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Insensitivity of Homocitrate Synthase in Extracts of *Penicillium chyrosogenum* to Feedback Inhibition by Lysine[1]

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We previously reported that lysine inhibits in vivo homocitrate synthesis in the lysine bradytroph, *Penicillium chrysogenum* L_1, and that such feedback inhibition could explain the known lysine inhibition of penicillin formation. In the present study, it was found that dialyzed cell-free extracts of mutant L_1 converted [1-^{14}C]acetate to homocitrate. This homocitrate synthase activity was extremely labile but could be stabilized by high salt concentrations. The pH optimum of the reaction was 6.9, and the K_m was 5.5 mM with respect to α-ketoglutarate. The reaction was also dependent upon the presence of Mg^{2+}, adenosine 5'-triphosphate, and coenzyme A. Surprisingly, the activity in these crude extracts was not inhibited by lysine. Benzylpenicillin at a high concentration (20 mM) partially inhibited the enzyme, an effect that was enhanced by lysine. Casein hydrolysate also partially inhibited the enzyme.

Penicillin and L-lysine appear to share a branched biosynthetic pathway in *Penicillium chrysogenum* (4a). The inhibition of penicillin production by lysine (3) might thus be explained by feedback inhibition of the first enzyme of the common path, homocitrate synthase, by lysine. In support of this hypothesis, we reported that addition of lysine to washed suspensions of *P. chrysogenum* inhibited incorporation of labeled valine into the antibiotic (16). To elucidate the site of lysine action, we isolated a lysine bradytrophic mutant of the penicillin-producing strain Wis. 54-1255. We have found that in vivo homocitrate accumulation in this early-blocked mutant is inhibited by lysine (4b). In the present communication, we report studies on homocitrate synthase activity in crude extracts of this mutant. Surprisingly, such activity is insensitive to lysine inhibition.

MATERIALS AND METHODS

Culture. The lysine bradytroph, *P. chrysogenum* L_1, was obtained by mutagenesis of *P. chrysogenum* Wis. 54-1255 (4b). It was maintained on slants of Sabouraud dextrose agar (Difco Laboratories, Inc., Detroit, Mich.) with 0.1% yeast extract.

Preparation of mycelia. Conidia of mutant L_1 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 to 72 h of growth (25 C, gyratory® shaker, 220 rpm, 2-in. [ca. 5.1 cm] diameter orbit), the mycelia were filtered and washed three times with a total of 6 volumes of sterile distilled water. The washed mycelia were resuspended in 50 ml of sterile distilled water. Fifty milliliters of glucose-citrate medium (citrate no. 2 medium of Yamamoto and Segel [21]) supplemented with 400 μg of lysine·hydrochloride per ml in 250-ml Erlenmyer flasks was inoculated with 2 ml of the mycelial suspension. The culture was grown on the shaker for 2.5 days.

Cell disruption. The mycelia were harvested and washed as described above and then disrupted by the following two methods. Method 1: Mycelia were frozen and disrupted in a Hughes press prechilled at −20 C. The broken cells were resuspended in 2 volumes of 0.1 M sodium phosphate buffer (pH 7.0) with 1 mg each of deoxyribonuclease (DNase) I and ribonuclease (RNase) A per 10 g (wet weight) of mycelium and incubated for 30 min at 25 C. Method 2: The washed mycelium was resuspended in 2 volumes of 0.1 M sodium phosphate buffer (pH 7.0). To this suspension were added 8 mg of chitinase and 1 mg each of DNase I and RNase A per 10 g (wet weight) of mycelium. The suspension was shaken for 4 h at 25 C and then homogenized in a Potter-Elvehjem tissue homogenizer.

