Propargylglycine as a Fungal Inhibitor: Effect on Sulphur Amino Acid Metabolism

By MAŁGORZATA PIOTROWSKA* AND ANDRZEJ PASZEWSKI

*Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 36 Rakowiecka,
02-532 Warszawa, Poland*

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The effect of propargylglycine (PPG) on four fungal species was studied. γ -Cystathionase was the major target of the antibiotic, as in mammalian but not in bacterial cells. Highly PPG-sensitive γ -cystathionase was found in *Aspergillus nidulans* and *Neurospora crassa*, less sensitive in *Saccharomyces cerevisiae* and insensitive in *Cephalosporium acremonium*. The effects of γ -cystathionase inhibition on growth depend on the role this enzyme plays in cysteine synthesis. Inhibition by PPG was reversed by cysteine, and also by other amino acids at high concentrations. The results of transport assays of wild-type strains and PPG-resistant mutants indicated that propargylglycine was taken up by amino acid permeases in *A. nidulans* and *S. cerevisiae*.

INTRODUCTION

L-Propargylglycine (2-amino-4-pentynoate, PPG), an antibiotic produced by *Streptomyces* sp. (Scannel *et al.*, 1971) causes growth inhibition of several bacteria, which can be reversed by addition of methionine to the growth medium. It is presumed that the administration of PPG leads to methionine requirement. Inactivation studies have indicated that cystathionine γ -synthase (see Fig. 1) is the major, if not sole, target in the bacterial methionine biosynthetic pathway (Marcotte & Walsh, 1975; Johnston *et al.*, 1979).

Effects of PPG in mammals have been studied both *in vivo* and *in vitro*. In rats it was found, with living organisms as well as with hepatocyte cells, that PPG interferes with cystathionine metabolism. If injected into rats it caused cystathioninuria and intracellular accumulation of cystathionine and several of its derivatives. The terminal enzyme of the cystathionine pathway, γ -cystathionase, is irreversibly inhibited by a so-called 'suicidal' inactivation (Abeles & Walsh, 1973; Beatty & Reed, 1980). The actual inactivator is generated enzymically at the active site – the enzyme catalyses the rearrangement of the acetylenic inhibitor to an allene, which reacts with the enzyme active site, forming a covalent linkage (Washtien & Abeles, 1977). Some other enzymes are also described as being affected by PPG, although to a lesser extent: e.g. alanine aminotransferase (Burnett *et al.*, 1980) and aspartate aminotransferase from pig heart (Tanase & Morino, 1976).

In fungi, apart from an early observation that synthetic PPG inhibits the growth of *Saccharomyces cerevisiae* (Gershon *et al.*, 1949), no information about its effects is available. It was of interest to establish how PPG operates in fungi, as it could eventually be a useful tool in studies of sulphur amino acid metabolism.

METHODS

Strains. The following strains of *Aspergillus nidulans* from our collection were used: *cysA1 nicA2 yA1*, lacking serine transacetylase; *cysB1 pyroA4 yA1* and *cysC1 chol7 yA1*, impaired in cysteine synthesis from *O*-acetylserine; *cysB1 cysC1 chol7*; *cysB1 cysD1 pyroA4* (*cysD* is impaired in homocysteine synthase: Paszewski & Grabski,

Abbreviation: PPG, propargylglycine.

Fig. 1. An outline of sulphur amino acid metabolism in fungi. Enzymes: (1) serine transacetylase, (2) cysteine synthase, (3) cystathionine γ -synthase, (4) β -cystathionase, (5) homocysteine synthase, (6) cystathionine β -synthase, (7) γ -cystathionase.

1975); and *methH pyroA4 yA1*, blocked in the last step of methionine biosynthesis. *pyroA4 yA1* was used as the reference wild-type (WT) strain. *Saccharomyces cerevisiae* A12-1c (*MAT α ade1*) was used as a standard wild type. *Neurospora crassa* was 74-OR23-1A. Both were obtained from Dr A. Kruszewska, Institute of Biochemistry and Biophysics, Warsaw, Poland. A wild-type *Cephalosporium acremonium* strain II-WT was obtained from the Biotechnology Research Development Centre, Warsaw, Poland.

