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Catabolism of Lysine in *Penicillium chrysogenum* Leads to Formation of 2-Aminoadipic Acid, a Precursor of Penicillin Biosynthesis

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***Penicillium chrysogenum* L2, a lysine auxotroph blocked in the early steps of the lysine pathway before 2-aminoadipic acid, was able to synthesize penicillin when supplemented with lysine. The amount of penicillin produced increased as the level of lysine in the media was increased. The same results were observed in resting-cell systems. Catabolism of [U-^{14}C]lysine by resting cells and batch cultures of *P. chrysogenum* L2 resulted in the formation of labeled saccharopine and 2-aminoadipic acid. Formation of [^{14}C]saccharopine was also observed in vitro when cell extracts of *P. chrysogenum* L2 and Wis 54-1255 were used. Saccharopine dehydrogenase and saccharopine reductase activities were found in cell extracts of *P. chrysogenum*, which indicates that lysine catabolism may proceed by reversal of the two last steps of the lysine biosynthetic pathway. In addition, a high lysine:2-ketoglutarate-6-aminotransferase activity, which converts lysine into piperideine-6-carboxylic acid, was found in cell extracts of *P. chrysogenum*. These results suggest that lysine is catabolized to 2-aminoadipic acid in *P. chrysogenum* by two different pathways. The relative contribution of lysine catabolism in providing 2-aminoadipic acid for penicillin production is discussed.**

2-Aminoadipic acid is a key intermediate in the biosynthesis of lysine and penicillin in β-lactam-producing fungi (Fig. 1) (2, 16, 21), as well as in the catabolism of lysine in mammals (9, 13) and in β-lactam-producing actinomycetes (17). The catabolism of lysine in a wide range of microorganisms, including *Pseudomonas* (10), *Flavobacterium* (35), *Streptomyces* (23), *Candida* (8, 33), *Neurospora* (34) and *Rhodotorula* (18) spp., has been studied. Lysine is catabolized to 2-ketoglutarate or acetate in these microorganisms by different pathways. In β-lactam-producing actinomycetes, lysine is converted to 2-aminoadipic acid by lysine-6-aminotransferase (LAT) (5, 23).

Very little is known about the catabolism of lysine in filamentous fungi (2), particularly in producers of β-lactam antibiotics. 2-Aminoadipic acid is a precursor of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, which is the first intermediate in the biosynthesis of β-lactam antibiotics (6, 24, 25). 2-Aminoadipic acid may be the limiting precursor for penicillin biosynthesis in *Penicillium chrysogenum* (14, 20, 28, 30). Addition of 2-aminoadipic acid (but not valine or cysteine) to resting-cell cultures of *P. chrysogenum* leads to an increase in the rate of penicillin biosynthesis (30).

2-Aminoadipic acid (an intermediate of the lysine biosynthetic pathway) is known to be synthesized in *P. chrysogenum* from 2-ketoglutarate and acetyl coenzyme A by homocitrate synthase. Feedback regulation of homocitrate synthase by lysine leads to a reduction in the pool of 2-aminoadipic acid and therefore to a decrease in the rate of penicillin biosynthesis (3, 22). However, lysine is known to increase the yield of cephalosporin in some *Cephalosporium acremonium* strains (27). This apparent contradiction was further stressed in preliminary studies with the mutant *P. chrysogenum* L2, a lysine auxotroph derived from *P. chrysogenum* Wis 54-1255, which is completely blocked before the 2-aminoadipic acid step in lysine biosynthesis (Fig. 1) and was still able to produce penicillin (21). We have further investigated this observation, and we describe in this report two different lysine catabolic pathways in *P. chrysogenum* which give rise to 2-aminoadipic acid.

