IRREVERSIBLE INHIBITION OF THE LIGHT ENZYME OF GRAMICIDIN S SYNTHETASE BY HALOGENOMETHYLKETONES OF PHENYLALANINE

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1. Introduction

Pseudosubstrates combining substrate-like features and a chemically reactive grouping can give information about the catalytic centres of enzymes. The substrate part of these reagents localize them at the active site and the chemically reactive part then forms a covalent bond with irreversible formation of an enzyme–inhibitor compound [1]. Halogenomethylketone analogs of substrate amino acids are an example of such inhibitors. They retain the main structural features of the amino acid and possess alkylating properties.

Phenylalanylhalogenomethylketones were used here to gain further information about the active centre of the multi-enzyme complex gramicidin S synthetase. This enzyme complex catalyzes the non-ribosomal biosynthesis of gramicidin S, and consists of a heavy and a light enzyme [2]. Biosynthesis of the peptide antibiotic is started by phenylalanine racemase (EC 5.1.1.11.), the light component of gramicidin S synthetase, which racemizes phenylalanine and activates it to the aminoacyl adenylate and in a second step to the thioester.

The results in this paper define the structural features necessary for an effective inhibitor of the light enzyme of gramicidin S synthetase. It is shown, that L-1-chloro-3-amino-4-phenyl-butan-2-one, the chloromethylketone derived from L-phenylalanine, is an irreversible inhibitor of phenylalanine racemase.

Abbreviations: Phe-CH_2Cl, chloromethylketone of phenylalanine; Phe-CH_2Br·HBr hydrobromide of the bromomethylketone of phenylalanine; Tos, *p*-toluenesulfonyl; Z, benzyloxycarbonyl; DTT, dithiothreitol.

2. Materials and methods

2.1. *Enzymes and activity measurement*

Enzymes were prepared as described by Vater et al. [3]. Formation of aminoacyl adenylate was measured according to Kleinkauf et al. [4] by determing the L- or D-phenylalanine-dependent ATP–$^{32}PP_i$ exchange reaction catalyzed by phenylalanine racemase obtained after hydroxylapatite chromatography. Concentrations of the incubation mixture were: triethanolamine buffer pH 7.8 (65 mM), EDTA (0.3 mM), $MgCl_2$ (6.5 mM), L-Phe (1.25 mM) (for Lineweaver-Burk plots in fig.2: D-Phe in various concentrations), ATP (1.25 mM) $Na_4P_2O_7$ (1.25 mM), and phenylalanine racemase in a rate limiting amount. Samples of 100 µl were incubated at 37°C for 15 min. Background radioactivity (obtained by omitting phenylalanine) was deducted.

Gramicidin S synthesis was estimated by the millipore filter assay according to Gevers et al. [5], in which incorporation of ^{14}C-labelled L-leucine into gramicidin S is measured. Concentrations of the incubation mixture were: triethanolamine buffer pH 7.8 (125 mM), EDTA (1.25 mM), $MgCl_2$ (25 mM), ATP (5 mM), Pro (5 mM), Val (5 mM), Orn (5 mM), [^{14}C]Leu ($1.4 \cdot 10^{-4}$ mM), the indicated amount of L-Phe, phenylalanine racemase after hydroxylapatite chromatography, and rate limiting amounts of the heavy enzyme of gramicidin S synthetase, obtained

after Sepharose 6-B chromatography. In control experiments, one non-radioactive substrate amino acid was omitted, which ensures that no antibiotic is formed. Control radioactivity values were deducted from radioactivity values obtained in synthesis experiments.

2.2. *Inhibition studies*

Since the halogenomethylketones of phenylalanine decompose in aqueous solutions after some minutes [6], inhibitor solutions were prepared freshly before use.

Inhibition of gramicidin S synthesis (3.1.) was studied by adding the inhibitor to incubation mixtures and measuring enzyme activities as described in 2.1.

In order to study the irreversibility of inhibition (3.2.) and protection against inactivation by substrates (3.3.), dialysis experiments were done: enzyme and inhibitor were preincubated at 37°C in 5 mM phosphate buffer pH 7.6, containing 6.5 mM $MgCl_2$ and 0.3 mM EDTA. After exhaustive dialysis at 4°C against 5 mM phosphate buffer containing 2.5 mM DTT, the enzyme solutions were assayed for ATP–PP_i exchange. To determine the rate of inhibition (3.4.), enzyme and inhibitor were incubated at 37°C in the same phosphate incubation buffer as described above. Aliquots were taken at different times and diluted tenfold with incubation mixture (without enzyme) and subsequently assayed for ATP–PP_i exchange. After this dilution, the excess of substrate over inhibitor abolishes a further inhibition.

