Accumulation of acyl-enzyme in DD-peptidase-catalysed reactions with analogues of peptide substrates

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Thioester substrates can be used to study the hydrolysis and transfer reactions catalysed by β-lactamases and DD-peptidases. With the latter enzymes, accumulation of the acyl-enzyme can be detected directly. The efficiency of various amines as acceptor substrates was in excellent agreement with previous results obtained with peptide substrates of the DD-peptidases. The results indicated the presence of a specific binding site for the acceptor substrates. Although most of the results agreed well with a simple partition model, more elaborate hypotheses will be needed to account for all the data presented.

INTRODUCTION

Penicillin-sensitive DD-peptidases and β-lactamases catalyse the hydrolysis of various depsipeptides (Pratt & Govardhan, 1984; Govardhan & Pratt, 1987; Adam *et al.*, 1990) and, if a suitable amine is supplied as carbonyl acceptor, a concomitant transfer reaction (Pratt & Govardhan, 1984). Recently some of those enzymes were found to hydrolyse efficiently simple thioesters such as carboxymethyl benzoylaminothioacetate, and the reaction could easily be continuously monitored either directly at 250 nm (Adam *et al.*, 1990) or by measuring the appearance of the thiol group of mercaptoacetate by reaction with a thiol-reactive reagent (J. A. Kelly, S. G. Waley & J.-M. Frère, unpublished work). Thus the introduction of those substrates represents a major practical advance in the study of DD-peptidases, for which activity assays had always been rather cumbersome (Frère *et al.*, 1976). Moreover, some of those substrates also exhibited relatively low K_m values with the DD-peptidases. Since two different substrates containing the same acyl group exhibited the same $k_{cat.}$ value with the R61 DD-peptidase, it could be suspected that for the first time with that enzyme a substrate with rate-limiting deacylation had been found.

In the present paper we have studied the acyl transfer reactions catalysed by the various enzymes with the thioesters as acyl donor substrates and α-amino acids, peptides and α-hydroxy acids as acceptors. We also describe successful attempts to detect directly the accumulation of acyl-enzyme and the influence of acceptors on the rate of utilization of the donor thioester.

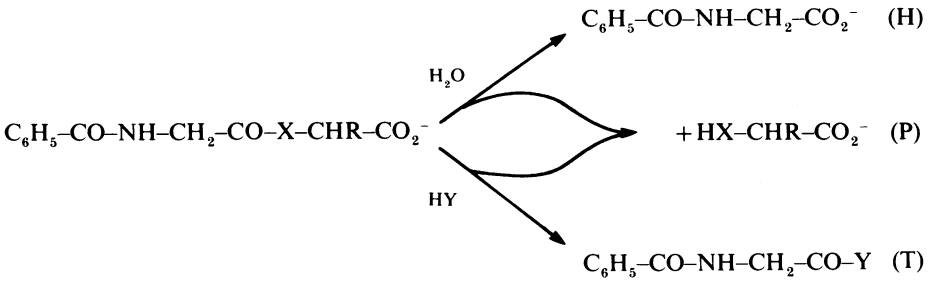
MATERIALS AND METHODS

Materials

The various enzymes were produced as described by Erpicum *et al.* (1990) and purified by the method of Matagne *et al.* (1990) for the β-lactamases and by the methods of Fossati *et al.* (1978) and Frère *et al.* (1974) for the DD-peptidases of *Streptomyces* R61 and *Actinomadura* R39 respectively.

The substrates were prepared as previously described (Adam *et al.*, 1990). The expected products of the transfer reaction with D-alanine and Gly-Gly were synthesized by benzoylation at pH 9.5 of commercially available Gly-D-Ala and Gly-Gly-Gly (Sigma Chemical Co., St. Louis, MO, U.S.A.) respectively. Both synthesized products were identified by m.s. with ionization by fast atom bombardment: $(M+H)^+ = 251$ (Fig. 1, P1) and 294 (Fig. 1, P2). The structures of the substrates and products are given in Fig. 1.

The various enzymes studied here catalysed both transfer and hydrolysis reactions with ester (X = O) and thioester (X = S) substrates according to the following scheme:



where HY can be an amine or an alcohol. In the following description the initial esters and thioesters are referred to as donor and HY as acceptor substrates. Similarly H and T represent the products of the hydrolysis (hippuric acid) and of the transfer reaction respectively. The same scheme applies to the substrate S2b.

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[T]/[H] ratio

The residual substrate and the products were separated by h.p.l.c. on an ET 250/8/4 Nucleosil-5 C_{18} column (Macherey–Nagel). The detailed conditions are described in Table 1. Quantification was achieved by integrating the areas of the various peaks with the use of hippuric acid and the synthesized products as standards.

Kinetic experiments with the DD-peptidases

All kinetics experiments were performed in 10 mM-sodium phosphate buffer, pH 7.0.

Absorbance measurements

The spectrophotometric measurements were made at 37 °C with a UVIKON 860 spectrophotometer linked to a microcomputer by an RS232 interface. The cuvette optical path length (1, 2, 4 or 10 mm) was chosen so that the initial absorbance was always below 1.5. Reactions were always initiated by adding enzyme. In hydrolysis and aminolysis reactions the disappearance of substrate S2a was monitored at 250 nm and kinetic parameters were computed by analysing the complete time courses (De Meester *et al.*, 1987). The $\Delta\epsilon$ at 250 nm for the hydrolysis of substrates S2a and S2c was 2000 $M^{-1} \cdot cm^{-1}$. In aminolysis reactions the $\Delta\epsilon$ for the global reaction depended on the structure of the acceptor and its concentration. This latter variation reflected the proportion of the reaction that was channelled through the aminolysis pathway. At each acceptor concentration the global $\Delta\epsilon$ was carefully determined.

