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Streptomyces K15 DD-peptidase-catalysed reactions with ester and amide carbonyl donors

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In water, the purified 26000-M_r membrane-bound DD-peptidase of *Streptomyces* K15 hydrolyses the ester carbonyl donor Ac_2-L-Lys-D-Ala-D-lactate (release of D-lactate) and the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala (release of D-alanine) with accumulation of acyl- (Ac_2-L-Lys-D-alanyl-)enzyme. Whereas hydrolysis of the ester substrate proceeds to completion, hydrolysis of the amide substrate is negligible because of the capacity of the K15 DD-peptidase for utilizing the released D-alanine in a transfer reaction (Ac_2-L-Lys-D-Ala-D-Ala + D-Ala → Ac_2-L-Lys-D-Ala-D-Ala + D-Ala) that maintains the concentration of the amide substrate at a constant level. In the presence of an amino acceptor X-NH_2 (Gly-Gly or Gly-L-Ala) related to the *Streptomyces* peptidoglycan, both amide and ester carbonyl donors are processed without detectable accumulation of acyl-enzyme. Under proper conditions, the acceptor activity of water and, in the case of the amide substrate, the acceptor activity of the released D-alanine can be totally overcome so that the two substrates are quantitatively converted into transpeptidated product Ac_2-L-Lys-D-Ala-NH-X (and hydrolysis is prevented). Experimental evidence suggests that the amino acceptor modifies both the binding of the carbonyl donor to the enzyme and the ensuing rate of enzyme acylation.

INTRODUCTION

For the purpose of defining the structure and functioning of the active sites of the DD-peptidases, enzymes have been isolated that cleave, with high efficiency, the *C*-terminal D-Ala-D-Ala linkage in the carbonyl-donor substrate analogue Ac_2-L-Lys-D-Ala-D-Ala, but that differ from each other with respect to their responses to β-lactam action (from extreme sensitivity to extreme resistance) and the effects that the presence of amino compounds in the reaction mixtures exerts on the fate and rate of consumption of the carbonyl-donor substrate (Ghuysen *et al.*, 1981, 1984; Frère & Joris, 1985). The 26000-M_r penicillin-binding protein that is present in the plasma membrane of *Streptomyces* K15 has been isolated to 95% homogeneity and characterized as a DD-peptidase (Nguyen-Distèche *et al.*, 1982). This K15 DD-peptidase is peculiar in that it effectively utilizes the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala only in the presence of a suitable amino acceptor, i.e. functions, essentially, as a strict DD-transpeptidase. The underlying mechanism has now been investigated.

MATERIALS AND METHODS

Enzymes

The K15 DD-peptidase, purified to 95% purity in the presence of 0.05% cetyltrimethylammonium bromide, was prepared as described previously (Nguyen-Distèche *et al.*, 1982). D-Amino acid oxidase and D-lactate dehydrogenase were from Boehringer, Mannheim, West Germany; catalase was from Sigma Chemical Co., St. Louis, MO, U.S.A.

Substrates (carbonyl donors and acceptors)

The amide carbonyl donor Ac_2-L-Lys-D-Ala was from UCB Bioproducts, Brussels, Belgium, and the ester carbonyl donor Ac_2-L-Lys-D-Ala-D-Lac was from Serva Feinbiochemica, Heidelberg, West Germany.

[^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (50 mCi/mmol) and [^{14}C]-Ac_2-L-Lys-D-Ala-D-Lac (50 mCi/mmol) were prepared from the corresponding non-acetylated compounds (synthesized by UCB Bioproducts). Aqueous solutions of L-Lys-D-Ala-D-Ala (or L-Lys-D-Ala-D-Lac) (6.25 μmol in 40 μl) were supplemented with 250 μl of acetonitrile, 5 μl of triethylamine, and 4.4 μmol of [^{14}C]acetic anhydride [5% (v/v) solution in toluene; specific radioactivity 114 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.] The reaction mixture was maintained for 16 h in an ice bath, supplemented with 20 μl of non-radioactive acetic anhydride and maintained at 0 °C for an additional 2 h. The radioactive diacetylated compounds were purified, and separated from small amounts of N^{α}-mono-acetylated compounds, by paper electrophoresis at pH 5.6.

Peptides 2–8 of Table 3, racemic diaminoadipate and peptides 7 and 19 of Table 4 were gifts from Professor H. R. Perkins, University of Liverpool, Liverpool, U.K. Peptides 9–18, 20 and 21 of Table 4 were from Sigma Chemical Co. The radioactive compounds 1–4 and 8 of Table 4 were from The Radiochemical Centre.

Enzymic determination of D-lactate

Samples (50 μl) containing 5–20 nmol of D-lactate were supplemented with (i) 250 μl of 0.5 M-glycine/0.4 M-hydrazine buffer, pH 9, containing 4 mg of NAD^+/ml

Abbreviation used: D-Lac (in sequences and equations), D-lactate.

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and (ii) 2 μl of lactate dehydrogenase (5 mg/ml). The mixtures were incubated for 30 min at 30 °C and the absorbances were measured at 340 nm.

Determination of the DD-peptidase activities

The catalysed reactions under consideration were hydrolysis:

Ac_2-L-Lys-D-Ala-D-Ala (or Ac_2-L-Lys-D-Ala-D-Lac) $+ H_2O \rightarrow Ac_2$-L-Lys-D-Ala + D-Ala (or D-Lac)

and transpeptidation:

Ac_2-L-Lys-D-Ala-D-Ala (or Ac_2-L-Lys-D-Ala-D-Lac) $+ NH_2$-X $\rightarrow Ac_2$-L-Lys-D-Ala-NH-X + D-Ala (or D-Lac)

They were carried out in 5 mM-potassium phosphate buffer, pH 7.5, containing 0.008% cetyltrimethylammonium bromide (usually in 30 μl final volume). Carbonyl-donor consumption was determined by measuring enzymically the amount of released D-alanine [by using the D-amino acid oxidase/peroxidase procedure described in Johnson *et al.* (1975)] or the amount of released D-lactate (by using the D-lactate dehydrogenase technique). More specifically, (i) hydrolysis was determined by measuring the amount of [^{14}C]Ac_2-L-Lys-D-Ala released from the amide [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (0.8 mCi/mmole) and ester [^{14}C]Ac_2-L-Lys-D-Ala-D-Lac (0.4 mCi/mmol) carbonyl donors, after separation by paper electrophoresis at pH 6.5, and (ii) transpeptidation was determined by measuring the amount of [^{14}C]Ac_2-L-Lys-D-Ala-NH-X formed from [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala or [^{14}C]Ac_2-L-Lys-D-Ala-D-Lac and a non-radioactive amino acceptor NH_2-X, or by measuring the amount of Ac_2-L-Lys-D-Ala-NH-[^{14}C]X formed from Ac_2-L-Lys-D-Ala-D-Ala or Ac_2-L-Lys-D-Ala-D-Lac and a labelled amino acceptor NH_2-[^{14}C]X. The reaction products were isolated by paper electrophoresis at pH 6.5 and 60 V/cm (radioactive carbonyl donors) or at pH 5.6 and 20 V/cm (radioactive acceptors).