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MATERIALS AND METHODS

Organisms and growth conditions. The *P. chrysogenum* strains used in this study were Wis 54-1255 and L2, a lysine auxotroph blocked in the conversion of homocitrate to 2-ketoadipate and derived from *P. chrysogenum* Wis 54-1255 (21). Spores were obtained as described previously (20). DIM, a defined medium (21), was used to obtain mycelia for the enzymatic assays; in some experiments ammonium sulfate was substituted by 30 mM lysine, and this medium is referred to as DIML. DPM, a defined medium for penicillin biosynthesis, contained the following (in grams per liter): sucrose, 10; lactose, 30; glucose, 10; citric acid, 10; acetic acid, 2.5; phenylacetic acid, 2; ethylamine, 3; $(NH_4)_2SO_4$, 5; KH_2PO_4, 1; $FeSO_4 \cdot 7H_2O$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.5; $ZnSO_4 \cdot 7H_2O$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.01; $MnSO_4 \cdot 4H_2O$, 0.01; $CoSO_4 \cdot 7H_2O$, 0.005; and NaCl, 0.001. The pH was adjusted to 5.5 with NaOH, and the medium was sterilized in an autoclave at 120°C for 20 min. The inoculum was developed in complex seed medium (29) and used to inoculate CPM (30), a complex medium for penicillin production. The mycelia were harvested by filtration, washed with 5 volumes of 0.9% NaCl, and stored at −20°C until use. Penicillin G assays were done by high-pressure liquid chromatography and bioassay as described previously (1, 30).

Penicillin biosynthesis by resting cells. Fresh spores of mutant L2 were grown for 72 h at 25°C with shaking in DIM supplemented with 0.5 mM lysine. A 2.5-ml portion of culture was transferred to 100 ml of DPM lacking ammonium sulfate and supplemented with 0.5 mM lysine. At 48 h, the mycelium was filtered under sterile conditions, washed with 0.9% NaCl, and resuspended at a concentration of 1 g of mycelium per 25 ml in nitrogen-limited suspension medium (26). The cell suspension was incubated at 25°C for 72 h with shaking.

Identification of products of catabolism of [U-^{14}C]lysine.