In alcoholysis reactions of substrate S2a the global reaction was monitored either directly at 250 nm or at 324 nm in the presence of 2 mM-4,4′-dithiobipyridyl (Sigma Chemical Co.), which reacts with the thiol group of the mercaptoacetic acid (J. A. Kelly, S. G. Waley & J.-M. Frère, unpublished work) ($\Delta\epsilon$ = 20000 $M^{-1} \cdot cm^{-1}$). Under those conditions the coupling reaction was faster than the disappearance of the donor substrate.

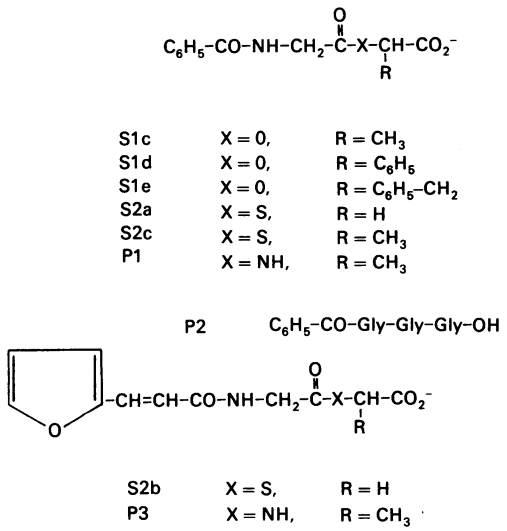


Fig. 1. Structures of the various substrates and products of the transfer reactions

All substrates containing an asymmetric carbon were racemic mixtures. As shown by Adam *et al.* (1990), only the D-isomer is utilized by the R61 DD-peptidase, with the possible exception of substrate S2c. This problem is not discussed further in the present paper.

Fluorescence experiments

Spectrofluorimetric measurements were made with a Perkin–Elmer MPF 44 fluorescence spectrophotometer equipped with a thermostatically controlled 0.4 cm × 1 cm cell. Excitation was at 280 nm and emission was at 320 nm. Experiments were performed at 25 °C.

Pre-steady-state kinetics

Under pre-steady-state conditions the reaction between S2a and the R61 DD-peptidase could be followed by monitoring either the absorbance at 250 nm or the intrinsic fluorescence of the protein.

Rapid kinetic measurements were performed with a Bio-Logic SFM3 stopped-flow apparatus. The mechanical system was enclosed in a thermostatically controlled water jacket. The syringe plungers were driven by stepping motors controlled by a Bio-Logic MPS51 microprocessor power supply (the dead time was about 5 ms). Kinetic data were accumulated and analysed with a Tandon microcomputer interfaced to the SFM3 module using the Bio-Logic BioKine hardware and software packages. One syringe contained the substrate and the other the enzyme solutions; 150 μl of each solution was used in each reaction. The total flow rate was 6 ml/s. In fluorescence experiments the photomultiplier tube was set up at a right-angle to the incident beam. A monochromatic light (290 nm) was used for excitation, and the fluorescence emission was recorded through a 310–490 nm-band-pass filter. In absorbance experiments a mono-

Table 1. H.p.l.c. conditions for separation of the various substrates and their hydrolysis and transfer products

The solvent systems were as follows: (*a*) A, 0.1 % (v/v) trifluoroacetic acid in water, and B, 0.1 % (v/v) trifluoroacetic acid in acetonitrile; (*b*) A, 0.015 % (v/v) triethylamine in 140 mM-sodium acetate buffer, pH 6.5, and B, 0.015 % (v/v) triethylamine in aq. 60 % (v/v) acetonitrile, pH 6.5; (*c*) 20 % (v/v) acetonitrile in 10 mM-sodium acetate buffer, pH 3.0. Flow rate was 1 ml/min in all experiments. S, T and H represent respectively the unchanged substrate, the product of transfer and that of hydrolysis, i.e. hippuric acid with all substrates except S2b.

Substrate	Detection (nm)	Solvent system	Concn. of buffer B in elution solvent (%, v/v)	Retention time (min)
Aminolysis reactions				
S1c	214	(*a*)	0–8 min, 0 %; 8–20 min, 0–70 %	H, 8.3; T, 12.7; S, 14.1
S1d	214	(*a*)	0–20 min, 0–35 %	H, 6.8; T, 8.4; S, 18.2
S1e	254	(*b*)	0–8 min, 0 %; 8–20 min, 0–70 %	H, 4.5; T, 7.3; S, 17.3
S2a	214	(*a*)	0–13 min, 0–17 %	H, 6.5; T, 8.2; S, 11.0
S2c	214	(*a*)	0–15 min, 0–60 %	H, 8.8; T, 13.0; S, 15.2
Alcoholysis reactions				
S2a	235	(*c*)	–	H, 9.4; T, 23.1; S, 29.2
S2b	235	(*c*)	–	H, 9.4; T, 23.1; S, > 30.0

chromatic light (250 nm) was used and the recorded signal was converted into absorbance values by the microcomputer.

