Biochem. J. (1992) 282, 361–367 (Printed in Great Britain)

Importance of the two tryptophan residues in the *Streptomyces* R61 exocellular DD-peptidase

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Modification of the *Streptomyces* R61 DD-peptidase by *N*-bromosuccinimide resulted in a rapid loss of enzyme activity. In consequence, the role of the enzyme's two tryptophan residues was investigated by site-directed mutagenesis. Trp^{271} was replaced by Leu. The modification yielded a stable enzyme whose structural and catalytic properties were similar to those of the wild-type protein. Thus the Trp^{271} residue, though almost invariant among the β-lactamases of classes A and C and the low-M_r penicillin-binding proteins, did not appear to be essential for enzyme activity. Mutations of the Trp^{233} into Leu and Ser strongly decreased the enzymic activity, the affinity for β-lactams and the protein stability. Surprisingly, the benzylpenicilloyl-(W233L)enzyme deacylated at least 300-fold more quickly than the corresponding acyl-enzyme formed with the wild-type protein and gave rise to benzylpenicilloate instead of phenylacetylglycine. This mutant DD-peptidase thus behaved as a weak β-lactamase.

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 the only penicillin-binding protein (PBP) for which three-dimensional-structure data are presently available (Kelly *et al.*, 1989). These indicate important similarities with the architecture of class A and class C β-lactamases (Samraoui *et al.*, 1986; Kelly *et al.*, 1986; Oefner *et al.*, 1990). The DD-peptidase contains Trp residues at positions 233 and 271. Although situated outside the active site, Trp^{271} is nearly invariant (box VI) among the β-lactamases of classes A and C and the low-M_r PBPs (Joris *et al.*, 1988; Ambler *et al.*, 1991).

Inactivation of the enzyme by β-lactam antibiotics (Frère *et al.*, 1975) and reactions with the thioester substrate carboxymethyl benzoylaminothioacetate (S2a) (Jamin *et al.*, 1991) were accompanied by a significant quenching of the fluorescence emission. Conversely, no such result was obtained in the presence of the peptide substrates (Nieto *et al.*, 1973*b*). With those latter compounds, no acyl-enzyme accumulates at the steady state, in contrast with what occurs with the β-lactams and the thioester. Those results thus indicate a modification of the Trp fluorophores' environment upon formation of the acyl-enzymes. In the present study we have investigated the role of the two Trp residues in chemical modification and site-directed mutagenesis.

MATERIALS AND METHODS

Buffers, enzymes, chemicals and antiserum

Unless otherwise stated, all the experiments were carried out in 10 mM-Tris/HCl buffer, pH 8, containing 50 μM-EDTA, except those using the thioester substrate (10 mM-sodium phosphate buffer, pH 7) and the chemical-modification experiments with *N*-bromosuccinimide (NBS) (10 mM sodium phosphate buffer, pH 6).

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.), Boehringer (Mannheim, Germany) and Amersham International, Amersham, Bucks., U.K.; [^{35}S]dATP (1350 Ci/mmol) was from NEN (Boston, MA, U.S.A.); benzylpenicillin was from Rhône-Poulenc (Paris, France), [^{14}C]benzylpenicillin (54 Ci/mol) from Amersham International, 6-aminopenicillanic acid and carbenicillin from Beecham Research Laboratories (Brentford, Middlesex, U.K.), cephaloridin, cephalothin, cephalexin and cephalosporin C from Eli Lilly and Co. (Indianapolis, IN, U.S.A.), cefuroxime from Glaxo Group Research (Greenford, Middx., U.K.), ampicillin from Bristol Benelux (Brussels, Belgium). All unlabelled β-lactam compounds were kindly given by the respective companies. 7-Aminocephalosporanic acid was purchased from Janssen Pharmaceutica (Beerse, Belgium), acrylamide from Boehringer (Mannheim, Germany), nitrocefin from Oxoid (Basingstoke, Hants., U.K.), *N*-bromosuccinimide from Janssen Chimica (Beerse, Belgium). The tripeptide Ac_2-L-Lys-D-Ala-D-Ala was from UCB Bioproducts (Braine-l'Alleud, Belgium). The synthesis of the poor substrate α-Ac-L-Lys-D-Glu-D-Ala was described previously (Nieto *et al.* 1973*a*). The thioester and ester substrates carboxymethyl benzoylaminothioacetate (S2a, C_6H_5–CO–NH–CH_2–CO–S–CH_2–CO_2^-), DL-mandelyl benzoylaminoacetate [S1d; C_6H_5–CO–NH–CH_2–CO–O–$CH(C_6H_5)$–CO_2^-], DL-

Abbreviations used: PBP, penicillin-binding protein; 6-APA, 6-aminopenicillanic acid; 7-ACA, 7-aminocephalosporanic acid; WT, wild-type; NBS, *N*-bromosuccinimide; Ac_2-L-Lys-D-Ala-D-Ala, diacetyl-L-lysyl-D-alanyl-D-alanine; α-Ac-L-lys-D-Glu-D-Ala, α-acetyl-L-lysyl-D-glutamyl-D-alanine; oligos, oligonucleotides.