Media, growth conditions and extract preparations. The minimal medium described by Paszewski & Grabski (1974) was used for *A. nidulans* strains, the minimal medium of Vogel (1964) for *N. crassa*, the minimal medium described by Nüesch *et al.* (1973) for *C. acremonium* and GO minimal medium as described by Świetlińska *et al.* (1978) for *S. cerevisiae*. These media were supplemented according to nutritional requirements. All strains were grown at 30 °C on a rotary shaker (150 r.p.m.) and harvested in the exponential phase of growth. Cell extracts were prepared as described by Paszewski *et al.* (1984), except for yeast, where cells were disrupted in a Braun homogenizer.

Enzyme assays. Cystathionine β -synthase (EC 4.2.1.22) was assayed according to Pieniżek *et al.* (1973). Homocysteine synthase (EC 4.2.99.10) was assayed using the reaction mixture described by Paszewski & Grabski (1973); homocysteine formed was estimated by the method of Kredich & Tomkins (1966). The activities of β -cystathionase (EC 4.4.1.8) and γ -cystathionase (EC 4.4.1.1) were determined as described by Paszewski & Grabski (1974). The procedure for determination of amino acid transport was essentially the same as that of Piotrowska *et al.* (1976). The incubation mixture contained 125–250 μ M-radioactive amino acid [0.2 μ Ci (7.4 kBq) per assay] and uptake was followed for 3 min for *A. nidulans* and 2 min for yeasts. Protein was estimated by the method of Bradford (1976).

Determination of pools of 35 S-labelled amino acids. *A. nidulans* strains were grown for 18 h under standard conditions, then 2 mM- $\text{Na}_2^{35}\text{SO}_4$ ($1\text{--}1.5 \times 10^6$ c.p.m. μmol^{-1}) was added to two parallel cultures, and simultaneously PPG to 10 μ M final concentration to one of them. After 6 h, cultures were harvested. *S. cerevisiae* was grown in the presence of 2 mM- $\text{Na}_2^{35}\text{SO}_4$ ($0.1\text{--}0.2 \times 10^6$ c.p.m. mol^{-1}); PPG (75 μ M) was added to one of the two identical cultures. Cultures were harvested in the late-exponential phase. The 35 S-labelled amino acid content of the cells was determined as described by Paszewski *et al.* (1984).

Reagents. O-Acetylserine and O-acetylhomoserine were synthesized according to Wiebers & Garner (1967). Other amino acids were from Sigma. PPG was the DL-product, from Sigma. Radioactive amino acids were the L-U- ^{14}C forms from UVVVR-Praha; [^{35}S]methionine was from Amersham. $\text{Na}_2^{35}\text{SO}_4$ was from the Institute of Nuclear Research, Świerk, Poland.

RESULTS

A. nidulans strains *cysB*, *cysC* and *cysA*, impaired in the main pathway of cysteine synthesis, were highly sensitive to PPG, as compared to the wild-type. Complete growth inhibition was observed with 1 μ M-PPG, whereas the growth of the wild-type was only partly inhibited by 1 mM-PPG (Table 1). When the mycelial mass increase of strain *cysB* in the presence of PPG was determined, no increase was observed during the first 6 h and only a 10% increase after 24 h.

A. nidulans mutants *cysB*, *cysC* and *cysA* are prototrophs, due to the functioning of the alternative pathway of cysteine synthesis involving homocysteine synthase, cystathionine β -synthase and γ -cystathionase (Paszewski & Grabski, 1975). Their higher sensitivity to PPG suggested that this pathway is blocked by the antibiotic. By analogy with mammalian systems, γ -cystathionase seemed the most likely target. In experiments *in vitro*, *A. nidulans* γ -cystathionase was inhibited to the following extent by different PPG concentrations: 1 μ M, 20%; 2 μ M, 38%;

Table 1. Effect of PPG on the growth of wild-type and *cysA*, *cysB* and *cysC* mutants of *A. nidulans*

The diameter of colonies grown for 48 h was measured: —, no growth; + to + + + +, increasing amounts of growth.

