JOURNAL OF BACTERIOLOGY, Dec. 1980, p. 869-876
0021-9193/80/12-0869/08$02.00/0

Vol. 144, No. 3

Inhibition and Repression of Homocitrate Synthase by Lysine in *Penicillium chrysogenum*

JOSE M. LUENGO, GLORIA REVILLA, MANUEL J. LÓPEZ, JULIO R. VILLANUEVA, AND JUAN F. MARTÍN*

Departamento de Microbiología, Facultades de Ciencias y de Farmacia, Universidad de Salamanca and Instituto de Microbiología Bioquímica, Consejo Superior de Investigaciones Cientificas, Salamanca, Spain

Homocitrate synthase is the first enzyme of the lysine biosynthetic pathway. It is feedback regulated by L-lysine. Lysine decreases the biosynthesis of penicillin (determined by the incorporation of [^{14}C]valine into penicillin) by inhibiting and repressing homocitrate synthase, thereby depriving the cell of α-aminoadipic acid, a precursor of penicillin. Lysine feedback inhibited in vivo the biosynthesis and excretion of homocitrate by a lysine auxotroph, L_2, blocked in the lysine pathway after homocitrate. Neither penicillin nor 6-aminopenicillanic acid exerted any effect at the homocitrate synthase level. The molecular mechanism of lysine feedback regulation in *Penicillium chrysogenum* involved both inhibition of homocitrate synthase activity and repression of its synthesis. In vitro studies indicated that L-lysine feedback inhibits and represses homocitrate synthase both in low- and high-penicillin-producing strains. Inhibition of homocitrate synthase activity by lysine was observed in cells in which protein synthesis was arrested with cycloheximide. Maximum homocitrate synthase activity in cultures of *P. chrysogenum* AS-P-78 was found at 48 h, coinciding with the phase of high rate of penicillin biosynthesis.

Lysine decreases the synthesis of penicillin by *Penicillium chrysogenum* (2) in low- and high-penicillin-producing strains (12). The inhibitory effect of lysine is reverted by the addition of α-aminoadipic acid (α-AAA) and other intermediates of the lysine biosynthetic pathway such as α-ketoadipate and homocitrate (5, 17). The nonprotein amino acid α-AAA is an intermediate of the lysine biosynthetic pathway in filamentous fungi. It is also a component of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, which is the direct precursor of isopenicillin N (10, 16). The role of α-AAA as an essential intermediate in penicillin biosynthesis has been unequivocally established by studying the behavior of lysine auxotrophs of *P. chrysogenum* (blocked before and after α-AAA) in relation to penicillin synthesis (8). Demain (3) postulated that the production of lysine and penicillin occurs through a branched pathway and that the "lysine effect" was due to a feedback regulation of homocitrate synthase.

Homocitrate synthase (acetyl-coenzyme A [CoA]: 2-ketoglutarate acetyl transferase, EC 4.1.3.21) forms homocitrate by condensation of acetyl-CoA and the tricarboxylic acid cycle intermediate α-ketoglutarate (Fig. 1). Masurekar and Demain (14) described that lysine inhibits in vivo the activity of homocitrate synthase but does not repress homocitrate synthase formation. However, the biosynthesis of lysine in yeast and fungi is subject to both feedback inhibition (7, 9, 13, 18) and feedback repression (9) at the homocitrate synthase level. It was therefore of interest to study whether repression by lysine is also exerted in *P. chrysogenum*.

Demain and Masurekar (4) described the inhibition by lysine of homocitrate synthesis in *P. chrysogenum* in vivo but failed to detect any in vitro effect. To elucidate the molecular mechanism of the lysine regulation of homocitrate synthase, we carried out experiments in vivo and in vitro using a low-producing *P. chrysogenum* strain, Wis 54-1255; a high-producing strain, AS-P-78; and a lysine auxotroph, *P. chrysogenum* L_2.

MATERIALS AND METHODS

Microbial strains. A low-penicillin-producing strain, *P. chrysogenum* Wis 54-1255 (of the Wisconsin family), was provided by A. L. Demain (Massachusetts Institute of Technology, Cambridge). *P. chrysogenum* L_2, a lysine auxotroph, was obtained as a spontaneous variant by selection of individual clones from a lysine bradytroph, L_1 (14). The high-producing strain AS-P-78 was kindly supplied by Antibioticos, S. A. (León, Spain). All strains were kept lyophilized or in liquid nitrogen.