RESULTS

Stability of the aminolysis and alcoholysis products

The products obtained by aminolysis of the various esters and thioesters were not substrates of β-lactamases, as observed by Pratt & Govardhan (1984), and were extremely poor substrates of DD-peptidases. For instance, after a 24 h incubation of 500 μM-hippuryl-D-alanine (Fig. 1, P1) at 37 °C in the presence of 11 μM *Streptomyces* R61 DD-peptidase, less than 5% of hippuric acid was produced (i.e. $k_{cat.} < 2.5 \times 10^{-5}\ s^{-1}$ if $K_m < 500\ \mu M$ or $k_{cat.}/K_m < 6 \times 10^{-2}\ M^{-1} \cdot s^{-1}$ if $K_m > 500\ \mu M$).

Conversely, the alcoholysis products were generally also substrates of the β-lactamases and DD-peptidases (Adam *et al.*, 1990). Moreover, the absorption coefficient of hippuric acid at 250 nm is larger than those of the esters and lower than those of the thioesters, and in consequence biphasic curves were observed

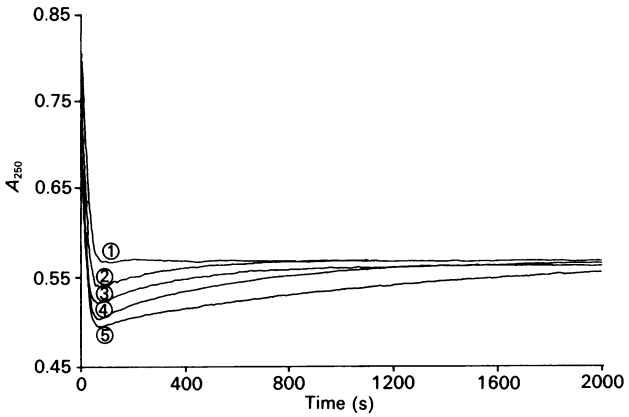


Fig. 2. Alcoholysis of substrate S2a by the R61 DD-peptidase

Substrate S2a (150 μM) and D-lactate at various concentrations [0 mM (1), 1 mM (2), 2 mM (3), 5 mM (4) and 10 mM (5)] were incubated at 37 °C. The reactions were initiated by addition of the R61 DD-peptidase (the final concentration of enzyme was 1 μM).

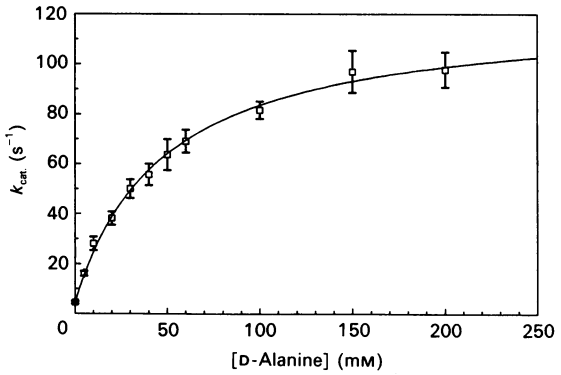


Fig. 3. Influence of the D-alanine concentration on the $k_{cat.}$ value for utilization of substrate S2a by the R61 DD-peptidase

Initial donor substrate concentrations were in the range $1.5 \times K_m$ to $3 \times K_m$. The cuvette optical-path length was adequately adjusted. Final enzyme concentrations ranged from 12 to 30 μg/ml. The curve is theoretical and was fitted to eqn. (1) by least-squares regression. The following values were obtained for the parameters: $a = 4.5 \pm 0.5\ s^{-1}$, $b = 2500 \pm 100\ M^{-1} \cdot s^{-1}$ and $c = 20.6 \pm 1.3\ M^{-1}$. The $k_{cat.}$ value at acceptor saturation was $117 \pm 3\ s^{-1}$.

Table 2. [T]/[H] ratio observed in experiments involving various enzymes and donor and acceptor substrates

In each case the ratio is given for various acceptor concentrations. The percentage in parentheses indicates the proportion of donor substrate utilized. The errors of the [T]/[H] ratios were ±20%.

Substrate	Concn. of donor (mM)	β-Lactamases			DD-Peptidases	
		E. cloacae P99	*S. albus* G	*B. licheniformis*	*Streptomyces* R61	*Actinomadura* R39
Acceptor: D-alanine						
S1c	0.5	N.D.	N.D.	N.D.	2 mM, 0.97 10 mM, 4.5 (20%)	N.D.
S1d	0.7	20 mM, 0.15 40 mM, 0.2 (40%)	20 mM, 0 40 mM, 0.1 (40%)	N.D.	10 mM, 5.9 20 mM, 10.1 (40%)	N.D.
S1e	0.3	20 mM, 0.10 40 mM, 0.20 (45%)	N.D.	N.D.	10 mM, 6.0 20 mM, 8.7 (35%)	N.D.
S2a	0.3	20 mM, 0.06 40 mM, 0.07 (15%)	40 mM, < 0.01 (25%)	40 mM, < 0.01 (25%)	10 mM, 2.5 20 mM, 4.4 (40%)	10 mM, 1.7 20 mM, 3.3 (25%)
S2c	0.5	N.D.	40 mM, < 0.01 (40%)	40 mM, 0.22 (40%)	10 mM, 3.7 (20%)	10 mM, 3.1 (20%)
Acceptor: D-lactate						
S2a	0.25	10 mM, 0.6 20 mM, 1.2 (< 10%)	N.D.	20 mM, 0.04 40 mM, 0.09 (< 10%)	10 mM, 11.7 (< 10%)	10 mM, 4.6 (< 10%)
S2c	0.25	10 mM, 0.45 (< 10%)	N.D.	20 mM, 0.03 (< 10%)	10 mM, 9.4 (< 10%)	10 mM, 4.6 (< 10%)
Acceptor: D-mandelate						
S2a	0.25	10 mM, 5.4 (< 10%)	N.D.	10 mM, 0.34 (< 10%)	10 mM, 2.9 (< 10%)	N.D.

