Lysine Inhibition of *in vivo* Homocitrate Synthesis in *Penicillium chrysogenum**

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SUMMARY

A lysine bradytroph of *Penicillium chrysogenum* Wis. 54-1255 was isolated. The mutant (L_1) grew with α -aminoadipic acid and excreted homocitrate. *In vivo* accumulation of homocitrate, which did not require protein synthesis, was markedly reduced by lysine. These results indicate that lysine diminution of penicillin production is caused by feedback inhibition of homocitrate synthase, thus limiting the supply of α -aminoadipic acid available for penicillin biosynthesis.

INTRODUCTION

For many years it has been known that lysine decreases the degree of penicillin biosynthesis in *Penicillium chrysogenum* (Demain, 1957). Since a biosynthetic lysine precursor, L - α -aminoadipic acid, is involved in penicillin formation (Arnstein & Morris, 1960), and since α -aminoadipic acid can reverse lysine diminution of penicillin formation (Somerson, Demain & Nunheimer, 1961), we postulated that a branched biosynthetic pathway was responsible for production of lysine and penicillin and that the 'lysine effect' was due to negative feedback control of an enzyme of the early common pathway (Demain, 1966). Goulden & Chattaway (1968) provided supporting evidence by showing that the concentration of α -aminoadipic acid in the intracellular pool of a late-blocked mutant of *P. chrysogenum* is low until lysine is depleted. Masurekar & Demain (1972) showed that, although prior growth in lysine stimulated penicillin formation by washed suspensions of prototrophic *P. chrysogenum*, the presence of lysine in the resting-cell suspension markedly inhibited penicillin biosynthesis. They concluded that the 'lysine effect' was caused by feedback inhibition of an early enzyme of lysine biosynthesis.

To determine whether homocitrate synthase, the initial lysine biosynthetic enzyme, is inhibited by lysine, we needed an early-blocked lysine auxotroph. Lysine auxotrophs were isolated (Masurekar, Kahgan & Demain, 1972), one of which was an early lysine bradytroph. We show here that *in vivo* homocitrate accumulation by this leaky auxotroph is markedly inhibited by lysine.

METHODS

Cultures. Nine lysine auxotrophs were isolated from *Penicillium chrysogenum* Wis. 54-1255 after 2 h ethylmethane sulphonate mutagenesis followed by pentachlorophenate enrichment (Masurekar *et al.* 1972). The lysine requirement of only one mutant, a leaky auxotroph, could be satisfied by α -aminoadipic acid. This bradytroph, designated as L_1 ,

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thus appears to be partially blocked before α -aminoadipic acid. The remaining auxotrophs presumably are either blocked between α -aminoadipic acid and lysine or unable to take up α -aminoadipic acid.

Penicillium chrysogenum strains Wis. 54-1255 and L_1 were maintained on slants of Sabouraud dextrose agar (Difco) with 0.1 % yeast extract.

Identification of accumulation product of mutant L_1 . Mutant L_1 and its parent (Wis. 54-1255) were grown for 3 days on the shaker at 25 °C in the glucose-citrate medium (citrate No. 2 medium of Yamamoto & Segel, 1966) supplemented with 250 μ g of L- α -aminoadipic acid/ml. The mycelia were filtered (Schleicher and Schuell No. 595 paper) and washed three times with a total of 6 vol. of sterile distilled water. The washed mycelia were resuspended in low glucose-citrate medium, i.e. glucose-citrate medium with glucose reduced from 40 g to 10 g/l; this reduction in the amount of sugar facilitated the subsequent concentration and extraction of the filtrates. To the medium, 0.5 μ Ci sodium [1- 14 C]acetate/ml was added. After 24 h of incubation on the shaker, the mycelia were removed by filtration and the clear filtrates were acidified with HCl to pH 2.0. The filtrates were concentrated *in vacuo* in a rotary evaporator to 5 ml, and 1 ml of the concentrated solutions was extracted with 3 ml of ether by vigorously mixing on a vortex mixer. The organic and aqueous phases were separated in a small separatory funnel.