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lactyl benzoylaminoacetate [S1c; C_6H_5–CO–NH–CH_2–CO–O–$CH(CH_3)CO_2^-$] and DL-phenyl-lactyl benzoylaminoacetate [S1e; C_6H_5–CO–NH–CH_2–CO–O– $CH(CH_2$–$C_6H_5)$–CO_2^-] were synthesized in our laboratory (Adam *et al.*, 1990). Rabbit anti-(R61 DD-peptidase) antiserum was from Gamma S.A. (Angleur, Belgium).

Oligonucleotides

Oligo-D-nucleotides (oligos) were obtained from Eurogentec (Liège, Belgium). The crude oligos were purified by PAGE on a 20% polyacrylamide/8 M-urea gel and desalted using a spun column (Maniatis *et al.*, 1982). The oligos used to introduce the mutations were as follows: W233L, CC ACC GAG CAG ACG GTG TCG T*T*G GCG CAG A; W233S, AG CAG ACG GTG TCG T*C*G GCG CAG A; W271L, G CAG ATG CAG CAG T*T*G ACG ACG G.

Recombinant DNA techniques

The procedures used were those described by 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 M13 dideoxy method using the USB Sequenase Kit.

Strains, plasmids and growth conditions

The *Escherichia coli* TG1 strain was used as a host for phage M13 and the *Streptomyces 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. pDML115 was the expression vector (A. M. Hadonou & J.-M. Frère, unpublished work). Growth conditions for the expression of the mutant proteins were as described by Jacob *et al.* (1990).

Mutagenesis, expression and purification

The general strategy for vector construction and site-directed mutagenesis has been described elsewhere (Hadonou *et al.*, 1992). A mutant clone for each mutagenesis was sequenced entirely to confirm that no undesired additional mutation had been introduced. After sequencing, the mutagenized inserts were released from their M13 vector and cloned into the corresponding sites of the pDML115 expression vector to replace the wild-type (WT) fragment. The presence of the mutations in those plasmids was confirmed by recloning the genes into M13mp18 and resequencing the zone of the expected mutation. Expression and purification of the mutant proteins were performed respectively as described by Erpicum *et al.* (1990) and Fossati *et al.* (1978), except that in the last step of the latter procedure, ion-exchange chromatography was performed with the help of a Pharmacia f.p.l.c. apparatus using Q-fast-flow as an ion-exchanger instead of DEAE-cellulose. During purification, the mutant proteins were detected by SDS/PAGE with the WT-DD-peptidase as the molecular-mass standard. Enzyme purity was also demonstrated by SDS/PAGE. Protein concentrations were routinely determined by absorbance measurements at 280 nm. Absorption coefficients of 38000 $M^{-1}\cdot cm^{-1}$ and 33000 $M^{-1}\cdot cm^{-1}$ were determined respectively for the WT enzyme and the Trp mutants on the basis of the amino acid compositions and the absorption spectra of the enzymes.

Stability measurements

The rate of protein denaturation was monitored by fluorescence spectroscopy. The decrease of fluorescence emission at 320 nm (excitation at 280 nm) was measured after increasing incubation times under fixed denaturation conditions. With the WT enzyme the stability was also determined by measuring the residual enzymic activity versus Ac_2-L-Lys-D-Ala-D-Ala after increasing incubation periods: inactivation and fluorescence quenching yielded similar denaturation rate constants.

Chemical and physical properties

C.d. spectra were recorded between 180 and 260 nm, at 25 °C, with protein concentrations of 20–60 μg/ml of Tris buffer and with a 1 mm optical path length, on a Jasco J-500A spectropolarimeter, and absorption spectra were recorded on a Varian Cary 219 spectrophotometer. Fluorescence lifetimes were measured at 25 °C with the pulse-fluorimetry technique. Excitation and emission were at 299.5 and 315 nm respectively. Electrospray m.s. was performed on a VG BIO-Q triple quadrupole atmospheric-pressure mass spectrometer equipped with an electrospray interface (VG BIOTECH, Altringham, Cheshire, U.K.). Isoelectric focusing was performed on a Pharmacia Phast system apparatus over the pH range 3–10. *N*-Terminal amino acid sequencing was done with a 470-A Applied Biosystems gas-phase sequenator as described previously (Joris *et al.*, 1985) and amino acid compositions were determined on a PICO-TAG analyser (Waters; Millipore). Excitation and emission spectra and the iodide and acrylamide quenching experiments were performed on a Perkin–Elmer 66 spectrofluorimeter; both emission and excitation spectra were corrected for the buffer blank. Quantum yields were determined by comparing the fluorescence of the samples with that of a pure L-tryptophan solution (quantum yield 0.14). The fluorescence quenching by NBS was monitored by using a Sigma ZWS-11 stopped-flow mixing unit (Biochem, Munich,) adapted to a Dia-Log Optical and detection set-up (Garching Instruments, Düsseldorf, Germany); all other fluorescence measurements were done with a Perkin–Elmer MPF 44 spectrofluorimeter.