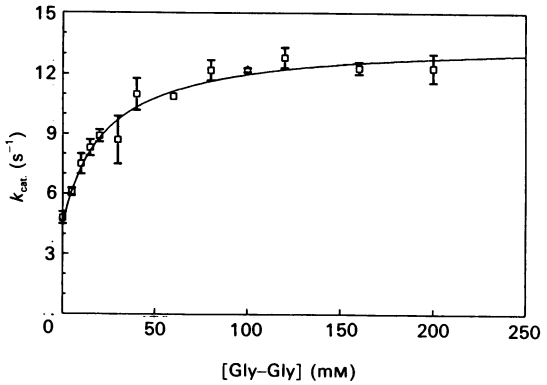


Fig. 4. Influence of the Gly-Gly concentration on the $k_{cat.}$ value for the utilization of substrate S2a by the R61 DD-peptidase

The continuous curve was fitted on eqn. (1): $a = 4.5 \pm 0.5\ s^{-1}$, $b = 600 \pm 80\ M^{-1} \cdot s^{-1}$ and $c = 44.4 \pm 6.8\ M^{-1}$. The $k_{cat.}$ value at acceptor saturation was $13.7 \pm 0.5\ s^{-1}$ and reflected the value of k_5 in the model.

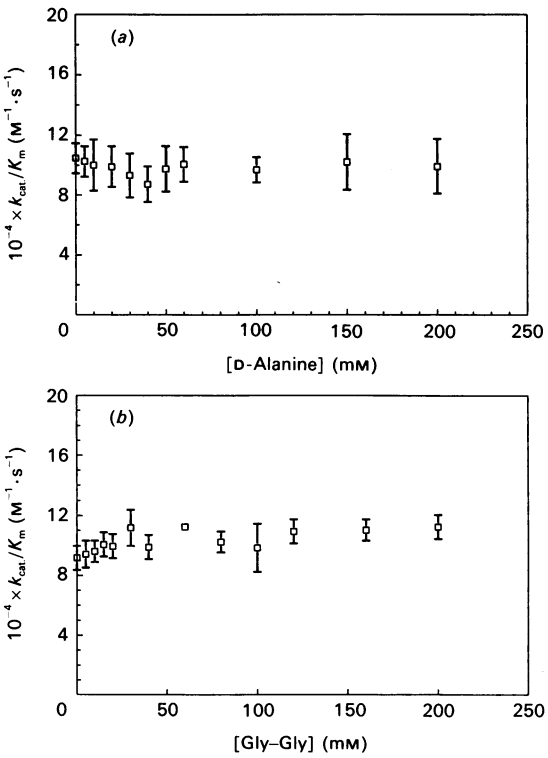


Fig. 5. Influence of D-alanine (*a*) and Gly-Gly (*b*) concentrations on the $k_{cat.}/K_m$ ratio with the R61 DD-peptidase

Conditions were as in Figs. 3 and 4. the $k_{cat.}/K_m$ values were computed from the individual $k_{cat.}$ and K_m values.

by monitoring the absorbance at 250 nm of mixtures containing the thioester, an adequate alcohol and the enzyme (Fig. 2). With the R61 DD-peptidase, during the first phase a rapid decrease of absorbance was recorded, this being due to the disappearance of the thioester and the accumulation of the ester. The second phase, during which the absorbance increased, corresponded to the hydrolysis of the ester formed during the first phase. The relative durations of the two phases reflected the fact that the thioesters

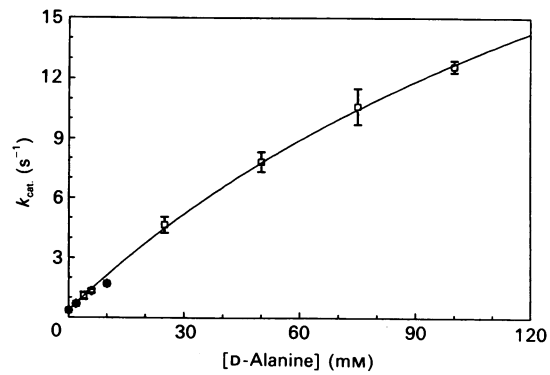


Fig. 6. Influence of D-alanine concentrations on the $k_{cat.}$ value for the utilization of substrate S2a by the R39 DD-peptidase

Enzyme concentration was 112 μg/ml. The continuous curve is theoretical and was fitted on eqn. (1): $a = 0.36 \pm 0.02\ s^{-1}$, $b = 210 \pm 18\ M^{-1} \cdot s^{-1}$ and $c = 6.8 \pm 1.6\ M^{-1}$. The $k_{cat.}$ value at acceptor saturation was $31 \pm 5\ s^{-1}$.