The ether extracts were chromatographed on paper in the following solvent systems (Bhattacharjee & Strassman, 1967*a*): (1) ethyl ether-benzene-formic acid-water (21:9:7:2); (2) 1-butanol-acetic acid-water (4:1:1); (3) 3-butanol-methyl ethyl ketone-formic acid-water (40:30:15:15); (4) 1-butanol-formic acid-water (5:1:1). After development, the strips were dried and examined in a Packard radio-chromatogram scanner to locate the radioactive areas. These strips were also sprayed with neutralized 0.04 % bromphenol blue in 95 % ethanol. This indicator reveals acids as yellow spots on a blue background. By the combination of scanning and spraying, radioactive acids were detected. Homocitric lactone, citric acid, α -ketoglutaric acid and succinic acid were spotted as standards. For co-chromatography, the ether extract of the broth of mutant L_1 was chromatographed in solvent system 1 and peaks corresponding to the homocitric acid and its lactone were located by scanning. In this solvent system, the faster-moving component is the lactone and the slower-moving component is the free acid. The portion of the strip corresponding to homocitric lactone was cut out and eluted with 95 % ethanol into a test tube, the elution being carried out in a desiccator saturated with ethanol. The eluate was concentrated *in vacuo* in a rotary evaporator and was co-chromatographed with authentic homocitric lactone in solvent systems 2, 3 and 4. The chromatograms were scanned and were sprayed with bromphenol blue for visualization of acid spots.

In vivo accumulation of homocitrate. Mutant L_1 conidia from a slant were inoculated into 50 ml of Sabouraud dextrose broth (Difco) supplemented with 0.1 % yeast extract in 250 ml Erlenmeyer flasks. After 48–72 h of growth, the mycelia were filtered and washed. The washed mycelia were resuspended in 50 ml of sterile distilled water and served as inoculum. Fifty ml of glucose-citrate medium supplemented with 400 μ g lysine.HCl/ml in 250 ml Erlenmeyer flasks were inoculated with 2 ml of mutant L_1 inoculum. After 72 h of growth, the mycelia were harvested, washed and resuspended in low glucose-citrate medium. Sodium [1- 14 C]acetate (0.5 μ Ci/ml) was added and the flasks were shaken for 24 h. The mycelia were harvested by filtration and dry mycelial weight was determined. Homocitrate accumulation in the filtrate was measured by paper chromatography in solvent system 1. The chromatograms were examined on the radioactive chromatogram scanner to locate the two radioactive peaks corresponding to homocitric acid and its lactone. The portions of the

chromatogram corresponding to these peaks were cut out and counted in a Beckman scintillation spectrometer, with toluene scintillation fluid [4 g of Omnifluor (New England Nuclear, Boston, Massachusetts, U.S.A.) in 1 l reagent-grade toluene].

We have used the incorporation of labelled acetate into homocitrate to indicate the *in vivo* activity of homocitrate synthase. It is, however, realized that we are only dealing with an approximation, since the amount of homocitrate formed is not necessarily directly proportional to the incorporation of [$1-^{14}\text{C}$]acetate into homocitrate. Direct proportionality exists only when the specific radioactivity of the intracellular acetate involved in synthesis (i.e. endogenous plus that from the extracellular fluid) is constant throughout the experiment.

Inhibition of protein synthesis by cycloheximide. The mycelium of mutant L_1 , grown in glucose-citrate-lysine medium, was suspended in the low glucose-citrate medium containing 100 000 c.p.m. L-[$U-^{14}\text{C}$]proline/ml (specific activity 195 mCi/mmol) with and without 10^{-4}M -cycloheximide. Samples were withdrawn periodically and the mycelia were treated with 20 ml of 5% cold trichloroacetic acid for 30 min. The mycelia were collected by filtration through Schleicher and Schuell 895-E filter paper and washed 3 times with a total of 3 vol. of 5% cold trichloroacetic acid containing 50 μg unlabelled L-proline/ml. To the mycelia were added 1 ml of water and 15 ml of Bray's solution (Bray, 1960). Radioactivity was determined in a Beckman scintillation spectrometer. After being counted, the mycelia were collected on tared Schleicher and Schuell 895-E filter papers and washed, first with acetone to remove the scintillation fluid, and then with water. The filter paper discs were dried under an infrared lamp and weighed to determine dry mycelial weight.

