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Importance of the His-298 residue in the catalytic mechanism of the *Streptomyces* R61 extracellular DD-peptidase

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Among the active-site-serine penicillin-recognizing proteins, the *Streptomyces* R61 extracellular DD-peptidase is the only one to have a His-Thr-Gly sequence [instead of Lys-Thr(Ser)-Gly] in 'box' VII. The His residue was replaced by Gln or Lys. Both mutations induced a marked decrease in the rates of both tripeptide substrate hydrolysis and acylation by benzylpenicillin and cephalosporin C. The rate of hydrolysis of the thioester hippuryl thioglycollate was less affected. The most striking result was the disproportionate loss of transpeptidation properties by both mutants, indicating an important role of His-298 in this reaction. We believe that this result represents the first modification of a DD-peptidase leading to a specific decrease of the transpeptidation-to-hydrolysis ratio.

INTRODUCTION

β-Lactamases of classes A, C and D, the low-M_r DD-peptidases and the penicillin-binding domains of the high-M_r penicillin-binding proteins (PBPs) appear to be members of a single superfamily of active-site-serine enzymes (Joris *et al.*, 1988; Ghuysen, 1991). All of these enzymes interact with β-lactams according to the same kinetic pathway, involving the formation of a covalent acyl-enzyme intermediate (model 1):

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E + P_H$$

where E is the enzyme, C the β-lactams, EC a non-covalent Henri–Michaelis complex and EC* the acyl-enzyme (Frère & Joris, 1985). The hydrolysis of peptides by DD-peptidases and of depsipeptides by some β-lactamases, DD-peptidases and PBPs appears to follow the same kinetic pathway (Pratt *et al.*, 1985; Varetto *et al.*, 1987*a*; Adam *et al.*, 1990; Jamin *et al.*, 1991).

There are, however, two major differences between the penicillin-sensitive PBPs and DD-peptidases and the β-lactamases: (1) the half-lives of the acyl-enzymes formed with β-lactams are generally much shorter with the β-lactamases, which in consequence efficiently hydrolyse these antibiotics; and (2) β-lactamases do not appear to interact with the peptide substrates of the DD-peptidases. Striking similarities have also been found in the 3-dimensional structures of several β-lactamases and the *Streptomyces* R61 DD-peptidase. Moreover, several conserved 'boxes' of residues have been detected in the primary structures of all of these proteins (Joris *et al.*, 1988).

One of these boxes (box VII) is a Lys-Thr-Gly or Lys-Ser-Gly triad which, in all known 3-dimensional structures of β-lactamases (4 class A and 1 class C), is situated on a piece of β-strand and forms one wall of the catalytic cavity (Kelly *et al.*, 1986; Dideberg *et al.*, 1987; Oefner *et al.*, 1990; Herzberg, 1991).

In the *Streptomyces* R61 DD-peptidase, and only in that enzyme, the first residue of the triad is a histidine. In this paper we have studied the role of this particular residue (His-298), which was postulated to facilitate the binding and the orientation of penicillins by forming a salt bridge with their carboxylate group (Kelly *et al.*, 1989).

MATERIALS AND METHODS

Mutagenesis

The exocellular DD-peptidase of *Streptomyces* R61 has been cloned and the enzyme expressed from a multicopy plasmid vector (pDML114) in *Streptomyces lividans* TK24 (Duez *et al.*, 1987). For the expression of the mutant proteins, another *Streptomyces* vector, pDML115, was constructed so that it presented unique *Sph*I and *Pst*I sites. In order to introduce oligonucleotide-directed changes, the *Sph*I–*Eco*RV fragment was subcloned in phage M13mp18 to provide single-stranded template DNA. The substitution of the His codon (CAC) with that for Gln (CAA) or Lys (AAG) was performed by the procedure of Eckstein (Taylor *et al.*, 1985; Nakamaye & Eckstein, 1986) with the help of the Amersham kit. The plasmid and the 21-base deoxyoligonucleotides used to obtain the mutations and the general strategy are depicted in Fig. 1. The oligonucleotides were purchased from Eurogentec (Liège, Belgium). The clones were screened by sequencing the complete fragment subcloned in M13 and by immunodetection after transformation of *S. lividans* TK24 cells by the plasmid containing the whole gene. The absence of any other mutation was confirmed by resequencing the DD-peptidase gene using deoxyoligonucleotides, with priming at regular intervals.