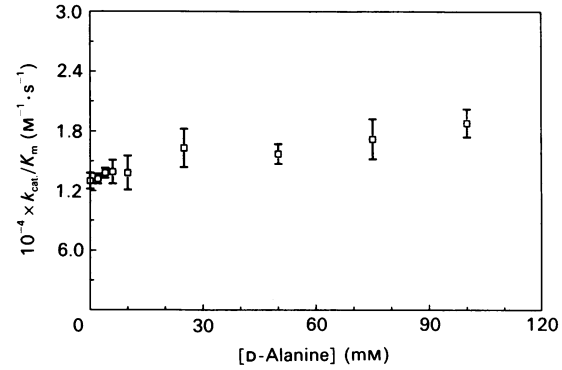


Fig. 7. Influence of D-alanine concentration on the $k_{cat.}/K_m$ ratio with the R39 DD-peptidase

The $k_{cat.}/K_m$ values were computed from the individual $k_{cat.}$ and K_m values.

were much better substrates of that enzyme than the esters, although the hydrolysis of the esters might have been considerably slowed down by the presence of free alcohol, which could compete with water.

Aminolysis and alcoholysis reactions

The ability of the different DD-peptidases and β-lactamases to perform acyl-transfer reactions was studied by measuring the aminolysis/hydrolysis and alcoholysis/hydrolysis ratios. The results shown in Table 2 indicated that the DD-peptidases performed the acyl-transfer reaction with a high efficiency. With the class C β-lactamase D-mandelic acid was the only good acceptor, and the class A β-lactamases were quite inefficient transferases.

Aminolysis of substrate S2a by the DD-peptidases

A detailed study of the concomitant hydrolysis and aminolysis reactions with substrate S2a and the R61 and R39 DD-peptidases was performed. Substrate S2a was chosen because the global reaction could be followed spectrophotometrically and the values of the individual parameters $k_{cat.}$ and K_m could easily be determined even at high acceptor concentrations.

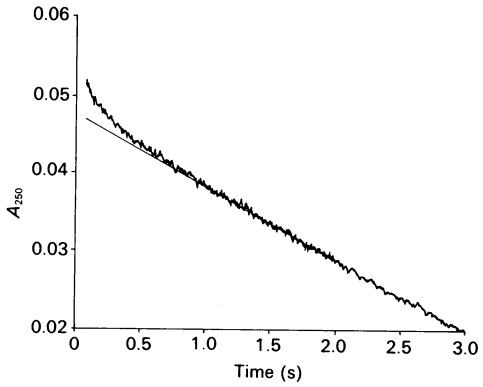


Fig. 8. Variation of the absorbance at 250 nm upon mixing of the R61 DD-peptidase and substrate S2a in the stopped-flow apparatus thermostatically controlled at 15 °C

Final concentrations were 13.2 μM-DD-peptidase and 250 μM-substrate. The treatment of the burst by a first-order fitting procedure yielded an apparent first-order rate constant of 5 ± 1 s^{-1}. Assuming $[S] \ll K'_S$, this would yield a value of 20000 ± 4000 $M^{-1} \cdot s^{-1}$ for the k_2/K'_S, which compares quite well with the $k_{cat.}/K_m$ value of 17500 ± 1000 $M^{-1} \cdot s^{-1}$ measured at the same temperature by analysing a complete time course.

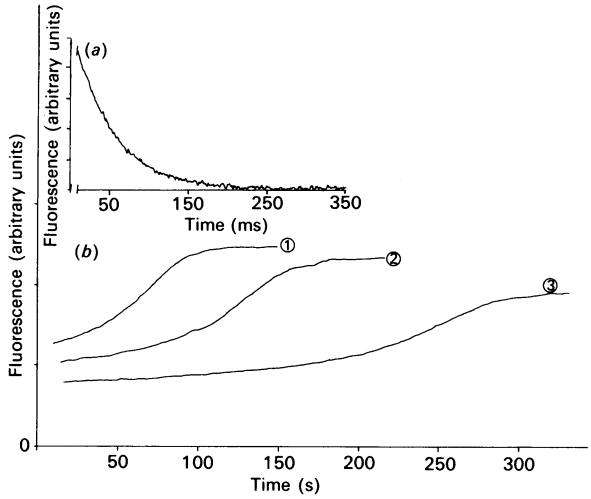


Fig. 9. (*a*) Change in the fluorescence of the enzyme upon mixing of the R61 DD-peptidase and substrate S2a in the stopped-flow apparatus at 30 °C and (*b*) time course of change in the fluorescence of the enzyme as a function of substrate S2a concentration

(*a*) Change in the fluorescence of the enzyme upon mixing of the R61 DD-peptidase and substrate S2a in the stopped-flow apparatus at 30 °C. The excitation wavelength was 290 nm and the emission signal was recorded through a 310–490 nm-band-pass filter. Final concentrations were 625 μM-substrate and 6.6 μM-R61 DD-peptidase. (*b*) Time course of change in the fluorescence of the enzyme as a function of substrate S2a concentration. Enzyme was incubated at 25 °C and reactions were initiated by adding the substrate. Final concentrations were: R61 DD-peptidase 1.6 μM and substrate S2a 0.25 mM (1), 0.50 mM (2) and 1.00 mM (3).

Global kinetic parameters

The global kinetic parameters $k_{cat.}$ and K_m for the hydrolysis of substrate S2a were 5 ± 1 s^{-1} and 50 ± 10 μM for the R61 DD-peptidase and 0.35 ± 0.05 s^{-1} and 30 ± 5 μM for the R39 DD-peptidase (Adam *et al.*, 1990).