Penicillin fermentation. Inoculum of *Penicillium chrysogenum* L_1 was prepared from conidia as described above. Two ml of inoculum were added to defined seed medium (50 ml in a 250 Erlenmeyer flask) containing (per litre): 40 g glucose, 13 g $(\text{NH}_4)_2\text{SO}_4$, 0.4 g L-lysine.HCl, 13 g CaCO_3 (sterilized separately), and 100 ml salts mixture. The salts mixture contained (per litre): 30 g KH_2PO_4 , 5 g Na_2SO_4 , 2.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.2 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.05 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. After 72 h on the shaker, 10 ml of seed were added to 250 ml flasks containing 40 ml of defined penicillin-production medium. The production medium was composed of (per litre): 30 g lactose, 10 g glucose, 6 g ammonium lactate, 3.5 g ammonium acetate, 1.8 g phenylacetyl-ethanolamine, 0.4 g L-lysine.HCl, 1 ml oleic acid and 100 ml salts mixture. The sugars were sterilized separately. At various times during the fermentation, flasks were removed and the mycelia harvested by filtration, washed and placed in a suspension medium containing 0.5 μCi of sodium [$1-^{14}\text{C}$]acetate/ml with and without 20 mM-lysine. The suspension medium contained (per litre): 48 g lactose (sterilized separately), 1.6 g sodium phenylacetate, 320 ml 1 M-sodium phosphate buffer (pH 7) and 160 ml salts mixture. After 24 h of incubation, the mycelia were harvested for the determination of dry mycelial weight and homocitrate accumulated in the broth was measured. Penicillin was measured by the hydroxamate method (Boxer & Everett, 1949) at the time of harvest of mycelium from the production medium.

Dry mycelial weight determination. The harvested mycelia were homogenized in a high-speed blender (Omnimixer, Sorvall and Co.) at 17 000 rev./min for 2 min. The absorbance of the homogenized mycelial suspension was measured in a Klett-Summerson photoelectric colorimeter with red filter (No. 66) after proper dilution. Dry mycelial weight was calculated from a calibration curve of absorbance against dry weight.

Chemicals. Cycloheximide, α -ketoglutaric acid and α -amino adipic acid were purchased from Calbiochem. Sodium [$1-^{14}\text{C}$]acetate, specific activity 51–57 mCi/mmol, was obtained from New England Nuclear. Phenylacetyl-ethanolamine and benzylpenicillin were generously

Table 1. R_f values of the product of the mutant and authentic homocitric lactone, citric acid, α -ketoglutaric acid and succinic acid

Solvent system no.	Mutant product	Homocitric lactone	Citric acid	α -Keto-glutaric acid	Succinic acid
1	0.25, 0.45	0.42	0.18	0.83	0.96
2	0.63	0.61	0.54	—	0.94
3	0.59, 0.72	0.70	0.53	0.84	0.87
4	0.54, 0.63	0.63	0.48	0.87	0.87

Fig. 1. Time-course of *in vivo* formation of homocitrate from sodium $[1-^{14}\text{C}]$ acetate by *Penicillium chrysogenum* L_1 .

donated by Dr D. W. Gallup and Dr J. Birnbaum of Merck and Co., respectively. Homocitric lactone was kindly supplied by Dr H. Broquist (Vanderbilt University, Nashville, Tennessee, U.S.A.) and by Dr A. Tucci (Albert Einstein Medical Centre, Philadelphia, Pennsylvania, U.S.A.).