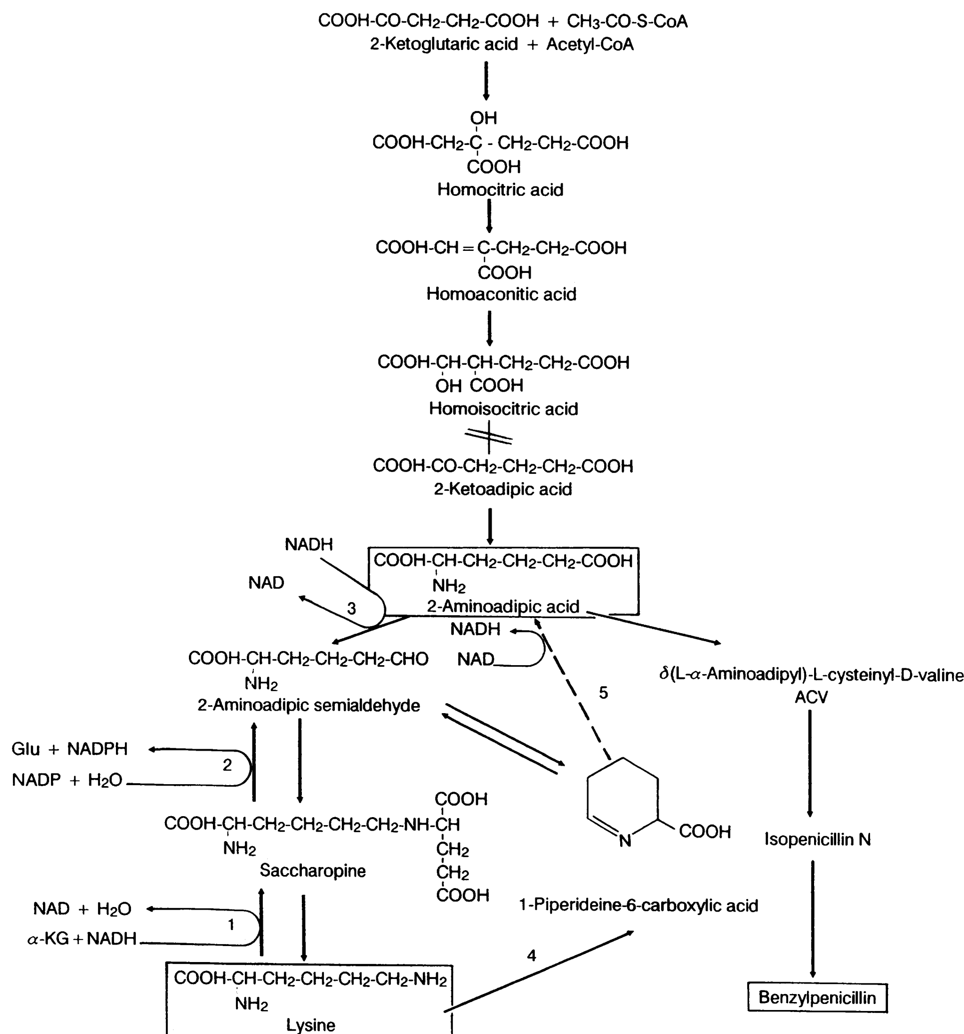

FIG. 1. The lysine and penicillin biosynthetic routes in *P. chrysogenum* and two catabolic routes for lysine which yield 2-aminoadipic acid as an intermediate. Solid arrows show reactions which have been demonstrated in this or other work. The dashed line shows a reaction deduced from the experimental evidence shown in this work. Enzyme activities are as follows: 1, saccharopine dehydrogenase; 2, saccharopine reductase; 3, 2-aminoadipic reductase; 4, lysine:2-ketoglutarate aminotransferase; 5, piperideine-6-carboxylic acid dehydrogenase (NAD^+ oxidoreductase). The L2 mutant strain accumulates homoisocitric acid. The branching-point intermediate for the lysine and penicillin pathways, 2-aminoadipic acid, and the final products of the pathways are boxed. Abbreviations: CoA, coenzyme A; α-KG, α-ketoglutarate.

Fresh spores of L2 mutant were inoculated into DIM lacking a nitrogen source and supplemented with (i) 10 μCi of L-[U-^{14}C]lysine, (ii) 10 μCi of L-[U-^{14}C]lysine and 0.5 mM carrier unlabeled lysine, or (iii) 10 μCi of L-[U-^{14}C] lysine and 1 mM carrier lysine. The cultures were incubated for 48 h before being harvested. The products of in vivo catabolism of lysine were identified, after cell disruption and extraction with 7.5% trichloroacetic acid, by thin-layer chromatography and electrophoresis. Extracts were centrifuged to remove debris and then passed through 10-ml Dowex AG 50W X8 H+ columns. After the columns were washed with 3 volumes of distilled water, the 2-aminoadipic acid was eluted with 4 volumes of 3 M pyridine, and, later, 5 volumes of 1 M NaOH were used to elute the lysine and saccharopine. The eluents from washing with distilled water and pyridine were lyophilized and dissolved in 200 μl of distilled water.

To further characterize the putative 2-aminoadipic acid and saccharopine spots after thin-layer chromatography separation, we extracted these compounds from the silica gel with distilled water adjusted to pH 10.5. This extraction was repeated five times to recover the products completely. The extracts were lyophilized and taken up in 50 μl of distilled water (made acidic with HCl to dissolve the saccharopine). Samples were then chromatographed in four different solvents: (i) butanol-acetic acid-water (4:1:1, vol/vol/vol); (ii) butanol-formic acid-water (7:3:3, vol/vol/vol); (iii) propanol-ammonium hydroxide (7:3, vol/vol); and (iv) chloroform-methanol-ammonium hydroxide (2:2:1, vol/vol/vol) (19).

Saccharopine was not easily separated from lysine by chromatography in eight different solvent systems. However, it was separated from lysine during thin-layer electrophoresis (0.5-mm Silica Gel 60 glass plates with an FBE-3000 Flat Bed Apparatus [Pharmacia]).

Saccharopine dehydrogenase and saccharopine reductase assays. Saccharopine dehydrogenase was assayed spectrophotometrically (based on the conversion of lysine into saccharo-

pine) as described by Broquist and coworkers (16, 32). The control reaction was performed without lysine. Mycelium was harvested, suspended in 50 mM potassium phosphate buffer (pH 7.0), and broken by ultrasonic disruption. Specific activity is given as units (micromoles of NADH oxidized per minute at 25°C) per milligram of protein (4).

