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INDUCTION OF PENICILLIN BIOSYNTHESIS BY L-GLUTAMATE IN *PENICILLIUM CHRYSOGENUM*

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The effect of L-glutamate on penicillin formation by *Penicillium chrysogenum* was investigated. This amino acid (10 mM) strongly stimulated the specific antibiotic formation in regard to systems containing similar concentrations of either NH_4Cl or L-glutamine as nitrogen source. No stimulation was achieved when in addition to glutamate the system contained cycloheximide. Nonmetabolizable analogues of the amino acid also stimulated antibiotic formation. At the enzymatic level, L-glutamate caused a 3.5 fold increase in δ(L-α-aminoadipyl) L-cysteine synthetase concentration, therefore we concluded that the amino acid stimulated penicillin formation through induction of this enzyme.

INTRODUCTION

It is becoming increasingly evident that secondary metabolism is induced mainly by primary metabolites. The classical work of Bu'Lock and Barr (1) and the studies of Vining (2) clearly demonstrated the participation of tryptophan in the induction of alkaloid formation in *Claviceps* sp. Later on, Krupinski *et al.* (3) gave the biochemical bases of this effect by showing that the amino acid induced dimethylallyl tryptophan synthetase, first enzyme of the ergot alkaloid biosynthetic pathway. Similarly, Drew and Demain (4) established the regulatory role of methionine on the formation of cephalosporin C, a β-lactam antibiotic produced by *Cephalosporium acremonium*. However, the biochemical bases of this action have not yet been elucidated.

In cells of *P. chrysogenum* grown under different experimental conditions, Hunter and Segel (5) have reported that the intracel—

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lular concentration of L-glutamate was higher than that of any other amino acid. We have confirmed these results and noted that the pool of glutamate increased at the end of the exponential growth phase. In addition, we observed that the glutamate increment preceded penicillin biosynthesis (6) and consequently, wondered about its physiological significance, in regard to antibiotic formation. In the present study, we have obtained experimental evidence for the involvement of glutamate in the induction of penicillin biosynthesis and give biochemical basis for its action.

METHODS

Organism, cultivation and antibiotic production. *P. chrysogenum* NRRL 1951 was kindly supplied by the Agricultural Research Service Culture Collection, Northern Regional Research Laboratory, Peoria, IL. 61604 U. S. A.

All cultures were grown for 36 h in a defined medium (DM), containing the standard salt mixture reported by Jarvis and Johnson (7), supplemented with 50 mM lactose, 39 mM acetate, 46 mM lactate, 3.6 mM phenylacetate and 8.5 mM NH_4Cl. The final pH of the medium was set up to 6.8 with 10 M NaOH.

For antibiotic production, the 36 h culture (100 ml) was harvested, washed with 2 vol distilled water and resuspended in 50 ml of antibiotic formation medium (AFM), contained in a 125 ml Erlenmeyer flask. The AFM medium was prepared with the same standard salt mixture (7), supplemented with 83 mM lactose, 3.6 mM phenylacetate and the desired nitrogen source. Under these conditions, the mycelium was incubated at 29°C for a further 36 hours on a rotary shaker at 160 rev min^{-1}. This procedure allowed determination of penicillin formation under well controlled conditions as well as some of the biochemical parameters involved in its biosynthesis.

Assay of penicillin. At desired times, penicillin was determined according to Aharonowitz and Demain (8), using benzylpenicillin as standard.

δ(L-α-aminoadipyl) L-cysteine synthetase activity. After growth for 36 h in DM medium and a further incubation for 36 h in AFM medium (500 ml contained in a 2800 ml Fernbach flask) supplemented or not with 10 mM L-glutamate, cell-free extracts of *P. chrysogenum* were prepared as described by Espin *et al.* (9) for *Neurospora crassa*, except that in all steps 0.1 M tris-HCl pH 7.0 was used. After dialysis against the same buffer (3 h at 4°C), the extract was used as the enzyme source. Activity was determined in 1 ml of a reaction system contained in 5 ml Thunberg tubes. The reaction mixture was prepared with 10 mM α-aminoadipate, 20 mM $MgCl_2.6H_2O$, 5 mM Na_2ATP and 50 mM 2-mercaptoethanol contained in 1 ml of tris-HCl (100 mM) pH 7.0. The reaction mixture and the enzyme (0.5-1.0 mg protein) were placed in the upper container of the tube. L-Cysteine was placed in the bottom of it. After elimination of the air (to avoid cysteine oxidation) the reaction was started by mixing the system with the cys-