Acrylamide and iodide fluorescence quenching

Solutions of proteins were mixed with 0–100 mM-KI or 0–500 mM-acrylamide. The 1 M-KI stock solution contained 0.5 mM-$Na_2S_2O_3$ to prevent I_3^- formation. KCl (100–0 mM was added to the samples to obtain constant ionic strength. Corrections for the inner-filter effect due to acrylamide absorption (Lakowicz, 1983) were calculated by using a value of 0.23 $M^{-1}\cdot cm^{-1}$ for the molar absorptivity of acrylamide at 295 nm (Tallmadge *et al.*, 1989). Fluorescence emission was monitored at 25 °C at 327 nm with excitation at 295 nm and was expressed as F_0/F, where F_0 is the fluorescence of the protein in the absence of quencher. The data were presented as direct Stern–Volmer plots of F_0/F versus the quencher concentration:

$$F_0/F = 1 + k_q T_0[Q] = 1 + (K_D[Q]) \quad (1)$$

where F_0/F characterizes the fractional decrease in fluorescence caused by the quencher at concentration [Q], K_D is the Stern–Volmer quenching constant, T_0 the fluorescence lifetime in the absence of quencher and k_q the bimolecular quenching constant.

Determination of kinetic parameters

Hydrolysis of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala was monitored by estimating the amount of released D-alanine by the D-amino acid oxidase procedure (Frère *et al.*, 1976). Utilization of the substrate did not exceed 10%, and the deduced initial rate values were used to determine $k_{cat.}$ and K_m on the basis of Hanes' linearization of the Henri–Michaelis equation. Hydrolysis and aminolysis of the thioester (S2a) and of the esters S1c, S1d and S1e was monitored spectrophotometrically as described by Adam *et al.* (1990). When possible a complete time course of the hydrolysis or aminolysis of substrates was recorded. The values of the kinetic parameters K_m and $k_{cat.}$ were then computed as

described by De Meester *et al.* (1987). When the reaction was too slow to obtain a complete time course within 15–20 min, initial rates were measured and Hanes plots were used to calculate K_m and $k_{cat.}$. When the K_m value was too high, only $k_{cat.}/K_m$ could be determined using first-order analyses of the time courses at $[S] \ll K_m$.

Depending on the enzyme and the β-lactam under study, inactivation experiments were performed using one of the following methods: (*a*) by mixing enzyme, reporter thioester substrate and inactivator and analysing the time course of thioester hydrolysis as described by De Meester *et al.* (1987); (*b*) by recording the decrease of protein fluorescence at 320 nm in the presence of the inactivator (excitation at 280 nm) (Frère *et al.* 1975); (*c*) by incubating the enzyme with various concentrations of the selected β-lactam and measuring the time-dependent decrease of activity on the tripeptide substrate (Frère *et al.*, 1984); (*d*) by using the β-lactam as substrate and monitoring the reaction at 230 nm (benzylpenicillin) or 482 nm (nitrocefin). The progress curves presented a 'burst' phase. The rate at steady state yielded $k_{cat.}$ ($[S] \gg K_m$) or $k_{cat.}/K_m = k_2/K$ ($[S] \ll K_m$), and analyses of the burst phase, performed as described by De Meester *et al.* (1987) and Galleni & Frère (1988), also allowed an independent computation of k_3 and k_2/K.

The deacylation rate constant (k_3) was determined: (*a*) as described in Frère *et al.* (1974); after complete inactivation of the enzyme and elimination of the excess of free β-lactam compound by addition of a small amount of the *B. licheniformis* β-lactamase, the time-dependent recovery of the enzyme activity was measured by assaying the activity on the tripeptide after increasing periods of time; (*b*) by similarly monitoring the recovery of the enzyme fluorescence after complete quenching; (*c*) together with the acylation rate as described above; (*d*) by using the β-lactam as substrate and obtaining the k_3 value as $k_{cat.}$ (at $[S] \gg K_m$).

All kinetic measurements were made at 37 °C (unless otherwise indicated) with a Uvikon 860 spectrophotometer coupled to a microcomputer via a RS232 interface or with a Perkin–Elmer MPF 44 spectrofluorimeter.