In the presence of an adequate acceptor substrate and at a saturating concentration of donor substrate an increase of the

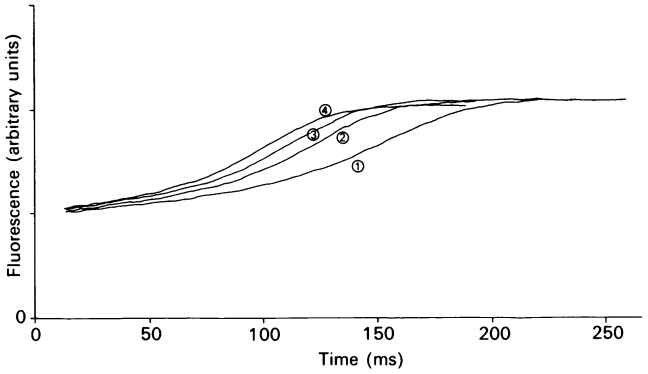


Fig. 10. Influence of acceptor substrate concentration (Gly-Gly) on the rate of enzyme fluorescence recovery

Enzyme and acceptor substrate were incubated at 25 °C and the reactions were initiated by adding substrate S2a. Final concentrations were R61 DD-peptidase 1.6 μM, substrate S2a 0.5 mM, and Gly-Gly 0 mM (1), 3.1 mM (2), 6.2 mM (3) and 12.4 mM (4).

global reaction rate was observed. The parameters $k_{cat.}$ and K_m for donor utilization were determined at different concentrations of D-alanine and Gly-Gly (Figs. 3 and 4). Both parameters increased with the acceptor concentration in a non-linear way. The dependence of the $k_{cat.}$ parameter fitted the empirical equation:

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

Conversely, the $k_{cat.}/K_m$ ratio remained constant within the investigated range of acceptor concentrations. Figs. 5(*a*) and 5(*b*) show the results obtained with D-alanine and Gly-Gly respectively as acceptor.

With the R39 enzyme results were obtained with two different donor substrates. In agreement with previously published results, the enzyme did not catalyse transpeptidation with Gly-Gly as acceptor (Perkins *et al.*, 1973) and experiments were only performed with D-alanine. The results obtained with substrate S2a are shown in Figs. 6 and 7 and are similar to those obtained with the R61 enzyme. An acceleration of the utilization of substrate S2b in the presence of D-alanine was also observed. Those results indicated that with the thioester substrates acylation was faster then deacylation for both enzymes.

Pre-steady-state kinetics

The hydrolysis of substrate S2a by the R61 DD-peptidase was followed by monitoring the absorbance at 250 nm in the stopped-flow apparatus. With a 250 μM concentration of substrate, i.e. $5 \times K_m$, a burst was observed (Fig. 8), the amplitude of which was proportional to the enzyme concentration. With that substrate, the absorbance decrease is due to the disappearance of the thioester bond, and that result indicated that the burst more probably corresponded to the accumulation of acyl-enzyme than of the Henri–Michaelis complex ES.

Fluorescence kinetics

Further evidence for acyl-enzyme accumulation was obtained by monitoring the fluorescence of the R61 enzyme during the reaction. A two-phase reaction was observed. The first phase, on the time scale of the stopped-flow apparatus, was a monoexponential decrease (Fig. 9*a*). The apparent first-order rate constant of this process, obtained by a non-linear least-square fitting procedure, increased with the concentration of donor substrate. At 30 °C the plot of the apparent rate constant k_a versus substrate

Table 3. Acceleration of utilization of substrate S2a by various potential acceptors with the R61 DD-peptidase

The acceleration factor is $\Delta k_{cat.}/\Delta[A]$ (see the text) when the conditions are such that $k_{cat.}$ varies linearly with [A].

Acceptor substrate	Acceleration factor ($M^{-1} \cdot s^{-1}$)
L-Alanine	< 5*
L-Phenylalanine	< 5*
Glycine	2200 ± 400
D-Alanine	3340 ± 350
D-2-Aminobutyrate	6440 ± 300
D-Norvaline	6360 ± 250
D-Norleucine	6450 ± 300
D-Phenylalanine	15500 ± 1600
D-Glutamine	12700 ± 1700
D-Asparagine	5600 ± 800
D-Serine	4000 ± 600
D-Aspartate	< 5*
D-Glutamate	< 5*
D-Valine	4900 ± 200
D-Leucine	9700 ± 500
D-Histidine	9500 ± 400
D-α-Aminoadipate	6500 ± 800†
Gly-Gly	490 ± 50
Gly-Gly-Gly	230 ± 30
Gly-L-Ala	+‡
Gly-L-Glu	+‡
Glycollate	280 ± 100
D-Lactate	1800 ± 400
D-Mandelate	2200 ± 400
D-Phenyl-lactate	1300 ± 300†

* Since the proposed model (see the Discussion section) does not imply that a transfer reaction be necessarily linked to an increase of $k_{cat.}$, the presence of the transfer product was also checked by h.p.l.c. At a 100 mM acceptor concentration, the [T]/[H] ratios were 0.1 (L-alanine), 0.01 (L-phenylalanine), 0.3 (D-aspartate) and 0.4 (D-glutamate).

† With D-α-aminoadipate and D-phenyl-lactate the linear part of the $k_{cat.}$ versus acceptor concentration was quite short and the given values are probably somewhat too low.

‡ The value of $k_{cat.}/K_m$ decreased with increasing acceptor concentration.