2.3. *Syntheses of halogenomethylketones of phenylalanine*

Purity of products synthesized were checked by thin-layer chromatography on silica gel plates in the following solvent systems: (I) butanone–acetone–water (60:6:10; by vol); (II) chloroform–methanol–water (65:25:4; by vol).

Benzyloxycarbonyl-D-phenylalanylchloromethylketone (D-Z-Phe-CH_2Cl) was prepared from D-Z-Phe by the anhydride method according to Fittkau [7], L-Z-Phe-CH_2Cl was obtained from Bachem Inc., Liestal, Switzerland, (R_F (I) = 0.79; R_F (II) = 0,87 for both chloromethylketones). The Z-group was removed by stirring D-or L-Z-Phe-CH_2Cl with a solution of hydrogen bromide in acetic acid (36%; w/v) for 30 min at room temperature. The reaction solution was evaporated to dryness (excess HBr was removed by adding ethyl acetate and ether before evaporating) and the residue was recrystallized from ethanol–ether (1:3; by vol). In the case of the D-isomer, 65% of D-Phe-CH_2Br·HBr was obtained, the L-isomer yielded 75% of L-Phe-CH_2Cl·HBr (R_F (I) = 0.76, R_F (II) = 0.85 for both isomers). Deblocking of D-Z-Phe-CH_2Cl had caused a displacement of all chlorine by bromide, perhaps due to raising of temperature during the deblocking reaction. The halogenomethylketones had correct elementary analyses (the halogen content was determined by potentiometric titration) and showed the expected infrared and n.m.r. spectra.

3. Results

3.1. *Inhibition of gramicidin S synthesis*

The effect of various pseudosubstrates of phenylalanine racemase on the biosynthesis of gramicidin S is shown in table 1. N-substituted halogenomethylketones of phenylalanine did not influence the enzyme activity of the whole synthetase complex, whereas D-Phe-CH_2Br and L-Phe-CH_2Cl are potent inhibitors of gramicidin S synthesis. The effect of D-phenylalanylbromomethylketone at different concentrations on gramicidin S synthesis is shown in fig.1.

3.2. *Irreversible inhibition of the phenylalanine-dependent ATP–PP_i exchange reaction*

The inhibition of gramicidin S synthesis as shown in 3.1. is expected to be the result of the inhibition

Table 1
Effect of phenylalanine derived pseudosubstrates on gramicidin S synthetase

Pseudosubstrate	Gramicidin S synthesis (%)
None	100 (5902 cpm)
L-Tos-Phe-CH_2Cl	120
D-Z-Phe-CH_2Br	95
D-Phe-CH_2Br	0
L-Phe-CH_2Cl	2

Gramicidin S synthesis was estimated by the millipore filter method at a ten-fold excess of inhibitor over the substrate L-phenylalanine (1 mM), other conditions are as described in 2.1.

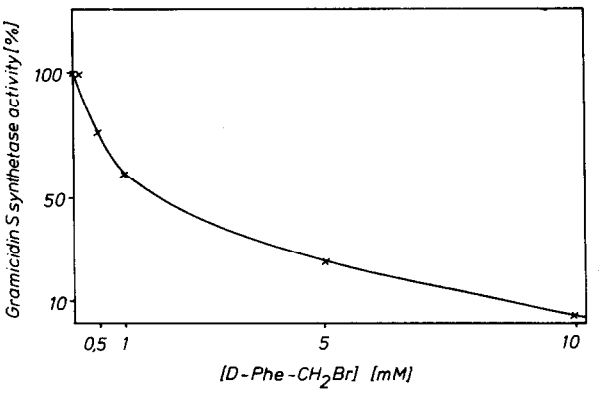

Fig.1. Inhibition by D-phenylalanylbromomethylketone of gramicidin S biosynthesis catalyzed by gramicidin S synthetase. Concentration of L-phenylalanine was 10^{-5} M (100% activity = 6000 cpm).