Denaturing buffer

Denaturing buffer referred to a 125 mM-Tris/HCl buffer, pH 6.8, containing 2% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.001% Bromophenol Blue (used as marker in gel electrophoresis).

Determination of [^{14}C]acyl-enzyme

The K15 DD-peptidase (4.6 μM unless otherwise stated) was incubated at 37 °C with various concentrations of [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (50 mCi/mmol) or [^{14}C]Ac_2-L-Lys-D-Ala-D-Lac (30 mCi/mmol) at 37 °C in 5 mM-potassium phosphate buffer, pH 7.5, containing 0.008% cetyltrimethylammonium bromide (15 μl final volume). The radioactive acyl-enzyme formed during the reaction was trapped and stabilized by adding 15 μl of denaturing buffer and maintaining the solution in a boiling-water bath for 30 s. The radioactive acyl-enzyme was separated by polyacrylamide-gel electrophoresis at pH 8.3 in the presence of SDS (Laemmli & Favre, 1973), detected by fluorography with preflashed Kodak X-Omat XR films and quantified by microdensitometry of the fluorogram in a Beckman DU8 spectrophotometer equipped with a gel-scanning device.

Controls were enzyme samples treated with [^{14}C]benzylpenicillin (50 mCi/mmol; The Radiochemical Centre) under conditions where the totality of the enzyme was converted into [^{14}C]benzylpenicilloyl-enzyme (Leyh-Bouille *et al.*, 1986). For this purpose, the enzyme (0.57–4.6 μM) was incubated with 0.18 mM-[^{14}C]benzylpenicillin (the specific radioactivity of which was identical with that of the radioactive amide or ester carbonyl donor) for 10 min at 37 °C, and the radioactive benzylpenicilloyl-enzyme was trapped by adding 5 μl of 0.1 M non-radioactive benzylpenicillin and 20 μl of denaturing buffer, and maintaining the solution in a boiling-water bath for 30 s.

Reaction models

The interactions between the K15 DD-peptidase and the ester substrate Ac_2-L-Lys-D-Ala-D-Lac and amide substrate Ac_2-L-Lys-D-Ala-D-Ala were interpreted on the basis of the reaction models shown below [where E = K15 DD-peptidase (or enzyme), K = dissociation constant of the Michaelis complex, k_{+2} and k_{+3} = first-order rate constants and k_{+2}/K = second-order rate constant of enzyme acylation]. Reference to the equations developed in Ghuysen *et al* (1986) is made with use of the original numbering.

Hydrolysis of the amide (ester) carbonyl-donor substrates.

$$E + Ac_2\text{-L-Lys-D-Ala-D-Ala (or -D-Lac)} \overset{K}{\rightleftharpoons} E \cdot Ac_2\text{-L-Lys-Ala-D-Ala (or -D-Lac)}$$

Michaelis complex

$$\xrightarrow{k_{+2}} Ac_2\text{-L-Lys-D-alanyl-E} \xrightarrow{k_{+3}} E + Ac_2\text{-L-Lys-D-Ala} \quad (1)$$

Acyl-enzyme

D-Ala or D-Lac $\qquad$ H_2O

Concomitant hydrolysis and transpeptidation of the amide (ester) carbonyl-donor substrates.

$$E + Ac_2\text{-L-Lys-D-Ala-D-Ala (or -D-Lac)} \overset{K}{\rightleftharpoons} E \cdot Ac_2\text{-L-Lys-D-Ala-D-Ala (or -D-Lac)}$$

Michaelis complex

$$H_2O \xrightarrow{k_{+3}} Ac_2\text{-L-Lys-D-Ala} \quad (2a)$$

$$\xrightarrow{k_{+2}} Ac_2\text{-L-Lys-D-alanyl-E} \rightarrow E$$

Acyl-enzyme

D-Ala or D-Lac

$$NH_2\text{-X} \xrightarrow{k_{+3}'} Ac_2\text{-L-Lys-D-Ala-NH-X} \quad (2b)$$

In such a simple system, partitioning of the enzyme activity depends only on the competition between NH_2-X and water (which is 55.5 M in an aqueous medium) at the level of the acyl-enzyme. If, in water, the acyl-enzyme accumulates at the steady state of the reaction, increasing concentrations of NH_2-X may increase the rate of acyl-enzyme breakdown, and hence may increase $k_{cat.}$ until the rate of enzyme acylation by the carbonyl-donor substrate (k_{+2}) becomes rate-determining (under which conditions the new $k_{cat.}$ becomes constant and equal to k_{+2}). Alternatively, if, in water, the acyl-enzyme does not accumulate, increasing concentrations of NH_2-X cannot increase $k_{cat.}$, but partitioning still occurs. For more details see Fersht (1985).

RESULTS

Table 1 gives the values of the constants K, k_{+2}, k_{+3}, $k_{cat.}$ and K_m obtained with the two carbonyl-donor substrates.

Reaction with the ester carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala-D-Lac: hydrolysis pathway (reaction 1)

As determined from Hanes plots of [Ac_2-L-Lys-D-Ala-D-Lac]/v versus [Ac_2-L-Lys-D-Ala-D-Lac], hydrolysis of the ester substrate proceeded with a K_m value of 0.8 mM and a $k_{cat.}$ value of 0.50 s^{-1}.

Incubation of the enzyme with various concentrations of [^{14}C]Ac_2-L-Lys-D-Ala-D-Lac for 20 s at 37 °C caused accumulation of the [^{14}C]Ac_2-L-Lys-D-alanyl-enzyme at the steady state of the reaction (Fig. 1*a*). Under saturating conditions, 40% of the enzyme occurred as acyl-enzyme. On the basis of eqn. (7) (Ghuysen *et al.*, 1986), the plot of $[E]_0/[E\text{–}D^*]_{ss}$ versus 1/[D] yielded a K value of 2.2 mM and a k_{+3}/k_{+2} ratio value of 1.4 (Fig. 2*a*). By using the $k_{cat.}$ value of 0.5 s^{-1}, eqns. (9), (10) and (5) (Ghuysen *et al.*, 1986) gave $k_{+2} = 0.86\ s^{-1}$, $k_{+3} = 1.20\ s^{-1}$ and $K_m = 1.3$ mM. This last value was close to that (0.8 mM) determined from the Hanes plot, demonstrating both the accuracy of the conditions used for trapping the acyl-enzyme and the inertness of the released D-lactate on the hydrolysis pathway.

At the ester carbonyl-donor concentrations used (from 0.5 to 5 mM), the 20 s of incubation was more than sufficient for the reaction to reach 99% of the steady-state level [eqn. (8) in Ghuysen *et al.* (1986)]. Moreover, that this situation actually applied was verified by showing that, at a constant ester carbonyl-donor concentration of 0.5 mM, the amount of acyl-enzyme trapped was independent of the incubation time (from 20 to 60 s).

Reaction with the ester carbonyl donor Ac_2-L-Lys-D-Ala-D-Lac: transpeptidation pathway (reaction 2b)

The presence of Gly-Gly or Gly-L-Ala (these two compounds have equivalent acceptor activity; see below) in the reaction mixture caused partitioning of the enzyme activity. As shown in Fig. 3(*a*), at a Gly-L-Ala concentration equivalent to that of the ester carbonyl donor (2 mM) the acceptor activity of water was almost completely abolished, the enzyme activity was channelled almost exclusively to transpeptidation and the rate of consumption of the carbonyl-donor substrate, when compared with that observed in the absence of amino acceptor, was considerably enhanced. Moreover, no acyl-enzyme accumulated at the steady state of the reaction.

