Regulation by Aromatic Amino Acids of the Biosynthesis of Candicidin by *Streptomyces griseus*

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The biosynthesis by *Streptomyces griseus* of candicidin, an aromatic polyene macrolide antibiotic, was inhibited by L-tryptophan, L-phenylalanine and, to a lesser degree, by L-tyrosine. A mixture of the three aromatic amino acids inhibited candicidin biosynthesis to a greater extent than did each amino acid separately. L-Tryptophan strongly inhibited the incorporation of the labelled precursors propionate or 4-aminobenzoic acid into candicidin. Incorporation of propionate into candicidin was 50% inhibited by 2.5 mM-tryptophan. Inhibition by tryptophan did not require protein synthesis as the same effect was observed in cells in which protein synthesis was prevented by chloramphenicol. The inhibitory effect of L-tryptophan was partially reversed by exogenous 4-aminobenzoic acid suggesting that this effect is exerted at the level of 4-aminobenzoic acid synthase.

**INTRODUCTION**

Candicidin is a polyene (heptaene) macrolide antifungal antibiotic produced by *Streptomyces griseus* IMRU 3570. It consists of a macrolide ring which contains a chromophore with seven double bonds. Candicidin also contains the amino sugar mycosamine (3-amino-3,6-dideoxy-α-mannopyranose) attached glycosidically, and a 4-aminoacetophenone moiety linked covalently to the macrolide ring (Martin & McDaniel, 1976a).

The 4-aminoacetophenone moiety may serve as starter of the head-to-tail condensation of malonyl-CoA and methylmalonyl-CoA in the biosynthesis of the macrolide ring of candicidin (Martin, 1977). This aromatic moiety is formed from chorismic acid via 4-aminobenzoic acid (PABA). That the intact ring and the carboxyl group of PABA are incorporated into candicidin was concluded from the quantitative incorporation of [ring-U-14C]PABA and [carboxy-14C]PABA (Liu et al., 1972; Martin & Liras, 1976). The branch-point compound of the aromatic pathway giving rise to PABA in bacteria is chorismic acid. In *Escherichia coli*, *Enterobacter aerogenes*, *Neurospora crassa* and *Bacillus subtilis*, chorismic acid is converted into PABA in the presence of L-glutamine as amino donor (Gibson et al., 1964; Huang & Gibson, 1970; Altendorf et al., 1971; Kane & O'Brien, 1975).

The aromatic pathway is a multi-branched pathway leading to the aromatic amino acids and to other quantitatively minor metabolic products such as PABA, 4-hydroxybenzoic acid and 2,3-dihydroxybenzoic acid (Gibson & Pittard, 1968). Different patterns of regulation of this pathway exist in different micro-organisms. Crossed feedback regulation (so-called ‘metabolic interlock’) by tryptophan of enzymes involved in tyrosine and phenylalanine biosynthesis has been described (Hagino & Nakayama, 1975). In *Streptomyces aureofaciens* the routes of phenylalanine and tyrosine biosynthesis are not influenced by their end-products. Rather, the concentration of tryptophan acts as the signal which controls the synthesis of aromatic amino acids (Lingens, 1976).
Liu et al. (1972) reported that the biosynthesis of candicidin is inhibited by a mixture of aromatic amino acids. However, the effect of each individual aromatic amino acid was not studied. Moreover, the experiments were carried out by adding the amino acids at the time of inoculation to growing cultures. Under these experimental conditions an effect of the aromatic amino acids on growth, indirectly affecting antibiotic synthesis, could not be excluded. It was therefore of interest to study the regulation of candicidin biosynthesis by each aromatic amino acid in resting cells using short-term experiments in which the biosynthesis of candicidin was determined by following the incorporation of the precursors [14C]PABA and [14C]propionate into the antibiotic.