A second, qualitative, assay for saccharopine dehydrogenase was carried out as described by Kurtz and Bhattacharjee (19), with 0.15 mM L-[U-^{14}C]lysine as the substrate (10 μCi/ml), except that 0.1 ml of reaction mixture was used and incubation was continued for 2 h. The positive control reaction mixture contained commercial saccharopine dehydrogenase of *Saccharomyces cerevisiae* (Sigma Chemical Co., St. Louis, Mo.), and negative control reactions were carried without enzyme extract. The reactions were stopped by adding 100 μl of cold methanol, and the protein was precipitated at −20°C. After 1 h the reaction mixture was filtered to remove protein and lyophilized. The dry residue was resuspended in 40 μl of acidified distilled water (pH about 3 with HCl, to dissolve saccharopine), and the saccharopine that formed was separated by thin-layer chromatography or electrophoresis. The plates were then dried well and placed with autoradiograph film for 4 days. After the film was removed, the plates were developed with ninhydrin to verify the position of unlabeled controls.

The saccharopine reductase assay was based on the catabolic reaction (conversion of saccharopine into 2-aminoadipic acid semialdehyde) (16). Specific activity is given as units (micromoles of NADP reduced per minute) per milligram of protein.

LAT and piperideine-6-carboxylic acid dehydrogenase assays. The LAT assay was essentially as described by Fothergill and Guest (10). Control reactions were carried out without 2-ketoglutarate and lysine. Specific activities (micromoles of piperideine-6-carboxylic acid formed per minute per milligram of protein) were calculated as described previously (10, 17).

The piperideine-6-carboxylic acid dehydrogenase assay was based on that of Fothergill and Guest (10). The reaction was followed by the NAD-dependent disappearance of piperideine-6-carboxylic acid or by the conversion of NAD to NADH. Since the substrate (piperideine-6-carboxylic acid) is not available, it was provided by the coupled LAT reaction with purified LAT (8a).

Radiochemicals. [U-^{14}C]lysine (324 mCi/mmol) and 2-[6-^{14}C]aminoadipic acid (55 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, United Kingdom, and CEA, Gif-sur-Yvette, France, respectively.

RESULTS

Penicillin production by mutant L2 is dependent on lysine supplementation. Growth of L2 mutant in lysine-supplemented media was similar at different lysine concentrations as long as lysine was present, but the amount of penicillin produced increased as the level of lysine in the medium was increased, up to 1 mM (Fig. 2). Higher concentrations of lysine (10 mM) retarded growth and did not cause a further increase of penicillin synthesis.

The stimulatory effect of lysine on penicillin production by the L2 mutant was more evident in the resting-cell studies (Fig. 3). There was a direct correlation between the amount of lysine added to the resting-cell cultures and the amount of penicillin produced. Cultures with no lysine added showed no penicillin production, whereas those with as little as 0.5 mM lysine showed antibiotic biosynthesis. The levels of penicillin synthesized became higher with increasing concentrations of lysine added to the resting-cell culture.

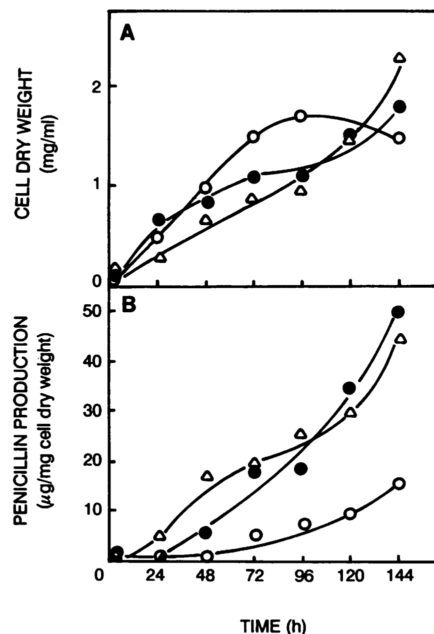

FIG. 2. Effect of increasing concentrations of lysine on growth of *P. chrysogenum* L2 (*lys*) (A) and penicillin production (B) in batch cultures in DPM. Symbols: ○, 0.05 mM lysine; △, 1 mM lysine; ●, 10 mM lysine.

