Streptomyces K15 DD-peptidase-catalysed reactions with suicide β -lactam carbonyl donors

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The values of the kinetic parameters that govern the interactions between the *Streptomyces* K15 DD-peptidase and β -lactam compounds were determined by measuring the inactivating effect that these compounds exert on the transpeptidase activity of the enzyme and, in the case of [14 C]benzylpenicillin and [14 C]cefoxitin, by measuring the amounts of acyl-enzyme formed during the reaction. K15 DD-peptidase binds benzylpenicillin or cefoxitin at a molar ratio of 1:1. Benzylpenicilloate is the major product released during breakdown of the acyl-enzyme formed with benzylpenicillin. Benzylpenicillin is not a better acylating agent than the amide $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ and ester $\text{Ac}_2\text{-L-Lys-D-Ala-D-lactate}$ carbonyl-donor substrates. β -Lactam compounds possessing a methoxy group on the α -face of the molecule show high inactivating potency.

INTRODUCTION

The *Streptomyces* K15 DD-peptidase is a membrane-bound penicillin-binding protein. This enzyme, purified to 95% homogeneity (Nguyen-Distèche *et al.*, 1982), performs effective transpeptidation reactions on carbonyl-donor and amino-acceptor co-substrates (Nguyen-Distèche *et al.*, 1986). Benzylpenicillin is recognized as carbonyl donor by the K15 DD-peptidase and immobilizes the enzyme in the form of a rather stable penicilloyl-enzyme. The interaction between the K15 DD-peptidase and β -lactam compounds has now been studied with the use of the equations developed in Ghuyssen *et al.* (1986). The results thus obtained are described below.

MATERIALS AND METHODS

Enzymes

The *Streptomyces* K15 DD-peptidase, obtained to 95% purity in the presence of 0.05% cetyltrimethylammonium bromide, was that used previously (Nguyen-Distèche *et al.*, 1982). The *Bacillus cereus* penicillinase I was from Whatman Biochemicals, Maidstone, Kent, U.K., and the *Enterobacter cloacae* P99 cephalosporinase was prepared as described by Ross (1975).

β -Lactam compounds

[14 C]Benzylpenicillin (50 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K., and [14 C]cefoxitin (2.7 mCi/mmol) was a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A. The non-radioactive β -lactams were from the following companies: carbenicillin, temocillin and ticarcillin, from Beecham, Brussels, Belgium; ampicillin and oxacillin, from Bristol Benelux, Brussels, Belgium; cephalixin, cephaloglycine, cephalothin and moxalactam, from Lilly Laboratories, Indianapolis, IN, U.S.A.;

sulfazecin, from Takeda Chemical Industries, Osaka, Japan; mecillinam, from Leo Pharmaceutical Products, Ballerup, Denmark; azthreonam and compounds SQ 81387, SQ 26559, SQ 81427, SQ 26630 and SQ 26324, from Squibb Institute for Medical Research, Princeton, NJ, U.S.A.; piperacillin, from Cyanamid Benelux, Ottignies, Belgium.

Detergent-containing phosphate buffer, pH 7.5

Unless otherwise stated, the reactions were carried out in 5 mM-potassium phosphate buffer, pH 7.5, containing 0.008% cetyltrimethylammonium bromide.

Determination of transpeptidase activity

The reaction catalysed was (Nguyen-Distèche *et al.*, 1986):

The assays were carried out by incubating the enzyme and the co-substrates in 30 μ l of detergent-containing phosphate buffer at 37 °C and measuring the amount of released D-Ala by the D-amino acid oxidase technique. In some cases [14 C]Gly-Gly was used and the transpeptidated product $\text{Ac}_2\text{-L-Lys-D-Ala-}^{14}\text{C-Gly-Gly}$ was determined after separation by paper electrophoresis. For more details see Johnson *et al.* (1975) and Leyh-Bouille *et al.* (1977).

