Mechanism of action of DD-peptidases: role of asparagine-161 in the *Streptomyces* R61 DD-peptidase

Jean-Marc WILKIN, Marc JAMIN, Bernard JORIS and Jean-Marie FRERE*

Centre d'Ingéniérie des Protéines and Laboratoire d'Enzymologie, Université de Liège, Institut de Chimie B6, 4000 Sart-Tilman, Liège, Belgium

The role of residue Asn-161 in the interaction between the *Steptomyces* R61 DD-peptidase and various substrates or β-lactam inactivators was probed by site-directed mutagenesis. The residue was successively replaced by serine and alanine. In the first case, acylation rates were mainly affected with the peptide and ester substrates but not with the thiol-ester substrates and β-lactams. However, the deacylation rates were decreased 10–30-fold with the substrates yielding benzoylglycyl and benzoylalanyl adducts. The Asn161Ala mutant was more generally affected, although the acylation rates with cefuroxime and cefotaxime remained similar to those observed with the wild-type enzyme. Surprisingly, the deacylation rates of the benzoylglycyl and benzoylalanyl adducts were very close to those observed with the wild-type enzyme. The results also indicate that the interaction with the peptide substrate and the transpeptidation reaction were more sensitive to the mutations than the other reactions studied. The results are discussed and compared with those obtained with the Asn-132 mutants of a class A β-lactamase.

INTRODUCTION

In the *Streptomyces* R61 DD-peptidase (EC 3.4.16.4), the Tyr-Ser-Asn triad, situated on a loop close to the active-site serine side chain, corresponds to the Ser-Asp-Asn triad in class A β-lactamases (EC 3.5.2.6) (Joris et al., 1991). In both cases, the side chains of the first and third residues point into the active site, whereas that of the second is embedded in the protein core, and the aspartate side chain of the *Streptomyces albus* G β-lactamase has been shown to play an essential role in maintaining protein stability (Jacob et al., 1990b). In a previous contribution (Wilkin et al., 1993), the importance of the Tyr-159 side chain in the interactions between the enzyme, its substrates and β-lactam antibiotics was examined. In the present paper, a similar analysis is presented for the Asn-161 residue. By site-directed mutagenesis, this residue was successively replaced by serine and alanine. The kinetic model on which the study was based was the same as before, i.e.

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

where E, C, EC, E-C* and P(s) are respectively the enzyme, the substrate or the β-lactam inactivator, the non-covalent Henri–Michaelis complex, the acylenzyme and the reaction products and where $K' = (k_{-1} + k_{+2})/k_{+1}$.

MATERIALS AND METHODS

Chemicals were of the same origin as in Wilkin et al. (1993), and Table 1 summarizes the structures of the various substrates.

Oligonucleotides were a gift from Dr. J. Brannigan, University of Sussex, Brighton, Sussex, U.K. The oligonucleotides utilized to introduce the mutations were purified with the help of the Oligonucleotide Purification Cartridge (Applied Biosystems). They had the following sequences:

Asn161Ser, GCCTATTCATACTCCTCCACGAACTTCGTC
AA

Asn161Ala, GCCTATTCATACTCCGCCACGAACTTCGTC
AA

where, at the level of the mutations, the two lower nucleotides are those of the wild-type gene.

Recombinant DNA procedures were as in Wilkin et al. (1993).

Production of the mutant DD-peptidases

Transformant *Streptomyces* colonies selected on thiostrepton-containing R2YE agar (Maniatis et al., 1982) were used to inoculate 250 ml precultures in TSB medium (30 g/l tryptic soy broth; Gibco, Paisley, U.K.) containing 25 mg/l thiostrepton. They were incubated at 28 °C in 1-litre baffled Erlenmeyer flasks with a 250 rev./min orbital agitation. After 24 h, these were used to inoculate (4%, v/v) the main cultures which were continued for 3 days under the same conditions.

Purification, determination of kinetic parameters, denaturation and h.p.l.c. experiments were carried out exactly as described in Wilkin et al. (1993). All experiments were performed in 10 mM sodium phosphate buffer, pH 7.0, unless otherwise stated.

RESULTS

Mutagenesis, production and purification

The two modified plasmids, pDML37 (Asn161Ala) and pDML38 (Asn161Ser), were purified, the genes sequenced and no additional mutations detected.