METHODS

Micro-organisms and growth conditions. Stock cultures of Streptomyces griseus IMRU 3570 were maintained in liquid nitrogen (gas phase). Cells were grown in 500 ml triple-baffled flasks containing 100 ml soya peptone/glucose (SPG) medium as described before (Martin & McDaniel, 1975a). Flasks were incubated at 32 °C in an orbital incubator at 250 rev. min⁻¹. Under these conditions candicidin production started after about 18 h growth. For candicidin determination, samples (250 μl) of complex medium were diluted with 2 ml distilled water and extracted with the same volume of butan-1-ol. Candicidin was determined spectrophotometrically taking into account the highest absorption peak (380 nm) after measuring an absorption spectrum of the polyene.

Preparation of phosphate-limited resting cells. Candicidin-producing cells were collected, washed twice with sterile saline and suspended in phosphate-free synthetic medium, as described previously (Martin & McDaniel, 1976b). Samples of cell suspension (5 or 10 ml) were incubated in 50 ml triple-baffled flasks in a water-bath orbital incubator at 32 °C and 250 rev. min⁻¹. The phosphate-limited resting cells in this system produced candicidin at a constant rate for 36 h without increase in cell dry weight. Candicidin was extracted from 250 μl samples of culture broth with 5 ml chloroform/methanol (1:1, v/v) and determined spectrophotometrically, as described above.

Incorporation of labelled precursors into candicidin. Incorporation of [carboxy-14C]PABA and [1-14C]-propionate was carried out as described previously (Martin & McDaniel, 1975b; Martin & Demain, 1976). Labelled candicidin was purified by thin-layer chromatography, visualized under u.v. light, scraped off the plates and counted. Cellular uptake of [14C]propionate and [14C]PABA was determined as described by Martin & McDaniel (1975b).

Aromatic amino acids (L-form) or chloramphenicol were added at the times indicated in the figure legends.

[carboxy-14C]PABA (30 to 50 Ci mol⁻¹; 1.1 to 1.9 TBq mol⁻¹) was obtained from ICN Chemical and Radiisotope Division, and sodium [1-14C]propionate (48.7 Ci mol⁻¹; 1.80 TBq mol⁻¹) from New England Nuclear. All other products were of reagent quality.

RESULTS

Effect of aromatic amino acids on candicidin production in complex medium. Production of candicidin by Streptomyces griseus in complex SPG medium was strongly inhibited by L-tryptophan and L-phenylalanine (10 mM) but was not affected by L-tyrosine (Fig. 1). None of the amino acids affected growth of the culture at the concentrations used. These results in complex medium prompted us to study the mechanism of this regulation in phosphate-limited resting cell cultures of S. griseus in which candicidin biosynthesis can be measured in short-term experiments (Martin & McDaniel, 1976b).

Effect of aromatic amino acids on candicidin production by phosphate-limited resting cells of Streptomyces griseus. L-Tryptophan was highly inhibitory (Fig. 2); at 10 mM, it inhibited total candicidin biosynthesis by 44%. Phenylalanine (10 mM) was also highly inhibitory; at 24 h it produced an inhibition of 28%. Tyrosine, on the contrary, was only slightly inhibitory (10%). These results fully agree with the results of long-term fermentations in complex medium (Fig. 1). When a mixture of the three aromatic amino acids (10 mM each) was added, the biosynthesis of candicidin was inhibited by 74%. This percentage inhibition is less than the sum of the percentages of inhibition produced by the individual aromatic amino acids (82%).
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Fig. 1. Effect of the aromatic amino acids on candicidin production in complex medium. Control (○), with 10 mM-tryptophan (△), 10 mM-tyrosine (●), 10 mM-phenylalanine (▲).

Fig. 2. Effect of the aromatic amino acids on candicidin production by phosphate-limited resting cells. Control (○), with 10 mM-tyrosine (●), 10 mM-phenylalanine (▲), 10 mM-tryptophan (△), 10 mM-tyrosine plus 10 mM-phenylalanine plus 10 mM-tryptophan (■).

Fig. 3. Effect of l-tryptophan on the incorporation of [14C]propionate into candicidin by resting cells. (a) Cells preincubated for 1 h in tryptophan before addition of [14C]propionate. Inset: cellular uptake of [14C]propionate. (b) Cells preincubated for 12 h in tryptophan. Control (○), with 10 mM-tryptophan (△).