Culture media and growth conditions. Strains Wis 54-1255 and L_2 were grown in complex seed medium (17). After incubation for 40 h at 25°C, 5 ml of

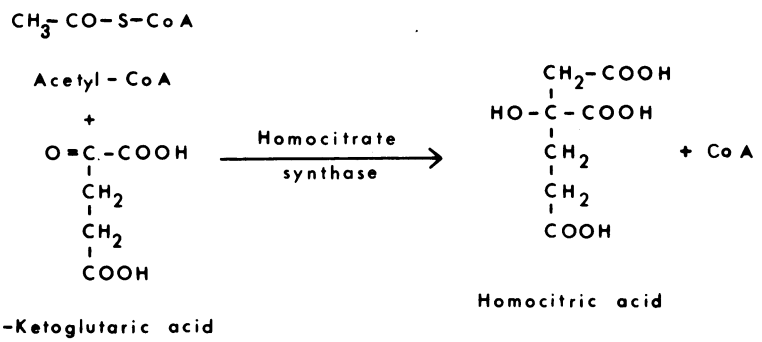

FIG. 1. *Reaction of acetyl-CoA and α-ketoglutaric acid catalyzed by homocitrate synthase.*

washed mycelium was used to inoculate 50 ml of complex production medium (17). In other experiments, growth of the microorganisms was carried out in defined seed medium containing the following (grams per liter): glucose, 40; $(NH_4)_2SO_4$, 13; H_2KPO_4, 3; $MgSO_4 \cdot 7H_2O$, 0.25; $FeSO_4 \cdot 7H_2O$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.02; Na_2SO_4, 0.5; $MnSO_4 \cdot H_2O$, 0.02; $CaCl_2 \cdot 2H_2O$, 0.05; $CaCO_3$, 13. After incubation for 60 h at 25°C, 5 ml of washed mycelium was used to inoculate the defined production medium Czapek-glucose-asparagine containing (grams per liter): glucose, 30; $NaNO_3$, 2; K_2PO_4H, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; asparagine, 0.2; $FeSO_4$, 0.01. Modified Yamamoto medium contained (grams per liter): glucose, 1; ammonium citrate, 10; sodium citrate $\cdot 2H_2O$, 4; K_2PO_4H, 30; NH_4Cl, 3; Na_2SO_4, 1; $MnCl_2$, 2; $MgCl_2 \cdot 6H_2O$, 50; $Na_2MoO_4 \cdot 2H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 2; $NaBO_4 \cdot 10H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.05; $Cu(NO_3)_2 \cdot 3H_2O$, 0.3; $ZnSO_4 \cdot 7H_2O$, 2; $CoNO_3$, 0.1; this medium was used for the study of the synthesis and excretion of homocitric acid by mutant L_2. After 34 h of growth, sodium [^{14}C]-acetate was added at 13-h intervals until 50 h. Cells were removed by filtration, and the filtrates were acidified with HCl to pH 2.0 (15). The auxotrophic requirement (L-lysine) was added to the medium when needed. Strain AS-P-78 was cultured as described previously (12).

Preparation of cell-free extracts. After 48 h of incubation in production medium (unless otherwise stated), cells were disrupted in a Braun homogenizer with glass beads in 0.05 M Tris-hydrochloride buffer (at pH 7.8 for strain AS-P-78 and pH 7.4 for the Wis 54-1255 and L_2 strains), containing 10% (vol/vol) glycerol and 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride. The suspension was then centrifuged for 15 min at 10,000 × *g*, and the supernatant was dialyzed for 2 h against 0.05 M Tris-hydrochloride buffer, pH 7.8 (or 7.4 depending on the strain as before), with glycerol and phenylmethylsulfonyl fluoride (7).

Homocitrate synthase assay. The assay of homocitrate synthase (EC 4.1.3.21) was based on the formation of [^{14}C]homocitric acid by condensation of α-ketoglutarate and [^{14}C]acetyl-CoA. The reaction mixture contained 4 μM acetyl-CoA, 50 μM α-ketoglutarate, 0.3 μM $MgCl_2$, 0.1 μCi of [1-^{14}C]acetyl-CoA, 0.5 mg of protein, and 0.05 M Tris-hydrochloride buffer (pH 7.8) in 1 ml. The incubation was continued for 1 h at 32°C and was stopped by addition of 2 μl of sulfuric acid (specific gravity, 1.84).

The reaction products were separated by thin-layer chromatography and identified as described by Bhattacharjee and Strassman (1) and Masurekar and Demain (15). One unit of activity is defined as 1 dpm of homocitrate formed per min of reaction. Specific activity is defined as the number of units per milligram of protein.

Incorporation of L-[*U*-^{14}C]valine into penicillin. *P. chrysogenum* AS-P-78 was grown in the complex production medium with and without L-lysine (10 or 50 mM) for 24 h. Cells were harvested by filtration, washed with sterile distilled water, and transferred to suspension medium (14).

After incubation for 90 min at 25°C, L-lysine (10 or 50 mM) was added to the medium, and L-[*U*-^{14}C]valine (0.25 μCi/ml) was supplemented at 120 min. Controls were carried out without lysine addition. Samples were taken over 60 min at 15-min intervals. Mycelium was removed by centrifugation (10 min at 2,000 × *g*), and the penicillin in 1 ml of supernatant was extracted with 0.5 ml of amylacetate previously acidified to pH 2.0 with phosphoric acid. The organic phase was chromatographed in Silica Gel G thin-layer plates using acetone-acetic acid (95:5) as the developing solvent. R_f values in this solvent system are 0.05 for valine and 0.87 for penicillin. Radioactivity was measured in a Packard Tri-Carb 3320 Scintillation Counter.