Maximum production was obtained after 72 h of growth, and the yield was 40 mg/l in both cases. Thus production in the TSB medium, although somewhat lower than in the modified YEME medium (Erpicum et al., 1990), is faster and offers an interesting alternative for production of the DD-peptidase mutants.

The purification steps are summarized in Table 2. As observed with the Tyr159Phe mutant (Wilkin et al., 1993), step 4 yielded

Abbreviations used: Ac_2KAA, $N^{\alpha}N^{\epsilon}$-diacetyl-L-lysyl-D-alanyl-D-alanine; Ac_2KALa, $N^{\alpha}N^{\epsilon}$-diacetyl-L-lysyl-D-alanyl-D-lactate; Ac_2KATLa, $N^{\alpha}N^{\epsilon}$-diacetyl-L-lysyl-D-alanyl-D-thiolactate.

* To whom correspondence should be addressed.

Table 1 Structures of the substrates

R^1-NH-CH(R²)-CO-X-CH(R^3)-COO⁻

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_2KATla	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_2(DL)	H	S

Table 2 Purification of the Asn-161 mutant proteins

Specific activities were determined with the tripeptide (Ac_2KAA) substrate at a concentration of 1 mM. The final preparations were ⩾ 95% pure as judged by SDS/PAGE and penicillin titration. Standard deviations were ⩽ 10%. Culture volumes were 5 and 4 l for the Asn161Ala and Asn161Ser mutants respectively. The purification steps were as described in Wilkin et al. (1993): step 1, mass adsorption/desorption on Amberlite CG50; step 2, chromatography on Q-Sepharose Fast Flow; step 3, filtration on Sephadex G-100; step 4, chromatography on prepacked Hi-Load 26/10 Q-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden).

	Asn161Ala			Asn161Ser		
Step	Total protein (mg)	Total enzyme (mg)	Specific activity (μmol/min per mg)	Total protein (mg)	Total enzyme (mg)	Specific activity (μmol/min per mg)
1	2800	150	0.007	2600	160	0.11
2	460	150	0.046	300	150	0.90
3	100	80	0.112	150	120	1.44
4	40	40	0.140	100	100	1.80

two forms of the Asn161Ala mutant, eluted respectively with 75 and 83 mM NaCl and in proportions of 70:30. The kinetic parameters for the hydrolysis of the S2a substrate were identical within the limits of experimental errors. In the experiments described below, performed with the mixture, no indications of heterogeneous behaviour were found and the two variants could be considered as kinetically equivalent.

Physical properties and stability

Absorption (230–320 nm), c.d. (200–350 nm) and fluorescence emission (excitation at 280 nm) spectra were superimposable on those of the wild-type enzyme. No major modifications of the stabilities of the mutants were found. At 60 °C, in buffer, the half-lives were 6.2±0.6 (wild-type), 3.0±0.3 (Asn161Ala) and 3.0±0.3 (Asn161Ser) min and at 56 °C in the presence of 6M urea 8.6±0.9 (wild-type), 3.0±0.3 (Asn161Ala) and 2.6±0.3 (Asn161Ser) min.

Kinetic properties

Carboxypeptidase activity

Table 3 shows the hydrolytic profiles of the two mutant enzymes. Fluorescence stopped-flow experiments performed at 20 °C yielded the values of k_{+2} and K' for the Asn161Ser mutant with substrate S2a: wild-type, $k_{+2} = 200 \pm 50$ (s^{-1}), $K' = 5.0 \pm 0.5$ (mM) (Jamin et al., 1991); Asn161Ser, $k_{+2} = 140 \pm 50$ (s^{-1}), $K' = 4.5 \pm 0.5$ (mM). Thus for this substrate, deacylation remained strongly rate-limiting (at 37 °C, $k_{+2} > 140$ s^{-1} and $k_{+3} = 0.33$ s^{-1}). For the same mutant, this was probably also true for substrate S2c, which forms the same acylenzyme and for which the $k_{cat.}$ value was the same as that observed with S2a, as expected if $k_{cat.}$ corresponds to k_{+3}. Moreover, both S2d and S2c quenched the fluorescence emission of the Asn161Ser mutant, indicating that, for these substrates, k_{+2} was similar to or larger than k_{+3}.

The pH-dependence of the kinetic parameters was studied for the Asn161Ser–S2a interaction. The results are presented in Figure 1.