Table 1. Kinetic constants for the interactions between the K15 DD-peptidase (E) and the ester carbonyl donor Ac_2-L-Lys-D-Ala-D-Lac and amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala in the presence and in the absence of the amino acceptor Gly-Gly

$$E + D \overset{K}{\rightleftharpoons} E\cdot \xrightarrow{k_{+2}} \text{acyl-enzyme} \xrightarrow{k_{+3}} E + \text{products}$$

In the presence of Gly-Gly, at the indicated concentrations, transpeptidation of the ester and amide carbonyl donors is (virtually) the only reaction catalysed. Values are given as means ± S.D.

Carbonyl donor	Acceptor	Hanes (H) and Dalziel (D) plots			Acyl-enzyme trapping					Second-order rate constant of enzyme acylation	
		$k_{cat.}$ (s^{-1})		K_m (mM)	$k_{cat.}$ (s^{-1})	K_m (mM)	K (mM)	k_{+2} (s^{-1})	k_{+3} (s^{-1})	$\frac{k_{+3}}{k_{+2}}$	k_{+2}/K ($M^{-1}\cdot s^{-1}$)	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)
Ac_2-L-Lys-D-Ala-D-Lac	H_2O	0.5 ± 0.04	(H)	0.8 ± 0.05		1.3	2.2	0.86	1.2	1.4	390	625
	Gly-Gly:											
	0.036–0.254 mM	2.4 ± 0.4	(D)	3.5 ± 0.5			No trapping					685
	5–10 mM	5.6 ± 0.7	(H)	8.9 ± 1.6			No trapping					630
Ac_2-L-Lys-D-Ala-D-Ala	H_2O	(See the text)			0.11	8	9	0.13	1.2	9.5	14	
	Gly-Gly:											
	2 mM	0.3 ± 0.01	(H)	4.6 ± 0.5			No trapping					65
	10 mM	0.55 ± 0.02	(H)	6 ± 0.7			No trapping					92

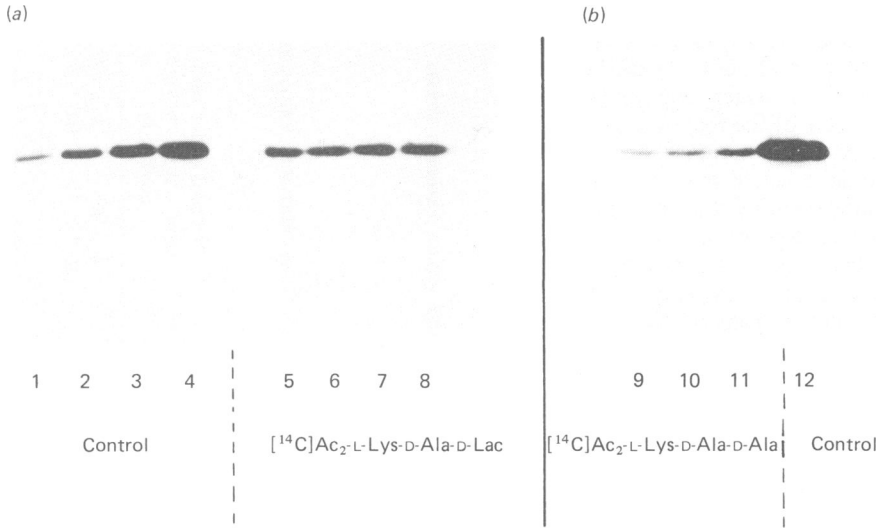

Fig. 1. Trapping of the acyl-enzyme formed during reaction between the K15 DD-peptidase and [^{14}C]benzylpenicillin (lanes 1–4 and 12), [^{14}C]Ac_2-L-Lys-D-Ala-D-Lac (lanes 5–8) and [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (lanes 9–11)

For lanes 1–4 and 12 the K15 DD-peptidase (0.57, 1.14, 2.3, 4.6 and 4.6 μM respectively) was incubated with 0.18 mM-[^{14}C]benzylpenicillin (a saturating concentration) for 10 min at 37 °C. For lanes 5–8 the K15 DD-peptidase (4.6 μM) was incubated with increasing concentrations of the ester carbonyl donor (1.5, 3, 4.5 and 6 mM respectively) for 30 s at 37 °C. For lanes 9–11 the K15 DD-peptidase (4.6 μM) was incubated with increasing concentrations of the amide carbonyl donor (2, 4 and 8 mM respectively) for 2 min at 37 °C. All the reactions were carried out in 5 mM-potassium phosphate buffer, pH 7.5, containing 0.008% cetyltrimethylammonium bromide (15 μl final volume). The reactions were stopped at the indicated times by the addition of SDS. The samples were submitted to polyacrylamide-gel electrophoresis in the presence of SDS. After fluorography of the gels, the darkness of the spots was measured by densitometry (for details see the Materials and methods section).

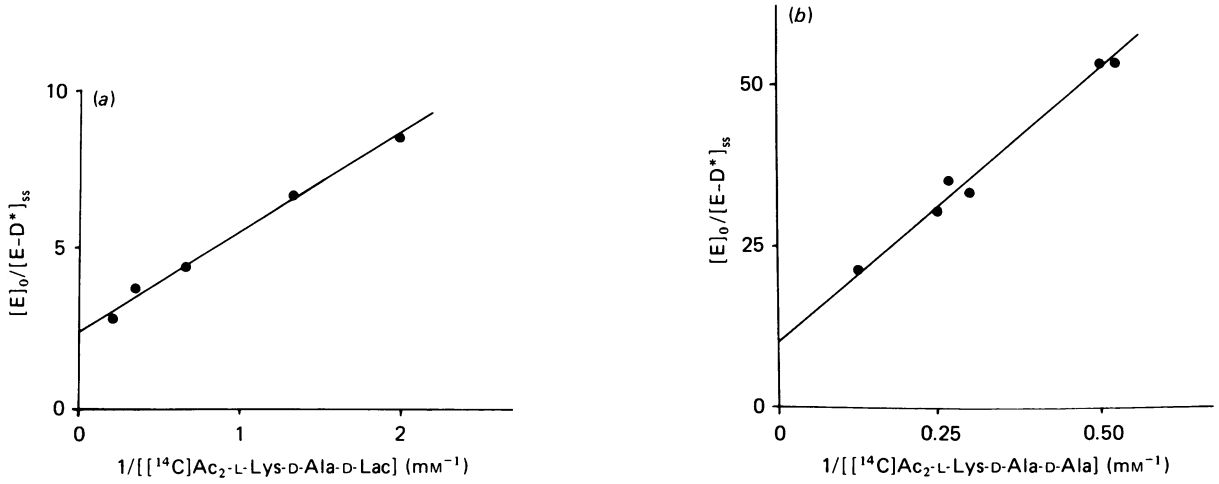

Fig. 2. Plot of $[E]_0/[E–D^*]_{ss}$ versus 1/[^{14}C]Ac_2-L-Lys-D-Ala-D-Lac (*a*) and 1/[^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (*b*)

$[E]_0$ = total enzyme and $[E–D^*]_{ss}$ = acyl-(Ac_2-L-Lys-D-alanyl-)enzyme at the steady state of the reaction. The plots were obtained from the data of Fig. 1 and other experiments. At a carbonyl-donor concentration of 2 mM about 25% and 2% of the enzyme occur as acyl-enzyme during reaction with Ac_2-L-Lys-D-Ala-D-Lac and Ac_2-L-Lys-D-Ala-D-Ala respectively.