Production and purification

The production of the modified DD-peptidases was achieved in a modified YEME medium as described by Erpicum *et al.* (1990). Concentrations of 100 mg/l were obtained when freshly transformed cells were used. Both mutants were found to exhibit a low level of activity with the tripeptide substrate Ac_2-L-Lys-D-Ala-D-Ala. Purification was as described previously (Fossati *et al.*, 1978) except that in the final steps, ion-exchange chromatography was performed with the help of a Pharmacia FPLC apparatus using Q-Sepharose fast-flow (Pharmacia, Uppsala, Sweden) as an ion-exchanger instead of DEAE-cellulose.

Enzymes, substrates, buffers and β-lactam compounds

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.);

Abbreviations used: PBP, penicillin-binding protein; T/H, transpeptidation/hydrolysis ratio; YEME, yeast extract/malt extract.

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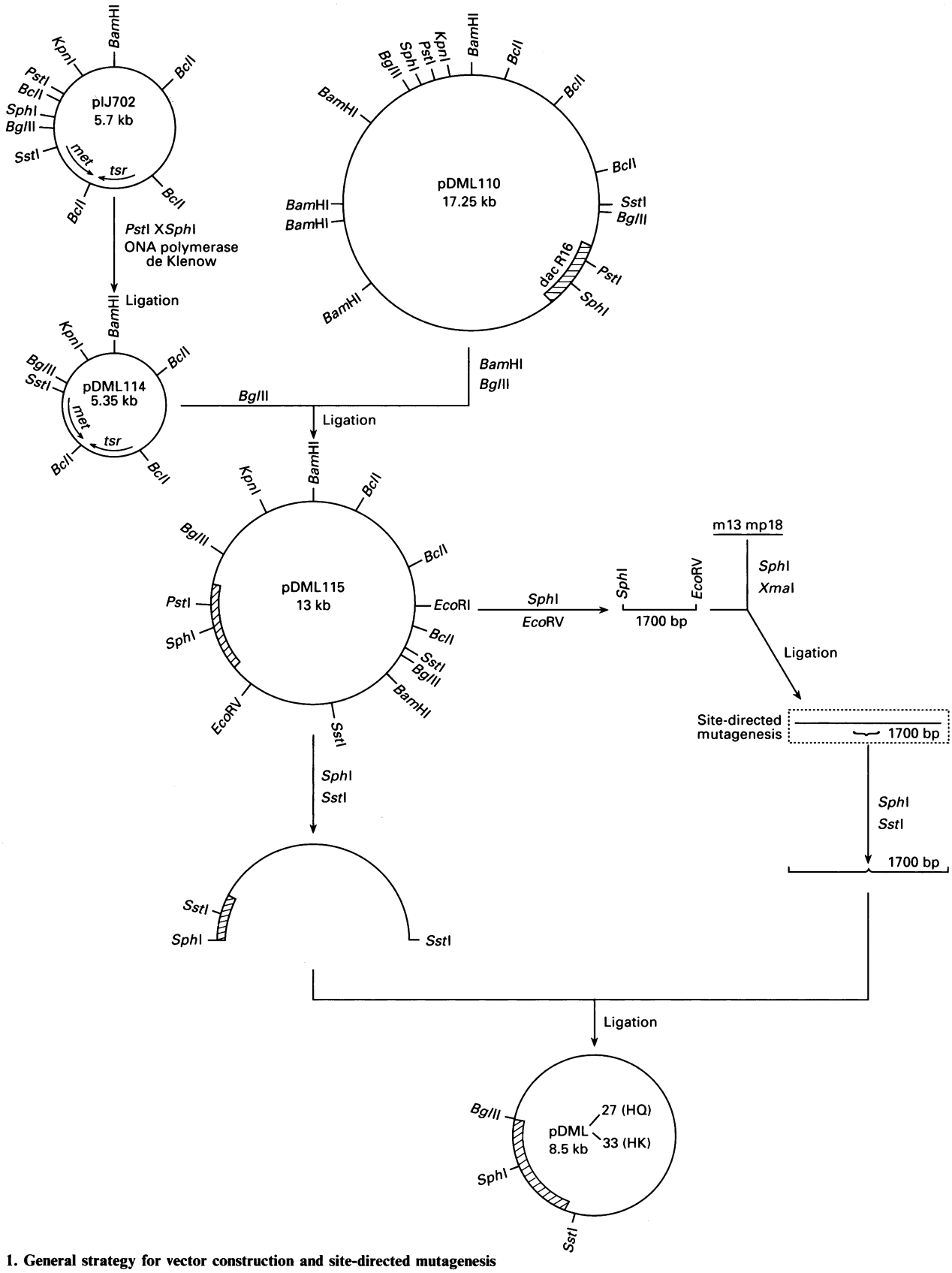