S2a concentration ([S] < 1 mM) was linear and the second-order rate constant $k_a/[S]$ deduced (63500 ± 5000 $M^{-1} \cdot s^{-1}$) agreed well the k_{cat}/K_m value (62000 ± 3000 $M^{-1} \cdot s^{-1}$) observed at the same temperature. Moreover, when k_a was measured at 15 °C and with 250 μM substrate a value of 6.1 s^{-1} was found, in excellent agreement with that determined on the basis of the burst at 250 nm (Fig. 8), thus indicating that the fluorescence decrease could be attributed to the acyl-enzyme accumulation. During the second phase, which was followed on a standard spectrofluorimeter, the fluorescence returned to its initial value (taking account of a small dilution effect and of a slight absorption of the emitted light by the substrate and by the products of the reaction). The time required for the final stabilization of the fluorescence intensity was of course dependent upon the initial thioester concentration (Fig. 9*b*). At constant thioester concentration the rate of fluorescence recovery increased with the concentration of acceptor, which showed that the disappearance of acyl-enzyme was accelerated by the acceptor (Fig. 10).

Similar results were obtained with the R39 enzyme.

Specificity for the acceptor substrate

At low acceptor concentration the $k_{cat.}$ value for the global reaction varied linearly with the concentration of acceptor substrate (Fig. 3). An acceleration factor could be defined as $\Delta k_{cat.}/\Delta[A]$, which was a reflection of the specificity of the enzyme for the various acceptors. The acceleration factors were measured with various α-amino acids, peptides and α-hydroxy acids (Table 3).

DISCUSSION

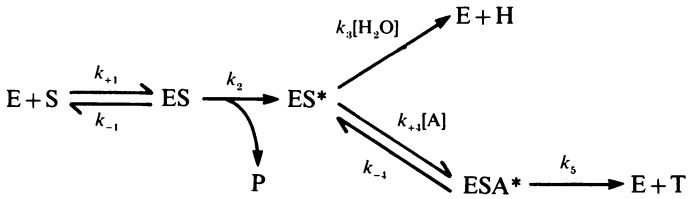
The relative efficiencies of the various enzymes for the catalysis of transfer reaction followed the decreasing order: DD-peptidases > class C β-lactamases > class A β-lactamases. This result was in agreement with those reported by Pratt & Govardhan (1984). However, in contrast with the esters, the thioesters were generally better substrates of the class A than of the class C enzymes when hydrolysis was measured (Adam *et al.*, 1990). This demonstrated that the class A β-lactamases were extremely inefficient catalysts for transferring the acyl group from the donor substrate on anything but water, even when they did efficiently hydrolyse that donor substrate. Since the catalysis of transfer reactions by a class C β-lactamase has been extensively studied by Pratt and co-workers (Pazhanisamy *et al.*, 1989; Pazhanisamy & Pratt, 1989*a*,*b*), we centred our study on the DD-peptidases. Both R61 and R39 enzymes were excellent catalysts of the transfer reaction, yielding very high [T]/[H] ratios under certain conditions. As shown previously with peptide donor substrates, the R39 enzyme only catalyses transfer reactions if the acceptor group is in the α-position relative to the free carboxylate group, i.e. dipeptides were not recognized (Perkins *et al.*, 1973). In contrast, the R61 enzyme could utilize a variety of amino acids and peptides. The enzyme exhibited a very strict stereospecificity for the acceptor molecule. Indeed, L-amino acids were not recognized, and Table 3 shows two or three orders of magnitude between the acceleration factors of D-alanine and L-alanine. Conversely, and as shown previously, free glycine, Gly-Gly and Gly-L-Xaa peptides were relatively good acceptors (Perkins *et al.*, 1973). Thus the results obtained with the thioesters were in perfect agreement with those previously reported with a peptide donor substrate.

Transesterification reactions could also be performed with α-hydroxy acids as acceptors. As shown previously, the products of those reactions were substrates for both DD-peptidases and β-lactamases, and transesterification with the help of R61 DD-peptidase appears to represent an easy method for synthesizing a variety of specific esters of hippuric acid, which might be assayed as substrates for a large number of penicillin-binding proteins and β-lactamases.

Surprisingly, at low concentrations D-alanine was not the best acceptor for R61 DD-peptidase, and the values of the acceleration factors were greater with D-amino acids containing large hydrophobic side chains, such as D-leucine and D-phenylalanine. Moreover, side-chain hydrophobicity was not a necessary factor, since D-histidine and D-glutamine were among the best acceptors. This clearly shows that the acceptor-binding site must be rather large to accommodate the bulky histidine and phenylalanine side chains. None of the three isomers of hydroxybenzoic acid was recognized by the R61 enzyme. Govardhan & Pratt (1987) have shown that the potential products of the reactions were substrates for the P99 β-lactamase but not for the R61 DD-peptidase. It could be assumed that for compounds of the same family a gross correlation exists between the acceptor and leaving-group properties. For example, L-alanine was not an acceptor and Ac_2-L-Lys-D-Ala-L-Ala was not significantly hydrolysed (Leyh-Bouille *et al.*, 1971).