Determination of protein synthesis. Protein synthesis was followed by measuring the incorporation of [^{14}C]leucine into trichloroacetic acid-insoluble material. The effect of the cycloheximide on protein synthesis was studied by adding cycloheximide to the flasks at the time indicated in the figures. Protein determination was carried out by the method of Lowry et al. (11).

Chemicals. L-Lysine, cycloheximide, homocitric acid lactone, acetyl-CoA, α-ketoglutarate potassium salt, and phenylmethylsulfonyl fluoride were from Sigma Chemical Co. (St. Louis, Mo.); toluene and Silica Gel G were from E. Merck AG (Darmstadt, Germany); sodium [*U*-^{14}C]acetate (57.6 mCi/mmol), [*U*-^{14}C]leucine (351 mCi/mmol), [1-^{14}C]acetyl-CoA (59.4 mCi/mmol), and L-[*U*-^{14}C]valine (280 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, England). Amylacetate and all other products were of reagent quality.

RESULTS

Effect of L-lysine on the incorporation of [^{14}C]valine into penicillin. The addition of 10 or 50 mM exogenous L-lysine to suspension cultures of *P. chrysogenum* greatly decreased the incorporation of [^{14}C]valine into penicillin. To study whether this was due to an inhibition of the activity of penicillin-synthesizing enzymes or to repression of such enzymes, we carried out experiments with cells grown in the absence of lysine and then supplemented with lysine in the suspension system for incorporation of precursors (Fig. 2a). Lysine at 10 mM or higher concentrations fully inhibited the incorporation of [^{14}C]valine into penicillin. Lysine when added to the suspension medium 30 min before [^{14}C]valine did not inhibit the uptake of labeled valine (Fig. 2b) as measured by the level of radioactive valine remaining in the medium.

Alternatively, we carried out experiments in which cells were grown in the presence of 10 or 50 mM lysine, washed exhaustively, and suspended in suspension medium for precursor incorporation without lysine (Fig. 2c). There was a slight stimulation of the incorporation of precursors by 10 mM lysine and a 60% reduction by 50 mM lysine. This result suggests that lysine, in addition to enzyme inhibition, represses the formation of enzymes involved in penicillin biosynthesis, but only at very high lysine concentrations (see below).

The inhibition exerted by L-lysine on the incorporation of [^{14}C]valine into penicillin was reversed by α-aminoadipic acid, which indicates that lysine is depriving the cell of this intermediate as previously reported.

Characterization of the lysine auxotroph L_2. *P. chrysogenum* mutant L_2 was characterized as a lysine auxotroph in both solid and liquid lysine assay medium (Difco) with and without supplementation with lysine. It grows but does not form penicillin in lysine assay medium supplemented with 0.4 mg of L-lysine per ml because this medium does not contain α-AAA, whereas the parental strain, Wis 54-1255, produces penicillin under these conditions. However, mutant L_2 synthesizes penicillin in complex medium, yeast extract (10 g/liter)-dextrose (10 g/liter), without lysine supplementation, suggesting that the specific branch of the biosynthetic pathway leading to penicillin biosynthesis is functional in this lysine auxotroph (Fig. 3).

This mutant was shown to excrete homocitrate as proved by the infrared spectrum and by cochromatography with a sample of authentic homocitric acid lactone (Sigma) (Fig. 4). From these data, mutant L_2 appears to be