Fig. 1. General strategy for vector construction and site-directed mutagenesis

Boehringer (Mannhein, Germany) and Amersham International (Amersham, Bucks., U.K.). The tripeptide Ac_2-L-Lys-D-Ala-D-Ala was from UCB Bioproducts (Braine-l'Alleud, Belgium). The thioester substrate hippuryl thioglycollate (C_6H_5-CO-NH-CH_2CO-S-CH_2-COO^-) was synthesized in this laboratory (Adam *et al.*, 1990). Benzylpenicillin was from Rhône–Poulenc (Paris,

France) and cephalosporin C was from Eli Lilly & Co. (Indianapolis, IN, U.S.A.). Experiments were performed in 10 mM-sodium phosphate, pH 7.0.

Determination of kinetic parameters

Tripeptide. Hydrolysis of the tripeptide was monitored by estimating the amount of D-Ala released using the D-Amino acid oxidase coupled assay (Frère *et al.*, 1976*c*). 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. In transpeptidation experiments, D-Leu was used as an acceptor. The residual substrate and reaction products, Ac_2-L-Lys-D-Ala and Ac_2-L-Lys-D-Ala-D-Leu, were separated and quantified by h.p.l.c. The reaction mixture was injected into a C_{18} reverse-phase column. Buffer A was 10 mM-sodium phosphate, pH 7, and solvent B was acetonitrile. The areas under the peaks were estimated by integration.

Thioester. Hydrolysis and aminolysis of the thioester were monitored spectrophotometrically at 250 nm, using a $\Delta\epsilon$ value of 2200 $M^{-1}\cdot cm^{-1}$. With the wild-type enzyme, deacylation was found to be the rate-limiting step of the hydrolysis reaction, and addition of a suitable acceptor such as D-Ala resulted in proportional increases of the $k_{cat.}$ and K_m values (Jamin *et al.*, 1991). At low acceptor concentrations ([A]), these increases were linear with [A] and the slope of the line obtained when $k_{cat.}$ was plotted versus the acceptor concentration characterized the quality of an acceptor ($\Delta k_{cat.}/\Delta[A]$). The relative acceleration was defined as $\Delta k_{cat.}/(k_{cat.}\cdot\Delta[A])$.

Formation of the acyl-enzyme was monitored by fluorescence spectroscopy (Jamin *et al.*, 1991) using a stopped-flow apparatus (Bio-Logic SFM-3).

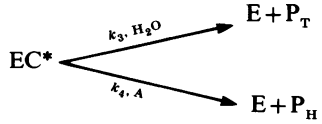
β-Lactams. Inactivation by the β-lactam antibiotics was monitored by measuring the decrease in enzyme fluorescence at 320 nm (Frère *et al.*, 1975) with a Perkin–Elmer MPF 44 spectrofluorimeter or by using the thioester as a reporter substrate (De Meester *et al.*, 1987).