ID₅₀ values

ID₅₀ values refer to the concentrations of the β -lactam compounds necessary to decrease the transpeptidase activity of the enzyme by 50%. With all the β -lactam compounds listed in Table 2, except compound SQ 26559, the enzyme (0.79 μ M), the co-substrates (at the concentrations aforementioned) and various concentrations of the selected β -lactam compound were mixed together in detergent-containing buffer (30 μ l final volume) and

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incubated at 37 °C for 30 min. The reaction was stopped by placing the solutions in a boiling-water bath for 1 min, and the released D-alanine was determined. In all cases the reaction was carried out under conditions where [β -lactam compound] was less, or very much less, than [enzyme]. With compound SQ 26559 (which is the most active β -lactam compound examined), in order to fulfil this latter condition, the enzyme (0.011 μ M), the co-substrates (at the concentrations aforementioned and with [14 C]Gly-Gly) and various concentrations of compound SQ 26559 were incubated for 120 min, after which time the amount of Ac₂-L-Lys-D-Ala-[14 C]Gly-Gly was determined.

Acyl-enzyme formation with [14 C]benzylpenicillin and [14 C]cefoxitin: saturation curves

The enzyme (1.59 μ M or 0.73 μ M in the experiments involving cefoxitin or benzylpenicillin respectively) and various concentrations of the selected radioactive β -lactam compound were incubated at 37 °C in detergent-containing phosphate buffer (30 μ l final volume) for 30 s ([14 C]cefoxitin) or 600 s ([14 C]benzylpenicillin). The reactions were stopped by adding 5 μ l of 0.1 M non-radioactive β -lactam and 30 μ l of denaturing buffer (Nguyen-Distèche *et al.*, 1986), and placing the solutions in a boiling-water bath for 60 s. After polyacrylamide-gel electrophoresis at pH 8.3 in the presence of SDS, the amounts of [14 C]acyl-enzyme formed were measured by microdensitometry of the fluorograms. For more details see Laemmli & Favre (1973), Laskey (1980) and Nguyen-Distèche *et al.* (1986).

Pseudo-first-order rate constant k_a of acyl-enzyme formation with non-radioactive β -lactam compounds

The enzyme (1.26 μ M) and various concentrations of the non-radioactive β -lactam compounds listed in Table 1 (except cefoxitin) were incubated at 37 °C in detergent-containing phosphate buffer (75 μ l final volume). At intervals samples (25 μ l) were removed and supplemented with a mixture containing the co-substrates at the aforementioned concentrations and, in order to destroy the excess of β -lactam present, sufficient amounts of P99 β -lactamase (in the case of cephalothin) or *B. cereus* β -lactamase I (in all the other cases). The reaction mixtures were incubated at 37 °C for 30 min (20 min in the case of piperacillin). The reaction was stopped by placing the solutions in a boiling-water bath for 60 s, and the released D-alanine was measured. Since breakdown of the acyl-enzyme (and enzyme recovery) proceeded during incubation with the co-substrates, a correction was introduced as described previously (Frère *et al.*, 1974).

Acyl-enzyme breakdown and enzyme recovery

With compound SQ 26324 and the β -lactam compounds listed in Table 1 except cefoxitin, samples of enzyme (0.95 μ M) in detergent-containing phosphate buffer (25 μ l final volume) were incubated for 10 min at 37 °C with a concentration of β -lactam compound more than sufficient to inhibit the enzyme activity completely. Excess β -lactam compound was destroyed by incubating the reaction mixtures for 5 min at 30 °C with the P99 β -lactamase (in the case of cephalothin and compound SQ 26324) or with the *B. cereus* β -lactamase I (in all other cases), and the samples, containing the acyl-enzyme, were maintained at

37 °C. At various intervals these incubation mixtures were supplemented with the co-substrates at the aforementioned concentrations, and incubated for an additional 30 min (20 min in the case of piperacillin), after which time the amount of released D-alanine was determined. The first-order rate constant of acyl-enzyme breakdown (and enzyme recovery) was calculated after correction as described previously (Frère *et al.*, 1974).