Preparation of cell-free extract. The cell debris was removed from the disrupted mycelial suspensions by centrifuging for 30 min at 27,000 × *g* followed by centrifugation of the supernatant fluid at 35,000 × *g*. The double centrifugation procedure facilitated complete removal of the cell debris. The supernatant fluid

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was dialyzed against 50 volumes of 0.1 M sodium phosphate buffer (pH 7.0) for 3 h, the buffer being replaced every 45 min. Longer periods of dialysis led to inactivation of the enzyme. This dialyzed cell-free extract was used for the assay of homocitrate synthase.

Assay of cell-free homocitrate synthase. The assay mixture contained 5 mM adenosine triphosphate, 5 mM magnesium chloride, 5 mM dithiothreitol, 5 mM potassium α-ketoglutarate, 0.3 mM coenzyme A, 5 μCi (0.1 mM) [1-^{14}C]sodium acetate, 0.3 ml of enzyme extract, and 0.1 M phosphate buffer (pH 7.0) in a total volume of 1.0 ml. Other additions or modifications are described in the Results section. The mixture was incubated at 30 C under a nitrogen atmosphere for 3 h. The reaction was terminated by the addition of one drop of concentrated sulfuric acid (specific gravity, 1.8). At this low pH, most of the homocitrate is present as homocitric acid lactone. The precipitated protein was removed by centrifugation. Homocitric acid lactone was determined by paper chromatography of the supernatant fluid using ethyl ether-benzene-formic acid-water (21:9:7:2, vol/vol vol/vol) as the developing solvent (1). The methods for locating the radioactive spot corresponding to homocitric acid lactone, elution, and counting were the same as previously described for in vivo homocitrate accumulation (4b). Ether extraction of the reaction mixtures was not necessary in these cell-free studies since there was no chromatographic inteference by dissolved solutes.

Protein determination. Protein content of the cell-free extracts was determined by the method of Lowry et al. (13).

Chemicals. Bovine pancreas RNase A and beef pancreas DNase I were from Sigma Chemicals (St. Louis, Mo.). α-Ketoglutaric acid, DL-α-aminoadipic acid, coenzyme A, and dithiothreitol were purchased from Calbiochem (Los Angeles, Calif.). [1-^{14}C]sodium acetate (specific activity, 51 to 57 mCi/mmol) was obtained from New England Nuclear, Boston, Mass., and chitinase was obtained from Schwarz/Mann, Orangeburg, N.Y. Homocitric acid lactone was kindly supplied by H. Broquist (Vanderbilt University, Nashville, Tenn.) and A. Tucci (Albert Einstein Medical Center, Philadelphia, Pa.).

RESULTS

Cell-free homocitrate synthase activity. Cell-free extracts of *P. chrysogenum* L_1 were capable of converting [1-^{14}C]acetate to homocitrate. It was noted that the enzyme activity was very labile, virtually complete losses being observed during overnight storage at −4, 8, and 25 C. Considerable losses in activity were also observed upon freezing and thawing.

The presence of homocitrate synthase activity in the cell-free extracts was dependent on the methods used for rupture of the cells. Active cell-free preparations were consistently obtained after chitinase disruption of the mycelium, whereas cells broken in the Hughes press sometimes yielded inactive extracts, possibly due to the freezing of the mycelia. In the experiments to be described, the extracts were prepared by breaking the cells enzymatically, unless otherwise specified.

The age of the culture used to prepare the cell-free extract was critical. There was a marked reduction in activity when extracts were prepared from older cultures. This observation is consistent with the in vivo results which showed that homocitrate synthesizing activity is maximal during the trophophase and decreases as growth slows down (4b). Therefore, the cell-free extracts were routinely prepared from mycelia grown for 2.5 days in the glucose-citrate medium.

Homocitrate synthase activity was found to require a high ionic strength. Either the disruption of cells in phosphate buffer at molarities lower than 0.1 M or dialysis against such buffers resulted in inactivation of the enzyme. The presence of mercaptoethanol (5 mM) in the buffer used for dialysis yielded slightly decreased activity.