Strain	Growth in the presence of PPG at the concn (μM) indicated								
	1000	100	10	1	0.5	0.1	0.01	0.001	0
<i>cysA</i>	—	—	—	—	+/-	+	+++	+++	+++
<i>cysB</i>	—	—	—	—	—	+/-	+	++	+++
<i>cysC</i>	—	—	—	—	—	+/-	+	++	+++
WT	++	++++	++++	++++	++++	++++	++++	++++	++++

Table 2. Effect of PPG on γ -cystathionase activity and generation time in *S. cerevisiae*

In experiments A, γ -cystathionase activity was measured in cells grown to mid-exponential phase in GO supplemented as indicated. In experiments B, PPG and methionine were added for 1 h to cultures grown in GO to exponential phase. Generation time was established in separate experiments. Values are means from at least three determinations.

Expt type	Medium supplementation		γ -Cystathionase [nmol min ⁻¹ (mg protein) ⁻¹]	Generation time (h)
	PPG	Methionine		
A	—	—	5.7	1.75
A	50 μM	—	4.2	2.75
B	100 μM	—	0.0.3	6.0
B	500 μM	—	0.0.2	6.0
B	1 mM	—	0	∞
A	500 μM	10 mM	1.1	2.33
B	1 mM	10 mM	0.2	5.6

5 μM , 65%; 10 μM , 68%; 20 μM , 86%; 200 μM , 100% (means from three experiments). No inhibition of cystathionine β -synthase or homocysteine synthase was observed at PPG concentrations up to 200 μM (the latter enzyme was inhibited about 50% by 2 mM-PPG). Since these two enzymes are not subject to PPG inhibition, we conclude that blockage of γ -cystathionase is the main effect of this antibiotic responsible for the growth inhibition of the *cys* mutants of *A. nidulans*.

Addition of PPG to cultures of *A. nidulans* resulted in a decrease of γ -cystathionase activity. Total inhibition was observed in extracts from mycelia collected 45 min after administration of 1 μM -PPG.

S. cerevisiae was more sensitive to PPG than the *A. nidulans* wild-type strain but more resistant than the *cys* strains. At 100 μM -PPG the generation time increased from 1.75 h to about 6 h (Table 2). Yeast γ -cystathionase tested *in vitro* was inhibited by PPG: 100 μM caused about 40% inhibition. Since no *S. cerevisiae* mutants corresponding to the *A. nidulans cysB* and *cysC* strains are available, it is difficult to establish whether γ -cystathionase activity is indispensable for this organism. The data in Table 2 show a fairly good correlation between the level of γ -cystathionase and prolongation of the generation time. This suggests that γ -cystathionase inhibition may be the cause of PPG toxicity, but we can not exclude the possibility that some target other than γ -cystathionase is responsible for the observed effect.

In *N. crassa* growth inhibition by PPG started at 0.5 mM-PPG, while the growth of *C. acremonium* was not affected even by 2 mM-PPG. *N. crassa* γ -cystathionase was inhibited 50% *in vitro* by 5 μM -PPG. No activity of the enzyme was found in mycelium grown in the presence of 1 μM -PPG. *N. crassa* therefore appeared to resemble *A. nidulans* with respect to PPG toxicity. γ -Cystathionase from *C. acremonium* was quite resistant to PPG: less than 50% inhibition *in vitro* was observed with 1 mM-PPG.

Table 3. Accumulation of ^{35}S -labelled amino acids in *A. nidulans* and *S. cerevisiae* strains in the presence of PPG

Values are given as nmol (g dry wt) $^{-1}$ and are means of four independent experiments $\pm$ SEM.