With cefoxitin (which is not susceptible to the *B. cereus* or P99 β -lactamase), the enzyme (17 μ M) and an excess of cefoxitin were incubated for 10 min at 37 °C in detergent-containing buffer (25 μ l final volume). The reaction mixture was supplemented with an equal volume of acetone precooled to -20 °C and maintained at -20 °C for 1 h. The suspension was centrifuged (20800 *g* for 20 min) at -10 °C and the pellet was dissolved in 25 μ l of buffer. Samples, containing the acyl-enzyme, were incubated at 37 °C for increasing times and the rate of enzyme recovered was determined as described above.

[14 C]Benzylpenicilloyl-enzyme breakdown: release of the acyl moiety

The enzyme (26 μ M) was incubated with 0.2 mM- [14 C]benzylpenicillin for 15 min at 37 °C in 30 mM-potassium phosphate buffer, pH 7.5, containing 0.05% cetyltrimethylammonium bromide (60 μ l final volume). Acetone (60 μ l) precooled to -20 °C was added and the reaction mixture was maintained at -20 °C for 1 h. The suspension was centrifuged at 20800 *g* for 20 min at -10 °C and the pellet was dissolved in 117 μ l of the same buffer as above. The yield in [14 C]benzylpenicilloyl-enzyme was 86%. Spontaneous breakdown, at 37 °C, of the radioactive acyl-enzyme gave rise to a mixture of [14 C]phenylacetyl-glycine and [14 C]benzylpenicilloate. The two reaction products were isolated and assayed (i) by paper electrophoresis at pH 6.5 (after 1 h and at 60 V/cm, phenylacetyl-glycine and benzylpenicilloate migrated 25 cm and 32 cm towards the anode respectively), and (ii) by chromatography on thin-layer Polygram Sil G (Macherey and Nagel Co., Düren, Germany) with either chloroform/methanol/acetic acid (44:5:1, by vol.) (under which conditions the R_F values of phenylacetyl-glycine and benzylpenicilloate were 0.49 and 0.0 respectively) or a water/butan-1-ol/acetic acid (5:4:1, by vol., upper phase) (under which conditions the corresponding R_F values were 0.67 and 0.60).

Table 1. Second-order rate constant k_{+2}/K of acyl-enzyme formation during reaction between the K15 DD-peptidase and β -lactam compounds and first-order rate constant k_{+3} of acyl-enzyme breakdown (at 37 °C and in detergent-containing potassium phosphate buffer, pH 7.5)

	k_{+2}/K ($M^{-1} \cdot s^{-1}$)	k_{+3} (s^{-1})	Half-life of acyl-enzyme (min)
Cephalothin	2	$< 0.25 \times 10^{-4}$	> 460
Oxacillin	8	1.2×10^{-4}	95
Ticarcillin	21	0.69×10^{-4}	165
Carbenicillin	37.5	0.26×10^{-4}	440
Benzylpenicillin	150	1×10^{-4}	115
Piperacillin	122	2.6×10^{-4}	45
Cefoxitin	860	0.17×10^{-4}	670

Fig. 1. Determination of the second-order rate constant k_{+2}/K of acylation of the K15 DD-peptidase by benzylpenicillin

(a) Plots of $\ln(A_t/A_0)$ versus time. (b) Secondary plots of k_a versus [benzylpenicillin]. A_t = residual enzyme activity after incubation in the presence of benzylpenicillin; A_0 = enzyme activity in the absence of benzylpenicillin. For experimental details see the Materials and methods section.

Reaction model

Inactivation of the K15 DD-peptidase by β -lactam compounds was interpreted on the basis of the reaction model:

where E = DD-peptidase, D (for carbonyl donor) = β -lactam, K = dissociation constant of the Michaelis complex $E \cdot D$, $E-D^*$ = acyl-enzyme, P = degradation product, 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.

RESULTS

Stoichiometry of the reaction and determination of the first-order rate constant k_{+3} of acyl-enzyme breakdown

Isolation of the [14 C]benzylpenicilloyl-enzyme formed by reaction with an excess of [14 C]benzylpenicillin showed that 1 mol of the 26000- M_r DD-peptidase bound 1 equivalent of penicilloyl moiety. Breakdown of the [14 C]benzylpenicilloyl-enzyme proceeded with a k_{+3} value of $0.91 \times 10^{-4} \text{ s}^{-1}$ and generated primarily [14 C]benzylpenicilloate ($k'_{+3} = 7.6 \times 10^{-5} \text{ s}^{-1}$) and, to a lesser extent, [14 C]phenylacetyl glycine ($k''_{+3} = 1.1 \times 10^{-5} \text{ s}^{-1}$).