The deacylation rate was determined by measuring the recovery of enzyme activity after complete inactivation and addition of β-lactamase to rapidly eliminate the excess of free antibiotic. Samples were then withdrawn after increasing periods of time and assayed for activity on the thioester substrate.

Thermal inactivation. The first-order rate constant for thermal inactivation was determined by monitoring the fluorescence decrease ($\lambda_{em.}$ = 320 nm; $\lambda_{exc.}$ = 280 nm) at 50 °C and 60 °C.

Kinetic background and behaviour of the wild-type enzyme

The interactions between the wild-type enzyme and substrate or β-lactam antibiotics have been shown to obey model 1 (see the Introduction section). In the presence of suitable acceptors, peptide and thioester substrates can undergo transpeptidation, which can be explained by a simple partition of the acyl-enzyme: where P_H and P_T are the products of hydrolysis and transpeptidation respectively. The various interactions present the following characteristics. (1) With the peptide substrate, k_2 is much smaller than k_3, so that no acyl-enzyme accumulates in the steady state (Varetto *et al.*, 1987*a*) and the presence of good acceptors fails to increase the rate of peptide utilization. In that case:



$$k_{cat.} = k_2, \text{ and } K_m = K' = (k_{-1}+k_2)/k_{+1} \quad (1)$$

(2) With the thioester substrate, k_2 is much larger than k_3, so that acyl-enzyme accumulates (Jamin *et al.*, 1991) and the presence of acceptors can increase the rate of thioester utilization if $[C_0]$ (i.e. concn. of substrate at time zero) is similar or larger than the K_m value of hydrolysis. In that case:

$$k_{cat.} = k_3\cdot\frac{(1+a[A])}{(1+b[A])}$$

and

$$K_m = (k_3/k_2)\cdot K'\frac{(1+a[A])}{(1+b[A])} \quad (2)$$

where a and b are groups of individual rate constants (Jamin *et al.*, 1991). The values of a and b were such that $k_{cat.}$ was linear versus [A] at low acceptor concentrations. (3) With β-lactam antibiotics, k_2 is much larger than k_3 but acceptors do not modify the rate of deacylation (Marquet *et al.*, 1979). Acylation and deacylation can be studied in separate experiments and, for $C_0 \gg E_0$, the pseudo-first-order rate constant for inactivation is:

$$k_a = \frac{k_2[C]}{K+[C]} \quad (3)$$

where $K = k_{-1}/k_{+1}$, since $k_2 \ll k_{-1}$. Note that, in all cases:

$$k_{cat.}/K_m = k_2/K' \quad (4)$$

RESULTS

Conformation of the proteins

The replacement of residue His-298 by either Gln or Lys did not seem to result in gross modifications of the protein structure. Firstly, the maximum of fluorescence emission was at 320 nm, the low value characteristic of the wild-type enzyme. Secondly, the stabilities of the proteins were not markedly different (see Table 1). Thirdly, the mutants retained penicillin-binding properties and a significant, although sometimes markedly decreased, activity with their substrates.

Interaction with the peptide substrate

The kinetic parameters for the hydrolysis of the tripeptide substrate are summarized in Table 2. The $k_{cat.}/K_m$ values for the mutants were decreased 100–200-fold. With the His298Gln mutant, no acceleration of tripeptide utilization was observed when 20 mM-D-Leu (a good acceptor; Jamin *et al.*, 1991) was

Table 1. Rates of thermal inactivation of wild-type and mutant enzymes

	Wild-type	His298Gln	His298Lys
$10^3\times k_d$ at 50 °C (s^{-1})	0.36±0.03	0.33±0.04	0.39±0.01
$10^3\times k_d$ at 60 °C (s^{-1})	3.3±0.4	3.6±0.7	3.6±0.6

Table 2. Kinetic parameters for the hydrolysis of the tripeptide substrate by the wild-type and mutant DD-peptidases at 37 °C

	Wild-type*	His298Gln	His298Lys
$k_{cat.}$ (s^{-1})	55	0.53±0.02	≫ 0.8
K_m (mM)	12	21±0.6	≫ 21
$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	4600	25±2	37±0.5

* Frère & Joris (1985).