Studies on the time-course of homocitrate synthesis (Fig. 1) showed an approximate 45-min lag before synthesis became linear, remaining linear for as long as 3 h. Incubations were carried out for 3 h in all the remaining experiments.

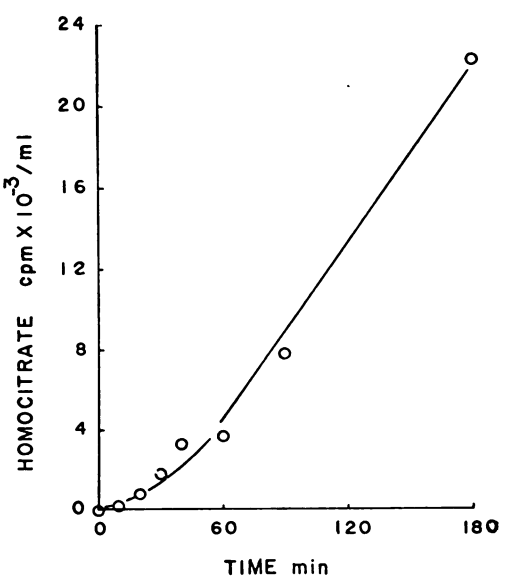

FIG. 1. *Time-course of in vitro formation of homocitrate from [1-^{14}C]sodium acetate by cell-free extracts of P. chrysogenum L_1.*

Product analysis showed that the in vitro synthesized product and authentic homocitric acid lactone have the same mobility in four solvent systems and that the eluted product co-chromatographs with the authentic material.

Effect of conditions on homocitrate synthase activity. The effect of cell-free extract concentration was determined by adding various amounts of extract to an incubation mixture containing a final concentration of 45 mM α-ketoglutarate. The extracts routinely contained about 5 mg of protein per ml. Table 1 shows that the activity was dependent on the addition of cell-free extract to 0.3 ml; higher concentrations resulted in inhibition.

The effect of pH was investigated by preparing enzyme extracts in buffers of various pH values and assaying the amount of homocitrate synthesized. Sodium phosphate was added to Tris(hydroxymethyl)aminomethane and bicine buffers to prevent enzyme inactivation. The pH optimum of homocitrate synthase was 6.9 (Fig. 2).

The concentration of potassium α-ketoglutarate was varied to determine its effect on homocitrate synthase activity. The concentration of potassium was maintained at a constant value. The results are shown in Fig. 3. The synthesis of homocitrate was completely dependent on the presence of α-ketoglutarate. The approximate K_m of homocitrate synthase as determined from the double reciprocal plot was 5.5 mM.

Dependence of homocitrate synthase on Mg^{2+}, adenosine 5'-triphosphate, and coenzyme A was studied by omitting them one at a time from the complete incubation mixture. Maximal homocitrate synthase activity was obtained only in the presence of all three cofactors (Table 2).

Lack of inhibition by lysine. To study the effect of lysine on homocitrate synthase activity, concentrations of lysine up to 20 mM were added to the complete incubation mixture, and homocitrate production was measured. Surpris-

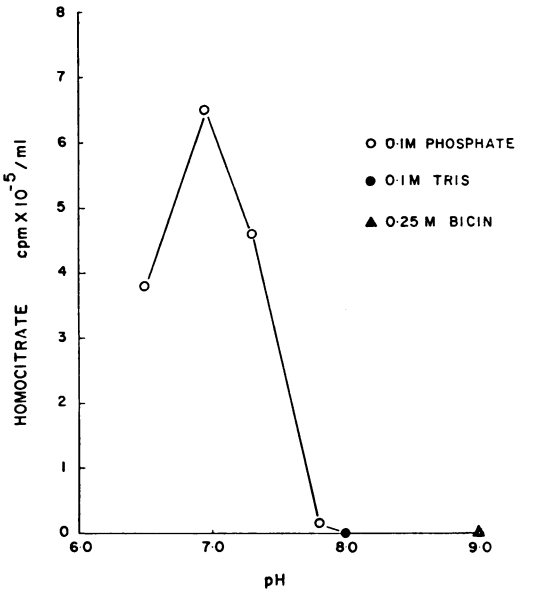

FIG. 2. *Effects of pH on activity of homocitrate synthase in cell-free extracts of P. chrysogenum L_1.*