Catabolism of [U-^{14}C]lysine to 2-aminoadipic acid via saccharopine. Labeled substances derived from [U-^{14}C]lysine were identified by thin-layer chromatography. Solvent system 1, methanol-toluene-acetic acid-distilled water (50:15:10:15), was found to be the best for separation of 2-aminoadipic acid from lysine and saccharopine. As shown in Fig. 4, a labeled spot which comigrates with unlabeled carrier 2-aminoadipic acid separates clearly from the other large radioactive spot which comigrates with unlabeled carrier lysine and saccharopine.

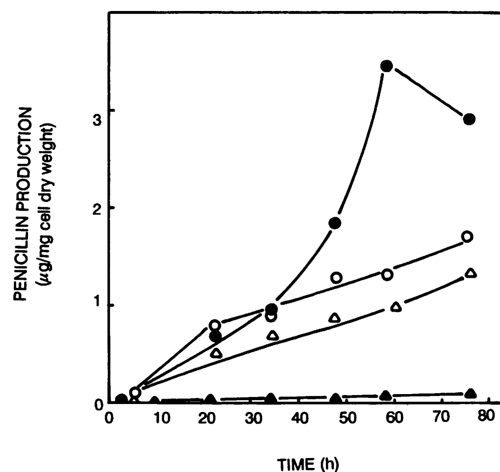

FIG. 3. Effect of increasing concentrations of lysine on penicillin production by nitrogen-limited resting cells of *P. chrysogenum* L2 (*lys*). Symbols: ▲, control without lysine; △, 0.5 mM lysine; ○, 10 mM lysine; ●, 50 mM lysine.

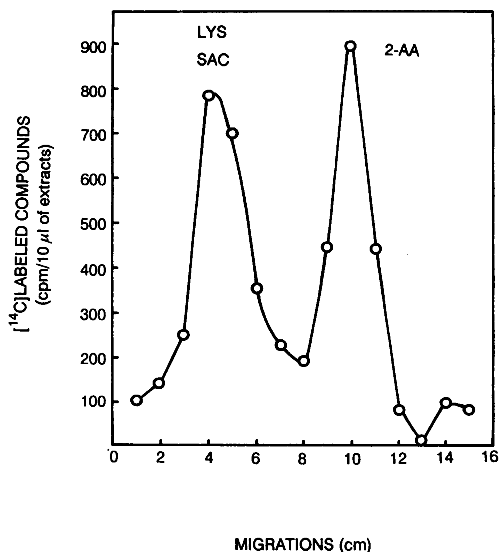

FIG. 4. Formation of 2-[^{14}C]aminoadipic acid (2-AA) by catabolism of [^{14}C]lysine. Extracts were prepared from resting cells of *P. chrysogenum* supplemented with [^{14}C]lysine (see the text for details). 2-Aminoadipic acid was separated from saccharopine (SAC) and from undegraded lysine (LYS) by thin-layer chromatography with solvent system 1, methanol-toluene-acetic acid-destilled water (50:15:10:15, by vol). Similar results were obtained when the catabolism of lysine was carried out in batch cultures of *P. chrysogenum*.

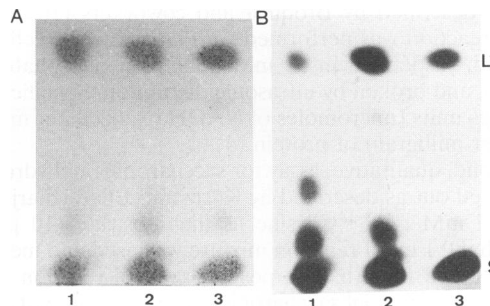

FIG. 5. Formation of [^{14}C]saccharopine by the saccharopine dehydrogenase of *P. chrysogenum*. Reactions (in 0.1 ml) were carried out with 0.15 mM L-[U[14]C]lysine (10 μCi/ml) as substrate or 0.15 mM L-[U-^{14}C]lysine supplemented with 50 mM carrier unlabeled lysine. After the enzymatic reaction was completed, 10-μl samples were electrophoresed on thin-layer plates stained with ninhydrin (A) and autoradiographed (B) (see the text). Lanes: 1, saccharopine dehydrogenase reaction with cell extract and labeled lysine as substrate; 2, reaction with both labeled and unlabeled (carrier) lysine as substrates; 3, control reaction with commercial *S. cerevisiae* saccharopine dehydrogenase (see the text). Abbreviations: S, saccharopine; L, lysine. Control reactions without enzyme showed only the lysine spot.

