Inhibition of gramicidin S synthetase 2 by L-phenylalanine chloromethyl ketone

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The inhibition of the gramicidin S-biosynthesis and the thioester formation activities of the multienzyme GS 2 by L-Phe-CMK, a substrate analogue of L-phenylalanine, were investigated. L-Phe-CMK inhibits irreversibly the activation of proline, valine, ornithine and leucine with about the same velocity and shows no specificity for hydrophobic amino acid binding sites, as could be expected. The gramicidin S-biosynthesis is inhibited about four times more rapidly than the thioester formations. Prevention of inhibition by the substrates opens the possibility of specific blocking of one or more active sites of the enzyme.

Gramicidin S synthetase *Phenylalanine activation* *L-phenylalanine chloromethyl ketone*

1. INTRODUCTION

The multienzyme system gramicidin S synthetase catalyzes the non-ribosomal biosynthesis of gramicidin S, a cyclic peptide antibiotic (cyclo(D-Phe → Pro → Val → Orn → Leu)$_2$). The system consists of a heavy (GS 2, M_r 280000) and a light multienzyme (SG 1, M_r 100000) [1]. The multienzymes activate amino acids by the formation of aminoacyl adenylates, which are accepted by specific thiol groups in an aminoacylation step. GS 1 activates phenylalanine and epimerizes L-phenylalanine to D-phenylalanine. GS 2 activates proline, valine, ornithine and leucine. Polymerization proceeds from these active esters by the 'protein–thiotemplate mechanism' [2]. The specificity of the gramicidin S synthetase system appears to be lower than the ribosomal system [1,3]. The ability of leucine to replace valine [4] and vice versa, as well as the activation and thioesterbinding of phenylalanine by GS 2 was shown [5]. This unexpected activation and thioesterbinding of phenylalanine prompted us to investigate the reaction of its pseudosubstrate L-Phe-CMK ($PhCH_2CH(NH_2)COCH_2Cl$) with GS 2. Its effective inhibitory properties for phenylalanine activation in the case of GS 1 were reported in [6]. Here, we report evidence, that L-Phe-CMK is not a specific inhibitor for one of the activities of GS 2. There is a similar inhibition of all 4 thioester formation reactions, which is prevented in the presence of the substrates. Thus, substrate protection may permit a selective inhibition of active enzyme sites.

Abbreviations: GS 2, gramicidin S synthetase 2; GS 1, gramicidin S synthetase 1; L-Phe-CMK, L-phenylalanine chloromethyl ketone; IAA, iodoacetamide; DTE, dithioerythritol; EDTA, ethylenedinitrilotetraacetic acid; PP_i, pyrophosphate; Boc, butyloxycarbonyl

2. MATERIALS AND METHODS

2.1. *Cell growth*

Bacillus brevis ATCC 9999 was obtained from the American Type Culture Collection and was grown as in [4].

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2.2. *Purification of gramicidin S synthetase 2*

2.2.1. Crude extract

200-g cells (wet wt) were suspended in 800 ml 50 mM sodium phosphate buffer (pH7.2) containing 2 mg lysozyme/ml, 2 mM DTE and 1 mM EDTA. The suspension was incubated at 30°C for 45 min and centrifuged for 20 min at $14000 \times g$. All the following steps were performed at 4°C. The supernatant was adjusted to 1% of streptomycine sulfate and after 5 min stirring, the suspension was centrifuged for 10 min at $26000 \times g$. The supernatant was adjusted to 45% saturation of ammonium sulfate by the addition of a saturated solution. After 20 min the precipitate was collected by centrifugation for 10 min at $26000 \times g$ (1180 mg protein) and dissolved in 35 ml buffer P (20 mM sodium phosphate (pH 7.2) 2 mM EDTA).

2.2.2. Ultrogel AcA 34 filtration

The predialysed crude extract was applied to a AcA 34 column (5×85 cm) equilibrated and eluted with buffer P. The enzymes (GS 1, GS 2) were localized by the amino acid-dependent ATP–PP_i-exchange (GS 2 fraction, 150 mg protein).

2.2.3. Further purification of the GS 2 fraction on DEAE-cellulose

The GS 2 fraction from Ultrogel AcA 34 was applied to a DE 52 column (2×6 cm) equilibrated with buffer P and eluted with 500 ml of a linear gradient of potassium phosphate (0.5 M) in buffer P. Active fractions were located by ATP–PP_i exchange and conductivity measurement (40 mg protein).

