-6}
Cefoxitin	k_2/K	1500*	20100
	k_3	5×10^{-5}*	8.3×10^{-6}

* From Frère & Joris (1985).
† k_2/K and k_3 values are in $M^{-1} \cdot s^{-1}$ and s^{-1} respectively.

tripeptide and thioester substrates were not drastically affected (Table 2). The transpeptidation/hydrolysis ratio determined by h.p.l.c. (Jamin *et al.*, 1991) after incubation of the enzymes with 290 μM-thioester substrate and 20 mM-D-alanine as acceptor was the same (8.5) for the wild-type and R103S enzymes. The R103S enzyme lost 98% of its hydrolytic activity after a 60 min treatment with 2 mM-phenylglyoxal. Re-activation of the enzyme was observed after elimination of the excess reagent if the active site was protected by benzylpenicillin before treatment with phenylglyoxal.

The characteristic parameters of the interaction of the mutant with β-lactam antibiotics also appeared to be similar to those of the wild-type enzyme with the striking exception of cefoxitin, which inactivated the mutant 10-fold faster than the wild-type enzyme (Table 2).

DISCUSSION

Modification of the R61 DD-peptidase by phenylglyoxal resulted in inactivation time courses similar to those obtained by Georgopapadakou *et al.* (1981); however, our results indicated that more than one enzyme residue was modified. Protection against inactivation was afforded by benzylpenicillin and the poor substrate Ac-L-Ala-D-Glu-Gly, suggesting the presence of a reactive arginine (or lysine) residue in the active site. Arg-99 was identified as the protected residue involved in the inactivation reaction and its role was further investigated by site-directed mutagenesis.

The R99L mutant protein was apparently underexpressed, segregated inside the cell and inactive. Such low-level expression of unstable mutant proteins has been reported in the case of the D131G mutant β-lactamase of *Streptomyces albus* G, where the conserved 'SDN' motif was modified (Jacob *et al.*, 1990), and in other instances (Schultz & Richards, 1986). Another totally inactive mutant R61 DD-peptidase (S62C) was expressed in normal amounts in the culture supernatant, and its folding appeared to be grossly correct (B. Joris & J.-M. Frère, unpublished work). The R99L mutation thus appeared to be highly detrimental to enzyme stability, reflecting a determining structural role for this residue. Yet, Arg-99 is not conserved among the active-site-serine penicillin-recognizing proteins, and β-lactamases even appeared to exhibit a deletion encompassing this position (Joris *et al.*, 1988). According to the available X-ray data, Arg-99 lies in the α-domain, outside the active site (Kelly *et al.*, 1989), and is involved in hydrogen bonds with the chain bearing the conserved 'YSN' motif (J. Lamotte-Brasseur, personal communication). The complete elimination of the side chain can be expected to induce some 'floppiness' of the 'YSN' loop.

Residue Arg-103 (box IV; Joris *et al.*, 1988) is strictly conserved or replaced by a lysine residue in most of the active-site-serine penicillin-recognizing proteins. Surprisingly, the modification to a serine residue yielded a stable enzyme, the catalytic properties of which were similar to those of the wild-type enzyme. Thus a positive charge at that level did not appear to be essential for the enzyme activity or structure. A totally unexpected result was the significant increase in the sensitivity of the mutant to one particular β-lactam, cefoxitin. Interpretation of this observation must await the refinement of the three-dimensional structure and the modelling of the interaction of the wild-type and mutant enzymes with various inactivating β-lactams.

From these results, it appeared that the important residue in this region of the R61 DD-peptidase primary structure was Arg-99 instead of Arg-103. In the analogous class A β-lactamases, a conserved lysine residue is found 40 residues after the active serine residue (Ambler *et al.*, 1991), forming box IV in the nomenclature of Joris *et al.* (1988). In the *Streptomyces* R61 DD-peptidase, Arg-99 and Arg-103 occur respectively 37 and 41 residues after the active-site serine residue. Our results suggest that Arg-99 is more likely to be an important residue in a structurally important conserved box. Finally, the present study also showed that protection afforded by inhibitors or substrates against modification by chemical reagents is not indisputable evidence for the presence of the target amino acid in the active site.

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