Transpeptidase activity

Figure 2 compares the effects of increasing D-alanine concentrations on the $k_{cat.}$ and K_m values of the wild-type and Asn161Ser protein. As observed with the wild-type enzyme, the $k_{cat.}$ curve fitted the empirical equation:

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

Table 3 $k_{cat.}$, K_m and $k_{cat.}/K_m$ values for the wild-type and Asn-161 mutant enzymes

Values for the wild-type enzyme are from Frère and Joris (1985)*, Varetto et al. (1987)† or Adam et al. (1990)‡. M, 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. ND, not determined. Standard deviations did not exceed 15%.

	Asn161Ala				Asn161Ser				Wild-type			
Substrate	$k_{cat.}$ (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	0.14	14500	10	B	1.7	13600	130	B	55*	14000	4000	B
Ac_2KALa	1.53	8000	190	B	13.0	44000	300	B	32†	40000	800	B
Ac_2KATLa	ND	ND	ND		ND	ND	9000	A	72	8000	9000	B
Sle	0.13	5000	30	B	0.33	1200	280	B	5‡	900	5500	A
S2a	2.20	660	3200	B	0.33	9	38000	A	5‡	50	100000	A
S2c	3.00	620	5000	B	0.30	6	50000	A	5‡	50	100000	A
S2d	12.0	50	240000	A	2.50	2	1250000	A	70	100	700000	A
S2e	ND	ND	ND		2.40	80	30000	A	70	560	80000	A
S2Val	1.5	70	21000	A	0.37	8	46000	A	4	500	8000	A

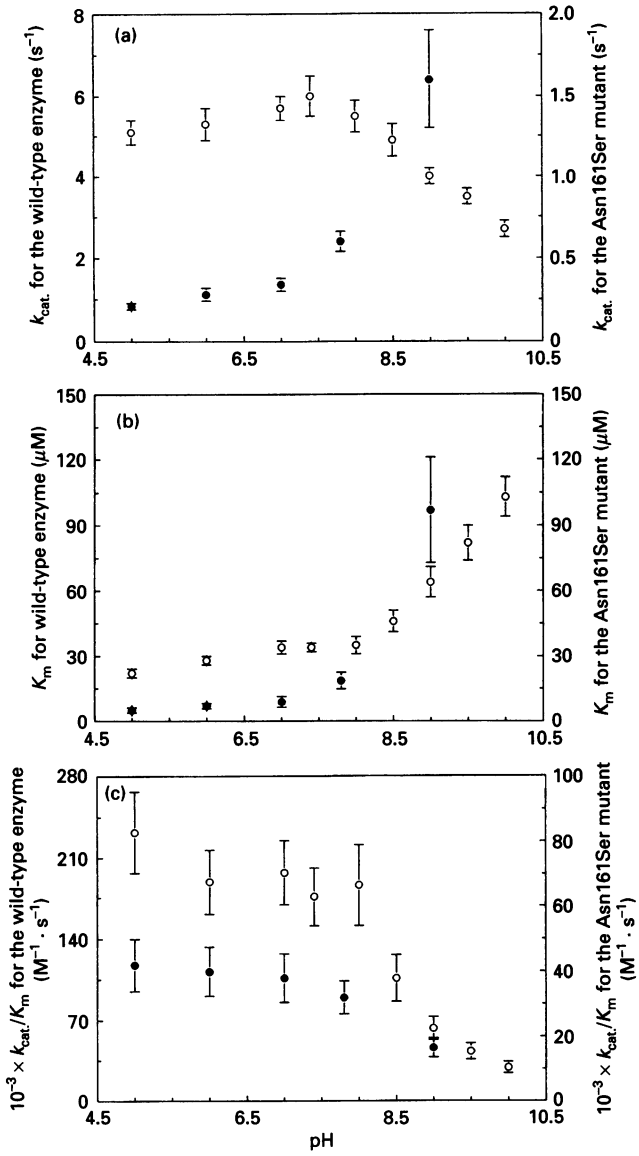

Figure 1 pH-dependence of the kinetic parameters for the hydrolysis of substrate S2a by the wild-type (○) and Asn161Ser (●) enzymes

(**a**) $k_{cat.}$ values; (**b**) K_m values; (**c**) $k_{cat.}/K_m$ values. These values were obtained by analysing complete time courses. Reactions were performed at 37 °C in the following buffers: pH 5.0 and 6.0, 10 mM and 13 mM sodium cacodylate/HCl respectively; pH 7.0 and 8.0, 10 mM and 7 mM sodium phosphate respectively and pH 9.0, 6 mM potassium borate/NaOH. The ionic strength was adjusted to the same value by addition of NaCl. The pH profile of the wild-type enzyme is from Varetto (1991). Results are means ± S.D. ($n = 6$ or more).