The rates of transpeptidation were measured with various concentrations of ester carbonyl donor (from 0.67 mM to 4 mM) and various concentrations of [^{14}C]Gly-Gly (from 0.036 mM to 0.254 mM). The enzyme concentration and the incubation times were such that in the absence of Gly-Gly and on the basis of the K_m (0.8 mM) and $k_{cat.}$ (0.5 s^{-1}) values for the hydrolysis pathway, 1%, at the most, of the carbonyl donor would be consumed. Under these conditions, the kinetic parameters ϕ_0 (equivalent to $1/k_{cat.}$), ϕ_D/ϕ_0 (equivalent to K_m for the carbonyl donor) and ϕ_A/ϕ_0 (equivalent to K_m for the amino acceptor) for the transpeptidation pathway (reaction 2b) were calculated by using the general initial rate equation (Dalziel, 1957) for an enzyme-catalysed bimolecular reaction ($S_1+S_2 \rightleftharpoons S_1'+S_2'$):

$$\frac{[E]_0}{v} = \phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_D}{[D]} + \frac{\phi_{AD}}{[A][D]}$$

where D = carbonyl donor and A = amino acceptor

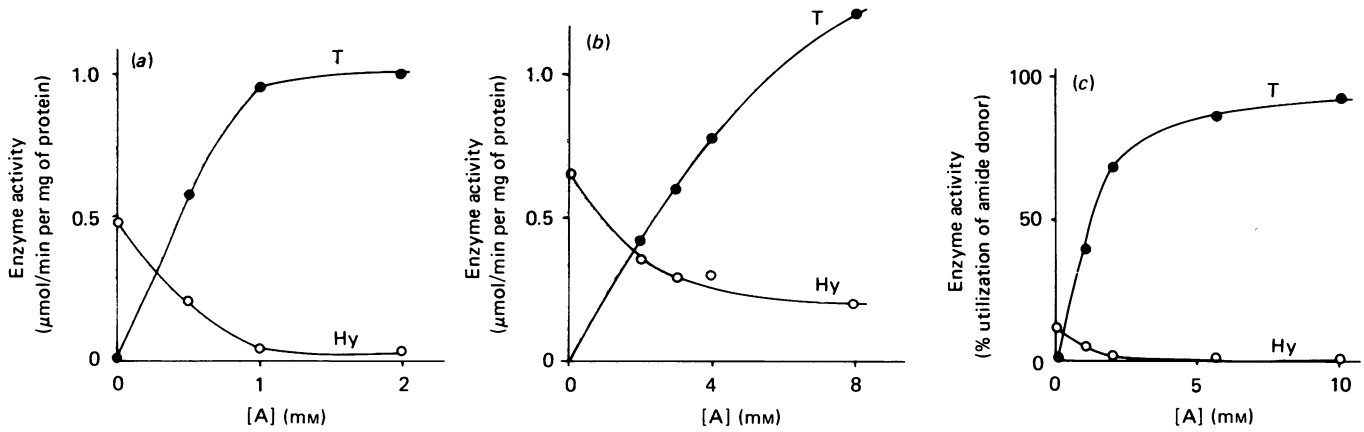

Fig. 3. Effects of increasing acceptor concentrations [A] on concomitant hydrolysis (Hy) and transpeptidation (T) of ester and amide carbonyl donors by the K15 DD-peptidase

The co-substrates were [^{14}C]Ac_2-L-Lys-D-Ala-D-Lac and Gly-L-Ala in (*a*), [^{14}C]Ac_2-L-Lys-D-Ala-D-Lac and Gly-L-Leu in (*b*) and [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala and Gly-Gly in (*c*). All the incubations were carried out at 37 °C in 5 mM-potassium phosphate buffer, pH 7.5, containing 0.008% cetyltrimethylammonium bromide (30 μl final volume). The enzyme concentrations and incubation times used were 1 μM and 15 min in (*a*), 0.5 μM and 30 min in (*b*) and 3.5 μM and 240 min in (*c*).

Gly-Gly [for more details see Dusart *et al.* (1977)]. The plots of $[E]_0/v$ versus 1/[A] for various [D] values (Fig. 4*a*) and of $[E]_0/v$ versus 1/[D] for various [A] values (Fig. 4*b*) gave rise to straight lines, the slopes and intercepts (on the ordinate axis) of which yielded $k_{cat.} = 2.4\ s^{-1}$, K_m (donor) = 3.5 mM and K_m (acceptor) = 0.07 mM. In turn, Hanes plots of [D]/*v* versus [D] in the presence of a saturated concentration (5–10 mM) of Gly-Gly yielded a $k_{cat.}$ value of 5.6 s^{-1} and a K_m value, for the carbonyl donor, of 8.9 mM.

Reaction with the ester carbonyl donor Ac_2-L-Lys-D-Ala-D-Lac: concomitant hydrolysis and transpeptidation (reactions 2a and 2b)

In order to study the effects that various concentrations of carbonyl donor [D] and various concentrations of amino acceptor [A] exerted on the ratio v_T/v_{Hy} (where v_T = initial rate of transpeptidation and v_{Hy} = initial rate of hydrolysis), Gly-L-Leu was used as amino acceptor because it had a weaker acceptor activity than Gly-L-Ala or Gly-Gly [compare Fig. 3(*b*) with Fig. 3(*a*)], thus permitting use of a broader range of amino-acceptor concentrations before hydrolysis was completely abolished. As shown in Fig. 5(*a*) and Fig. 5(*b*), at a fixed 4 mM-Gly-L-Leu concentration the ratio v_T/v_{Hy} decreased as [D] increased (from 1 to 8 mM), and at a fixed 4 mM-Ac_2-L-Lys-D-Ala-D-Lac concentration v_T/v_{Hy} was directly proportional to [A] (from 2 to 8 mM).

In addition, at a fixed 4 mM-Ac_2-L-Lys-D-Ala-D-Lac concentration the plot of v_{Hy} versus v_T/[A] obeyed the equation $v_{Hy} = k(v_T/[A])$ (Fig. 5*c*). Hence the hydrolytic activity of the enzyme preparation was a genuine property of the K15 DD-peptidase and was not due to the presence of a contaminating hydrolase (that lacks transpeptidase activity). Indeed, in this latter case v_{Hy} would be equal to the sum of the hydrolytic activity of the transpeptidase and that of the contaminating hydrolase ($v_{Hy(0)}$), the plot of v_{Hy} versus v_T/[A] would give rise to a straight line having the same slope but intercepting the ordinate axis, and the value of this intercept would be equal to the rate of hydrolysis by the contaminating hydrolase [$v_{Hy} = v_{Hy(0)} + k(v_T/[A])$].