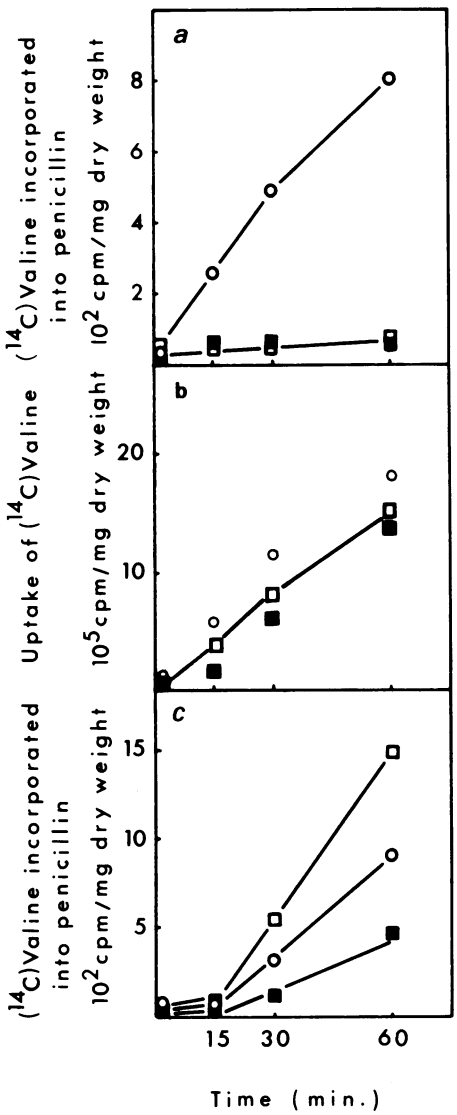

FIG. 2. *Effect of L-lysine on the cell uptake and incorporation of [^{14}C]valine into penicillin. (a) Inhibition of penicillin biosynthesis by L-lysine. P. chrysogenum AS-P-78 was grown in the complex fermentation medium without lysine. Cells were collected, washed, and suspended in suspension medium. Flasks were supplemented with 10 mM (□) or 50 mM (■) L-lysine 30 min before addition of [^{14}C]valine. Control without lysine (○). (b) Uptake of [^{14}C]valine by cells of P. chrysogenum AS-P-78 in the suspension medium. Control without lysine (○); 10 mM lysine (□); 50 mM lysine (■). (c) Repression of penicillin biosynthesis by high levels of lysine. P. chrysogenum AS-P-78 was grown with 10 mM (□) or 50 mM (■) lysine or without it (○). Cells were collected, washed, and suspended without lysine for precursor incorporation.*

blocked in the conversion of homocitric acid to oxaloglutaric acid (Fig. 3).

Effect of L-lysine on the formation and excretion of homocitrate by the lysine auxotroph L_2. The accumulation of radioactive homocitrate from [^{14}C]acetate in vivo by the *lys* auxotroph L_2 was studied in Yamamoto medium. Cell suspensions prepared from cultures supplemented with 50 mM lysine at inoculation time formed 43% less homocitric acid than the control without lysine supplementation. When lysine (50 mM) was added daily, there was a 100% inhibition of homocitrate formation (Fig. 5a and b). However, neither penicillin nor 6-aminopenicillanic acid, the end product of the other branch of the biosynthetic pathway, exerted any inhibitory effect at levels of 1,000 μg/ml (Fig. 5c and d).

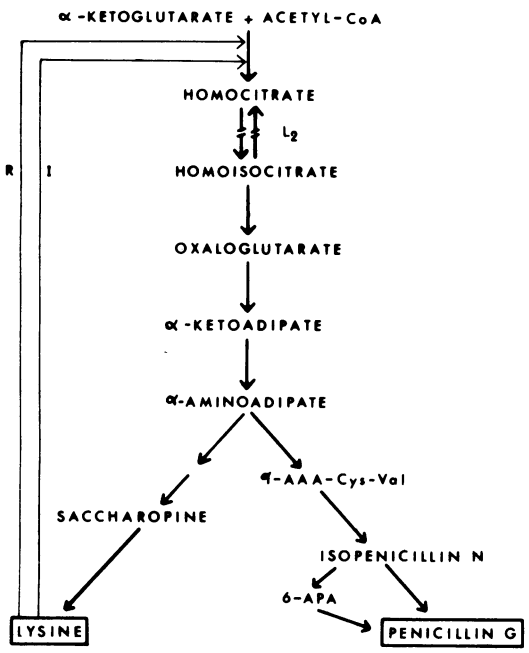

FIG. 3. *Branched pathway leading to lysine and penicillin. α-AAA is the branching intermediate. Mutant L_2 is blocked before homoisocitrate and therefore accumulates homocitrate. The first enzyme of the pathway is repressed (R) and inhibited (I) by L-lysine but not by penicillin G. 6-APA, 6-Aminopenicillanic acid.*

Regulation by lysine of homocitrate synthase activity in the auxotroph L_2. The previous experiments suggest that lysine feedback regulates the homocitrate synthase activity since it inhibits the in vivo formation of homocitrate. To check whether repression of this enzyme was involved, homocitrate synthase activity was measured in cell-free extracts of cultures grown with and without lysine in antibiotic-producing complex medium. The inhibitory effect of lysine added directly to the enzyme assay system was also tested.

The results shown in Table 1 indicate that addition of 50 mM lysine during the enzyme assay produced a 22% inhibition. When the cells were grown in lysine (daily supplementations) a 42% inhibition of homocitrate synthase activity was observed, and when lysine was added during both growth and enzyme assay an additive 67% inhibition was obtained. The results suggest that