RESULTS AND DISCUSSION

Tryptophan side-chain oxidation with NBS

Oxidation of the tryptophan side-chain by NBS at pH 6 was accompanied by (i) a rapid 83% decrease of the enzyme fluorescence at 320 nm (Fig. 1*a*) and (ii) a complete loss of the enzyme hydrolytic activity as assayed on the synthetic tripeptide Ac_2-L-Lys-D-Ala-D-Ala (Fig. 1*b*). The fluorescence quenching was about 60 times faster than the loss of activity. The inactivated enzyme also became unable to covalently bind [^{14}C]benzylpenicillin. SDS/PAGE analysis failed to reveal any nicking of the polypeptide chain in the oxidized enzyme. Under the modification conditions of Fig. 1, (i) no decrease in the enzyme fluorescence by NBS was measured at 320 nm when benzylpenicllin was covalently bound to the active site; (ii) inactivation by NBS was considerably slowed down in the presence of 10 mM-α-Ac-L-Lys-D-Glu-D-Ala, a very poor substrate (Nieto *et al.*, 1973*a*). After 5 min, 80% of the enzyme activity was still detected instead of 0% in the absence of the peptide; (iii) re-activation of the DD-peptidase after contact with NBS was complete if the enzyme was preliminarily incubated with benzylpenicillin. Protection against inactivation and fluoresecence quenching was thus afforded if benzylpenicillin or the poor substrate α-Ac-L-Lys-D-Glu-D-Ala occupied the enzyme active site. Finally, the absorption spectrum of the enzyme after treatment with NBS (decreased and increased absorption at 280 nm and 250 nm respectively) showed that the modification was restricted to the Trp side chains and did not affect Tyr residues (Tyr can be brominated by NBS, which results in an absorption increase at 280 nm).

Those experiments indicated that one or both Trp residues were important for maintaining a functional active site. Their respective roles were further investigated by site-directed mutagenesis. Trp^{233} was replaced by Leu and Ser, and Trp^{271} by Leu. Replacing Trp with Leu constituted a 'safe' mutation (Bordo &

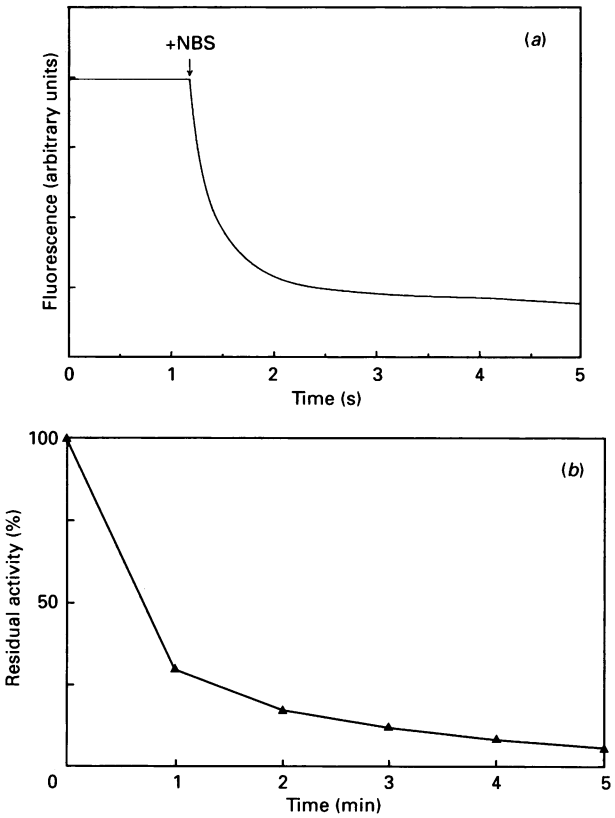

Fig. 1. Effect of NBS (18 μM) on the R61 DD-peptidase (2.2 μM)

(*a*) Quenching of fluorescence followed by stopped flow at 20 °C. Excitation was at 295 nm and emission at 320 nm. (*b*) Hydrolytic activity towards Ac_2-L-Lys-D-Ala-D-Ala at 37 °C;

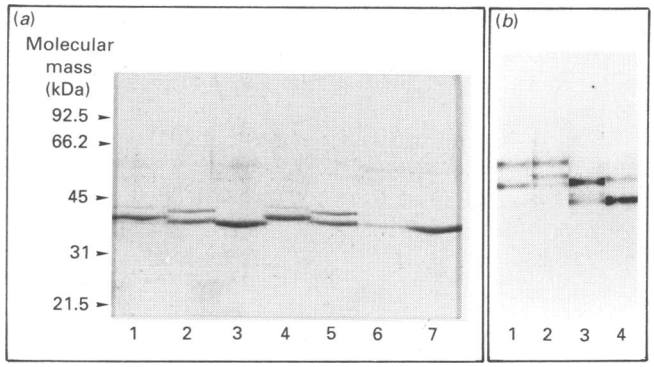

Fig. 2. Coomassie Blue staining of the purified DD-peptidases

(*a*) SDS/PAGE: lane 1, WT + W233S; lane 2, WT + W233L; lane 3, WT + W271L; lane 4, W233S; lane 5, W233L; lane 6, WT; lane 7, W271L. (*b*) PAGE under non-denaturing conditions: lane 1, W233S; lane 2, W233L; lane 3, W271L; lane 4, WT.

Argos, 1991). The mutation to Ser was chosen to retain the possibility of H-bond formation.

Expression of the mutant DD-peptidases

Excretion of the wild-type *Streptomyces* R61 DD-peptidase is accompanied by the elimination of both an *N*-terminal 31-residue signal peptide and the *C*-terminal 26-residue peptide (Gly^{350}–Asp^{375}) of the enzyme precursor (Duez *et al.*, 1987).