Effect of tryptophan on the incorporation of labelled precursors into candicidin. Further experiments were carried out only with tryptophan. To check whether tryptophan affects total candicidin yields by inhibiting its formation rather than increasing its degradation, we studied the effect of tryptophan on the incorporation of [14C]propionate and [14C]PABA into candicidin. The results for [14C]propionate are shown in Fig. 3. When the resting cells were kept for 12 h in the presence of tryptophan, precursor incorporation was inhibited by...
80%, but when tryptophan was added just before the label no effect on precursor incorporation was shown in the first hour, although inhibition of incorporation occurred thereafter. These results suggested that a period of 1 to 2 h was required to exert the inhibitory effect. The addition of tryptophan did not affect the uptake of [14C]propionate by S. griseus (Fig. 3, inset).

Similar results were obtained when we measured the effect of tryptophan on the incorporation of [14C]PABA into candicidin (Fig. 4). The inhibitory effect was clearly seen after 2 h incubation in the presence of tryptophan. There was no significant effect of tryptophan on the uptake of [14C]PABA (Fig. 4, inset).

**Effect of incubation time in the presence of tryptophan on the candicidin biosynthetic activity.** As observed in Fig. 4, the extent of inhibition of candicidin biosynthesis was dependent upon the period of incubation in the presence of tryptophan. In order to establish the influence of the incubation time in the presence of tryptophan on the candicidin biosynthetic activity of phosphate-limited resting cells, we conducted experiments in which cells were preincubated with tryptophan for 0 (control), 1, 3, 6 and 8 h. The results (Fig. 5) indicated that as the preincubation period in tryptophan was increased, the candicidin biosynthetic activity was increasingly inhibited. This suggested that either the intracellular tryptophan pool was built up slowly or, more likely, that there was a repression by tryptophan of the formation of candicidin-synthesizing enzymes which are continuously replenished in the resting cell system.

**Determination of the inhibition of candicidin biosynthesis by different concentrations of tryptophan.** Tryptophan at 1 mM inhibited candicidin biosynthesis by 23% when the amino acid was added at the time of suspension of the cells in the resting cell system (8 h incubation in presence of tryptophan). At a tryptophan concentration of 2.5 mM, 50% inhibition was observed. However, concentrations above 10 mM were required to achieve more than 80% inhibition.

**Reversal of the tryptophan effect by 4-aminobenzoic acid.** Tryptophan (or other aromatic amino acids) might inhibit candicidin biosynthesis by depriving the cell of PABA. This would be the case if tryptophan inhibited PABA synthase which converts chorismic acid into
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Fig. 6

Reversal by PABA of the inhibition of candicidin biosynthesis by tryptophan in resting cells.
Control (○), with 0.5 mM-PABA (●), 2.5 mM-tryptophan plus 0.5 mM-PABA (▲), 2.5 mM-tryptophan (△), 10 mM-tryptophan plus 0.5 mM-PABA (■), 10 mM-tryptophan (□).

Fig. 7

Inhibitory effect of tryptophan on candicidin biosynthesis in resting cells in which protein synthesis was prevented. Control (○), with chloramphenicol (50 μg ml⁻¹) (●), 10 mM-tryptophan (△), 10 mM-tryptophan plus chloramphenicol (50 μg ml⁻¹) (▲).

PABA. To check whether this was the case, experiments were carried out to measure the incorporation of propionate into candicidin in flasks supplemented with tryptophan alone or in combination with PABA. The results of these experiments (Fig. 6) indicated that the inhibitory effect on candicidin biosynthesis produced by low concentrations of tryptophan (2-5 mM) was reversed by PABA (0.5 mM) when both effectors were added at the same time. However, with high concentrations of tryptophan (10 mM) the inhibitory effect was only slightly reversed.