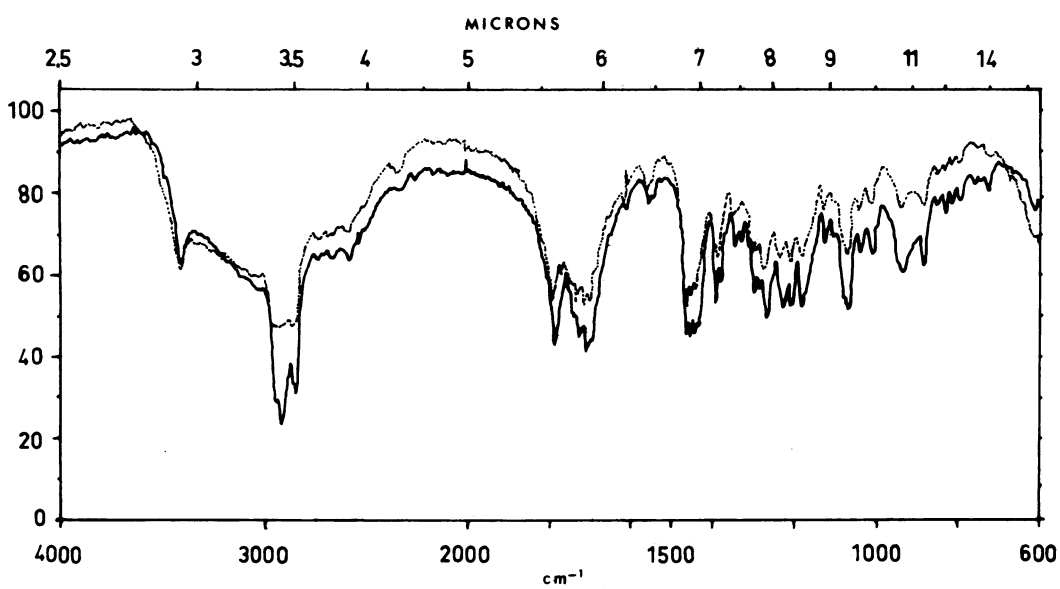

FIG. 4. *Infrared spectra of the lactone of homocitric acid excreted by the lysine auxotroph L_2 (·····) as compared to commercial homocitric acid lactone obtained from Sigma (——).*

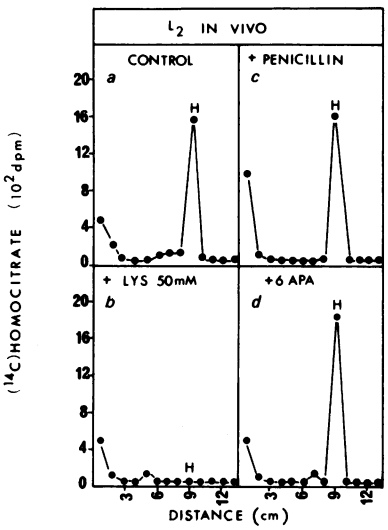

FIG. 5. *Effect of L-lysine, penicillin G, and 6-aminopenicillanic acid on the in vivo formation of homocitrate by the lysine auxotroph L_2. Radioactive acetate was added to the cultures, the broth was acidified to pH 2, and the labeled homocitrate formed was extracted with ether, purified by thin layer chromatography, and counted. The peak of homocitrate appears at 9 cm in the plates. (a) Control without additions. (b) Supplemented with 50 mM lysine. (c) Supplemented with 1,000 μg of penicillin G per ml. (d) Supplemented with 1,000 μg of 6-aminopenicillanic acid (6-APA) per ml.*

homocitrate synthase activity is inhibited and repressed by high concentrations of lysine.

Due to the relative insensitivity of homocitrate synthase to lysine in complex medium (a maximum 67% reduction of activity was obtained even at lysine concentrations as high as 50 mM), we studied the same effect in a synthetic medium. Czapek-asparagine medium (supplemented with a low concentration of lysine because strain L_2 is a lysine auxotroph) supported good mycelial growth but did not favor penicillin production; a 98% repression of homocitrate synthase activity occurred when cultures in Czapek-asparagine medium were grown in the presence of 50 mM lysine (Table 1). Addition of lysine only during the enzyme assay resulted in a 48% inhibition of homocitrate synthase activity.

Regulation by lysine of the homocitrate synthase activity in the low-penicillin-producing strain Wis 54-1255. Experiments on lysine regulation of homocitrate synthase using the low-producing prototroph strain Wis 54-1255 in complex and defined Czapek-asparagine media gave results very similar to those of the auxotroph mutant L_2 (Table 1). The homocitrate synthase activity of this strain is subject to both inhibition and repression in cells grown in either complex or defined medium. However, the homocitrate synthase activity of cells grown in Czapek-asparagine was much more sensitive to lysine than the enzyme of cells grown in complex medium.

The similar sensitivity to lysine in the low-producing strain Wis 54-1255 and the lysine auxotroph L_2 reflects the fact that the former is the parental strain of the auxotroph. Introduction of the mutational block in the lysine pathway after homocitrate does not affect the enzymatic activity or the regulatory properties of the homocitrate synthase.

Regulation by lysine of the homocitrate synthase activity in the high-penicillin-producing strain AS-P-78. In vivo studies of the high-producing strain AS-P-78 as compared to the low-producing strain have shown that lysine inhibits total penicillin synthesis to a similar extent. However, onset of penicillin synthesis occurred even at a high lysine concentration in the high-producing strain, whereas in the low-producing strain lysine had to be depleted before penicillin production started (12).