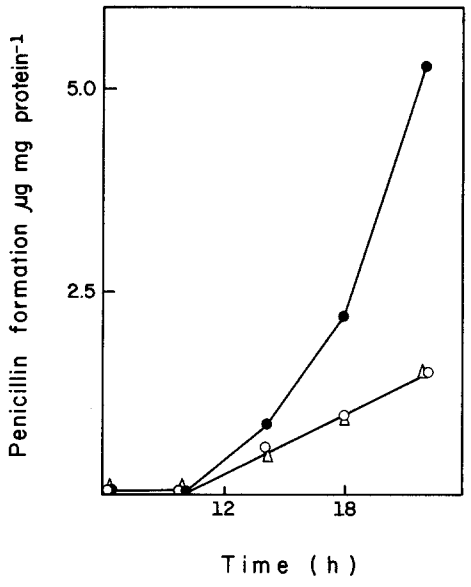

Figure 1. Effect of different nitrogen sources on penicillin formation. The total antibiotic formed was measured in cells (previously grown for 36 h in DM medium) incubated at 29°C in AFM medium supplemented with 8.5 mM NH_4Cl (○); 10 mM L-glutamine (△); and 10 mM L-glutamate, (●).

teine crystals (10 mM final concentration). Activity was carried out at 37°C for 1 to 40 min and terminated with 1 ml 0.3 M trichloroacetic acid. The inorganic phosphate formed was determined from the absorbance at 595 nm by the method of Taussky and Shorr (10). Appropriate controls in which enzyme and substrates were separately omitted were carried out. Specific activity was expressed in μkat Kg protein^{-1}.

Protein determination. Protein was measured by the Lowry method.

Determination of amino acid pools. L-Glutamate, L-valine and L-α-aminoadipate were extracted, separated in an Aminco amino acid analyzer (Silver Springs, MD. 20852 U. S. A.) and quantified in an Aminco Radio Fluorometer after coupling with *O*-phthaldialdehyde as reported by Espin *et al.* (9).

RESULTS

P. chrysogenum NRRL 1951 produced penicillin when incubated in AFM medium supplemented with different nitrogen sources. As can be seen in the Fig. 1, when the system was supplemented with 10 mM L-glutamate, an important stimulation of penicillin production was observed relative to medium supplied with similar concentrations of either NH_4Cl or L-glutamine (4 times more antibi-

Table 1. *Effect of L-glutamate on the intracellular concentration of several amino acids*[a]

	Amino acid concentration μmol mg protein^{-1}	
	Control[b]	L-Glutamate[c]
L-Alanine	0.2	2.2
L-α-Aminoadipate	0.03	0.04
L-Glutamate	0.8	3.8
L-Glutamine	0.01	0.1
L-Valine	0.05	0.1

[a] Determined after 36 h incubation in AFM medium at 29°C.
[b] Contained only AFM medium.
[c] After being filter sterilized it was added to the AFM medium at a final concentration of 10 mM.

otic at 24 h incubation). Under these conditions, cells fed with glutamate brought about an important increase in the alanine, glutamine and glutamate pools, without significant changes on other amino acids including the amino precursors of the penicillin moiety (Table 1).