of the light enzyme of gramicidin S synthetase. Therefore the inhibition by phenylalanylhalogenomethylketones with free amino groups of the phenylalanine-dependent ATP–PP_i exchange catalyzed by the light enzyme was studied in detail. Other possible inhibitory effects of halogenomethylketones on other partial reactions of gramicidin S biosynthesis were not examined. As shown in table 2 for L-phenylalanylchloromethylketone, the exchange reaction is also inhibited, and no reactivation of the enzyme can be observed after dialysis. L-Phe-CH_2Cl and D-Phe-CH_2Br exhibit a non-competitive type of inhibition, as expected of an irreversible inhibitor (fig.2). Inhibition of gramicidin S synthesis by

Table 2
Inhibition of the L-Phe dependent ATP–PP_i exchange reaction catalyzed by phenylalanine racemase

L-Phe-CH_2Cl (M)	Relative ATP–PP_i exchange Enzyme dialyzed (%)	Enzyme not dialyzed (%)
None	100 (7500 cpm)	100
10^{-2}	1.4	12.5

Enzyme was pretreated in phosphate buffer for 15 min with chloromethylketone in every case. ATP–PP_i exchange activity of the undialyzed and dialyzed enzyme were estimated at the same time.

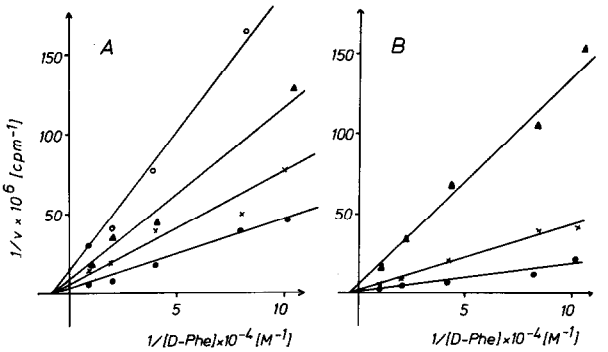

Fig.2. Lineweaver-Burk plots for the inhibition of the D-Phe dependent ATP–PP_i exchange of phenylalanine racemase by halogenomethylketones of phenylalanine. The concentration of D-Phe was varied at defined concentrations of inhibitor. (A) D-Phe-CH_2Br, (B) L-Phe-CH_2Cl; 2 mM (○), 1 mM (▲), 0.5 mM (×), absence of inhibitor (●).

Table 3
Protective effects of substrates of the light enzyme of gramicidin S synthetase against inhibition by L-phenylalanylchloromethylketone

Preincubation with protecting agent	Incubation with L-Phe-CH_2Cl	Relative ATP–PP_i exchange (%)
None	None	100 (16 280 cpm)
None	1 mM	26
L-Phe (1 mM)	1 mM	27
ATP (1.25 mM)	1 mM	31
L-Phe(1 mM)+ATP(1.25 mM)	1 mM	89

Phenylalanine racemase was preincubated with the indicated protecting agents for 10 min at 37°C in phosphate buffer before the inhibitor was added for a further incubation (25 min). After dialysis the L-Phe dependent ATP–PP_i exchange was assayed.

D-Phe-CH_2Br also shows a non-competitive mechanism (results not shown).

3.3. *Protection of phenylalanine racemase against inhibition*

In order to protect the light enzyme against alkylation by the chloromethylketone of L-phenylalanine, the enzyme and its substrates were incubated together before adding the inhibitor. After dialysis it could be shown, that preincubation with both substrates L-Phe and ATP protects the enzyme nearly completely against inactivation, whereas each substrate alone has a poor protective effect (table 3).

3.4. *Rate of inhibition*

The expected reaction scheme for the action of an irreversible substrate-like alkylating agent on phenylalanine racemase is:

$$E + I \overset{K_I}{\rightleftharpoons} EI \xrightarrow{k_2} E\text{–}I$$

where EI is the reversible intermediate enzyme–inhibitor complex and k_2 the rate constant for the conversion of EI into the alkylated enzyme E–I. A kinetic analysis as described by Kitz and Wilson [8] was used to study the concentration dependence of the inactivation by L-Phe-CH_2Cl (fig.3). Inhibitor was added in large excess over enzyme to provide apparent first order inactivation of the enzyme.