RESULTS

Accumulation product of mutant L_1 . The ether extracts from mutant L_1 and the prototrophic parent Wis 54-1255 were chromatographed in four solvent systems as described under Methods. The chromatograms were scanned to detect radioactive peaks and were sprayed with bromphenol blue to visualize the acid spots. When the chromatograms developed in solvent systems 1, 3 and 4 were examined, the chromatograms for mutant L_1 were found to contain two radioactive acid spots not present in those of the parent. The R_f values of the faster-migrating spot were similar to those obtained for authentic homocitric lactone in the same solvent systems (Table 1). In solvent systems 1, 3 and 4, homocitric acid and its lactone migrate at different rates; the faster-moving component is the lactone and the slower-moving component is the free acid. In solvent system 2, both migrate as a single spot and the R_f is the same as that of the single extra radioactive acid spot of mutant L_1 (Table 1). To confirm these observations, we eluted the mutant peak

Fig. 2. Inhibition of *in vivo* biosynthesis of homocitrate by lysine in *Penicillium chrysogenum* L₁.

Table 2. Effect of cycloheximide on the *in vivo* synthesis of homocitrate

Cycloheximide concentration (M)	Homocitrate (c.p.m./mg dry mycelial wt)
0	235
10 ⁻⁴	201

corresponding to homocitric lactone with ethanol from the chromatograms developed in solvent system 1. The ethanol extract was co-chromatographed with authentic unlabelled homocitric lactone in solvent systems 2, 3 and 4. The R_F values of the radioactive peaks and the acid spots were identical. These results confirm that the mutant accumulates homocitrate.

In vivo synthesis of homocitrate. Studies on the time-course of *in vivo* homocitrate synthesis were carried out by growing mutant L₁ in the glucose-citrate-lysine medium and re-suspending the mycelium in the low glucose-citrate medium containing sodium [¹⁴C]acetate. The flasks were removed periodically and the label incorporated into homocitrate was measured. The results (Fig. 1) showed a linear formation of homocitrate from 3 to 24 h. For the remaining experiments, *in vivo* synthesis of homocitrate was determined after 24 h.

The relatively slow *in vivo* formation of homocitrate made it important to determine whether protein synthesis was involved or whether homocitrate was produced by pre-formed enzymes. For this purpose, we measured homocitrate production in the presence and absence of 10⁻⁴ M-cycloheximide. This concentration of cycloheximide was sufficient to inhibit protein synthesis as determined by inhibition of the incorporation of [¹⁴C]proline into the trichloroacetic acid-insoluble fraction. Table 2 shows that there is no difference in the amount of homocitrate produced in the presence or in the absence of cycloheximide. Hence protein synthesis is not required for the accumulation of homocitrate in the 24 h suspension system, which can thus be used to investigate lysine inhibition of homocitrate synthesis.

Effect of lysine on in vivo homocitrate synthesis. The effect of lysine was examined by determining homocitrate formation in the low glucose-citrate medium supplemented with

Fig. 3. Lack of lysine effect on uptake of sodium [^{14}C]acetate by *Penicillium chrysogenum* L₁.
 ○, With 20 mM-lysine; □, without lysine.

Fig. 4. Ability of *Penicillium chrysogenum* L₁ mycelia taken at various times from a penicillin fermentation to produce homocitrate from sodium [^{14}C]acetate. Penicillin was measured at the time of harvest of the mycelium from defined production medium. Dry mycelial weight was determined after 24 h incubation in the suspension medium. Homocitrate was measured at the end of the 24 h incubation in the suspension medium. It is expressed on the basis of dry cell weight. Open bar, homocitrate synthesis in the absence of lysine; closed bar, homocitrate synthesis in the presence of lysine.
 □—□, Dry cell weight; ○—○, penicillin.

various concentrations of lysine. The results shown in Fig. 2 demonstrate that lysine strongly inhibited the *in vivo* formation of homocitrate. In this experiment, 50 % inhibition occurred at a lysine concentration of 0.3 mM.

To make sure that lysine did not interfere with the transport of labelled acetate into the mycelium, we measured the uptake of radioactivity from the medium during suspension in the low glucose-citrate medium. As shown in Fig. 3, there was no difference in the uptake of acetate in the presence or in the absence of 20 mM-lysine. Thus, lysine reduced accumulation of homocitrate by an inhibitory action inside the cell, presumably by inhibiting homocitrate synthase.