Finally, the acceleration factor did not give a full representation of the behaviour of an acceptor. It corresponds to $k_{cat.}/K_m$ in a simple model and should be completed by the determination of the individual $k_{cat.}$ and K_m values. This was done with D-alanine

and Gly-Gly, which exhibited strikingly different behaviours. Indeed, the $k_{cat.}$ value observed at acceptor saturation was much higher with D-alanine, and parameter b in eqn. (1) was 2500 $M^{-1} \cdot s^{-1}$ compared with 600 $M^{-1} \cdot s^{-1}$ with the dipeptide. Such an acceleration of donor utilization in the presence of acceptors has been described by Pazhanisamy & Pratt (1989*b*) with *Enterobacter cloacae* P99 β-lactamase and interpreted on the basis of a complex model, where the acceptor binds to the Henri–Michaelis complex. That model does not seem to apply in the case of the DD-peptidases. Indeed, with the thioester substrates deacylation was clearly rate-limiting and, for the first time, the accumulation of an enzymically competent acyl-enzyme could be monitored in two different ways: firstly, a burst was observed in the disappearance of that substrate, and, secondly, the enzyme fluorescence was transiently quenched upon its addition. This was reminiscent of observations obtained in the presence of penicillins and cephalosporins, with which the accumulation of a stable acyl-enzyme could be measured by following the time-dependent fluorescence decrease (Nieto *et al.*, 1973; Frère *et al.*, 1975; Fuad *et al.*, 1976). However, in the present case the acyl-enzyme was not stable, and the fluorescence returned to its initial value within a time scale that was in agreement with the known kinetic parameters. Moreover, the presence of the acceptor not only increased the overall rate of the reaction but also accelerated the rate of the fluorescence recovery. All those results could be interpreted by assuming a rather simple partition model:



where S is the donor substrate, ES is the Henri–Michaelis complex, ES* is the acyl-enzyme, A is the acceptor substrate HY, P is the product arising from the leaving group (HX–CHR–CO_2^-), H is hippuric acid and T is the hippuryl-Y transfer product. The model nicely explains the following experimental observations. (1) With $k_2 > k_3$, the acyl-enzyme accumulates at saturating donor concentrations; (2) Under those conditions, the presence of the acceptor accelerates the turnover of the substrate and the acyl-enzyme disappearance provided that $k_5 > k_3$. (3) The values of $k_{cat.}$ and K_m are as follows:

$$k_{cat.} = \frac{\{k_3 + (k_5[A]/K'_A)\}k_2}{k_2 + k_3 + \{(k_2 + k_5)[A]/K'_A\}}$$

$$K_m = \frac{\{k_3 + (k_5[A]/K'_A)\}K'_S}{k_2 + k_3 + \{(k_2 + k_5)[A]/K'_A\}}$$

where $K'_S = (k_{-1} + k_2)/k_{+1}$ and $K'_A = (k_{-4} + k_5)/k_{+4}$ and, as expected for a model in which the acceptor binds to the acyl-enzyme, the $k_{cat.}/K_m$ ratio ($= k_2/K'_S$) is independent of the presence of the acceptor, as observed for D-alanine and Gly-Gly (Fig. 5). It can easily be seen that the value of $k_{cat.}$ given above corresponds to empirical equation (1) with $a = k_2 \cdot k_3/(k_2 + k_3)$, $b = k_2 \cdot k_5/(k_2 + k_3) \cdot K'_A$ and $c = (k_2 + k_5)/(k_2 + k_3) \cdot K'_A$. (4) The formation of the ternary ESA* complex is demonstrated by the fact that, at saturation of acceptor substrate, $k_{cat.}$ is different with D-alanine and Gly-Gly. Indeed, if the acceptor were just responsible for a direct attack on the acyl-enzyme (ES* + A → E + T), the same limiting value of $k_{cat.}$ ($= k_2$) would be observed independently of the structure of the acceptor. Thus it is likely that k_2 is equal to or larger than 120 s^{-1} and that k_5 for Gly-Gly is about 12 s^{-1}. The strict preference of the *Actinomadura* R39 enzyme for α-D-amino acid acceptors also indicates the presence of a specific acceptor-binding site on that enzyme.

However, other results obtained in the present study indicate that a more complicated model is necessary to account for all our observations, as follows. (1) All the compounds listed in Table 2 yield the same acyl-enzyme, and the [T]/[H] ratios with a given acceptor should be independent of the structure of the leaving group. Those systems should be further analysed by using ranges of acceptor concentrations that would yield [T]/[H] ratios not far from 1, under which conditions the measurements would be most accurate. (2) Some of the acceptors listed in Table 3 appeared to influence the $k_{cat.}/K_m$ ratio. This could be explained by the model proposed by Pratt and co-workers (see above). However, with the DD-peptidases, and in contrast with the β-lactamases studied by those authors, we observed acyl-enzyme accumulation and an apparently direct influence of the presence of the acceptor on the half-life of that acyl-enzyme. In consequence we have no reason to believe that acceptors bind to the enzyme before the formation of the acyl-enzyme. The acceptors that influence $k_{cat.}/K_m$ might exceptionally form dead-end complexes in the early stages of the pathway. (3) the [T]/[H] ratio has been shown to be a very sensitive indicator for the validity of a model (Frère, 1973). In an analysis of results previously obtained with a peptide donor substrate, we suggested that the dependency of that ratio on the acceptor concentration could be explained on the basis of a model where the acceptor would bind to the enzyme before the donor (Frère *et al.*, 1973). However, we failed to detect enzyme–acceptor complexes. The simple partition model implies that the [T]/[H] ratio remains strictly proportional to the acceptor concentrations. Thus our earlier results also disagree with that simple model.

In conclusion, the simple model presented here must certainly be modified. However, since it explains well most of the results, it can serve as a basis on which a more elaborate model can be built. This will require a more detailed analysis of the enzyme–substrate interaction, including transient-state studies. In that regard, and as shown in the present study, the thioester substrates should allow a very easy analysis of the transfer and hydrolysis reactions.

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