We studied the sensitivity of homocitrate syn-

TABLE 1. *Effect of L-lysine on homocitrate synthase activity*

Strain	Medium	Lysine supplementation during:		Enzyme sp act	
		Growth	Enzyme assay	U/mg of protein	% Inhibition
L_2[a]	Complex	None	None	3,067	0
		50 mM	None	1,779	42
		None	50 mM	2,362	22
		50 mM	50 mM	1,012	67
	Defined[b]	None	None	1,734	0
		50 mM	None	38	98
		None	50 mM	896	48
		50 mM	50 mM	8	100
Wis 54-255[c]	Complex	None	None	3,528	0
		50 mM	None	1,975	44
		None	50 mM	2,677	24
		50 mM	50 mM	1,016	71
	Defined[b]	None	None	1,640	0
		50 mM	None	33	98
		None	50 mM	912	44
		50 mM	50 mM	0	100
AS-P-78[d]	Complex	None	None	1,849	0
		50 mM	None	1,204	34
		None	50 mM	1,541	15
		50 mM	50 mM	1,031	54

[a] Auxotrophic mutant.
[b] Defined Czapek-asparagine medium.
[c] Low-producing strain.
[d] High-producing strain.

thase of the high-producing strain AS-P-78 in the complex medium (but not in Czapek-asparagine medium, where the strain AS-P-78 grows very poorly). The enzyme of the high-penicillin-producing strain was still partially sensitive to lysine inhibition and repression, although to a lesser extent than that of low-producing strain Wis 54-1255 (Table 1).

Effect of the intracellular pool of lysine on the in vitro activity of homocitrate synthase. The large reduction of homocitrate synthase activity of cells grown in the presence of lysine appears to be due to repression of homocitrate synthase formation. However, the possibility that the enzyme was formed and its activity was inhibited during the in vitro assay by the increased pool of lysine existing in lysine-grown cells could not be excluded a priori. Results of experiments in which denatured extracts of cells grown in 50 mM lysine were added to cell-free extracts containing active homocitrate synthase activity showed that the lysine pool of cells grown at a high lysine concentration was not enough to inhibit homocitrate synthase in vitro. This result indicates that the reduction of homocitrate synthase observed in cells grown in a high lysine concentration is not due to inhibition of enzyme activity by the lysine pool during the in vitro assay but to repression of the formation of this enzyme.

Time sequence of homocitrate synthase formation. The homocitrate synthase is an enzyme involved in lysine biosynthesis as well as in penicillin formation. Studies on the time sequence of enzyme activity during the culture (Fig. 6a) indicate that the maximum homocitrate synthase activity in the cells existed at about 48 h of incubation, coinciding with the phase of rapid penicillin biosynthesis. A high level of homocitrate synthase was observed until 96 h of incubation. Thereafter, both homocitrate synthase activity and penicillin synthesis decreased sharply. This result suggests that, after growth, a high level of homocitrate synthase activity is maintained, presumably to synthesize α-AAA for penicillin formation.

The time sequence of homocitrate synthase activity of cells grown in the presence of 50 mM lysine is shown in Fig. 6b. Lysine-grown cells clearly showed lower levels of enzyme than control cultures, but the time sequences of enzyme formation appeared to be similar. Significant levels of homocitrate synthase were formed even in cells grown in 50 mM lysine. It seems, therefore, that lysine only partially represses homocitrate synthase formation.

Effect of cycloheximide on protein synthesis and homocitrate synthase formation in strain AS-P-78. To establish whether lysine affects penicillin biosynthesis in cells in which protein synthesis has been blocked with protein synthesis inhibitors, we studied first the effect of cycloheximide on protein synthesis in cultures of *P. chrysogenum* in defined production medium. As shown in Fig. 7a, cycloheximide at 70 μg/ml fully inhibited incorporation of [^{14}C]leucine into proteins for at least 6 h.

When protein synthesis was inhibited, a slight decrease of the homocitrate synthase activity in the cell was observed. In control cultures the specific activity of the enzyme increased slightly over the 6-h period, whereas in cultures in which protein synthesis had been inhibited there was a small decrease in homocitrate synthase activity (Fig. 7b). Identical results were obtained in complex production medium. These results suggest that the homocitrate synthase in vivo is relatively stable and that there is only a small

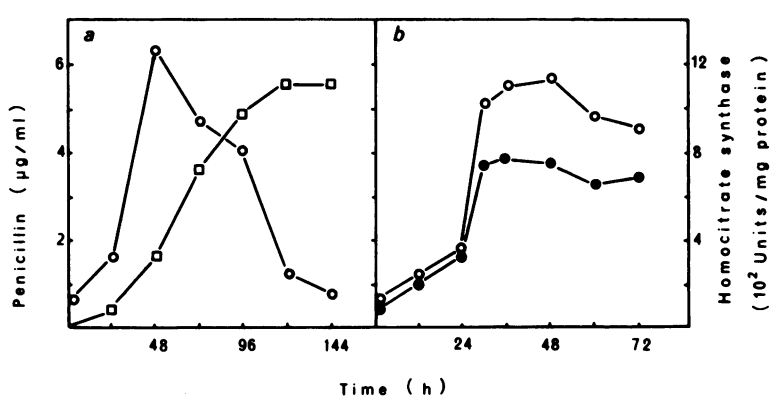

FIG. 6. *Time sequence of activity of homocitrate synthase in cultures of P. chrysogenum AS-P-78. (a) Long-term control cultures: homocitrate synthase* (○), *penicillin* (□). *(b) Homocitrate synthase activity in cultures without* (○) *and with 50 mM lysine* (●) *supplementation.*