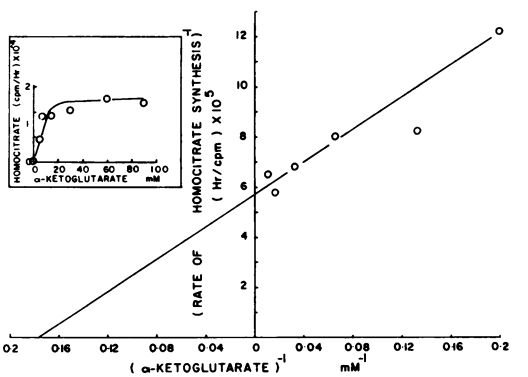

FIG. 3. *Effect of concentration of potassium α-ketoglutarate on homocitrate synthase activity in cell-free extracts of P. chrysogenum L_1.*

TABLE 1. *Effect of concentration of cell-free extract on homocitrate synthesis*

Cell-free extract[a] (ml)	Homocitrate (counts per min per ml) $\times 10^{-2}$
0	0
0.05	31.0
0.1	113.4
0.3	513.4
0.5	369.2
1	82.7

[a] Volume added per 1.1 ml total volume.

TABLE 2. *Dependence of homocitrate synthase activity on magnesium ions, adenosine 5'-triphosphate, and coenzyme A*

Omission from complete system[a]	Homocitrate (counts per min per ml) $\times 10^{-2}$
None	51.9
Mg^{2+}	28.0
ATP	18.8
CoA	22.3

[a] ATP, adenosine 5'-triphosphate; CoA, coenzyme A.

ingly, no inhibition was observed. One possible reason for the lack of inhibition could be a decrease in the enzyme affinity for lysine occurring during cell-free extract preparation; hence, higher concentrations of lysine were tested in a second experiment. Table 3 shows that concentrations as high as 100 mM failed to inhibit homocitrate synthase, but there was a curious stimulation of homocitrate synthase by concentrations of lysine 20 mM and above. Stimulation was not observed upon the addition of the same concentrations of sodium phosphate or ornithine (data not shown).

Since L-α-aminoadipate is presumed to be at the branch point for biosynthesis of lysine and penicillin, its effect was also tested. It showed no effect on homocitrate synthase activity at concentrations of 10 and 20 mM of the DL-mixture, either in the absence or presence of 20 mM L-lysine. Benzylpenicillin was also tested since it is considered to be the other end product of the branched pathway. As shown in Table 4, 20 mM benzylpenicillin inhibited the enzyme by 55%. Even more interesting was the finding that the inhibition was enhanced by lysine.

All work to this point had been carried out on extracts prepared by chitinase treatment of mycelia grown on a glucose-citrate medium. An attempt was made to confirm the effects of the compounds studied above with extracts from an actual penicillin fermentation. The fermentation was conducted in a defined production medium as described previously (4b). After 2 days, the mycelia were harvested, washed, and disrupted in a Hughes press. The press was used to bypass the possibility that the chitinase treatment desensitized homocitrate synthase to lysine inhibition. The results shown in Table 5 confirm the mild lysine stimulation, the lack of effect by aminoadipate, and the inhibitory action of benzylpenicillin on homocitrate synthase. An amino acid mixture was also included in this experiment to explore the possibility that the simultaneous presence of lysine and some other amino acid might be required for homocitrate synthase inhibition; 0.2% salt-free and vitamin-free casein hydrolysate inhibited the enzyme by about 50%. Further work is necessary to determine the particular amino acids responsible for the inhibitory effect. We attempted to carry out the identical experiment with mycelia from a later stage of the fermentation (i.e., at mid-idiophase). However, as previously observed in in vivo studies (4b), the homocitrate synthase activity dropped markedly after trophophase. Thus, activity was so low that the extracts, whether they were prepared in the Hughes Press or with chitinase, could not be used.