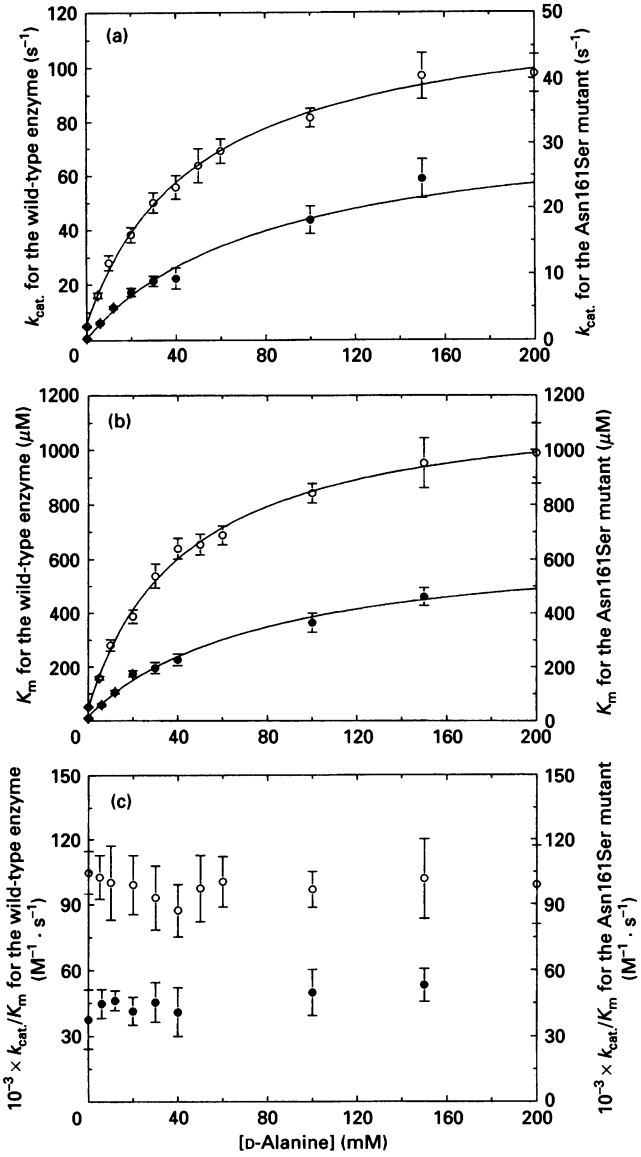

Figure 2 Evolution of the $k_{cat.}$, K_m and $k_{cat.}/K_m$ values for the hydrolysis of S2a with increasing concentrations of D-alanine for the wild-type (○) and Asn161Ser (●) enzymes

Values were determined by using the complete time-course method (De Meester et al., 1987) at 37 °C in 10 mM sodium phosphate buffer, pH 7.0. Values for the wild-type enzyme are from Jamin et al. (1991). For other details, see the legend of Figure 1.

The D-alanine concentration corresponding to half-saturation was about 80 mM ($[A]_{50} = 1/c$) which is not significantly different from the wild-type value ($[A]_{50} = 50$ mM). Since K_m increased according to a similar equation, the $k_{cat.}/K_m$ was not significantly modified in the presence of D-alanine, as found for the wild-type enzyme. Thus the behaviour of the mutant seemed to be similar to that of the wild-type enzyme. At low acceptor concentrations, $k_{cat.}$ varied linearly with the acceptor concentrations, and the quality of an acceptor could be characterized by the $\Delta k_{cat.}/\Delta[A]$ ratio or by the relative acceleration, $\Delta k_{cat.}/(\Delta[A]^* k_{cat.})$. As shown in Table 4, the specificity profile of the mutant for various potential acceptors also closely paralleled that of the wild-type enzyme. However, the relative acceleration was sometimes significantly higher for the mutant although it was lower with D-lactate.

With the Asn161Ala mutant enzyme [for technical reasons (the K_m value should be adequately low), these experiments were performed with substrate S2d], the specificity profile for the acceptor was again similar to that of the wild-type enzyme. With substrate S2a and D-alanine, the $\Delta k_{cat.}/\Delta[A]$ was 340 $M^{-1} \cdot s^{-1}$ and the relative acceleration 150 M^{-1}, a value close to that observed for the wild-type enzyme and the same S2a substrate and for the mutant and S2d substrate.

