Transport of neutral amino acids and penicillin formation in *Penicillium chrysogenum*

ROSA DEL CARMEN MATEOS and SERGIO SANCHEZ*

Departamento de Biotecnología del Instituto de Investigaciones Biomedicas, Universidad Nacional Autonoma de Mexico, Apartado Postal 70228, Mexico DF 04510, Mexico

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The production of penicillin was inhibited by neutral amino acids in a resting cell system of *Penicillium chrysogenum* with cycloheximide. The inhibitory action was prevented by preincubation with glutathione, even though this stimulated uptake of the neutral amino acid glutamine. Chromatographic analysis of extracts obtained from cells incubated with labelled glutamine revealed that radioactivity was taken up through the formation of two intermediates; L- γ -glutamyl-L-glutamine and δ -(L- α -aminoadipyl)-L-glutamine. In a resting cell system prepared from cultures previously grown in the presence of 50 mM-L-lysine, a condition which restrains α -aminoadipate and penicillin formation, uptake of glutamine was 80% inhibited. We propose that the penicillin precursor δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine, which is structurally related to glutathione, is also utilized in the uptake of neutral amino acids.

Introduction

Glutamine is not only a precursor in the synthesis of primary and secondary metabolites, but also a key regulator in the synthesis and degradation of several enzymes and other compounds (Marzluf & Fu, 1988; Mora *et al.*, 1988). The regulation of penicillin formation by nitrogen compounds has recently received much attention (Sanchez *et al.*, 1988), and glutamine has been reported to inhibit the formation of penicillin in a resting-cell system of *Penicillium chrysogenum* with cycloheximide. The addition of 1 mM-glutathione (GSH) prevented this inhibition but did not cause a reduction of glutamine uptake (Mateos *et al.*, 1984). The extent of this inhibition was related neither to the availability of the amino acid precursors of the antibiotic moiety, nor to the changes in activity of δ -(L- α -aminoadipyl)-L-cysteine synthetase, thought to be the first enzyme of the penicillin formation pathway, although recent studies suggest that δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine synthetase is the first enzyme in penicillin formation in other micro-organisms (Banko *et al.*, 1987; Van Liempt *et al.*, 1989). The present report examines further physiological effects of glutamine on penicillin formation, and the basis of the GSH effect.

Abbreviations: GSH, glutathione; ACV, δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine.

Methods

Organism and cultivation. *P. chrysogenum* NRRL 1951 was kindly supplied by the Agricultural Research Service Culture Collection, Northern Regional Research Laboratory, Peoria, Ill., USA. All fermentations were done in a defined medium (DM) as previously reported (Lara *et al.*, 1982).

Resting cell system studies. A 36 h culture (25 ml) was harvested, washed with 2 vols sterile distilled water, and resuspended in 25 ml of antibiotic formation medium (AFM) (Mateos *et al.*, 1984). Under these conditions, cells produced penicillin linearly during 36 h in the presence of cycloheximide (100 μ g ml⁻¹), without increasing in dry weight.

Penicillin assay. Penicillin was determined at specified times according to Lara *et al.* (1982), using benzylpenicillin as a standard.

Determination of glutamine transport. One millilitre of L-[U-¹⁴C]glutamine (1.68 μ Ci mmol⁻¹ ml⁻¹; 62.2 kBq mmol⁻¹ ml⁻¹) was added to 24 ml of a resting cell system with cycloheximide (100 μ g ml⁻¹) and samples were analysed at specified times.

Separation of peptides and amino acids. Intracellular peptides and amino acids were extracted by homogenizing mycelia (from 25 ml of a resting cell system) with 10 ml 80% (v/v) ethanol as reported by Espin *et al.* (1979). Soluble peptides and amino acids (10 μ l samples) were separated by two-dimensional thin-layer chromatography (A, 1-propanol/NH₄OH 85:37, v/v; and B, phenol/water 80:20, v/v) according to Brenner *et al.* (1969). After separation, the chromatograms were visualized with ninhydrin reagent. When labelled glutamine was used, the spots were cut out from the chromatographic plates, and their radioactivity was estimated by scintillation counting.

Reproducibility of results. The experiments reported were repeated at least once (two independent experiments) in duplicate and the results reported are the mean values. The observed variations were consistently less than 10%.

Results

Effect of different amino acids on antibiotic formation

In a resting cell system with cycloheximide, cells of *P. chrysogenum* produced penicillin for more than 24 h. Under these conditions, antibiotic formation was inhibited by the addition of 10 mM-glutamine. Other neutral amino acids suppressed penicillin production to a similar extent (Table 1). On the other hand, none of the acidic or basic amino acids tested inhibited idiolite formation. The inhibitory action of neutral amino acids was prevented by preincubation of the system for 1 h with 1 mM-GSH.

Effect of GSH on glutamine uptake

Considering that neutral amino acids other than glutamine also inhibited penicillin formation and that this effect was prevented by GSH, a more general mechanism was investigated as a possible cause of inhibition. For this purpose, the uptake of [14 C]glutamine by cells previously incubated with GSH at different concentrations was determined (Fig. 1). Glutamine was linearly incorporated for more than 2 h by a resting cell system of the fungus. Preincubation with GSH stimulated glutamine uptake according to the GSH concentration used.

To explore the stimulation of glutamine uptake by GSH further, chromatographic analyses were conducted on extracts from resting cells previously incubated with [14 C]glutamine. After incubation for 1 h with labelled glutamine (Table 2), radioactivity was taken up as L- γ -glutamyl-L-glutamine and δ -L-(α -amino adipyl)-L-glutamine. As shown in the same table, a slow increase of radioactivity was detected in the glutamine position. Chromatographic analysis of cell extracts incubated with other neutral amino acids was limited by the availability of appropriate γ -glutamyl standards.