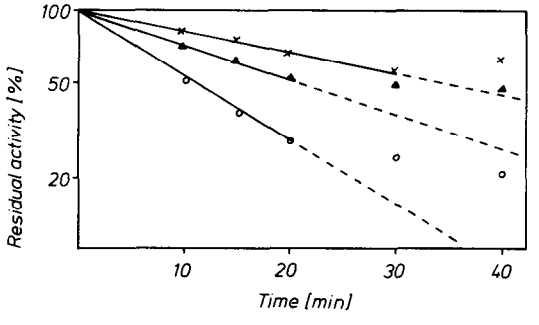

Fig.3. Inactivation of phenylalanine racemase at various concentrations of L-Phe-CH_2Cl. Loss of L-Phe-dependent ATP–PP_i exchange activity was measured at least with a 5000-fold excess of inhibitor over enzyme. The dashed lines indicate the plots expected, assuming no decomposition of the inhibitor. Concentrations of inhibitor are: 0.5 mM (×), 1 mM (▲), 2 mM (○).

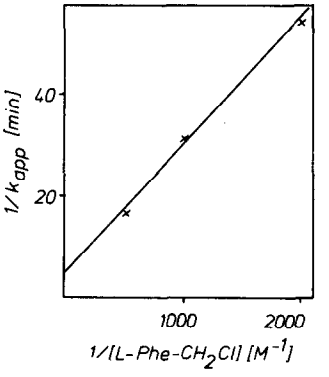

Fig.4. Dependence of the k_{app} of inactivation on concentration of inhibitor. The apparent first-order rate constants k_{app} are replotted in double reciprocal fashion at various L-Phe-CH_2Cl concentrations that provide first-order inactivation kinetics (fig.3).

Semilogarithmic plots of inactivation deviated from the expected straight lines. The cause of the non-linearity at longer time periods appeared to be decomposition of the chloromethylketone, which reduced its concentration. However, deviations during shorter time periods (20 min) used in the determination of the apparent first order rate constants for irreversible inactivation (k_{app}) were so small that corrections were unnecessary. A reciprocal plot of k_{app} against inhibitor concentration (fig.4) gave a positive intercept on the Y-axis, showing that the formation of the reversible complex EI preceeds that of the irreversible complex E–I. From fig.4, k_2 = 0.2 min^{-1} and $K_i = 5.2 \cdot 10^{-3}$ M.

4. Discussion

As shown previously, the amino group of phenylalanine is essential for its binding to the aminoacyl adenylate activation site of the light enzyme of gramicidin S synthetase, whereas the carboxyl group seems not to be bound to the enzyme [3]. In line with these conclusions, the results in table 1 show that effective halogenomethylketone inhibitors of the light enzyme must contain a free amino group, thus providing evidence that inactivation is not due to unspecific effects.

The alkylating reagents used in our studies were

active-site directed in their design and we hoped that they would function as irreversible inhibitors at the amino acid activation site(s) of phenylalanine racemase. The data shown in 3.2. indicate that both optical isomers L-Phe-CH_2Cl and D-Phe-CH_2Br inhibit the light enzyme of gramicidin S synthetase irreversibly, which could be expected for an enzyme activating both L- and D-phenylalanine. The inhibition of the Phe-tRNA-synthetase by halogenomethylketones of phenylalanine is reversible and competitive [9].

The experiments described in 3.3. and 3.4. seem to point to an action of L-phenylalanylchloromethylketone at the active centre of the light enzyme, because the substrates L-Phe and ATP, both added to the incubation mixture, protect the enzyme against inactivation. The formation of an intermediate complex during inactivation indicates that L-Phe-CH_2Cl acts as a substrate-like alkylating agent. The precise site of the inactivation must be determined by further studies.

The present work opens up the way for use of the chloromethylketone of L-phenylalanine as an affinity label for the light enzyme of gramicidin S synthetase.

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References

[1] Shaw, E. (1970) in: The Enzymes (Boyer, P. D., ed.) Vol. 1, pp. 91–146, Academic Press, New York.
[2] Lipmann, F. (1973) Acc. Chem. Res. 6, 361–367.
[3] Vater, J. and Kleinkauf, H. (1976) Biochim. Biophys. Acta, in the press.
[4] Kleinkauf, H., Gevers, W. and Lipmann, F. (1969) Proc. Nat. Acad. Sci. US 62, 226–233.
[5] Gevers, W., Kleinkauf, H. and Lipmann, F. (1968) Proc. Nat. Acad. Sci. US 60, 269–276.
[6] Fittkau, S. (1972) Acta biol. med. germ. 28, 259–267.
[7] Fittkau, S. (1973) J.f. prakt. Chem. 315, 1037–1044.
[8] Kitz, R. and Wilson, I. B. (1962) J. Biol. Chem. 237, 3245–3249.
[9] Kovaleva, G. K. (1975) Biokhim. 39, 1036–1039.