The radioactive product eluted from thin-layer chromatography plates as indicated in Materials and Methods had the same R_f values as 2-aminoadipic acid in the four solvent systems A to D (R_f values, 0.04, 0.09, 0.47, and 0.40, respectively). The radioactive spot was reisolated from the chromatography plates, and, as expected, it migrated with the same mobility as authentic radioactive 2-aminoadipic acid.

A second product of catabolism of [^{14}C]lysine, different from 2-[^{14}C]aminoadipic acid, was noticed after thin-layer electrophoresis. It comigrated with a control of pure saccharopine. The results indicated that both [^{14}C]saccharopine and 2-[^{14}C]aminoadipic acid were present in extracts as products of catabolism of lysine.

Conversion of lysine to saccharopine in vitro. As shown in Fig. 5, the presence of [^{14}C]saccharopine was detected along with unconverted L-[U-^{14}C]lysine in all samples. Reactions which involved only labeled lysine (0.15 mM) (without carrier) as the substrate showed the greatest conversion of lysine. In reactions with both labeled (0.15 mM) and unlabeled (50 mM) lysine as carrier, more labeled lysine remained unconverted after the reaction was stopped. There was a conversion in positive controls with commercial saccharopine dehydrogenase but not in reactions without cell extract. These results clearly show that lysine is converted to saccharopine by cell extracts of *P. chrysogenum*. In reactions with crude extracts of *P. chrysogenum* (but not in reactions with purified commercial *S. cerevisiae* saccharopine dehydrogenase), other radioactively labeled products were also formed; these have not been identified. These labeled products do not react with ninhydrin (Fig. 5) and are probably deaminated products formed from lysine catabolism. They were present in larger amounts in assays without carrier lysine since they are isotopically diluted in reactions with carrier lysine.

Saccharopine dehydrogenase and saccharopine reductase activities. Saccharopine dehydrogenase activity was higher in *P. chrysogenum* cultures at 48 and 72 h cultures than at 96 h both in defined DIM and in DIM supplemented with lysine (Table 1). Similar results were observed when the saccharopine reductase activity was quantified at different times of the cultures. These results indicate that the ability of *P. chrysogenum* to convert saccharopine into 2-aminoadipic acid semialdehyde by these enzymes is greater during the period preceding the onset of penicillin synthesis.

P. chrysogenum **shows LAT and piperideine-6-carboxylic acid dehydrogenase activities.** An activity converting lysine into piperideine-6-carboxylic acid was detected in extracts of the parental *P. chrysogenum* Wis 54-1255 (Table 1) and L2. L2 and Wis 54-1255 grown in DIML showed very similar specific activities. The enzyme activity has been purified (8a) and behaves functionally as an LAT.

Piperideine-6-carboxylic acid dehydrogenase activity was

TABLE 1. Enzyme activities in batch cultures of *P. chrysogenum* Wis 54-1255 grown in DIML and DIM

Medium[a] and reaction time	Activity (mU/mg of protein) of[b]:		
	Saccharopine dehydrogenase[c]	Saccharopine reductase[c]	Lysine aminotransferase[d]
DIML			
48 h	134.6	3.4	3.2
72 h	40.4	4.8	1.3
96 h	20.0	0.5	1.7
DIM			
48 h	25.9	2.7	0.2
72 h	47.7	1.8	1.3
96 h	0	0	0.8

[a] DIML contains lysine as the only source of nitrogen; DIM contains ammonium sulfate as the only source of nitrogen.

[b] Two enzyme determinations were made from duplicate flasks; the enzyme activities are the mean of the four measurements.

[c] Specific activities of saccharopine dehydrogenase or saccharopine reductase are given as units (micromoles of NADH oxidized or NADP reduced per minute, respectively) per milligram of protein.