Table 5 shows the transpeptidation/hydrolysis ratios (T/H ratios) observed for the two mutants under steady-state conditions (10% utilization of the S2a substrate). As observed for

Table 4 Acceleration factors ($\Delta k_{cat.}/\Delta[A]$) and relative accelerations ($\Delta k_{cat.}/\Delta[A]k_{cat.}$) for the wild-type and mutant enzymes

ND, not determined; meso-A_2pm, mesodiaminopimelic acid. The complete time-course method (De Meester et al., 1987) was used to calculate the $k_{cat.}$ values. Standard deviations did not exceed 15%.

	Donor substrate = S2a				Donor substrate = S2d	
	Asn161Ser		Wild-type		Asn161Ala	
Acceptor	Acceleration factor ($M^{-1} \cdot s^{-1}$)	Relative acceleration (M^{-1})	Acceleration factor ($M^{-1} \cdot s^{-1}$)	Relative acceleration (M^{-1})	Acceleration factor ($M^{-1} \cdot s^{-1}$)	Relative acceleration (M^{-1})
L-Alanine	< 5	–	< 5	–	≪ 5	–
D-Alanine	345	1050	3340	670	1140	100
D-Phenylalanine	5500	16500	15500	3100	25300	2100
D-Glutamine	970	2900	5600	1100	ND	ND
D-Glutamate	< 5	–	< 5	–	ND	ND
Meso-A_2pm	1300	4000	6540	1300	800	700
Glycylglycine	40	120	490	100	ND	ND
D-Lactate	25	75	1800	360	11	10

Table 5 T/H ratios observed for the reactions with 250 μM S2a as donor and D-alanine as acceptor substrates

[D-Alanine] (mM)	Asn161Ala	Asn161Ser	Wild-type
5	0.45 ± 0.09	0.30 ± 0.06	–
10	–	–	2.7 ± 0.1
25	1.17 ± 0.25	0.60 ± 0.08	4.3 ± 0.6
35	1.03 ± 0.25	0.82 ± 0.10	–
50	1.95 ± 0.30	0.90 ± 0.10	6.0 ± 0.8
75	2.00 ± 0.4	–	–
100	2.00 ± 0.4	0.80 ± 0.10	–
150	–	1.05 ± 0.20	–
200	–	0.90 ± 0.10	–

the wild-type enzyme, these data indicate a clear saturation effect but the T/H ratios remained low. Combined with the observed increases in the $k_{cat.}$ values, these results show that the presence of the acceptor also increased the rate of the hydrolysis reaction, in contrast with what had been observed for the wild-type enzyme, at least at low acceptor concentrations.

Interaction with β-lactams

Table 6 gives an overview of the parameters characteristic of acylation (k_{+2}/K') and deacylation (k_{+3}), and Table 7 details the individual values of k_{+2} and K' for carbenicillin. These values were also determined for the interaction between cloxacillin and the Asn161Ala mutant indicating that the k_{+2} value was somewhat decreased (0.020 ± 0.001 s^{-1} for the mutant and 0.16 ± 0.2 s for the wild-type proteins), whereas the K' value was slightly increased for the mutant (4.0 ± 0.5 mM versus 2.1 ± 0.3 mM for the wild-type).

At pH 7.0, both mutants exhibited a significant increase in the penicilloic acid/phenylacetylglycine ratio during the re-activation step: 2 ± 0.2 versus < 0.1 for the wild-type enzyme.

DISCUSSION

The asparagine residue which corresponds to the *Streptomyces* R61 DD-peptidase Asn-161 is nearly invariant in all penicillin-

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

The numbers in parenthesis refer to the various procedures described in Wilkin et al. (1993): (1a) fluorescence quenching, (1b) reporter substrate method, (2) reporter inactivator method and (3) reactivation of the isolated acylenzyme. Standard deviations did not exceed 15%. ND, not determined; 7-ACA, 7-aminocephalosporanic acid.