Inhibition of the activity of candicidin-synthesizing enzymes by tryptophan. From the previous experiments it was concluded that tryptophan was most probably inhibiting candicidin biosynthesis at the stage of PABA synthesis. In order to check whether this effect was due to repression (requiring protein synthesis) or whether it was exerted by inhibition of enzyme activity, experiments were carried out in which protein synthesis was inhibited by adding 50 μg chloramphenicol ml⁻¹ (Martín et al., 1977). Tryptophan exerted its inhibitory effect even in cells in which protein synthesis was inhibited by chloramphenicol (Fig. 7), suggesting that the effect is due to inhibition of the activity of candicidin-synthesizing enzymes. The decrease in the rate of candicidin formation (as determined by the incorporation of [¹⁴C]propionate into candicidin) caused by chloramphenicol probably reflects a turnover of candicidin-synthesizing enzymes which is prevented in the presence of chloramphenicol (Martín et al., 1977).

DISCUSSION

The results described in this paper confirm an earlier report about the regulation of the biosynthesis of candicidin by aromatic amino acids (Liu et al., 1972). Crossed feedback regulation (metabolic interlock) was exerted by tryptophan and phenylalanine whereas tyrosine was only slightly inhibitory.

The percentage inhibition by the mixture of the three aromatic amino acids suggests that it may be a cumulative feedback type of regulation (Fig. 2). The higher inhibition by tryptophan seems reasonable if, as suggested by the results of Fig. 6, the regulatory effect is exerted
Fig. 8. Schematic representation of the biosynthetic pathways for tryptophan and candicidin.

The inhibitory effect of tryptophan was by depriving the cell of PABA, a precursor of candicidin (Fig. 8). This result could also be explained on the basis of inhibition of the whole aromatic pathway at the DAHP synthase level, but this second alternative seems less likely because the DAHP synthase of at least seven *Streptomyces* species is insensitive to tryptophan (Jensen & Rebello, 1969). Only the DAHP synthase from *S. aureofaciens* appeared to be inhibited by tryptophan (Lingens, 1976). Studies on the activity of PABA synthase *in vitro* have indicated that this enzyme is subject to tryptophan regulation (J. A. Gil & J. F. Martin, unpublished results).

The PABA synthase of *Bacillus subtilis* has a subunit in common with the anthranilate synthase (the enzyme that synthesizes 2-aminobenzoic acid, an intermediate in tryptophan biosynthesis). Subunit A has aminase but not amidotransferase activity. The second subunit, X, which is also a component of the anthranilate synthase, has no PABA synthase activity but forms a complex with subunit A to give amidotransferase activity (Kane & O’Brien, 1975). Tryptophan feedback inhibits and/or represses the anthranilate synthase (the first enzyme of the branch leading to tryptophan) in a large number of micro-organisms including *Streptomyces* (Francis et al., 1978). It is tempting to speculate on the role that the similar-
Regulation of candicidin biosynthesis may play in the regulation by tryptophan of its own biosynthetic pathway (at anthranilate synthase) and that of candicidin (at PABA synthase).

Tryptophan inhibited the incorporation of labelled propionate and PABA into candicidin with high specificity (Figs 3 and 4). The apparent $K_i$ of tryptophan in resting cells was 2-5 mM. Inhibition of the incorporation of propionate into candicidin may be explained on the basis of starvation of the cell for PABA, thus preventing the activity of the candicidin synthase (Fig. 8). It is more difficult to explain the inhibition of the incorporation of exogenous PABA into candicidin. Since tryptophan does not affect the uptake of labelled PABA, a reduction in the synthesis of endogenous PABA should not affect the incorporation of exogenous PABA into candicidin. Since tryptophan does not affect the uptake of labelled PABA, a reduction in the synthesis of endogenous PABA should not affect the incorporation of exogenous PABA, unless the inhibitory effect is also taking place at a later stage, e.g. at the activation of PABA to 4-aminobenzoyl-CoA or at the chain polymerization stage (dashed line in Fig. 8). A system for the study of the biosynthesis of candicidin in vitro is now being investigated to clarify this point.

REFERENCES