The mutant DD-peptidases were purified from culture supernatants with reasonable yields (25%). The proteins thus obtained were > 90% pure as estimated by SDS/PAGE. As shown by SDS/PAGE, the purified W233L and W233S proteins contained respectively 33 and 12% of larger species which reacted with rabbit anti-(R61 DD-peptidase) antiserum and were able to bind penicillin. The M_r difference was about 2600. Sequencing of the mutant gene showed that it did not contain any insertion. Further analysis of the purified preparations yielded the following results: (*a*) the *N*-terminal sequences of all three mutants were identical with that of the WT and were homogeneous; thus a misprocessing of the *N*-terminal signal peptide could not account for the electrophoretic behaviour of the W233 mutants; (*b*) after reduction with 4 mM-dithiothreitol, the two free cysteine residues (Cys^{291} and Cys^{344}) thus produced were carboxymethylated by reaction with [14C]iodoacetate and the proteins cleaved with CNBr. For the W233L larger species, this resulted in the detection of a *C*-terminal peptide larger than that obtained with both the smaller species and the WT enzyme; (*c*) the M_r difference between the larger species of the W233L protein and that of the WT DD-peptidase, as measured by electrospray m.s., was 2476 ± 20 Da. This was close to the size of the *C*-terminal portion which is eliminated in the wild-type enzyme (2680 as calculated from the amino acid sequence).

From those results it could be assumed that, for unknown reasons, the *C*-terminal portions of the W233 mutant enzymes were abnormally processed and that a large part or the totality of the *C*-terminal peptide was not cleaved in a significant proportion of the mutant proteins. This misprocessing did not seem to affect the enzyme excretion, as indicated by the level of production in the culture supernatant.

Physico-chemical comparisons

PAGE, when performed under non-denaturing conditions, demonstrated an identical charge heterogeneity for the WT and the W271L enzymes and different patterns for the W233 mutants (Fig. 2*b*). Preliminary results did not indicate any significant differences between the catalytic properties of the WT enzyme charge variants (M. Jamin & J.-M. Frère, unpublished work).

On isoelectric focusing the W233 mutants displayed major bands corresponding to pI values slightly different from those of the WT and W271L enzymes and additional minor bands. Those results could reflect conformation modifications with W233 mutants.

From c.d. spectra, an α-helical content of 30% was found for the WT and W271L enzymes, in agreement with the crystallographic data. The W233L and W233S exhibited a somewhat higher α-helical content, but the difference might not be significant.

Fluorescence study

Table 1 shows that, when only W233 was present (W271L mutant), quenching upon β-lactam binding occurred as in the WT; by contrast, when W271 was the only fluorophore (W233L mutant), no such result was obtained. This suggested that W233 might be the residue involved in the fluorescence-quenching phenomenon. According to these results, the β-lactam side chain also seemed to play a significant role: inactivators devoid of a side chain [6-aminopenicillanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA)] were much poorer quenchers than those containing an acyl side chain (benzylpenicillin and cephalosporin C respectively). Binding of dansyl-6-APA to the WT and W271L enzymes led to a non-radiative fluorescence transfer from the tryptophan residues to the dansyl moiety (excitation wavelength 280 nm; emission wavelength 535 nm).

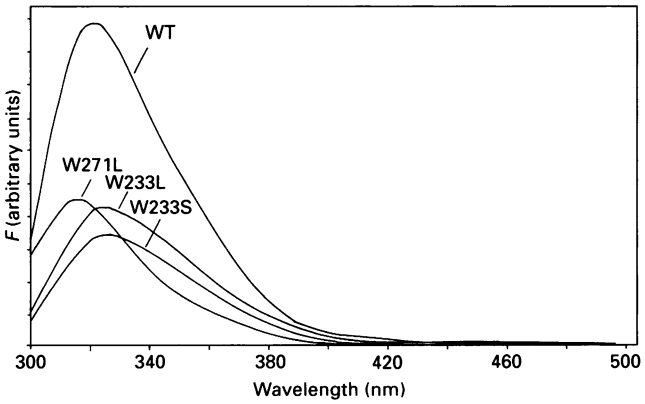

Fig. 3. Fluorescence emission spectra of WT and mutant DD-peptidases at 25 °C

Excitation was at 295 nm and the bandwidth was 2 nm; *F* is the fluoresence adjusted for the same protein absorption at 295 nm for all samples. The absorption was corrected for scattering. Concentrations, emission maxima ($\lambda_{max.}$) and quantum yields (*Q*) were as follows:

Peptidase	Concn. (μM)	$\lambda_{max.}$ (nm)	*Q*
WT	11.8	322 ± 2	0.23
W271L	14.7	316 ± 2	0.09
W233L	10.8	325 ± 2	0.11
W233S	9.04	326 ± 2	0.09

The emission fluorescence spectra of the WT and mutant DD-peptidases upon excitation at 295 nm are shown in Fig. 3. Compared with that of the WT, the fluorescence emission spectrum of the W271L mutant was significantly blue-shifted, indicating a more hydrophobic environment for the remaining Trp^{233} residue. The absorption at the excitation wavelength being the same for all samples, the emission spectrum of the wild-type was much more intense than those of the mutants, and the calculated quantum yield was about twice as high. In all cases the maximum excitation wavelength was 281 ± 2 nm (result not shown).