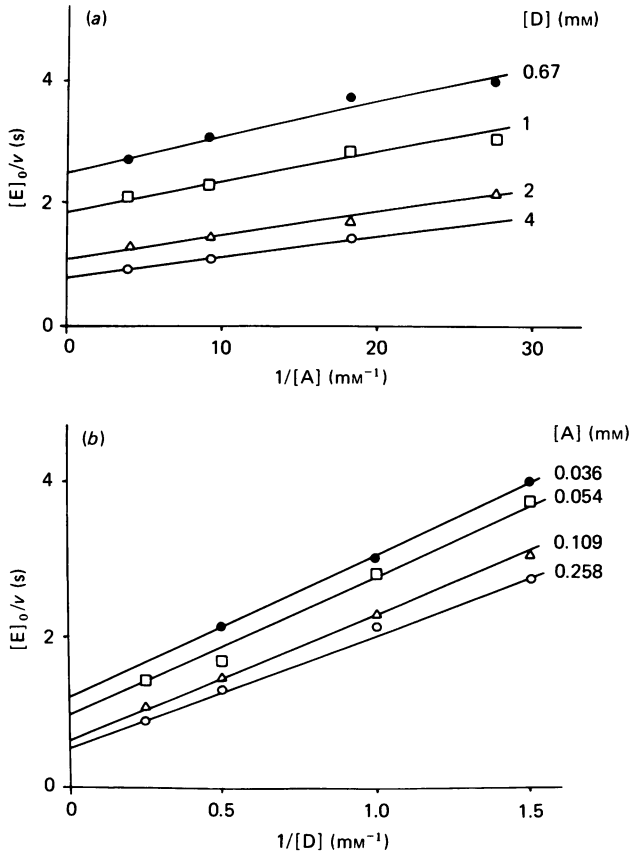

Fig. 4. K15 DD-peptidase-catalysed transpeptidation reaction with Ac_2-L-Lys-D-Ala-D-Lac as carbonyl donor and [^{14}C]Gly-Gly as amino acceptor

(*a*) Double-reciprocal plot of $[E]_0/v$ versus 1/[acceptor] for various concentrations of donor; (*b*) double-reciprocal plot of $[E]_0/v$ versus 1/[donor] for various concentrations of acceptor. The enzyme (0.021 μM) was incubated for 20 min at 37 °C in 5 mM-potassium phosphate buffer, pH 7.5, containing 0.008% cetyltrimethylammonium bromide (30 μl final volume) with various concentrations of donor and acceptor as indicated. The product of the reaction was Ac_2-L-Lys-D-Ala-[^{14}C]Gly-Gly.

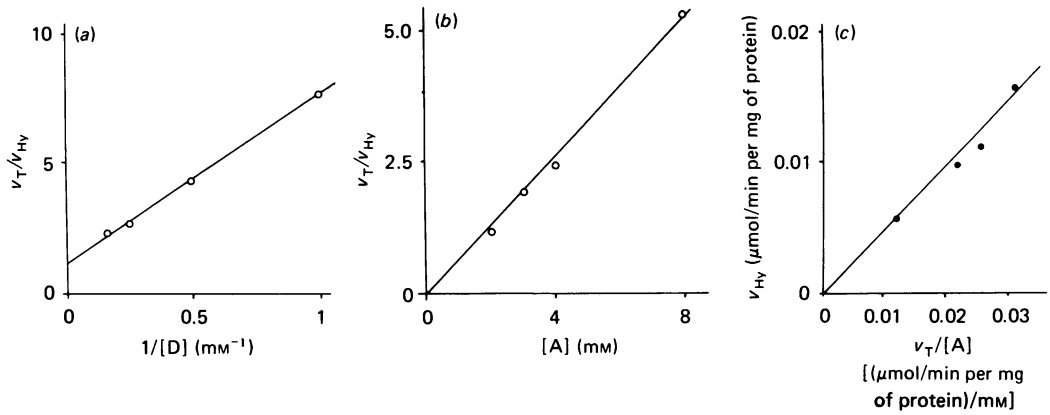

Fig. 5. K15 DD-peptidase-catalysed hydrolysis and transpeptidation with [^{14}C]Ac_2-L-Lys-D-Ala-D-Lac as ester carbonyl donor and Gly-L-Leu as amino acceptor

(*a*) Plot of v_T/v_{Hy} versus 1/[D] in the presence of a fixed (4 mM) Gly-L-Leu concentration; (*b*) plot of v_T/v_{Hy} versus [A] at a fixed (4 mM) Ac_2-L-Lys-D-Ala-D-Lac concentration; (*c*) plot of v_{Hy} versus v_T/[A] at a fixed (4 mM) Ac_2-L-Lys-D-Ala-D-Lac concentration. In all cases the DD-peptidase (0.5 μM) and the various substrates, as indicated, were incubated together for 15 min at 37 °C in 5 mM-potassium phosphate buffer, pH 7.5, containing 0.008% cetyltrimethylammonium bromide (30 μl final volume). The reaction products were [^{14}C]Ac_2-L-Lys-D-Ala (hydrolysis) and [^{14}C]Ac_2-L-Lys-D-Ala-Gly-L-Leu (transpeptidation).

Reaction with the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala: hydrolysis pathway (reaction 1)

As observed with the ester carbonyl donor, incubation of the enzyme with various concentrations of [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala for 120 s at 37 °C caused accumulation of the [^{14}C]Ac_2-L-Lys-D-alanyl-enzyme at the steady state of the reaction (Fig. 1*b*). Under saturating conditions 10% of the enzyme occurred as acyl-enzyme (instead of 40% with the ester substrate). The plot of $[E]_0/[E\text{–}D^*]_{ss}$ versus 1/[D] yielded a K value of 9 mM and a k_{+3}/k_{+2} ratio value of 9.5 (Fig. 2*b*). Since the same acyl-([^{14}C]Ac_2-L-Lys-D-alanyl-)enzyme was formed, the k_{+3} value of 1.20 s^{-1} determined for the interaction with the ester substrate applied to the interaction with the amide substrate. With knowledge of the ratio k_{+3}/k_{+2} and k_{+3}, it followed that $k_{+2} = 0.13$ s^{-1}. In turn, eqns. (4) and (5) (Ghuysen *et al.*, 1986) gave $k_{cat.} = 0.11$ s^{-1} and $K_m = 8$ mM.

On the basis of these $k_{cat.}$ and K_m values, hydrolysis of 2 mM-Ac_2-L-Lys-D-Ala-D-Ala by the K15 DD-peptidase (used at a 3.3 μM-concentration) should proceed according to line 1 in Fig. 6. In fact, hydrolysis proceeded according to curve 3 in Fig. 6, i.e. at a much lower rate. The reason for this discrepancy was that, after an initial burst of hydrolysis, the D-alanine endogenously released from Ac_2-L-Lys-D-Ala-D-Ala was re-utilized in a transpeptidation reaction (Ac_2-L-Lys-D-Ala-D-Ala + D-Ala → Ac_2-L-Lys-D-Ala-D-Ala + D-Ala) that maintained the carbonyl-donor concentration at a constant level. This interpretation rested upon the three following observations.