	Asn161Ala		Asn161Ser		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	230 (1a)	5.1×10^{-4} (3)	14000 (1a)	4.3×10^{-5} (3)	14000 (1a)	1.4×10^{-4} (3)
Carbenicillin	10 (1a)	2.4×10^{-4} (3)	1200 (1a)	4.3×10^{-5} (3)	800 (1a)	1.4×10^{-4} (3)
Ampicillin	7 (1a)	2.5×10^{-4} (3)	170 (1a)	1.7×10^{-5} (3)	110 (1a)	1.4×10^{-4} (3)
Cloxacillin	5 (1a)	2.3×10^{-5} (3)	ND	ND	80	1.0×10^{-3} (3)
Nitrocefin	440 (2)	4.9×10^{-4} (3)	1800 (1a)	2.1×10^{-3} (3)	4100 (1a)	3.0×10^{-4} (3)
Cephalosporin C	35 (1a)	3.1×10^{-5} (3)	500 (1a)	1.8×10^{-6} (3)	1500 (1a)	1.0×10^{-6} (3)
Cefuroxime	320 (1a)	2.0×10^{-5} (3)	1200 (1a)	6.1×10^{-6} (3)	350 (1a)	4.0×10^{-6} (3)
Cefotaxime	30 (1a)	1.9×10^{-5} (3)	ND	ND	16 (1a)	$< 1.0 \times 10^{-6}$ (3)
7-ACA	ND	ND	30 (1b)	4.0×10^{-3} (3)	30 (1b)	1.6×10^{-3} (3)

Table 7 Individual k_{+2}, K' and k_{+2}/K' values for the wild-type and mutant enzymes

The determinations of these parameters were realized as described in detail by Wilkin et al. (1993) by monitoring the quenching of fluorescence emission (1a) or by the reporter substrate method (1b) (De Meester et al., 1987); *values from Frère and Joris (1985). ND, not determined.

Antibiotic	Kinetics parameters	Asn161Ala	Asn161Ser	Wild-type
Carbenicillin	k_{+2} (s^{-1})	0.07 ± 0.02 (1a)	0.31 ± 0.04 (1a)	0.09 ± 0.01*
	K' (mM)	5.00 ± 0.40 (1a)	0.25 ± 0.05 (1a)	0.11 ± 0.01*
	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)	10 ± 2.0 (1a)	1200 ± 300 (1a)	830 ± 80 (1a)
Cloxacillin	k_{+2} (s^{-1})	0.020 ± 0.001 (1b)	ND	0.16 ± 0.02 (1b)
	K' (mM)	4.0 ± 0.5 (1b)	ND	2.10 ± 0.20 (1b)
	k_{+2}/K' ($M^{-1} \cdot s^{-1}$)	5.5 ± 0.90 (1b)	ND	80 ± 8 (1b)

recognizing enzymes (Joris et al., 1988, 1991). The only known exception is β-lactamase III of *Bacillus cereus* where a serine residue is found in this position. The available three-dimensional structural data indicate that it is positioned on one of the walls of the active-site cavity, and, in class A β-lactamases, Asn-132 has been hypothesized to form an important hydrogen bond with the carbonyl oxygen of the 6β-acylamido side chain of the substrate (Herzberg and Moult, 1987; Moews et al., 1990). The replacement by a serine, giving the Asn132Ser mutant in the *Streptomyces albus* G β-lactamase (Jacob et al., 1990a,b), however, yielded unexpected results: whereas the modified enzyme remained a rather good penicillinase, it became a very poor cephalosporinase. In contrast, the Asn132Ala mutant was severely impaired with all substrates, but again much more so with cephalosporins. Surprisingly, the interactions with 6-aminopenicillanate, where such a hydrogen bond cannot be involved, were more affected than those with the other penicillins, and with both mutants. Moreover, $k_{cat.}$ seemed to be the most altered parameter, indicating a more drastic effect on the transition state than on the initial recognition of the substrate. The equivalent residue in *Escherichia coli* penicillin-binding protein 3 is Asn-361, and its replacement by a serine resulted in an increased resistance of the bacterium to cephalosporins, but not to benzylpenicillin or aztreonam, an observation that could be correlated with those discussed above for the β-lactamase (Frère et al., 1992).

The detailed analysis presented here of the Asn161Ser and Asn161Ala mutants of the *Streptomyces* R61 DD-peptidase indicates a probably much more complex situation. The mutations did not seem to decrease the stabilities significantly or alter the structures of the mutant proteins, which was in agreement with a catalytic rather than a structural role and with the significant catalytic activities or penicillin-binding properties measured in some cases.