2.2.4. Sucrose gradient centrifugation

Highly purified enzyme (>90%) was obtained by precipitation of DE 52-purified enzyme with twice its volume of a saturated ammonium sulfate solution. The precipitate was dissolved in 1 ml buffer P, dialysed for 30 min against 0.5 l buffer P and layered onto a linear sucrose gradient (5–20%, w/total v) in buffer P (32 ml). Centrifugation was carried out in a SW 27-rotor for 24 h at 0°C and 27000 rev./min. The amount of pure enzyme was 10 mg.

2.3. *Measurement of thioester formation*

The incubation mixture (0.15 ml) contained 20 mM sodium phosphate (pH 7.2), 5 mM ATP, 20 mM $MgCl_2$, 1 mM EDTA, 5 μM amino acid, 2 μCi/assay ^{3}H-labeled amino acid (5 μM), 60 mM DTE and enzyme. The reaction was carried out at 37°C for 10 min and stopped by adding 1 ml of 5% trichloroacetic acid. The mixture was filtered on a millipore filter, washed with 2×20 ml of 5% trichloroacetic acid and 20 ml water and dried at 110°C for 10 min. Radioactivity was measured by adding 5 ml toluene-based scintillation liquid in a scintillation counter.

2.4. *Estimation of gramicidin S synthesis*

Gramicidin S synthesis was estimated by the millipore filter assay described in [7].

2.5. *ATP–$^{32}PP_i$-exchange reaction*

Amino acid activation was measured as in [8] by determining the amino acid-dependent ATP–PP_i-exchange reaction.

2.6. *Inhibition studies*

Due to the instability of halomethyl ketones, inhibitor solutions were prepared freshly before use. GS 2 (from the sucrose gradient centrifugation) was dialysed against 4×0.5 l of 20 mM sodium phosphate buffer (pH 7.2), 1 mM EDTA under nitrogen atmosphere to remove the DTE. Inhibition was carried out by adding 50 μl inhibitor to 50 μl enzyme and incubation for the appropriate time at 0°C. The reaction was stopped by adding 20 μl of 0.5 M DTE-solution and incubating for 5 min at 37°C. Residual activity was measured by adding 50 μl of the appropriate mix (see sections 2.3–2.5).

2.7. *Synthesis of L-phenylalanine chloromethyl ketone*

L-Phe-CMK was prepared from *N-t*-butyloxycarbonyl-L-phenylalanine diazomethyl ketone by removal of the *t*-butyloxycarbonyl (Boc) group with hydrochloric acid. Concurrent with the cleavage of the *N*-terminal moiety, the diazomethyl group is transformed to a chloromethyl group yielding the desired unsubstituted chloromethyl ketone.

$$\text{Boc-aa-OH} \xrightarrow[\text{2. } CH_2N_2]{\text{1. mixed anhydride}} \text{Boc-aa-}CHN_2 \xrightarrow[\text{dioxane/HCl}]{} \text{aa}CH_2Cl \cdot HCl$$

2.7.1. *N-t*-Butyloxycarbonyl-L-phenylalanine diazo ketone (1-diazo-3-*N-t*-butyloxycarbonyl-4-phenyl-butan-2-one) [9]

The intermediate diazomethyl ketone was prepared from a mixed anhydride by reaction of Boc-L-Phe with *i*-butyl chloroformate, triethylamine and (after removal of salt precipitates by filtration) diazomethane [9]. From 8 g (30.1 mM) Boc-L-Phe in 100 ml anhydrous tetrahydrofurane, 4.2 ml (30.1 mM) triethylamine, were obtained 6.03 g (69.1% yield) of product. Recrystallisation from ether/petrolether. m.p. 79–81°C, $[\alpha]_D^{20} = -35.0°$ ($c = 1.2$ EtOH), IR (KBr): 2110 cm^{-1} (diazo), 1630 cm^{-1} (C=O), thin-layer chromatography: $R_F = 0.63$(A), $R_F = 0.83$(B), anal. calcd for: $C_{15}H_{19}N_3O_3$ (289.36); C, 62.26; H, 6.63; N, 14.52. Found: C, 62.15; H, 6.91; N, 14.77.