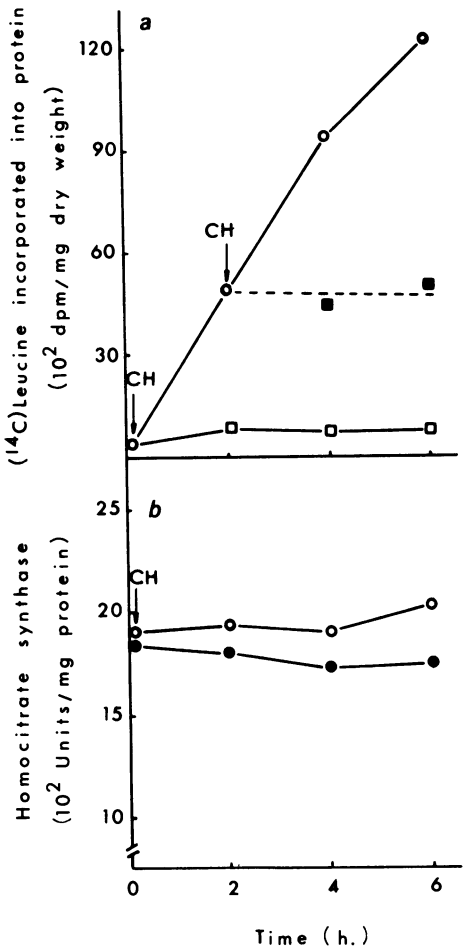

FIG. 7. *Effect of cycloheximide on protein synthesis and homocitrate synthase activity in cells of P. chrysogenum AS-P-78. Cells were grown in defined production medium. (a) Cycloheximide was added at the time indicated by the arrows. (b) After addition of cycloheximide at 0 h, cells were broken at 0, 2, 4, and 6 h, and the homocitrate synthase was measured in the cell-free extracts.*

decrease in enzyme activity when protein synthesis is blocked with cycloheximide, probably as the result of enzyme turnover.

Inhibition by L-lysine of homocitrate synthase activity in cells in which protein synthesis has been blocked by cycloheximide. Control suspension cultures of cells grown without lysine had a homocitrate synthase activity of 1,950 U, which increased very little during a 6-h incubation period. Cultures grown in 50 mM lysine and then suspended in lysine-supplemented medium had an activity of 1,000 to 1,200 U, whereas cells grown in medium with lysine and then suspended in medium without lysine showed an increased enzyme activity, up to 1,350 to 1,450 U. This result suggests that there was an increase in activity after the removal of lysine.

When synthesis of proteins was prevented by cycloheximide, the homocitrate synthase activity (1,850 U/mg of protein) was not greatly affected during the 6-h period, but if lysine was added to cells with blocked protein synthesis, a small reduction of homocitrate synthase activity (1,370 U/mg of protein) was observed. This result is explained on the basis of inhibition of enzyme activity when protein synthesis is blocked.

DISCUSSION

The biosynthesis of lysine in yeast and fungi is feedback-regulated by high concentrations of lysine. Similar results have been described in *P. chrysogenum* (12, 14). However, different authors disagree as to whether this control is exerted by feedback inhibition or feedback repression. In all cases, the control of the lysine biosynthetic pathway is primarily exerted at the homocitrate synthase level.

In *Saccharomyces cerevisiae* (18, 19) and *Neurospora crassa* (9) homocitrate synthase is both feedback inhibited and repressed by lysine, whereas in *Saccharomycopsis lipolytica* only inhibition has been reported (7). In *P. chrysogenum*, Demain and Masurekar (4) described in vivo inhibition but not repression of homocitrate synthase by lysine, and they failed to detect any in vitro effect (15). Our results both in vivo and in vitro suggest that lysine inhibits and represses homocitrate synthase (Fig. 3) (see Table 1). The detection of in vitro activity is probably the result of a careful cell disruption (see Materials and Methods). In vivo experiments using the lysine auxotroph L_2 blocked after homocitrate indicated that homocitrate formation by this strain is feedback-regulated by lysine (Fig. 5). In vitro experiments using cell-free extracts of low- or high-penicillin-producing strains indicated that the homocitrate synthase of *P. chrysogenum* was only partially sensitive to lysine when cells were grown in complex medium, requiring 50 mM lysine to get 50% repression of enzyme activity (Table 1). However, in cells grown in defined medium a 100% repression of the homocitrate synthase activity was obtained using the same lysine concentration. Also, 100% repression of homocitrate formation in vivo was observed in the defined Yamamoto medium. Interestingly, cells grown in defined medium showed a lower specific homocitrate synthase activity. The existence of two different isoenzymes with contrasting properties as occurs in *S. cerevisiae* (19) might explain the differences

observed in activity and feedback sensitivity of homocitrate synthase when cells are cultivated in complex or defined media. Other amino acids at 50 mM concentrations did not affect homocitrate synthase activity (J. M. Luengo, manuscript in preparation).