To distinguish whether or not glutamate itself was responsible for this action, the effect of nonmetabolizable glutamate analogues was tested on penicillin formation. As shown in Table

Table 2. *Effect of L-glutamate analogues on penicillin biosynthesis.*

Conditions[a]	Penicillin formation[b] μg mg protein^{-1}
Control	1.75
L-Glutamate	5.12
L-Glutamic acid γ-mono-hydroxamate	5.80
γ-Benzyl-L-glutamate	11.20
L-Glutamate plus cycloheximide	1.0

[a] L-Glutamate and its analogues were filter sterilized and added at a final concentration of 5 mM. The control contained 8.5 mM NH_4Cl. Cycloheximide was used at a final concentration of 0.35 mM.
[b] Determined after 36 h incubation in AFM medium at 29°C.

Table 3. *Effect of L-glutamate on δ(L-α-aminoadipyl) L-cysteine synthetase formation*[a]

Condition	Specific activity μkat Kg protein^{-1}
Control	114
L-Glutamate[b]	403

[a] Determined after 36 h incubation in AFM medium at 29°C.
[b] After being filter sterilized it was added to the AFM medium at a final concentration of 10 mM.

2, glutamate and its analogues stimulated antibiotic formation between 3 and 6 fold, relative to a control with NH_4Cl. In addition, when protein synthesis was inhibited, no stimulation by L-glutamate was accomplished on penicillin biosynthesis.

To investigate this problem further, we looked for an effect of this amino acid on the synthesis of δ(L-α-aminoadipyl) L-cysteine synthetase (first enzyme of the penicillin formation pathway). As can be seen in Table 3, when the AFM medium was supplemented with 10 mM glutamate, a 3.5 fold increase in enzyme concentration was achieved relative to mycelia incubated in the absence of the amino acid.

DISCUSSION

The results obtained in the present work have shown a direct relationship between L-glutamate and the ability of *P. chrysogenum* to produce penicillin. Since L-glutamate did not significatively increase the pools of the penicillin amino precursors and because this amino acid by itself is not incorporated into the antibiotic moiety, only a regulatory relationship might be expected to exist between glutamate and penicillin formation. This seems to be the case considering that stimulation was prevented in the presence of cycloheximide and that glutamate analogues also stimulated antibiotic formation.

Stimulatory effects by L-glutamate have been also reported in *Aspergillus ochraceus* on the synthesis of ochratoxin A (11). According to this work, the amino acid exerted an inducer-like effect on mycotoxin biosynthesis. Gräfe *et al.* (12) also reported a striking connection between glutamate dehydrogenase activity (NADPH-dependent, EC 1.4.1.4) and the ability of *Streptomyces noursei* to synthesize nourseothricin. Therefore, it seems evident that in different microorganisms, glutamate can be a link between primary and secondary metabolism by triggering the synthesis of antibiotic. This role might be important from the industrial point of view since it is predictable that a *P. chrysogenum* strain with high capability to produce glutamate, might also be a better antibiotic producer. This seems to be the case for the synthesis of a penicillin related antibiotic: Queener *et al.* (13) have demonstrated a correlation between glutamate dehydrogenase activity and the ability of one selection line of *C. acremonium* to produce cephalosporin C.

Experimental evidence was recently presented which suggested that the first enzyme implicated in the formation of glutathione is also involved in the first step of the penicillin biosynthetic pathway (14). This enzyme (γ-glutamyl-cysteine synthetase EC 6.3.2.2.), catalyzes the formation of γ-glutamyl cysteine from L-cysteine and L-glutamate and is inhibited by glutathione (15). In agreement with these results, we have recently found that in *P. chrysogenum* glutathione caused a strong inhibition of δ(L-α-aminoadipyl) L-cysteine synthetase activity. Furthermore, we also observed that glutamate induced both γ-glutamyl-cysteine synthetase and δ(L-α-aminoadipyl) L-cysteine synthetase formation (Vazquez, G., Mateos, R.C. and Sanchez, S. unpublished). These observations further support the idea of one enzyme catalyzing both reactions and, on the other hand, give a clear meaning to the results obtained in the present study.

It seems reasonable to conclude with the above statements, that the increase observed in the pool of glutamate at the end of the logarithmic growth phase of *P. chrysogenum*, represented the biochemical signal that caused induction of δ(L-α-aminoadipyl) L-cysteine synthetase formation and thereby, elicited the biosynthesis of penicillin.

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