The k_{+3} values reported in Table 1 were calculated on the basis of the recovery of the enzyme activity as described in the Materials and methods section.

Determination of the second-order rate constant k_{+2}/K of acyl-enzyme formation

Two procedures were used, indicated below.

(1) The values of the pseudo-first-order rate constant k_a of protein acylation were determined by monitoring the disappearance of the enzyme activity at three β -lactam compound concentrations and three incubation times. In

Fig. 2. Saturation curves of the K15 DD-transpeptidase by [14 C]benzylpenicillin (○) and [14 C]cefoxitin (●)

For experimental details see the Materials and methods section. $\triangle$ and $\blacktriangle$, Theoretical curves calculated on the basis of a k_{+2}/K value of $150 \text{ M}^{-1} \cdot \text{s}^{-1}$ for benzylpenicillin and $850 \text{ M}^{-1} \cdot \text{s}^{-1}$ for cefoxitin, and of a k_{+3} value of $1 \times 10^{-4} \text{ s}^{-1}$ for benzylpenicillin and $1.7 \times 10^{-5} \text{ s}^{-1}$ for cefoxitin (for more details see Ghuysen *et al.*, 1986).

all cases the k_a values were at least 10 times larger than the k_{+3} values [showing that eqn. (17) in Ghuysen *et al.* (1986) applied] and the plots of k_a versus [β -lactam compound] gave rise to straight lines (showing that the condition [β -lactam compound] $\ll K$ also applied). Consequently the k_{+2}/K values reported in Table 1 (except for cefoxitin) were calculated from the slopes of the lines thus obtained [eqn. (18) in Ghuysen *et al.* (1986)]. Figs. 1(a) and 1(b) illustrate the data obtained with benzylpenicillin. Note that at the lowest (20 μM) benzylpenicillin concentration used the k_a value ($3.1 \times 10^{-3} \text{ s}^{-1}$) is 31-fold larger than the k_{+3} value ($1 \times 10^{-4} \text{ s}^{-1}$).

(2) The second procedure was based on saturation curves obtained by measuring the amounts of radioactive

Fig. 3. Lineweaver-Burk plots of $1/P$ versus $1/[Ac_2-L-Lys-D-Ala-D-Ala]$ in the presence of a fixed (2 mM) Gly-Gly concentration and in the absence and in the presence of 3 μM -, 6 μM - and 10 μM -benzylpenicillin

The enzyme (0.47 μM) and the various reactants were incubated for 60 min at 37 °C in detergent-containing phosphate buffer (30 μl final volume). P = reaction product $Ac_2-L-Lys-D-Ala-Gly-Gly$.

acyl-enzyme formed by reaction between the enzyme and various concentrations of [^{14}C]benzylpenicillin for 600 s or of [^{14}C]cefoxitin for 30 s. Note that the incubation times were 11.5-fold smaller (in the case of benzylpenicillin) and very small (in the case of cefoxitin) when compared with the half-lives of the corresponding acyl-enzyme derivatives. As shown in Fig. 2, [^{14}C]benzylpenicillin concentrations of 3.4, 7.6 and 17 μM , and [^{14}C]cefoxitin concentrations of 13.5, 26 and 58 μM , acylated the enzyme by 30%, 50% and 75% respectively. From the extents of protein acylation and the corresponding β -lactam compound concentrations, eqn. (18) in Ghuysen *et al.* (1986) yielded k_{+2}/K values of 150, 175 and 135 $M^{-1}\cdot s^{-1}$ in the case of benzylpenicillin (average value 150 $M^{-1}\cdot s^{-1}$) and 885, 880 and 800 $M^{-1}\cdot s^{-1}$ in the case of cefoxitin (average value 860 $M^{-1}\cdot s^{-1}$). On the basis of the k_{+2}/K values thus obtained, and the known k_{+3} values, eqn. (11) in Ghuysen *et al.* (1986) showed that, at the steady state of the reactions, benzylpenicillin at 3, 6, 10 and 30 μM concentrations would acylate the enzyme by 82%, 90%, 94% and 98% respectively. At the lowest cefoxitin concentration used (7.5 μM) and at the steady state of the reaction, cefoxitin would acylate the enzyme by 99.7%. These examples illustrate cases where the range of β -lactam compound concentrations and incubation times used justify satisfactorily (benzylpenicillin) or perfectly (cefoxitin) the use of eqn. (18) in Ghuysen *et al.* (1986).