Relationship of glutamine uptake to the formation of penicillin intermediates

The structural similarity of GSH (L- γ -glutamyl-L-cysteinyl-glycine) to the tripeptide intermediate of the penicillin biosynthetic pathway δ -(L- α -amino adipyl)-L-cysteinyl-D-valine (ACV), suggested a possible role of the latter in the uptake of neutral amino acids. To explore this possibility, *P. chrysogenum* was grown in the

Fig. 1. Transport of 5 mM-L-[U 14 C]glutamine (0.6 μ Ci mmol $^{-1}$; 22.2 kBq mmol $^{-1}$) by resting cell systems previously incubated for 1 h with 1 mM (●), 5 mM (○) or 10 mM (□) GSH. Δ , Control without GSH.

Table 1. Effect of several amino acids on the formation of penicillin by resting cell systems of *P. chrysogenum* with cycloheximide

Antibiotic formation was estimated after 18 h incubation.

Amino acid (10 mM)	Penicillin produced [μ g (mg protein) $^{-1}$]	
	By resting cells	By resting cells preincubated for 1 h with 1 mM-GSH
Control	0.86	0.83
L-Alanine	0.00	0.60
L-Arginine	0.70	0.65
L-Asparagine	0.00	0.60
L-Aspartate	1.50	1.30
L-Cysteine	0.00	0.65
Glycine	0.00	0.57
L-Glutamate	0.82	0.80
L-Glutamine	0.00	0.70
L-Serine	0.00	0.61
L-Tryptophan	0.00	0.61
L-Tyrosine	0.00	0.61
L-Valine	0.00	0.65

presence of 50 mM-L-lysine, which inhibits the synthesis of α -amino adipate (Masurekar & Demain, 1972) and so the formation of ACV. As shown in Fig. 2, the uptake of glutamine was also inhibited in resting cells of that culture. As expected, chromatographic analyses revealed the absence of α -amino adipate in mycelia grown in the presence of L-lysine (not shown).

Fig. 2. Transport of 10 mM-L-[U¹⁴C]glutamine (1.68 μ Ci mmol⁻¹; 62.2 kBq mmol⁻¹) by resting cell systems prepared from cultures previously grown with 8.5 mM-NH₄Cl (●) or 50 mM-L-lysine (○).

Table 2. Intracellular fate of the radioactivity from [¹⁴C]glutamine as a function of the incubation time

A and B are the solvent mixtures used in the two-dimensional thin-layer chromatography; see Methods.

Spot	Compound	R _F values		Radioactivity incorporated [c.p.m. (mg protein) ⁻¹]		
		A	B	1 h	3 h	6 h
A	L-Cysteine	0.28	0.13	116	129	183
B	L-Glutamine	0.49	0.28	258	343	637
C	L-Valine	0.6	0.31	177	173	263
D	Glycine	0.39	0.18	65	188	103
E	L-γ-Glutamyl-L-glutamine	0.3	0.19	749	1450	1958
F	δ-(L-α-Aminoadipyl)-L-glutamine	0.55	0.40	621	907	1790

Discussion

Using a resting-cell system of *P. chrysogenum* we found that glutamine and other neutral amino acids strongly inhibit penicillin formation when used at concentrations higher than 5 mM. The inhibitory effect on antibiotic biosynthesis was prevented by preincubation with 1 mM-GSH through a mechanism apparently unrelated to amino acid exclusion, since GSH strongly stimulated the uptake of glutamine. It seems that GSH can mediate translocation of neutral amino acids across mammalian

Fig. 3. Proposed model for the incorporation of neutral amino acids using an intermediate of the penicillin biosynthetic pathway. The valine molecule may assume the L-form when free, and the D-form when attached to the peptides. AAA, Amino adipic acid.

cell membranes (Meister, 1983). This mechanism, which involves transfer of the GSH γ -glutamyl moiety to the amino acid by the action of γ -glutamyl transpeptidase, results in the formation of the γ -glutamyl amino acid derivative (Meister, 1983). This also seems to be true for the transport of neutral amino acids by resting cell systems of *P. chrysogenum*, since glutamine was taken up through the formation of two intermediates, one of which corresponded to L- γ -glutamyl-L-glutamine. Considering the structural similarity between GSH and ACV, the tripeptide intermediate of the penicillin biosynthetic pathway, the possibility that this intermediate could be used in amino acid transport processes was envisaged as a mechanism to explain the decrease in penicillin formation caused by neutral amino acids (Fig. 3). Experimental evidence supporting this hypothesis is that L-lysine (reported to inhibit penicillin formation by Masurekar & Demain, 1972) caused a significant reduction in the transport of glutamine (Fig. 2). Furthermore, chromatographic analysis of extracts obtained from cells incubated with labelled glutamine showed incorporation not only in the L- γ -glutamyl-L-glutamine position of the plate but also in another spot which corresponded to δ -(L- α -amino adipyl)-L-glutamine. It is postulated that the valine molecule may recover the L-form when it is free, and assume the D-form when attached either to L-cysteinyl-D-valine (Cys-Val) or to ACV (α -AAA-Cys-Val).

Meister & Anderson (1983) have reported that once the L- γ -glutamyl-glutamine is produced, the amino acid

is released by the action of γ -glutamyl-cyclotransferase with the concomitant formation of 5-oxoproline. This compound is a highly stable cyclized form of glutamate, and an intermediate of the γ -glutamyl cycle, which is then converted to glutamate by the action of 5-oxoprolinase (Meister & Anderson, 1983). In agreement with our results, a cyclized form of α -aminoadipate (6-oxopiperidine-2-carboxylate) has also been detected in the fermentation broths of *P. chrysogenum* grown in the presence of neutral amino acids (Brundidge *et al.*, 1980).

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