2.7.2. L-Phenylalanine chloromethyl ketone hydrochloride (1-chloro-3-amino-4-phenyl-butan-2-one hydrochloride)

N-t-Butyloxycarbonyl-L-phenylanine diazo ketone 4.95 g (17 mM) was added with stirring under cooling to a solution of 4 N HCl/dioxane. After 2 h the resulting crystals were filtered and recrystallized from anhydrous methanol-ether. A 75% yield (3.0 g) of product were recovered with m.p. 176°C and $[\alpha]_D^{20} = +30.2°$ ($c = 1.3$ MeOH). Ref. [10] m.p. 176°C and $[\alpha]_D^{20} = +30.2°$ ($c = 2$ in 0.1 N HCl). Rev. [11] m.p. 169–170°C. IR (KBr): 1740 cm^{-1} (C=O). The product was shown to be pure by thin-layer chromatography on silica gel G plates: $R_F = 0.84$(A), $R_F = 0.92$(B), anal. calcd for: $C_{10}H_{13}NOCl_2$ (234.14); C, 51.29; H, 5.60; N, 5.98; Found: C, 51.10; H, 5.48; N, 6.04. Solvent A: C_6H_6/MeOH/AcOH = 15:2:1, solvent B: Cyclohexane/$CHCl_3$/acetic acid = 45:45:10 (by vols).

3. RESULTS AND DISCUSSION

3.1. *Inhibition of gramicidin S biosynthesis and thioester formation by L-phenylalanine chloromethyl ketone*

The effect of increasing concentrations of L-Phe-CMK on the biosynthesis of gramicidin S and the thioester formation of proline, valine, ornithine and leucine is shown in fig.1. The 4 thioester formation activities of the enzyme are inhibited in a similar manner, whereas he biosynthesis of gramicidin S is evidently more strongly inhibited.

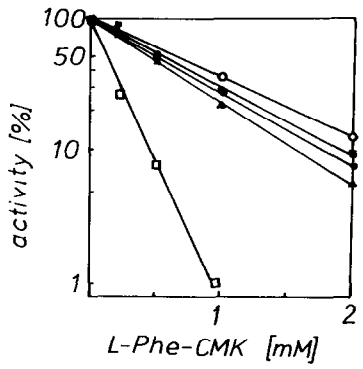

Fig.1. Inhibition of gramicidin S biosynthesis (□—□) by L-Phe-CMK at 0°C and of thioester formation of proline (▲—▲), valine (■—■), ornithine (●—●) and leucine (○—○) as catalyzed by framicidin S synthetase.

3.2. *Rate of inhibition*

The reaction scheme for an irreversible substrate analogue alkylating agent of GS 2 is:

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E \cdot I \xrightarrow{k_2} E - I \qquad (1)$$

whereas for an unspecific irreversible inhibitor it is:

$$E + I \xrightarrow{k} E - I \qquad (2)$$

A kinetic analysis of the first case is described in [12] and of the second case in [13]. The formation of a E·I complex means, that a plot of $\ln \frac{100}{x}/t$ against the concentration of L-Phe-CMK results in a straight line with an intercept on the vertical axis. In the cases of the 4 thioester formation activities and the gramicidin S biosynthesis we found straight lines passing through the origin. Thus, the inhibition kinetics of L-Phe-CMK follows the pattern expected for an unspecific irreversible inhibitor. The results are shown in fig.2 (in the case of ornithine thioester formation) and in table 1. We observed no specific inhibition of leucine thioester formation, as expected [5]; therefore we have to assume, that L-Phe-CMK is not a specific inhibitor for one of the active sites of GS 2. The biosynthesis is inhibited about 4-times more rapidly than one of the thioaminoacylation reactions. It is obvious that inactivation of a single site results in loss of the overall reaction. In comparison, we have determined the apparent second-order rate constant for the inhibition of gramicidin S biosynthesis by the irrever-

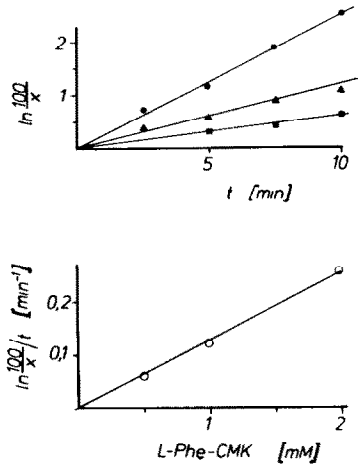

Fig.2. Top: Determination of the second order rate constant for the inactivation of ornithinethioester formation of GS 2 by L-Phe-CMK at 0°C. Similar patterns were obtained for the respective proline-, valine- and leucine reactions. (X) = residual activity in 1%; L-Phe-CMK concentrations: (■—■) 0.5 mM; (▲—▲) 1.0 mM; (●—●) 2.0 mM. Bottom: Replot of slopes $\ln(\frac{100}{x})/t$ to demonstrate unspecific irreversible inhibition.