Competition between benzylpenicillin and the carbonyl-donor substrate $Ac_2-L-Lys-D-Ala-D-Ala$

The presence of 3 mM- and 6 mM- $Ac_2-L-Lys-D-Ala-D-Ala$ in the reaction mixture decreased the value of the

second-order rate constant of enzyme acylation by benzylpenicillin from 150 $M^{-1}\cdot s^{-1}$ to 90 and 65 $M^{-1}\cdot s^{-1}$ respectively. In addition, the Lineweaver-Burk plots of $1/[P]$ versus $1/[Ac_2-L-Lys-D-Ala-D-Ala]$ (Fig. 3) as obtained in the presence of 2 mM-Gly-Gly, both in the absence and in the presence of various concentrations of benzylpenicillin, indicated an apparent competitive phenomenon between benzylpenicillin and the carbonyl-donor substrate $Ac_2-L-Lys-D-Ala-D-Ala$. However, given I = benzylpenicillin and S = $Ac_2-L-Lys-D-Ala-D-Ala$ and assuming that the dissociation constant of the ternary complex ($ESI \rightleftharpoons ES + I$) was of the same order of magnitude as K , both competitive and non-competitive models would give rise, for $[I] \ll K$ and within the limits of experimental errors, to the same competitive plot (Frère & Joris, 1985). Therefore, under the experimental conditions used, it was not possible to distinguish between the two models.

Effect of Gly-Gly

At 2 mM concentration the amino acceptor Gly-Gly had no effect on the value of the second-order rate constant of enzyme acylation by benzylpenicillin. Similarly, it had no effect on the values of the first-order rate constant of breakdown of the acyl-enzyme formed with benzylpenicillin and with compound SQ 26324 ($k_{+3} = 0.2 \times 10^{-4} s^{-1}$; half-life 575 min; results not shown in Table 1).

Specificity profile of the K15 DD-peptidase for β -lactam compounds

The profile of the enzyme was investigated by determining the concentration of the β -lactam compounds (ID_{50} values) necessary to inhibit the transpeptidase activity by 50% when the enzyme, the co-substrates $Ac_2-L-Lys-D-Ala-D-Ala$ and Gly-Gly, and the selected β -lactam were mixed together and incubated for a given period of time. Comparison of the data of Tables 1 and 2 showed that there was at least an approximate parallelism between the k_{+2}/K values and the corresponding ID_{50} values.

DISCUSSION

The 26000- M_r protein that is present in the plasma membrane of *Streptomyces* K15 is a powerful transpeptidase on well-defined co-substrates (Nguyen-Distèche *et al.*, 1986) and binds penicillin in a 1:1 molar ratio. In these two respects this protein differs from the high- M_r penicillin-binding proteins isolated from bacterial membranes (in particular the proteins 1A, 1B, 2 and 3 of *Escherichia coli*), which show very low or no detectable transpeptidase activity and bind much less than 1 mol of penicillin per mol of protein (Frère & Joris, 1985).