TABLE 3. *Effect of high lysine concentrations on in vitro homocitrate synthesis*

Concentration of L-lysine (mM)	Homocitrate (counts per min per ml) $\times 10^{-2}$
0	28.7
20	35.8
40	48.0
60	50.9
80	34.9
100	41.8

TABLE 4. *Effect of benzylpenicillin on activity of homocitrate synthase*

Additions	Homocitrate (counts per min per ml) $\times 10^{-2}$	Activity (% of control)
None (control)	28.7	100
10 mM Benzylpenicillin	25.4	88
20 mM Benzylpenicillin	13.0	45
20 mM L-Lysine·HCl (control)	35.8	100
20 mM L-Lysine·HCl + 10 mM benzylpenicillin	18.6	52
20 mM L-Lysine·HCl + 20 mM benzylpenicillin	10.9	30

TABLE 5. *Effect of lysine, α-aminoadipic acid, penicillin, and an amino acid mixture on homocitrate synthase prepared from the trophophase of a penicillin fermentation*

Additions	Homocitrate (counts per min per ml) $\times 10^{-2}$	Activity (% of control)
None	15.4	100
10 mM L-lysine·HCl	18.3	119
20 mM L-lysine·HCl	19.3	125
10 mM DL-α-aminoadipic acid	17.0	110
20 mM DL-α-aminoadipic acid	14.7	95
10 mM Benzylpenicillin	12.3	80
20 mM Benzylpenicillin	8.6	56
20 mM[a] Acid-hydrolysed casein	8.2	53

[a] The concentration of acid-hydrolyzed casein was calculated assuming an average amino acid molecular weight of 100; i.e., it was added at 0.2% (wt/vol).

DISCUSSION

The lability of homocitrate synthase from *P. chrysogenum* and its sensitivity to freezing was

unexpected in view of the stability of this enzyme from *Saccharomyces cerevisiae* (15) and *Neurospora crassa* (10). The yeast and *Neurospora* enzymes do not require high ionic strength for stability.

The inhibition of homocitrate synthesizing activity by increasing the amounts of the crude enzyme extract above 0.3 ml (1.4 mg of protein) might be due to carryover of some inhibitor. A similar observation was reported for 6-methylsalicyclic acid synthetase in *Penicillium patulum* (18).

An optimal pH value has not been previously reported for any homocitrate synthase. Therefore, the results obtained with the *P. chrysogenum* enzyme cannot be compared with literature values. However, the K_m for α-ketoglutarate was virtually identical to the figure of 5×10^{-3} M observed with homocitrate synthase from *Candida lipolytica* (P. Maldonado, L. Poirer, and H. Heslot, Abstr. Fourth Internat. Ferment. Symp., p. 243, 1972).

The dependence of the *P. chrysogenum* enzyme on Mg^{2+}, adenosine 5'-triphosphate, and coenzyme A appears to be partial. With the *Neurospora* enzyme, there is total adenosine 5'-triphosphate dependence, but the Mg^{2+} and coenzyme A requirements are only partial (10). The partial dependencies observed in the present studies with the *Penicillium* enzyme could be due to incomplete removal of the cofactors during dialysis because of binding to some macromolecule.

The total lack of lysine inhibition of homocitrate synthase in cell-free extracts of *P. chrysogenum* is contrary to the observations on this enzyme in *Saccharomyces* (15, 19) and *Neurospora* (10). A number of possible explanations can be considered which could explain our findings. These are as follows: (i) Lysine is not the true effector but it regulates the level of the effector or is converted into such a compound. (ii) Lysine exerts concerted feedback inhibition in combination with another ligand. (iii) Lysine is the true inhibitor but the optimal conditions for in vitro homocitrate synthase activity are so different from that occurring in the cell that lysine has no effect in the in vitro assay. (iv) The enzyme is desensitized during liberation from the mycelia.