sible inhibitor iodoacetamide. The constant is smaller than that of L-Phe-CMK, but of the same order as the inhibition constant for the condensing reaction of fatty acid synthetase (1.3 $s^{-1}.M^{-1}$) [13] and about the same value as the constants for the partial reactions.

3.3. *Irreversibility of L-Phe-CMK-inhibition and substrate protection*

After complete inhibition of the enzyme by L-Phe-CMK and exhaustive dialysis against 20 mM phosphate buffer at 4°C no enzyme activities were retained. Therefore, L-Phe-CMK is an irreversible inhibitor of GS 2. Incubation of GS 2 in the presence of substrates ([ATP] = 20 mM, [aa] = 5 mM, $[Mg^{2+}]$ = 50 mM) and inhibitor (1 mM) and separation of the enzyme from low M_r components by gel filtration shows complete protection of the 4 thiol-aminoacylation reactions and of the gramicidin S biosynthetic activity (not shown).

Table 1

Inactivation constants for the inhibition of thioester formation and gramicidin S biosynthesis by L-Phe-CMK and iodoacetamide at 0°C

Reaction, inhibited	$k.s^{-1}.M^{-1}$	Inhibitor
Proline thioester formation	2.17	L-Phe-CMK
Valine thioester formation	1.67	L-Phe-CMK
Ornithine thioester formation	2.08	L-Phe-CMK
Leucine thioester formation	2.00	L-Phe-CMK
Gramicidin S biosynthesis	7.33	L-Phe-CMK
Gramicidin S biosynthesis	1.20	IAA

3.4. *Inhibition of thioester formation by L- and D-phenylaline*

Assuming, that phenylaline is thioesterbound to the leucine site of GS 2 [5], we have to expect an inhibition of leucine-thioester formation by L- and D-phenylaline. The results are shown in table 2. The inhibition of the partial activities seems to be specific of the leucine site at low phenylalanine concentrations. At higher concentrations valine- and leucine-thioester binding are reduced significantly more than that of proline and ornithine. This inhibition behaviour of L- and D-phenylalanine may arise from an interaction with the binding sites of these hydrophobic amino acids.

3.5. *Activation of D- and L-phenylalanine by GS 2*

Table 3 shows the amino acid-dependent ATP–PP_i-exchange reaction for the cognate amino acids and L- and D-phenylaline. A very small, but significant activation of L- and D-phenylalanine can be observed.

Table 2

Effect of L- and D-phenylalanine on the thioester formation of proline, valine, ornithine and leucine by GS 2[a]

Inhibitor	Conc. (mM)	Inhibition in %			
		Proline	Valine	Ornithine	Leucine
L-Phenylalanine	1	2	3	0	12
	10	25	55	0	65
	30	28	70	0	80
D-Phenylalanine	1	0	2	0	12
	10	5	70	15	75
	30	12	85	15	85

[a] Concentrations of amino acids are 2 mM

Table 3

Amino acid-dependent ATP–$^{32}PP_i$-exchange of GS 2

Amino acid (2 mM)	Exchange of $^{32}PP_i$ (mol PP_i . mol enzyme^{-1} . min^{-1})
Proline	135
Valine	255
Ornithine	131
Leucine	263
L-Phenylalanine	4.1
D-Phenylalanine	1.7

3.5. *Concluding remarks*

L-Phe-CMK, a substrate analogue of L-phenylalanine, inhibits the 4 thioester formation activities of GS 2 with about the same velocity and shows no specificity for the leucine site, as could be expected [5]. A small, but significant amount of ATP–$^{32}PP_i$-exchange activity in the presence of L- or D-phenylalanine could be confirmed, as in [14]. A solution to the question whether phenylalanine can be used instead of leucine in gramicidin S synthesis, may be obtained by synthesizing the respective gramicidin S analogue followed by product analysis. Prevention of inhibition by the substrates opens the possibility of specific blocking of one or more active sites of the enzyme.

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