The K15 DD-peptidase is a target of benzylpenicillin of moderate sensitivity. Acylation of the enzyme by benzylpenicillin ($k_{+2}/K = 150 M^{-1}\cdot s^{-1}$) is hardly more effective, whether Gly-Gly is present or not in the reaction mixture, than acylation by the amide carbonyl donor $Ac_2-L-Lys-D-Ala-D-Ala$ in the presence of Gly-Gly ($k_{+2}/K = 92 M^{-1}\cdot s^{-1}$) and is less effective than acylation by the ester carbonyl donor $Ac_2-L-Lys-D-Ala-D-Lac$ ($k_{+2}/K = 625 M^{-1}\cdot s^{-1}$) (Nguyen-Distèche *et al.*, 1986). Kinetically benzylpenicillin and the carbonyl donor $Ac_2-L-Lys-D-Ala-D-Ala$ seem to compete for the same

enzyme active site, but a choice between competitive and non-competitive model would need further experiments. The amino acceptor Gly-Gly has no effect on the rate of breakdown of the acyl-enzyme formed with benzylpenicillin or the monocyclic β -lactam compound SQ 26324. It has been suggested (Waxman *et al.*, 1983) that the presence of a thiazolidine (dihydrothiazine) ring in the penicilloyl-(cephalosporoyl)-enzyme immobilizes the DD-peptidases by preventing access of the nucleophilic acceptor to or functioning of the enzyme acceptor site. The present studies show that a monocyclic β -lactam causes the same effect.

Table 2. Specificity profile of the K15 DD-peptidase for β -lactam compounds as expressed by the β -lactam concentrations (ID₅₀) necessary to inhibit the transpeptidase activity by 50% (at 37 °C and in detergent-containing potassium phosphate buffer, pH 7.5)

Temocillin, cefoxitin, compound SQ 26630, sulfazecin, moxalactam and compound SQ 26559 have a methoxy group on the α -face of the molecule. The structures of compounds SQ 81387, SQ 26559, SQ 81427, SQ 26630 and SQ 26324, ticarcillin, temocillin and benzylpenicillin are shown in Fig. 4.

β -Lactam	ID ₅₀ (M)
Azthreonam	$> 10^{-3}$
Cephalexin	$> 10^{-3}$
Mecillinam	$> 10^{-3}$
Cephaloglycine	6×10^{-4}
Compound SQ 26324	4.5×10^{-4}
Cephalothin	4.3×10^{-4}
Compound SQ 81387	2.7×10^{-4}
Compound SQ 81427	2.6×10^{-4}
Oxacillin	1.6×10^{-4}
Ticarillin	8×10^{-5}
Carbenicillin	3.5×10^{-5}
Temocillin	2×10^{-5}
Ampicillin	1.8×10^{-5}
Benzylpenicillin	1.1×10^{-5}
N-Formimidoylthienamycin	9.6×10^{-6}
Piperacillin	9×10^{-6}
Cefoxitin	2.6×10^{-6}
Compound SQ 26630	1.5×10^{-6}
Sulfazecin	1.4×10^{-6}
Moxalactam	1.3×10^{-6}
Compound SQ 26559	2.5×10^{-7}

the pair compound SQ 81427–compound SQ 26630 and the pair compound SQ 81387–compound SQ 26559, the occurrence of an α -methoxy group at C-3 of the monobactams causes a 200–300-fold increased inactivating potency. Cefoxitin (7 α -methoxylated cephalosporin) is also an efficient inactivator. Similarly, the occurrence of an α -methoxy group at C-3 confers on certain monobactams a 10–100-fold increased efficacy of binding to the R61 exocellular DD-peptidase (Georgopapadakou *et al.*, 1982) and the penicillin-binding proteins 4 of *Staphylococcus aureus* and 4, 5 and 6 of *Escherichia coli* (Georgopapadakou *et al.*, 1983). Cefoxitin also has a high affinity for these latter proteins (Curtis *et al.*, 1979).

(iv) Among the various β -lactam compounds tested, cefoxitin, compound SQ 26630, sulfazecin, moxalactam and compound SQ 26559 are the most potent inactivators of the K15 DD-peptidase; they all possess an α -methoxy group in an equivalent position. In this respect, the K15 DD-peptidase differs markedly from the exocellular R61 and R39 serine-active-site DD-peptidases, for which benzylpenicillin is one of the most efficient inactivators tested (Georgopapadakou *et al.*, 1982; Frère & Joris, 1985).

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