Let us first examine the results obtained with the serine mutant. The rate of the acylation reaction was only significantly modified with the peptide substrate (Ac_2KAA) and with the ester hippuryl D-phenyl-lactate (S1e) and very little with all the other substrates and β-lactams tested. In fact, with some compounds, these rates were even increased (substrates S2d and S2Val, carbenicillin, ampicillin and cefuroxime). With the peptide substrate and the wild-type enzyme, acylation was rate-limiting (Varetto et al., 1987) and $K_m = K'$. It was likely that the $k_{cat.}$ value for the mutant also reflected k_{+2} since the K_m value was not altered. The value of k_{+3} might also decrease, but this remained impossible to assess. Conversely, with the hippuryl D-phenyl-lactate, the decrease in k_{+2}/K' ($= k_{cat.}/K_m$) was accompanied by a decrease in k_{+3}, which remained rate-limiting, as indicated by the similarity of the $k_{cat.}$ values for the three substrates exhibiting the same acylating hippuryl group (S1e, S2a and S2c). With all the thiol esters, there was a 15–16-fold decrease in the $k_{cat.}$ value, whereas the k_{+2}/K' value was only slightly affected, and sometimes increased (interestingly, the k_{+2}/K' value observed in the interaction between S2d and the Asn161Ser mutant was the highest observed so far between a substrate and a DD-peptidase), which could be interpreted as a major impact of the mutation on the deacylation rate with a much more limited influence on k_{+2} and K'.

The acceptor profile, visualized on the basis of the acceleration factors, was not significantly modified, and the values of the relative accelerations seemed to indicate that the mutation did not interfere with the transpeptidation reaction. This conclusion was, however, falsified by the T/H ratios which were decreased about 7-fold for the mutant (Table 5). The somewhat unexpected consequence of these observations was that, in the latter case, a substantial portion of the increase in the donor substrate utilization was in fact due to an increased rate of the hydrolysis reaction. It can indeed be computed that, at a 5 mM D-alanine concentration, the $k_{cat.}$ values for the hydrolysis and transpeptidation were 1.54 and 0.46 (s^{-1}) respectively, and the former value was thus significantly larger than that observed in the absence of acceptor (0.33 s^{-1}). This apparent paradox will be discussed in another contribution where a complete kinetic model will be proposed for the transpeptidation reaction (Jamin et al., 1993).

The pH-dependence of the parameters for substrate S2a is not easy to interpret. That of the $k_{cat.}$ was drastically altered and K_m increased more markedly above pH 8.0, which resulted in a $k_{cat.}/K_m$-dependence very similar to that of the wild-type enzyme. One could tentatively assume that $k_{cat.}$ and K_m curves mainly reflected the behaviour of k_{+3} and that the other parameters, k_{+2} and K', followed the wild-type pattern. However, the reasons for the very different pH-dependence of k_{+3} in the wild-type and Asn161Ser enzymes remain a complete mystery.

The Asn161Ala mutant was more generally impaired, but not with all the compounds studied. Again, the most drastic decreases in the acylation rates were recorded with the peptide and hippuryl D-phenyl-lactate substrates (400- and 200-fold), but some penicillins and cephalosporins also became less efficient acylating agents. Nevertheless, and surprisingly, acylation produced by S2d, S2Val, cefuroxime and cefotaxime exhibited either little modification or even an increased efficiency (S2Val and cefotaxime). With all the thiol esters, and in contrast with the situation for Asn161Ser, the $k_{cat.}$ value, corresponding to k_{+3}, was only slightly decreased. With S2a and S2c, a concomitant increase in K_m was observed, which was the major factor responsible for the decrease in $k_{cat.}/K_m$. Conversely, with S2d and S2Val, K_m decreased, leading to slightly decreased (S2d) or increased (S2Val) values of $k_{cat.}/K_m$. Hippuryl D-phenyl-lactate

(S1e) produced completely modified parameters. The $k_{cat.}$ value decreased below that for S2a, which has the same acylating group, and the only possible conclusion is that, in this case, k_{+2} had now become smaller than k_{+3} and that $k_{cat.}$ reflected k_{+2}. Such a large decrease in k_{+2} also explained the drastic decrease in $k_{cat.}/K_m$ ($= k_{+2}/K'$). However, since the K_m value only increased 5–6-fold, one has to assume that, with this substrate and the wild-type enzyme, the k_{+2}/k_{+3} ratio was not very large, probably between 4 and 6.

Transpeptidation was not drastically affected: the acceptor profile was not greatly modified (if one excepts D-lactate) and the T/H ratios were only slightly lower than those observed with the wild-type enzyme.