The fluorescence lifetime distributions of the four proteins (results not shown) indicated that both tryptophan residues exhibited heterogeneous fluorescence decay kinetics. This is becoming a common observation, even for single-tryptophan-containing proteins (Hutnik & Szabo, 1989; Royer *et al.*, 1990). Trp^{271} (W233 mutants) seemed to be characterized by a somewhat higher average fluorescence-lifetime value than that of Trp^{233} (W271L mutant) (Table 2).

The solvent accessibility of the Trp residues in each protein was probed by measuring the fluorescence quenching by iodide ions and acrylamide (charged and uncharged quenchers respectively). Stern–Volmer plots for the quenching were linear in all cases. The smallest k_q quenching constant (lowest accessibility) was obtained with the W271L mutant (whose emission spectrum

Table 1. Residual fluorescence of the DD-peptidases after convalently binding various β-lactams and S2a substrate

Excitation was at 295 nm; emission was at 324 nm. All values are percentages of that for the free enzyme (25 °C). A small amount of β-lactam or S2a substrate was added to the enzyme solution and the fluorescence decrease was monitored. Quenching due to direct re-absorption by β-lactam or substrate at 324 nm (and not linked to acyl-enzyme formation) was negligible.

β-Lactam or substrate	Concn.	Peptidase...	Fluorescence (%)		
			WT (4.4 μM)	W271L (5.9 μM)	W233L (5.3 μM)
6-Aminopenicillanic acid	3 mM		96	97	–
Benzylpenicillin	100 μM		80	65	96
7-Aminocephalosporanic acid	100 μM		63	65	–
Cephalosporin C	10 μM		50	48	80*
Cephalexin	100 μM		89	–	–
Ethylpenicillin	12 μM		66	–	–
S2a substrate	280 μM		55	50	–

* The cephalosporin C concentration was 50 μM.

Table 2. Fluorescence quenching by acrylamide and iodide, and fluorescence lifetimes

K_D is the Stern–Volmer quenching constant and k_q is the bimolecular quenching constant. The fluorescence decay kinetics were analysed as sums of exponentials: $I(t) = \Sigma \alpha_i e^{-t/\tau_i}$ and the average fluorescence lifetime value was calculated as: $\langle T \rangle = \Sigma \alpha_i \tau_i^2 / \Sigma \alpha_i \tau_i$. S.D. values were within 5%.

Peptidase	Acrylamide quenching		Iodide quenching		⟨T⟩ (ns)
	K_D (M^{-1})	$10^{-9} \times k_q$ ($M^{-1} \cdot s^{-1}$)	K_D (M^{-1})	$10^{-9} \times k_q$ ($M^{-1} \cdot s^{-1}$)	
WT	3.54	1.26	4.62	1.65	2.8
W271L	1.90	0.68	1.74	0.62	2.8
W233L	4.55	1.10	4.41	1.08	4.1
W233S	5.27	1.32	4.40	1.10	4.0

was blue-shifted) for both quenchers, indicating no charge effect on the accessibility of the W233 residue; it was actually buried, as opposed to being shielded from I^- ions by electrostatic repulsion by a negatively charged group (Table 2).

Stability of the mutant DD-peptidases

The half-lives for the WT and mutant DD-peptidases during denaturation experiments are shown in Table 3. The modification at position 271 yielded a protein whose stability was similar to that of the WT enzyme, whereas the W233 mutants were significantly less stable at 60 °C. Moreover, the nature of the substituting side chain in position 233 (Leu or Ser) seemed to influence the enzyme's stability in urea. Residue 233 is thus likely to play a role in the enzyme stability.

Table 3. Half-inactivation times of the mutant DD-peptidases (determined by fluorescence measurements)

S.D. values were within 15%.

Denaturation conditions	Peptidase...	Half-lives (min)			
		WT	W271L	W233L	W233S
37 °C		> 1500	–	267	397
60 °C		12	12	0.6	0.8
56 °C + 6 M-urea		9	8	0.5	≪ 0.5
56 °C + 0.5 M-urea		–	–	–	0.5

Kinetic study

The behaviour of the W271L mutant was in all cases quite similar to that of the WT enzyme (Tables 4, 5 and 6). It was also rapidly inactivated by NBS and could be protected by the presence of the benzylpenicilloyl moiety at the active site. In consequence, that residue did not appear to fulfil any essential function in the catalytic process. However, the W271L mutation conserved the hydrophobic character of the residue which seemed to be important for the enzyme functionality, since NBS oxidation of the Trp^{271} side chain led to a complete loss of the weak hydrolytic activity of the W233L mutant.