In vivo homocitrate-synthesizing activity at various phases of a penicillin fermentation. Since the results described above showed that lysine inhibits *in vivo* homocitrate formation, we felt it desirable to determine the homocitrate-synthesizing activity and its sensitivity to inhibition by lysine at various times in a medium capable of supporting penicillin production. Penicillin, a secondary metabolite, is produced in a distinct phase of the fermentation (idiophase) subsequent to the growth phase (trophophase). Mutant L₁ was useful for such an experiment, since its 'leakiness' allows it to produce penicillin as well as accumulate homocitrate.

Mutant L₁ was grown in the defined penicillin production medium containing lysine. At various times, flasks were removed, the mycelia harvested and divided between flasks of a lactose-phenylacetate suspension medium either containing or lacking lysine. Penicillin was measured at the time of the harvest of the mycelia from the production medium. Homocitrate and dry mycelial weight were determined at the end of a 24 h suspension period. The results of this experiment are shown in Fig. 4. Homocitrate-synthesizing activity was maximal during the trophophase and decreased drastically during the idiophase. The activity in both phases was strongly (65 to 95 %) inhibited by lysine.

DISCUSSION

The distribution of lysine auxotrophs isolated after ethylmethane sulphonate mutagenesis was somewhat surprising in that eight out of the nine auxotrophs appeared to be blocked late in lysine biosynthesis, i.e. after aminoadipic acid. Fortunately, the ninth mutant (L₁) was blocked early in the sequence and accumulated homocitrate.

Previous studies with other fungi on the *in vivo* accumulation of intermediates of the lysine biosynthetic pathway and the effect of lysine on such accumulation have contributed significantly to our understanding of the regulation of lysine biosynthesis (Bhattacharjee & Strassman, 1967*a, b*). Control of lysine biosynthesis via feedback regulation occurs in *Saccharomyces*, *Rhodotorula* and *Neurospora* (Trupin & Broquist, 1965; Maragoudakis, Holmes & Strassman, 1967; Bhattacharjee, 1970; Glass & Bhattacharjee, 1971). Lysine produces feedback repression of homocitrate synthase (Hogg & Broquist, 1968; Tucci, 1969) and aminoadipic semialdehyde-glutamate reductase (Sinha, Kurtz & Bhattacharjee, 1971) and feedback inhibition of homocitrate synthase (Maragoudakis *et al.* 1967; Hogg & Broquist, 1968). The present studies showed that lysine inhibits homocitrate synthase activity in *Penicillium chrysogenum*.

Penicillium chrysogenum mutant L₁, an early-blocked lysine bradytroph, accumulates homocitrate rather slowly and we chose a duration of 24 h for our experiments. Because of this long duration, we felt that diminution of homocitrate formation by lysine could be explained by repression of a homocitrate synthase undergoing rapid turnover, or by feedback inhibition. We found, however, that cycloheximide did not diminish homocitrate formation,

thus proving that the normal production of homocitrate in our 24 h system did not involve protein synthesis. In view of this finding, diminution of homocitrate formation by lysine cannot be due to lysine repression of homocitrate synthase; feedback inhibition is apparently the true mechanism.

Homocitrate-synthesizing activity in *Penicillium chrysogenum* was maximal during the rapid growth phase and drastically decreased as the growth slowed down. A number of enzymes of primary metabolism showed similar behaviour (Kisumi, Komatsubara & Chibata, 1971; Hostalek *et al.* 1969; Hampton, McCormick, Behforouz & Freese, 1971). There are two aspartokinases in *Bacillus subtilis* (Hampton *et al.* 1971), only one of which is sensitive to lysine inhibition. As growth slows down, the level of the sensitive enzyme decreases while that of insensitive aspartokinase (which is low during rapid growth) increases. In the case of homocitrate synthase in *Penicillium chrysogenum*, such a dramatic difference in regulation is not readily apparent since lysine strongly inhibits *in vivo* homocitrate accumulation in cells taken from both the growth and the penicillin-production phases. There was some variation in the degree of inhibition observed at the various times; this could be due to fluctuations in the level of the intracellular lysine pool or to experimental variation. An important implication of these observations is that penicillin synthesis should be extremely sensitive to lysine present during the production phase.

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