From the crystallographic studies of *Staphylococcus aureus* and *Bacillus licheniformis* class A β-lactamases (Herzberg and Moult, 1987; Moews et al., 1990), it has been postulated that Asn-132 is involved in a hydrogen bond (via its $-NH_2$ amide group) with the carbonyl oxygen of the C-6 β-lactam side chain, thus allowing the proper positioning of the substrate molecule in the catalytic site of the enzyme. Yet, the mutation of Asn-132 to alanine or serine in the *S. albus* G class A β-lactamase resulted in alterations in the $k_{cat.}$ values rather than the K_m values; the transition state was thus also stabilized by Asn-132 (Jacob et al., 1990a,b). Modelling of benzylpenicillin in the cavity of the *S. albus* G β-lactamase accordingly showed that Asn-132 plays two important roles in the enzymic mechanism (Lamotte-Brasseur et al., 1991): (1) orientation of the antibiotic by forming a hydrogen bond between the $-NH_2$ group of Asn-132 and the carbonyl oxygen of the β-lactam side chain; (2) orientation of various residues (Ser-130, Lys-73 and Ser-70) directly involved in the catalytic process by formation of additional hydrogen bonds. These assumptions were recently reinforced by the study of the TEM-1 class A β-lactamase (Strynadka et al., 1992).

If we assume that the two roles of Asn-132 also hold for Asn-161 in the Streptomyces R61 DD-peptidase, the alterations in the activity profiles of the Asn161Ala and Asn161Ser mutants can be partially explained. For instance, carbenicillin produced an affinity constant K' that was either slightly altered for the Asn161Ser mutant, in which the serine could possibly replace the asparagine (as observed in the Asn132Ser mutant of the *S. albus* G β-lactamase), or greatly increased for the Asn161Ala mutant, in which no hydrogen bond can be formed. In contrast, cloxacillin produced a larger alteration in the k_{+2} (8-fold decrease) than in the K' value (2-fold increase). Thus it appears that, depending on the exact structure of the antibiotic, the balance between the binding and catalytic contributions of the Asn-161 side chain might be modified, but this distinction might also be considered as somewhat arbitrary. In contrast, the variations in the acylation and deacylation rates of the various substrates remain more difficult to explain.

Nevertheless, our data again underline possible similarities between the catalytic mechanism of β-lactamases and DD-peptidases, but these relationships are neither simple nor straightforward, as indicated for instance by the fact that the degradation of benzylpenicilloyl intermediates formed with both Asn161Ser and Asn161Ala mutants occurred preferentially according to the direct hydrolysis pathway, whereas, with the wild-type enzyme, the same reaction first involved the hydrolysis of the C-5–C-6 bond of the antibiotic moiety. Thus the Asn-161 mutants exhibited behaviours similar to that of the Tyr-159 mutants studied previously (Wilkin et al., 1993). Moreover, the k_{+3} value for nitrocefin also increased 7-fold for the Asn161Ser mutant. Although interesting, these observations remain unexplained.

In conclusion, most of the results obtained with the two mutants described here can be reasonably interpreted on the basis of the variations in the individual rate constants. Except for the acylation and the binding of the β-lactam inactivators, it remains, however, impossible to relate these variations directly to a well-defined role of the Asn-161 residue in the catalytic process. It is noteworthy that the interaction with the peptide substrate is always much more sensitive to the mutations than those with the esters and thiol esters or with the β-lactam inactivators. Surprisingly, the rate of deacylation (k_{+3}) with the substrates containing a benzoyl side chain was more affected by the Asn161Ser mutation than by the Asn161Ala mutation, as if the hydrogen bonds which can still be formed with the former were capable of inducing a distortion of the acylenzyme, making it somewhat more resistant to hydrolysis. As shown by the behaviour of the Asn161Ala mutant, the Asn-161 side chain plays a very minor role in the deacylation rate with these substrates. It is, in contrast, quite important for the acylation by the normal peptide substrate. In this context, it would be interesting to examine thoroughly the catalytic properties of the Asn361Ser mutant of *E. coli* penicillin-binding protein 3. Similarly, and also in agreement with the results obtained with the Tyr-159 mutants, the transpeptidation reaction also seemed to be more sensitive to modifications of the active-site geometry and properties. This additional observation is certainly not devoid of interest, since this reaction is probably the physiologically most important one.

Finally, our results again support the need to perform a complete analysis of the kinetic properties of mutants. Indeed, a very limited and probably irrelevant picture would have emerged if we had restricted our studies to one substrate and one β-lactam.

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