Table 3. Characteristics of the transpeptidation reaction catalysed by the wild-type and mutant enzymes at 37 °C

For measurement of the tripeptide T/H ratio, the tripeptide and D-Leu concentrations were 6 mM and 60 mM respectively. The thioester T/H ratio was determined on the basis of the increase of the $k_{cat.}$ value. The acceptor (D-Leu) concentration was 3 mM. S.D. values for the various measurements were less than 10% unless otherwise indicated.

Enzyme	Tripeptide T/H ratio	Thioester T/H ratio	Thioester $\Delta k_{cat.}/\Delta[A]$ ($mM^{-1}\cdot s^{-1}$)	Thioester Relative acceleration $\Delta k_{cat.}/k_{cat.}\cdot\Delta[A]$ (mM^{-1})
Wild-type	5.40±0.07	2.45	4.74	0.78
His298Gln	0.072±0.004	0.2	0.07	0.05
His298Lys	0.27±0.05	0.6	0.20	0.21

Table 4. Kinetic parameters for the hydrolysis of the thioester substrate by the wild-type and mutant DD-peptidases at 37 °C

	Wild-type	His298Gln	His298Lys
$k_{cat.}$ (s^{-1})	6±0.2	1.44±0.03	0.96±0.03
K_m (μM)	50±2	187±6	24±1
$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	120000	10000±600	44000±3000

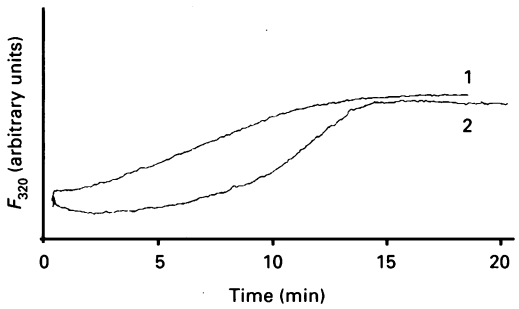


Fig. 2. Recording of fluorescence obtained by manually mixing samples of mutant enzymes (1 μM) with 400 μM-thioester substrate

Final concentrations are given. The experiments were performed in 1 ml of 10 mM-sodium phosphate, pH 7.0. Curve 1, His298Gln; curve 2, His298Lys.

added to an incubation mixture containing 21 mM-tripeptide, indicating that acylation remained rate-limiting with the modified enzyme. With the same acceptor, the P_T/P_H (or T/H) ratios were decreased in both mutants when compared with the wild-type enzyme (Table 3).

Interaction with the thioester substrate

For the mutants, the thioester (Table 4) remained a better substrate than the peptide. In all cases, the k_3 values were smaller than the k_2 values, as indicated by the accumulation of acyl-enzyme which could be visualized both by fluorescence (Fig. 2) and by the increase in $k_{cat.}$ upon addition of D-Ala (a good acceptor; Jamin *et al.*, 1991).

With the His298Gln mutant, the formation of acyl-enzyme was followed after rapid mixing in the stopped-flow apparatus. Under these conditions, the pseudo-first order rate constant for acyl-enzyme accumulation is given by:

$$k_a = k_3 + (k_2[C])/(K' + [C]) \quad (5)$$

Fig. 3(*a*) shows a time course of the fluorescence decrease after mixing of the His298Gln mutant with the thioester substrate, and Fig. 3(*b*) shows the variation of k_a versus [S]. Fitting of these experimental data to eqn. (5) yielded $k_2 = 81\ s^{-1}$, $K' = 12$ mM and a value of $6700\ M^{-1}\cdot s^{-1}$ for k_2/K' at 30 °C, in excellent agreement with that determined by steady-state kinetics ($10000\ M^{-1}\cdot s^{-1}$ at 37 °C; Table 4). The individual values of k_2 and K' could be compared with those obtained with the wild-type enzyme: $480\ s^{-1}$ and 8 mM respectively at 30 °C (M. Jamin & J.-M. Frère, unpublished work). If the hydrolytic activity remained high, both mutants became very poor transpeptidases. With the His298Gln mutant, the T/H ratio with D-Ala was decreased by a factor of more than 10 when compared with the wild type (Table 3). Note that, with both mutants, the T/H ratio decreased by the same factor as the relative acceleration, i.e. 12–15-fold for His298Gln and 4-fold for His298Lys.