(1) Under the experimental conditions of curve 3 in Fig. 6, 25% of the carbonyl donor was consumed after 24 h at 37 °C. At this time the reaction mixture contained 1.5 mM residual Ac_2-L-Lys-D-Ala-D-Ala, 0.5 mM released D-alanine and 0.5 mM-Ac_2-L-Lys-D-Ala. That the observed very low rate of hydrolysis was due to transpeptidation of the carbonyl donor to the accumulated D-alanine was demonstrated by incubating fresh DD-peptidase (at the same concentration as above), 1.5 mM-Ac_2-L-Lys-D-Ala-D-Ala and 0.5 mM-D-[^{14}C]alanine. Under these conditions synthesis of the radioactive tripeptide Ac_2-L-Lys-D-Ala-D-[^{14}C]Ala (reaction catalysed: Ac_2-L-Lys-D-Ala-D-Ala + D-[^{14}C]Ala → Ac_2-L-Lys-D-Ala-D-[^{14}C]Ala + D-Ala) proceeded at an average rate of 98 nmol/min per mg of

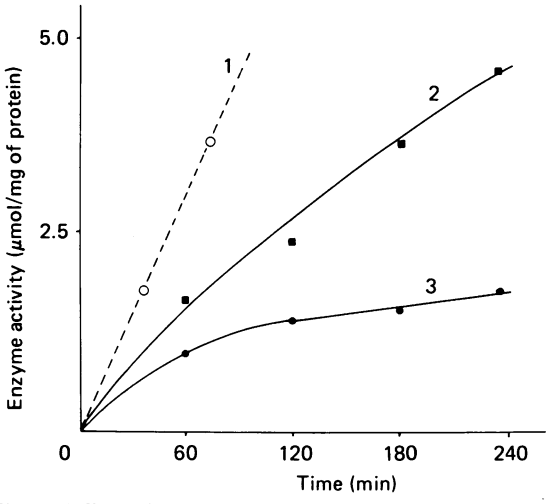

Fig. 6. Effect of D-amino acid oxidase on the K15 DD-peptidase-catalysed hydrolysis of [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala into [^{14}C]Ac_2-L-Lys-D-Ala

The enzyme (3.3 μM) and [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (2 mM) were incubated for increasing periods of time at 37 °C, in the absence (curve 3) and in the presence (curve 2) of D-amino acid oxidase and catalase, in 5 mM-potassium phosphate buffer, pH 7.5, containing 0.008% cetyltrimethylammonium bromide (30 μl final volume). Line 1 shows the theoretical rate of hydrolysis on the basis of $K_m = 8$ mM and $k_{cat.} = 0.13$ s^{-1}. For experiments related to curve 2, the reaction mixtures were supplemented with 1 μl of a D-amino acid oxidase solution (5 mg of protein/ ml; previously dialysed against 0.1 M-Tris/HCl buffer, pH 8.0, containing 50 μg of FAD/ml) and 1 μl of a catalase solution (4.5 mg of protein/ml). Dialysis of the D-amino acid oxidase was carried out to eliminate the $(NH_4)_2SO_4$ present in the commercially available preparation.

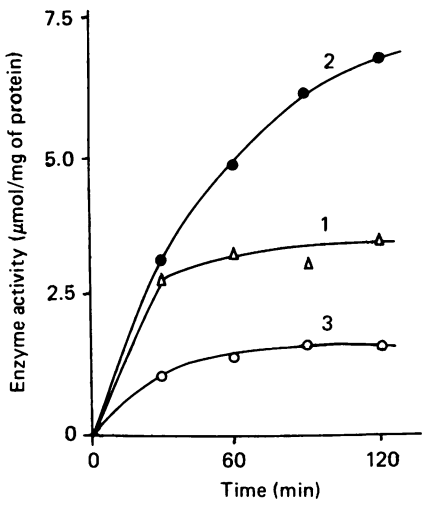

Fig. 7. Time course of the K15 DD-peptidase-catalysed conversion of 1.5 mM-Ac_2-L-Lys-D-Ala-D-Ala into Ac_2-L-Lys-D-Ala-D-[^{14}C]Ala in the presence of 0.5 mM-D-[^{14}C]alanine (curve 1) and in the presence of both 0.5 mM-D-[^{14}C]alanine and 1.5 mM-Gly-Gly (curve 3) and comparison with the catalysed conversion of 1.5 mM-Ac_2-L-Lys-D-Ala-D-Ala into Ac_2-L-Lys-D-Ala-[^{14}C]Gly-Gly in the presence of both 0.5 mM-D-alanine and 1.5 mM-[^{14}C]Gly-Gly (curve 2)

The enzyme (3.3 μM) and the various reactants, as indicated, were incubated for increasing periods of time at 37 °C in 5 mM-potassium phosphate buffer, pH 7.5, containing 0.008% cetyltrimethylammonium bromide (30 μl final volume). At zero time all the reaction mixtures contained 0.66 mM-Ac_2-L-Lys-D-Ala.

protein during the first 30 min of incubation and then slowed down considerably (curve 1 in Fig. 7). At this time half (0.25 mM) of the initially available D-[^{14}C]alanine was consumed and replaced by an equal amount of non-radioactive D-alanine. As a consequence of the progressive dilution of D-[^{14}C]alanine and the competition between the non-radioactive and radioactive D-alanine as acceptors for the transfer reaction, the net synthesis of radioactive Ac_2-L-Lys-D-Ala-D-[^{14}C]Ala stopped (although the enzyme continued to work).

(2) Assuming that the above interpretation was correct, the immediate elimination of free D-alanine once it was released from the carbonyl-donor substrate should maintain the initial rate of hydrolysis. The addition of D-amino acid oxidase (converting D-alanine into pyruvate) to the reaction mixture partially fulfilled these conditions. As shown by curve 2 in Fig. 6, the yield of hydrolysis was enhanced. However, it remained smaller than that expected on the basis of the K_m and $k_{cat.}$ values as measured by trapping the acyl-enzyme (i.e. under true conditions of initial velocity, since trapping was carried out after a very short time, 120 s, of incubation).

(3) Another prediction of the model was that the D-alanine, as it was released from the carbonyl donor, should increase the rate of acyl-enzyme breakdown, and in parallel to this should decrease the amount of acyl-enzyme present. Moreover, this effect should be at least diminished if the reaction was carried out in the presence of D-amino acid oxidase. The data (Table 2) confirmed the prediction.

Reaction with the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala: transpeptidation pathway (reaction 2b)

As shown in Fig. 3(*c*), incubation of mixture of 2 mM-Ac_2-L-Lys-D-Ala-D-Ala and 10 mM-Gly-Gly with the K15 DD-peptidase (3 μM) caused quantitative conversion of Ac_2-L-Lys-D-Ala-D-Ala into Ac_2-L-Lys-D-Ala-Gly-Gly. The enzyme functioned (almost) exclusively as a DD-transpeptidase, and no acyl-enzyme accumulated at the steady state of the reaction. After 240 min at 37 °C the yield of the reaction was 92%, and at this time residual Gly-Gly (8.16 mM; i.e. $20 \times K_m$) and released D-alanine (1.84 mM) were present in a molar ratio of about 4.4:1.