As shown in Table 4, the W233 mutant enzymes exhibited drastically decreased activities ($k_{cat.}/K_m$) values ranging from 0.1 to 6% of those of the WT). No significant differences were observed between the two mutants (Leu or Ser at position 233). Inactivation by β-lactams (Table 5) also became considerably slower with the enzymes modified at position 233; surprisingly, the deacylation rates (Table 6) with the W233 mutants were considerably increased (up to 300-fold with benzylpenicillin and the W233L mutant). Analysis by t.l.c. of the reaction mixture after degradation of about 0.25 nmol of [^{14}C]benzylpenicillin by the W233L mutant enzyme revealed the presence of 97% of [^{14}C]benzylpenicilloate and only 3% of [14C]phenylacetylglycine. The involvement of an exogenous contaminating β-lactamase (either active-site-serine or zinc-dependent) was unlikely, since similar results were obtained with nitrocefin in the presence of a stoichiometric concentration of either β-iodopenicillanic acid or EDTA. This meant that with a k_3 value of 4.6×10^{-2} s^{-1}, the W233L mutant DD-peptidase behaved as a weak β-lactamase (the k_3 values are generally $< 10^{-3}$ s^{-1} for the DD-peptidases and sometimes ≥ 1000 s^{-1} for the β-lactamases). Moreover, the deacylation pathway was the same as that utilized by β-lactamases

Table 4. Kinetic parameters for the interaction with substrates

Unless otherwise indicated, S.D. values are 10–20%. Values in parentheses are percentages of the WT ones. T/H is the ratio of transpeptidation/hydrolysis determined by h.p.l.c. after incubation of the enzyme with 290 μM-S2a and 20 mM-D-alanine. The kinetic parameters for the disappearance of the thioester (S2a) substrate (290 μM) were determined in the presence of 10 mM-D-alanine (transpeptidation). Sources of values: [a]from Frère and Joris (1985); [b]from initial rate values and Hanes' plot; [c]computed from complete time courses (De Meester *et al.*, 1987); [d]from the values of $k_{cat.}$ and K_m; [e]from Adam *et al.* (1990).

Parameter	Enzyme... WT	W271L	W233S	W233L
Carboxypeptidase activity against Ac_2-L-Lys-D-Ala-D-Ala				
K_m (mM)	14[a]	10±3[b]	23±8[b]	17[b]
$k_{cat.}$ (s^{-1})	55[a]	8.5±3[b]	(9.5±3)×10^{-2}[b]	(7.2±1)×10^{-2}[b]
$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	3900[a]	845±250 (22%)[b]	4.1±2 (0.1%)[b]	4.3±2 (0.1%)[b]
Esterase activity against:				
S2a				
K_m (μM)	41±4[c]	63±7[c]	322[b]	200[b]
$k_{cat.}$ (s^{-1})	5±0.3[c]	3.1±0.3[c]	0.3[b]	0.55[b]
$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	120000[d]	49000 (41%)[d]	930 (0.8%)[d]	2750 (2%)[d]
S1d				
$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	2400±600[e]	1300±350 (54%)[c]	140±10 (6%)[b]	160±20 (7%)[b]
S1e				
$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	2600±200[e]	440±60 (17%)[c]	30±5 (1%)[b]	40±5 (1.5%)[b]
S1c				
$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	1500±100[e]	450±150 (30%)[b]	50±30 (3%)[b]	60±10 (4%)[b]
Transpeptidase activity with S2a and D-alanine:				
K_m (μM)	308±55[c]	225±64[c]	1000[b]	717[b]
$k_{cat.}$ (s^{-1})	47±13[c]	12.5±2[c]	1.3[b]	2.3[b]
$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	153000[d]	56000 (37%)[d]	1250 (0.8%)[d]	3200 (2%)[d]
T/H	8.5	5.2	6	4.7

Table 5. k_2/K ($M^{-1}\cdot s^{-1}$) values for the interaction with β-lactams

Sources of values: [a]from Frère & Joris (1985); [b]measured with the tripeptide Ac_2-L-Lys-D-Ala-D-Ala; [c]by fluorescence measurement; [d]with S2a as reporter substrate; [e]β-lactams as substrate, k_2/K computed as $k_{cat.}/K_m$; [f]nitrocefin as substrate, k_2/K measured as k_i with establishment of a steady state.

β-Lactam	Peptidase... WT	W271L	W233S	W233L
Benzylpenicillin	13000 (25 °C)[a]	2400±200[d]	30±10[d]	60±15[e]
Ampicillin	110[a]	115±15[b]	< 5[b]	< 5[b]
Nitrocefin	4100±650[c]	1000±200[c]	46[f]	3000±1000[e]
Cephaloridine	200±100[b]	140±25[b]	< 5[b]	< 5[b]
Cephalosporin C	1500[a]	900±100[b]	40[b]	< 5[b]
Cefuroxime	1400±450[b]	600±60[b]	–	–
Cephalotin	840±130[b]	340±40[b]	–	–

yielding penicilloate, a behaviour quite different from that of the WT DD-peptidase, whose re-activation involves the cleavage of the C-5–C-6 bond of penicillins, yielding *N*-acylglycine.