Interaction with β-lactams

Inactivation by benzylpenicillin and cephalosporin C also became considerably slower with both modified enzymes, but the second-order rate constants for acylation (k_2/K) were somewhat larger with the His298Lys mutant than with the His298Gln mutant (Table 5). With the former protein, the rate of recovery of the enzymic activity was also significantly increased.

DISCUSSION

The most remarkable result in the present study was the fact that the ability of the two mutant enzymes to catalyse transpeptidation reactions was severely affected. In fact, both proteins behaved almost exclusively as hydrolases. This was particularly striking in the case of the thioester substrate, with which both acylation and deacylation were not very strongly affected in the mutants, an observation which might be related to the fact that thioesters are not very specific substrates for the peptidases, since they are also hydrolysed by the β-lactamases of classes A and C. With the wild-type enzyme, transpeptidation reactions performed with the thioester as acyl donor substrate are particularly easy to visualize (Jamin *et al.*, 1991), and impaired transpeptidation activities of the mutants were immediately apparent.

At neutral pH a very large proportion of the acceptor amino group is protonated, and it was suggested a long time ago that an enzyme side chain of pK ~ 7 could be involved in the deprotonation of this group (Frère *et al.*, 1973), acting as a general base

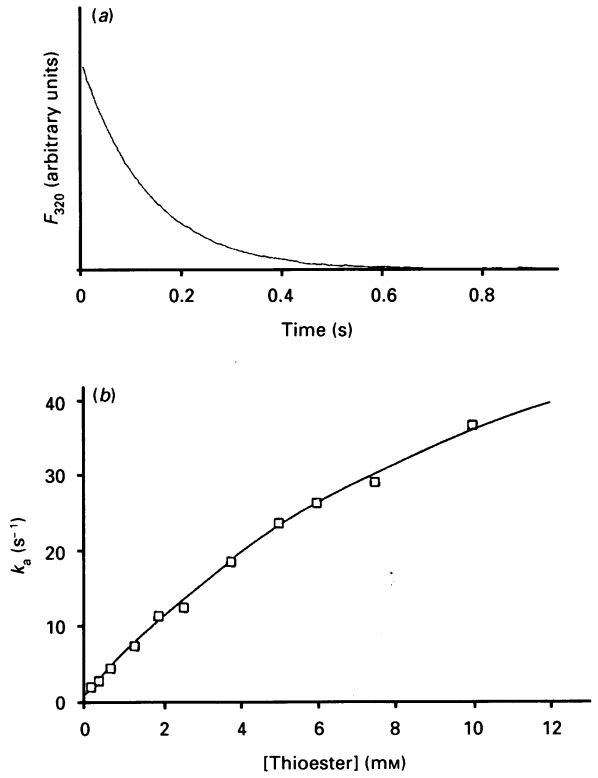


Fig. 3. Measurement of the k_2 value for the His298Gln mutant and the thioester

(*a*) Quenching of fluorescence was monitored in the stopped-flow system. The enzyme (150 μl, 3.3 μM) was mixed with the substrate (150 μl, 2500 μM) and the fluorescence was recorded through a 310–490 nm band-pass filter at 30 °C. The excitation wavelength was 290 nm. (*b*) Plot of k_a versus thioester concentration. The data were fitted to eqn. (4) using a k_3 value of 1.44 s^{-1}, as determined by steady-state kinetics. The solid line is the theoretical curve obtained with the k_2 and K' values given in the text.

Table 5. Kinetic parameters for the interaction between β-lactams and the wild-type and mutant enzymes at 37 °C

ND, not done.