However, when the enzyme, used at the same concentration as above, was incubated with 2 mM-Ac_2-L-Lys-D-Ala-D-Ala and 2 mM- (instead of 10 mM-)Gly-Gly, the yield of conversion into transpeptidated product after 240 min of incubation was only 69% (instead of 92%), and remained virtually unchanged after prolonged incubation (results not shown). Reaction mixtures were then reconstituted which mimicked the situation where 25% of the tripeptide had been consumed [so that residual Gly-Gly (1.5 mM) and released D-alanine (0.5 mM) occurred in a molar ratio of 3:1]. For this purpose 1.5 mM-Ac_2-L-Lys-D-Ala-D-Ala and fresh enzyme were incubated together in the presence of either a mixture of 1.5 mM-Gly-Gly and 0.5 mM-D-[^{14}C]alanine or a mixture of 1.5 mM-[^{14}C]Gly-Gly and 0.5 mM-D-alanine. After 120 min at 37 °C, the carbonyl donor was converted into both Ac_2-L-Lys-D-Ala-[^{14}C]Gly-Gly (44%; Fig. 7, curve 2) and Ac_2-L-Lys-D-Ala-D-[^{14}C]Ala (11%; Fig. 7, curve 3).

From the foregoing it thus followed that, under certain conditions, the K15 DD-peptidase was able to function strictly as a transpeptidase, i.e. to catalyse the transfer of the electrophilic group Ac_2-L-Lys-D-alanyl exclusively to the amino acceptor Gly-Gly, until the carbonyl donor was completely consumed, in the absence of hydrolysis and without interference by the released D-alanine. The conditions were that the concentration of free Gly-Gly in the reaction mixture remained sufficiently high to maintain saturation of the enzyme acceptor site at all times and to maintain a [free Gly-Gly]/[released D-alanine] molar ratio of at least 5:1.

Specificity profile of the K15 DD-peptidase for amide carbonyl-donor peptides

Under standard conditions and with Gly-Gly (2 mM) as the acceptor, the ability of a series of peptides to react as donor for transpeptidation by the K15 DD-peptidase was compared with that of Ac_2-L-Lys^1-D-Ala^2-D-Ala^3 (Table 3). These data indicated that transpeptidase activity (i) strictly required a D-alanine residue at position 2, (ii) was decreased but not abolished when glycine or a D-amino acid other than a D-alanine residue was at position 3 (L-alanine at this position abolished donor activity), and (iii) was decreased when the neutral side chain of the L-amino acid residue at position 1 was shortened.

Specificity profile of the K15 DD-peptidase for amino acceptors

The profile of the K15 DD-peptidase for acceptors was examined with Ac_2-L-Lys-D-Ala-D-Ala as the carbonyl donor (Table 4). These data showed the following. (i) D-Lactate was a very weak acceptor, explaining why the enzyme-catalysed hydrolysis of the ester carbonyl donor

Table 2. Effect of the presence of D-amino acid oxidase on the amount of acyl-([^{14}C]Ac_2-L-Lys-D-alanyl-)enzyme formed during reaction between the K15 DD-peptidase and [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala

The enzyme (3.2 μM) was incubated with 2 mM-[^{14}C]Ac_2-L-Lys-D-Ala-D-Ala for various times at 37 °C in the presence and in the absence of 2.5 μg of D-amino acid oxidase and 4.5 μg of catalase (see Fig. 6 legend) in 5 mM-potassium phosphate buffer, pH 7.5, containing 0.008% cetyltrimethylammonium bromide (15 μl final volume). The reactions were stopped as indicated in Fig. 1 legend. After separation by gel electrophoresis, the radioactive acyl-enzyme was estimated by densitometry of the fluorograms. The area values thus obtained are expressed in arbitrary units. [^{14}C]Benzylpenicillin was used to trap the enzyme quantitatively as [^{14}C]benzylpenicilloyl-enzyme and the corresponding area served as a control (see the Materials and methods section).

Carbonyl donor	Presence of D-amino acid oxidase	Incubation time (min)	Area (arbitrary units
[^{14}C]Ac_2-L-Lys-D-Ala-D-Ala	0	2	0.64
[^{14}C]Ac_2-L-Lys-D-Ala-D-Ala	0	30	0.27
[^{14}C]Ac_2-L-Lys-D-Ala-D-Ala	0	60	0.24
[^{14}C]Ac_2-L-Lys-D-Ala-D-Ala	+	30	0.41
[^{14}C]Ac_2-L-Lys-D-Ala-D-Ala	+	60	0.39
[^{14}C]Benzylpenicillin (control)	0	10	34

Table 3. Specificity profile of the K15 DD-peptidase for tripeptide carbonyl donors in transpeptidation reactions with Gly-Gly as amino acceptor

Reaction catalysed:

R^1-R^2-NH-R^3 + [^{14}C]Gly-Gly → R^1-R^2-[^{14}C]Gly-Gly + NH_2-R^3

The enzyme (0.92 μM), each of the selected tripeptides (2 mM) and [^{14}C]Gly-Gly (2 mM) were incubated together at 37 °C in 5 mM-potassium phosphate buffer, pH 7.5, containing 0.008% cetyltrimethylammonium bromide (30 μl final volume). The incubation time was 1 h with Ac_2-L-Lys-D-Ala-D-Ala and 5 h in all the other cases. Abbreviation: A_2Bu, diaminobutyric acid.

Donor	Specific activity (nmol of reaction product/min per mg of protein)	(%)
(1) Ac_2-L-Lys-D-Ala-D-Ala	300	100
(2) Ac_2-L-A_2Bu-D-Ala-D-Ala	120	40
(3) Ac_2-L-Lys-D-Ala-D-Leu	25	8
(4) Ac_2-L-Lys-D-Ala-Gly	25	8
(5) Ac_2-L-Lys-D-Ala-D-Lys	10	4
(6) Ac_2-L-Lys-D-Ala-L-Ala	0	0
(7) Ac_2-L-Lys-D-Leu-D-Ala	0	0
(8) Ac_2-L-Lys-Gly-D-Ala	0	0

Ac_2-L-Lys-D-Ala-D-Lac was not affected by endogenously released D-lactate. (ii) *meso*-Diaminopimelate, either as such or with substituents on its amino group at the L-centre, D-aminoadipate and glycine were acceptors of lower efficacy than D-alanine; L-alanine had no detectable activity. (iii) Of the amino compounds tested the dipeptides Gly-Gly and Gly-L-Ala exhibited the highest acceptor activity; dipeptides with L-amino acid other than L-alanine or with a D-alanine residue at the *C*-terminal position had decreased or very weak activity, and dipeptides with an L-alanine or a D-alanine residue at the *N*-terminus had no detectable activity; Gly-Gly-Gly and Gly-Gly-L-Ala were weak acceptors, suggesting that the distance between the *N*- and *C*-termini of the molecule was important.

DISCUSSION

Catalysis of transpeptidation reactions by the active-site-serine proteinases such as chymotrypsin (Johnston *et al.*, 1950) and trypsin (Waley & Watson, 1954) is a well-known phenomenon. Basically, the 26000-M_r membrane-bound DD-peptidase of *Streptomyces* K15 behaves similarly. It operates via the transitory formation of an acyl-enzyme and, in the presence of a suitable amino compound, it catalyses hydrolysis and transpeptidation of the carbonyl donor on a competitive basis. The K15 DD-peptidase has specificity profiles for carbonyl donors and amino acceptors that reflect the structural features of the wall peptidoglycan. In these respects it resembles the exocellular R61 and R39 DD-carboxypeptidases/transpeptidases excreted by *Streptomyces* R61 and *Actinomadura* R39 respectively (Ghuysen *et al.*, 1981, 1984; Frère & Joris, 1985). However, the K15 DD-peptidase is peculiar in several respects.