The presence of a dual inhibition-repression regulatory mechanism insures fine control of the lysine biosynthetic pathway as occurs in the biosynthesis of many other amino acids. One important observation is that homocitrate synthase of *S. cerevisiae* is much more sensitive to lysine. It is strongly inhibited by 1 mM lysine, whereas the homocitrate synthase of the cephalosporin producer *Cephalosporium acremonium* is totally insensitive to lysine (Luengo, in preparation). It is interesting to speculate on the low degree of regulation of homocitrate synthase by lysine in β-lactam-producing fungi as compared with *S. cerevisiae*. Production of high levels of penicillin and cephalosporin by these fungi may have evolved thanks to the partial insensibility of homocitrate synthase to lysine, making possible the synthesis of large amounts of α-AAA required for β-lactam biosynthesis.

Cells grown in lysine, or grown in absence of this amino acid but supplemented with lysine in the suspension medium, showed a greatly decreased penicillin biosynthetic activity, as measured by the incorporation of [^{14}C]valine into penicillin (Fig. 2), because lysine is depriving the cells of α-AAA. This inhibitory effect of lysine is reverted by α-AAA, which appears to be the limiting amino acid for penicillin biosynthesis (G. Revilla et al., manuscript in preparation) (6).

ACKNOWLEDGMENTS

This investigation was supported by a grant from Antibioticos S.A. (León, Spain).

We are greatly indebted to J. García-Jimeno, F. Holguera, J. M. Fernández-Sousa, and J. L. Fernández for their interest and collaboration in this research project.

LITERATURE CITED

1. **Bhattacharjee, J. K., and M. Strassman.** 1967. Accumulation of tricarboxylic acids related to lysine biosynthesis in a yeast mutant. J. Biol. Chem. **242**:2542–2546.
2. **Demain, A. L.** 1957. Inhibition of penicillin formation by lysine. Arch. Biochem. Biophys. **67**:244–245.
3. **Demain, A. L.** 1974. Biochemistry of penicillin and cephalosporin fermentations. Lloydia **37**:147–167.
4. **Demain, A. L., and P. S. Masurekar.** 1974. Lysine inhibition of *in vivo* homocitrate synthesis in *Penicillium chrysogenum*. J. Gen. Microbiol. **82**:143–151.
5. **Friedrich, C. G., and A. L. Demain.** 1977. Homocitrate synthase as the crucial site of the lysine effect on penicillin biosynthesis. J. Antibiot. **30**:760–761.
6. **Friedrich, C. G., and A. L. Demain.** 1978. Uptake and metabolism of α-aminoadipic acid by *Penicillium chrysogenum* Wis 54-1255. Arch. Mikrobiol. **119**:43–47.
7. **Gaillardin, C. M., L. Poirier, and H. Heslot.** 1976. A kinetic study of homocitrate synthase activity in the yeast *Saccharomycopsis lipolytica*. Biochim. Biophys. Acta **422**:390–406.
8. **Goulden, S. A., and F. W. Chattaway.** 1968. Lysine control of α-aminoadipate and penicillin synthesis in *Penicillium chrysogenum*. Biochem. J. **110**:55.
9. **Hogg, R. W., and H. P. Broquist.** 1968. Homocitrate formation in *Neurospora crassa*. J. Biol. Chem. **243**: 1839–1845.
10. **Konomi, T., S. Herchen, J. E. Baldwin, M. Yoshida, N. A. Hunt, and A. L. Demain.** 1979. Cell-free conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine into an antibiotic with the properties of isopenicillin N in *Cephalosporium acremonium*. Biochem. J. **184**:427–430.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. **Luengo, J. M., G. Revilla, J. R. Villanueva, and J. F. Martín.** 1979. Lysine regulation of penicillin biosynthesis in low producing and industrial strains of *Penicillium chrysogenum*. J. Gen. Microbiol. **115**:207–211.
13. **Maragoudakis, M. E., H. Holmes, and M. Strassman.** 1967. Control of lysine biosynthesis in yeast by a feedback mechanism. J. Bacteriol. **93**:1677–1680.
14. **Masurekar, P. S., and A. L. Demain.** 1972. Lysine control of penicillin biosynthesis. Can. J. Microbiol. **18**: 1045–1048.
15. **Masurekar, P. S., and A. L. Demain.** 1974. Insensitivity of homocitrate synthase in extracts of *Penicillium chrysogenum* to feedback inhibition by lysine. Appl. Microbiol. **28**:265–270.
16. **O'Sullivan, J., R. C. Bleaney, J. A. Huddleston, and E. P. Abraham.** 1979. Incorporation of ^{3}H from δ-(L-α-amino-[4,5-^{3}H]adipyl)-(L-cysteinyl-D-[4,4-^{3}H])valine into isopenicillin N. Biochem. J. **184**:421–426.
17. **Somerson, N. L., A. L. Demain, and T. D. Nunheimer.** 1961. Reversal of lysine inhibition of penicillin production by α-aminoadipic acid. Arch. Biochem. **93**:238–241.
18. **Tucci, A. F.** 1969. Feedback inhibition of lysine biosynthesis in yeast. J. Bacteriol. **99**:624–625.
19. **Tucci, A. F., and L. N. Ceci.** 1972. Homocitrate synthase from yeast. Arch. Biochem. Biophys. **153**:742–750.