	Wild-type*	His298Gln	His298Lys
Benzylpenicillin			
k_2 (s^{-1})	180	0.14	0.086
K (mM)	13	1.27	0.36
k_2/K ($M^{-1} \cdot s^{-1}$)	13000	118±10	240±11
$10^4 \times k_3$ (s^{-1})	1.4	0.94±0.2	5.5±0.08
Cephalosporin C			
k_2 (s^{-1})	> 1	0.013	N.D.
K (mM)	> 1	0.6	N.D.
k_2/K ($M^{-1} \cdot s^{-1}$)	1500	26±3.5	120
$10^6 \times k_3$ (s^{-1})	1	1.3±0.04	63±2.6

* Frère *et al.* (1975).

and thus transforming it into a suitable nucleophile for attacking the acyl-enzyme. That the His298Lys mutant was a somewhat better transpeptidase than the His298Gln mutant could be explained by the fact that a small proportion of the Lys side chain might be deprotonated at neutral pH.

However, acylation of the mutant enzymes by the peptide and β-lactams was also markedly impaired, suggesting an important role for His-298 in acyl-enzyme formation with these compounds. It has been shown that the rate of acylation by the peptide and the β-lactam antibiotics decreases at high pH, an observation which suggested an interaction of the substrate or inactivator carboxylate with a positively charged group of pK 9–9.5 (Varetto *et al.*, 1987*a*, *b*). If this group were assumed to be His-298, its pK would be 3 pH units larger than that of a free imidazolium group. Moreover, in the acyl-enzyme, the pK of the same His side chain should return to a normal value, close to 7, to explain its role in the catalysis of transpeptidation, where the deprotonated form would be the efficient one.

In all other known penicillin-recognizing enzymes, the corresponding residue is a Lys. The study of Lys → His mutants in other PBPs will thus be of particular interest. Unfortunately, no such mutant has yet been studied, but the mutation has been performed in the class A β-lactamase of *Streptomyces albus* G (Brannigan *et al.*, 1991). At low pH (< 6), the mutant was nearly as active as the wild-type enzyme, and the pH-dependence of the $k_{cat.}$ suggested the participation of a group of pK ~ 6.5, which could be identified as the newly introduced His residue. These results indicated that this residue played a prominent role in transition state stabilization. Conversely, the mutant did not exhibit increased transpeptidation catalysis.

These results underlined the importance of a positively charged group in that particular position, but comparison of the two pairs of enzymes (His-298 and Lys-298 transpeptidase; His-234 and Lys-234 β-lactamase) did not yield a coherent picture, suggesting different roles for residues situated in identical positions in the two types of enzymes.

Finally, another interesting observation was that the His298Lys mutation somewhat increased the rate of recovery of enzyme activity after inactivation by the β-lactams. Indeed, the mutation made the peptidase much more similar to β-lactamases, but for most PBPs, where this residue is also a Lys, the k_3 value is generally low or very low. Moreover, degradation of the benzylpenicilloyl-R61 adduct does not occur by a simple hydrolysis reaction (Frère *et al.*, 1976*b*), and the k_3 value cannot be directly compared with those observed with β-lactamases.

In conclusion, residue His-298 appears to play two distinct roles in the catalytic mechanism of the *Streptomyces* R61 DD-peptidase. Firstly, it contributes a factor of 200 to the rate of acylation by the tripeptide and a factor varying from 12 (cephalosporin C, His298Lys) to 100 (benzylpenicillin, His298Gln) to the rate of acylation by β-lactams, whereas with the thioester its participation seems to be less significant. Secondly, although this residue does not seem to play a major role in the deacylation phenomenon when only water is involved (the $k_{cat.}$ value for the thioester which directly reflects k_3 is not strongly decreased and, unfortunately, the k_3 value for the peptide cannot be determined), it appears to play a specific and essential role in the proper positioning and/or reactivity of the amino group of the acceptor substrate. Furthermore, comparison with the class A β-lactamase indicates that identical residues might not play identical roles in the two enzymes.

Hopefully, the elucidation of the 3-dimensional structure of the R61 DD-peptidase (Kelly *et al.*, 1989) will make it possible in the near future to assess the validity of those hypotheses by molecular modelling.

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