When H_2O is the only nucleophile available, the acyl-enzyme accumulates detectably at the steady state of the reaction with both the ester carbonyl donor Ac_2-L-Lys-D-Ala-D-Lac and the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala. However, although the released D-lactate has no detectable effect on hydrolysis of the ester substrate (which proceeds to completion), released D-alanine is re-utilized as acceptor in a transfer reaction so that hydrolysis of the amide substrate is prevented, a situation similar to that observed by Waley & Watson (1954) with trypsin.

In the presence of an amino acceptor (Gly-Gly or Gly-L-Ala) related to the natural wall peptidoglycan acceptor, the ester and carbonyl-donor substrates undergo partitioning between hydrolysis and transpeptidation, and acyl-enzyme does not accumulate. Hence, assuming that Gly-Gly acted only as an alternate nucleophile at the level of the acyl-enzyme (see reaction models in the Materials and methods section), the K_m and $k_{cat.}$ values derived from Hanes plots of [Ac_2-L-Lys-D-Ala-D-Lac (or -D-Ala)]/v versus [Ac_2-L-Lys-D-Ala-D-Lac (or -D-Ala)] in the presence of Gly-Gly should be

Table 4. Specificity profile of the K15 DD-peptidase for D-lactate and amino acceptors in transfer reactions with Ac_2-L-Lys-D-Ala-D-Ala as carbonyl donor

Reaction catalysed:

$$Ac_2\text{-L-Lys-D-Ala-Ala} + \text{acceptor} \rightarrow Ac_2\text{-L-Lys-D-Ala-acceptor} + \text{D-Ala}$$

[^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (2 mM) was used as the donor except for acceptors 1, 2, 3, 4 and 8 (in which cases the non-radioactive tripeptide was used). The enzyme (at the concentrations indicated), Ac_2-L-Lys-D-Ala-D-Ala and each of the acceptors (at the indicated concentrations) were incubated in 5 mM-potassium phosphate buffer, pH 7.5, containing 0.008% cetyltrimethylammonium bromide (30 μl final volume) at 37 °C. Abbreviation: A_2pm, diaminopimelate.

Acceptor	[Acceptor]/[Donor]	[Enzyme] (μM)	Incubation time (h)	Specific activity (nmol of reaction product/min per mg of protein)	Specific activity (%) (relative to Gly-Gly)
(1) D-[^{14}C]Lac	5:1	0.29	2	8	3
(2) [^{14}C]Gly	1:1	0.29	2	65	22
(3) D-[^{14}C]Ala	1:1	0.29	2	150	60
	5:1	0.29	2	205	68
(4) L-[^{14}C]Ala	1:1	1.84	6	2	1
(5) *rac*-A_2pm	5:1	0.29	2	180	60
(6) *meso*-A_2pm	5:1	0.29	2	150	50
(7) L-Ala-γ-D-isoGln-*meso*-A_2pm-D-Ala	1:1	0.92	6	60	20
(8) [^{14}C]Gly-Gly	1:1	0.29	2	300	100
(9) Gly-L-Ala	1:1	0.29	2	300	100
(10) Gly-L-Glu	1:1	0.29	2	150	50
	5:1	0.29	2	175	60
(11) Gly-L-Leu	1:1	0.29	2	100	33
	5:1	0.29	2	190	60
(12) Gly-D-Ala	5:1	0.92	6	9	3
(13) L-Ala-Gly	5:1	0.92	6	0	0
(14) L-Ala-L-Ala	5:1	0.92	6	0	0
(15) L-Ala-D-Ala	5:1	0.92	6	0	0
(16) D-Ala-Gly	5:1	0.92	6	0	0
(17) D-Ala-D-Ala	5:1	0.92	6	0	0
(18) D-Ala-L-Ala	5:1	0.92	6	0	0
(19) N^{α}-Ac-L-Lys-D-Ala	5:1	0.92	6	0	0
(20) Gly-Gly-Gly	1:1	0.92	6	3	1
	5:1	0.92	6	8	3
(21) Gly-Gly-L-Ala	1:1	0.92	6	5	1.5
	10:1	0.92	6	20	7

equal to the K and k_{+2} values of the hydrolysis pathway respectively, as determined by trapping the accumulated acyl-enzyme. The data shown in Table 1 are at variance with this simple model. With the ester substrate, the 4-fold increased K value (from 2.2 mM to 8.9 mM) and the 6.5-fold increased k_{+2} value (from 0.86 s^{-1} to 5.6 s^{-1}) caused by the presence of Gly-Gly almost compensate for each other, with the consequence that the second-order rate constant of enzyme acylation (from 390–625 $M^{-1} \cdot s^{-1}$ to 630 $M^{-1} \cdot s^{-1}$) is not much affected. With the amide substrate, 10 mM-Gly-Gly causes a 1.5-fold decreased K value (from 9 mM to 6 mM) and a 4-fold increased k_{+2} value (from 0.13 s^{-1} to 0.55 s^{-1}), resulting in a substantial increase of the second-order rate constant of enzyme acylation (from 14 $M^{-1} \cdot s^{-1}$ to 92 $M^{-1} \cdot s^{-1}$). Hence, to all appearances, the amino acceptor does not behave as a simple alternate nucleophile but it influences both the initial binding between the enzyme and the carbonyl donor and the ensuing enzyme-acylation step. Therefore hydrolysis and transpeptidation do not proceed through identical pathways. That the v_T/v_{Hy} ratio decreases as the carbonyl-donor concentration increases also suggests that the amino acceptor, and not the carbonyl donor, may bind first to the enzyme (Frère, 1973; Frère *et al.*, 1973). In turn, that the v_T/v_{Hy} ratio is directly proportional to the amino-acceptor concentration suggests that the enzyme does not bind more than one molecule of acceptor (Frère, 1973; Frère *et al.*, 1973).

A striking property of the K15 DD-peptidase is that under certain conditions the amino acceptor completely suppresses the acceptor activity of water and, in the case of the amide carbonyl donor, successfully competes with the acceptor activity of the released D-alanine. Quantitative conversion of both types of carbonyl donors into transpeptidated products then proceeds until complete consumption, thus conferring on the K15 DD-peptidase the property of functioning as an effective strict DD-transpeptidase.

The above analyses of the catalytic properties of the K15 DD-peptidase offer a possible explanation to the well-known observation that certain proteins, though assumed to be DD-peptidases because they bind penicillin,

have no detectable or weak enzymic activity on carbonyl donors more or less structurally related to wall peptidoglycan (Suzuki *et al.*, 1980; Tomioka *et al.*, 1982, 1983; Ishino & Matsuhashi, 1981; see also Table 18 in Frère & Joris, 1985). By analogy with the K15 DD-peptidase, such proteins may lack hydrolytic potency on D-Ala-D-Ala-terminated carbonyl donors because they re-utilize the released D-alanine as acceptor of the transfer reaction. In addition, they may have very low intrinsic catalytic activity unless a suitable amino acceptor is present, which enhances the efficacy with which the carbonyl donor acylates the protein.

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