[d] One unit of lysine aminotransferase activity corresponds to 1 μmol of piperideine-6-carboxylic acid formed per min.

found in extracts of *P. chrysogenum* by using a coupled assay. This activity led to the complete disappearance of the piperideine-6-carboxylic acid generated by the LAT. Controls which did not have lysine and 2-ketoglutarate added to the first (LAT) reaction showed only basal conversion of NAD to NADH in the coupled reaction; i.e., the dehydrogenase activity is dependent on piperideine-6-carboxylic acid.

DISCUSSION

Feedback regulation of the lysine biosynthetic pathway by lysine decreases penicillin biosynthesis (7, 15, 21). However, in *Cephalosporium acremonium*, lysine stimulated cephalosporin production, which argues against the hypothesis that high concentrations of lysine decrease the biosynthesis of β-lactam antibiotics in this microorganism by limiting the availability of 2-aminoadipic acid (27). Attempts were made to block the lysine branch of the pathway to direct the pool of 2-aminoadipic acid toward penicillin (26, 28). In *P. chrysogenum* H, a mutant strain which was defective in saccharopine dehydrogenase, lysine increased rather than decreased penicillin production (28). All these unexpected results are now explained on the basis of the conversion of lysine to 2-aminoadipic acid.

The conversion of lysine to 2-aminoadipic acid explains the observation that mutant L2 (a lysine auxotroph which is blocked before 2-aminoadipic acid) is able to synthesize increasing concentrations of penicillin when the level of lysine added to the culture is augmented.

Lysine is catabolized to 2-aminoadipic acid in *P. chrysogenum*, as in other yeasts and fungi, apparently by two different pathways. Studies reported here with labeled [U-^{14}C]lysine clearly indicate that lysine is converted to saccharopine and then to 2-aminoadipic acid. Two distinct enzymes, saccharopine dehydrogenase (lysine-forming EC 1.6.1.7) and saccharopine reductase (also named saccharopine dehydrogenase glutamate-forming EC 1.5.1.10), are known to catalyze fully reversible reactions in yeasts (2, 11, 12, 32, 36). We have found that lysine is converted to saccharopine in *P. chrysogenum*. Studies with mutant yeasts indicate that a single enzyme catalyzes both the forward and the reverse reactions (2).

The second catabolic pathway involves an enzyme with L-lysine:2-ketoglutarate 6-aminotransferase activity, which results in the direct formation of 2-aminoadipate semialdehyde (which is in equilibrium with the cyclic form, piperideine-6-carboxylic acid). This enzyme has also been found in *Rhodotorula glutinis* (18). It is interesting that this enzyme is functionally identical to the LAT which exists in *Streptomyces clavuligerus* (23, 31), *Nocardia lactamdurans* (5), and other β-lactam-producing actinomycetes (5). In actinomycetes, the 2-aminoadipic acid required for β-lactam biosynthesis is obtained by catabolism of lysine through the action of LAT (17, 23). The gene encoding this enzyme is located in the cluster of cephamycin biosynthetic genes (5, 37) and is missing in nonproducers of β-lactam antibiotics (5). It is intriguing that eucaryotic as well as procaryotic β-lactam-producing organisms have a functionally similar enzyme to form 2-aminoadipic acid. This might suggest that the role of LAT in β-lactam biosynthesis is more important than previously considered.

Little information is available on the conversion of piperideine-6-carboxylic acid (or, in linear form, 2-aminoadipic semialdehyde) to 2-aminoadipic acid. The formation of 2-[^{14}C]aminoadipic acid from [^{14}C]lysine proves that such a conversion occurs in *P. chrysogenum*. The lack of the commercially available substrate has prevented purification of the enzyme so far. However, using a coupled reaction in which piperideine-6-carboxylic acid was obtained by the action of purified LAT, we have observed an NAD-dependent oxidation of the 2-aminoadipate semialdehyde.

The relative contribution of the lysine catabolic pathways to the 2-aminoadipate pool for penicillin biosynthesis is unclear. It is well established that the lysine biosynthetic pathway provides 2-aminoadipic acid for penicillin biosynthesis. However, when protein synthesis ceases, enough lysine may be available from the turnover and degradation of proteins to provide 2-aminoadipic acid for penicillin biosynthesis through the catabolism of this amino acid.

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