We have no data to evaluate the first possibility, but feedback inhibition in other systems is sometimes effected via amino acid derivatives rather than the amino acids themselves (5, 11). The second possibility is quite common in pathways of primary metabolism but has never been studied in a pathway involving a secondary metabolite. The inhibitory effect of an amino acid mixture observed in the present study supports such a possibility; however, whether lysine is involved must still be determined. Another finding which merits further study is the ability of benzylpenicillin to inhibit homocitrate synthase and the apparent enchancement of this effect by lysine. At present, we are somewhat suspicious of the physiological significance of the penicillin effect due to the high concentrations required for inhibition (i.e., 10 to 20 mM or 3.5 to 7.0 g/liter). Although industrial strains of *P. chrysogenum* produce even higher extracellular concentrations of penicillin, the intracellular concentration is thought to be much lower than that of the extracellular fluid (4). However, if compartmentation exists in *P. chrysogenum*, it is possible that high penicillin concentrations exist in such a compartment. High levels of penicillin are also required to inhibit net penicillin synthesis in intact mycelia of *P. chrysogenum* (7), but this could be due to a permeation problem. The third possibility is known in other systems. For example, pH is an important factor for feedback inhibition of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase of *Streptomyces aureofaciens* (8) and inosine-5'-monophosphate dehydrogenase of *Bacillus subtilis* (20). In the case of the *Streptomyces* enzyme, the optimal pH for feedback inhibition is different from the optimum for enzyme activity. The effect of pH on the regulation of homocitrate synthase remains to be examined. The inorganic salt concentration is another factor which is often critical for feedback inhibition. For example, homoserine dehydrogenase of *Rhodospirillum rubrum* is insensitive to feedback inhibition at high (200 mM KCl) salt concentrations (2). Unfortunately, homocitrate synthase of *P. chrysogenum* is unstable at low salt concentrations and had to be studied at high salt concentrations. Another factor to be considered in feedback inhibition is the substrate concentration (20). However, even when we lowered the concentration of α-ketoglutarate, we observed no inhibition by lysine. The fourth possibility, that of enzyme desensitization during preparation of extracts, has been observed in other systems (6, 9, 12, 14). Glatzer et al. (6) reported that mitochondrial acetohydroxyacid synthetase of *N. crassa* was strongly inhibited by valine in an in vivo system. However, in extracts, the same enzyme was unstable and was completely insensitive to feedback inhibition by valine. A similar situation involving 6-methylsalicylic acid synthetase was reported by Light (12) in *P. patulum*. This enzyme was sensitive to inhibition by 6-methylsalicylic acid

in vivo but was insensitive to inhibition and unstable in vitro. In *P. chrysogenum*, Goulden and Chattaway (9) reported that preparation of permeabilized suspensions in serum albumin-sucrose-phosphate results in complete desensitization of acetohydroxyacid synthetase to feedback inhibition by valine. The same cells, permeabilized by suspension in 20% glycerol with 10% benzene, possessed enzyme inhibitable by valine.

In our studies, instead of inhibiting the enzyme, high concentrations of lysine activated homocitrate synthase. Similar activation was not observed with the same concentration of sodium phosphate or of ornithine. Activation by a known feedback inhibitor, isoleucine, has been observed with threonine dehydratase of *Brevibacterium flavum* (17). This enzyme has an activation site to which valine normally binds; apparently, isoleucine can also bind to this site. In the desensitized 6-methylsalicylic acid synthetase system mentioned above, Light (12) observed that 6-methylsalicylic acid stimulated, rather than inhibited, enzyme activity.

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