The hydrolysis of benzylpenicillin and nitrocefin by the W233L mutant was monitored under initial-rate conditions at 230 nm and 482 nm respectively, and separate values of $k_{cat.}$ and K_m could be obtained (Table 7). Trp^{233} was thus likely to be important for the enzyme active-site conformation, since a modification of that residue appeared to influence β-lactam and substrate binding. The results could also be interpreted by assuming an equilibrium between a small percentage of active enzyme and a major proportion of inactive protein exhibiting a slightly modified conformation.

Conclusions

Both Trp residues were most likely responsible for the fluorescence emission of the wild-type DD-peptidase (the maximum of the emission spectrum being at an intermediate wavelength between that of the Trp^{233} and the Trp^{271} residues), but only the W233 fluoresence was quenched upon binding of the β-lactams. Both residues were oxidized by NBS with subsequent loss of enzymic activity. However, the structural requirements for the side chains at positions 233 and 271 appear to be quite different. As indicated by the sequence comparisons and the catalytic properties of the W271L mutant, which were very similar to those of the WT enzyme, the presence of a hydrophobic residue at that position might be sufficient for maintaining a functional structure. Accordingly, oxidation of the Trp^{271} sidechain of the W233L mutant into a hydrophilic oxindole destroyed the residual activity of that protein. In contrast, a Trp residue appears to be necessary in position 233, since another hydrophobic residue does not seem to be able to efficiently replace it. Conversely, a replacement by a residue with H-bond-forming properties is also unable to maintain a functional structure. Trp^{233} is thus likely to play a unique role in the architecture of the protein. Moreover, the available crystallographic data indicate that this residue is located close to the putative binding site for the substrate and β-lactam side chains. The proteins modified at that position were

Table 6. k_3 (s^{-1}) values for the interaction with β-lactams

Sources of values: [a]from Frère & Joris (1985); [b]β-lactam as substrate, k_3 computed as $k_{cat.}$; [c]measured with the tripeptide Ac_2-L-Lys-D-Ala-D-Ala; [d]by fluorescence measurements; [e]β-lactam as substrate, k_3 measured from the steady-state velocity, v_{ss}. S.D. values were within 10–20%.

β-Lactam	Peptidase... WT	W271L	W233S	W233L
Benzylpenicillin	1.4×10^{-4a}	6×10^{-5c}	4×10^{-2e}	4.6×10^{-2b}
Ampicillin	1.4×10^{-4a}	1.2 ± 10^{-4c}	1×10^{-2e}	4×10^{-2e}
Carbenicillin	1.4×10^{-4a}	–	1.6×10^{-2e}	2×10^{-2e}
Nitrocefin	3×10^{-4a}	3×10^{-4c}	1×10^{-2e}	3.4×10^{-2b}
Cephaloridine	4×10^{-5d}	7×10^{-6c}	6.7×10^{-5d}	6×10^{-5d}
Cephalosporine C	1×10^{-6a}	4×10^{-5d}	5×10^{-5d}	4.5×10^{-5d}
Cefuroxime	4×10^{-6a}	3×10^{-5c}	$<10^{-5c}$	$<1\times10^{-5d}$

Table 7. $k_{cat.}$ and K_m values for the interaction of the W233L enzyme with benzylpenicillin and nitrocefin

β-Lactam	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)
Nitrocefin	$(3.4\pm0.9)\times10^{-2}$	10 ± 9	3000 ± 1000
Benzylpenicillin	$(4.6\pm2)\times10^{-2}$	800 ± 500	60 ± 15

also much more unstable. Unexpectedly, mutation of residue W233 yielded a protein whose catalytic properties represented a first step towards the transformation of a DD-peptidase into a β-lactamase: decreased peptidase accompanied by increased β-lactamase activities. Significantly, esterase activity was less affected, and β-lactamases are known to exhibit non-negligible esterase and no peptidase activities.

Note added in proof (received 2 December 1991):

During contact between NBS and the WT enzyme, the loss of activity was concomitant with that of the ability to bind labelled penicillin.

We thank Dr. Y. Engelborghs (KUL, Leuven, Belgium) for useful comments on the manuscript. This work was supported in part by an Action Concertée with the Belgian Government (convention 86/91-90), the Fonds de la Recherche Scientifique Médicale (contract n° 3.4537.88), a Convention Tripartite between the Région Wallone, SmithKline Beecham (U.K.) and the University of Liège and the Belgian National Incentive Program on Fundamental Research in Life Sciences (contract BIO 22). This paper presents research results of the Belgian programme on Interuniversity Poles of attraction initiated by the Belgian State Prime Minister's Office, Science Policy Programming. Scientific responsibility is assumed by the authors. B.J. is a Chercheur Qualifié of the Fonds National de la Recherche Scientifique (FNRS) (Brussels, Belgium), J.M.W. is fellow of the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (IRSIA) and C.B.B. performed a large part of the work described here as a Fellow of IRSIA (Brussels, Belgium).

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Received 24 June 1991/22 August 1991; accepted 3 September 1991