Organism	Strain	PPG	Cysteine	Homocysteine	Glutathione	Cystathionine
<i>A. nidulans</i>	WT	–	99 $\pm$ 26	148 $\pm$ 42	1173 $\pm$ 435	350 $\pm$ 112
		10 μM	127 $\pm$ 49	171 $\pm$ 127	971 $\pm$ 350	386 $\pm$ 142
	<i>cysB</i>	–	239 $\pm$ 76	166 $\pm$ 75	509 $\pm$ 52	819 $\pm$ 191
		10 μM	41 $\pm$ 17	78 $\pm$ 24	51 $\pm$ 28	250 $\pm$ 59
<i>S. cerevisiae</i>		–	68 $\pm$ 12	71 $\pm$ 29	1666 $\pm$ 400	139 $\pm$ 67
		75 μM	105 $\pm$ 11	218 $\pm$ 73	2055 $\pm$ 538	458 $\pm$ 267

Table 4. Reversal of PPG-induced growth inhibition of *A. nidulans* by amino acids

Medium supplementation		Growth* of strain:			
PPG	Amino acid	WT	<i>methH</i>	<i>cysB cysD</i>	<i>cysB</i>
–	–	++++	+/–	–	++++
–	Methionine 100 μM	++++	+++	++	++++
1 μM	–	++++	+/–	–	–
1 μM	Methionine 100 μM	++++	+++	–	–
1 μM	Methionine 1 mM	++++	+++	+	+
1 μM	Methionine 10 mM	++++	+++	+++	++++
–	Cysteine 100 μM	++++	+/–	++	++++
1 μM	Cysteine 100 μM	++++	+/–	++	++++
1 μM	Cysteine 500 μM	++++	+/–	+++	++++
1 μM	Serine 10 mM	++++	+/–	–	–
1 μM	Serine 100 mM	++++	+/–	–	++

* Measured as described for Table 1.

Effect of propargylglycine upon sulphur amino acid pools

In the *A. nidulans* wild-type strain the presence of 10 μM -PPG in the growth medium had no effect on the pools of sulphur amino acids (Table 3). This observation strongly supports the conclusion that γ -cystathionase does not play a significant physiological role in this strain. On the other hand, in the *cysB* mutant, in which cysteine synthesis depends on γ -cystathionase activity, a considerable decrease of the sulphur amino acid pools was observed. It was most pronounced for glutathione. Cysteine was also diminished, whereas the cystathionine pool, although low, was relatively high in comparison to other sulphur amino acids. It should be noted that this small accumulation of these amino acids took place during growth inhibition.

With *S. cerevisiae* the presence of 75 μM -PPG in liquid culture resulted in some increase of the cystathionine and homocysteine pools (Table 3). However, cysteine and glutathione were not diminished, so it is not clear why PPG at this concentration slowed the growth rate. One possibility is that the growth rate is adjusted to keep the cysteine and glutathione pools relatively constant.

Reversal of PPG-induced growth inhibition by amino acids

Reversal of the PPG-induced growth inhibition of *A. nidulans* by several amino acids was observed (Table 4). Cysteine auxotrophy should arise from the combination of a *cysB* block with γ -cystathionase inhibition by 1 μM -PPG. Indeed, cysteine at a concentration (100 μM) sufficient to supply the auxotrophic requirement of the *cysB cysD* strain restored the growth of *cysB*. Methionine reversed the inhibition, but, as may be seen from tests with auxotrophic mutants *methH* and *cysB cysD*, only at a much higher concentration than that needed for supplementing

Fig. 2

Fig. 2. Transport of (a) L-[¹⁴C]leucine and (b) L-[¹⁴C]arginine in the presence and absence of PPG in strain *cysB cysC* of *A. nidulans* and a PPG-resistant mutant of this strain. ○, *cysB cysC*; ●, *cysB cysC*, 1 mM-PPG present; △, PPG-resistant mutant. The results are from one representative experiment.

Fig. 3

Fig. 3. Transport of (a) L-[³⁵S]methionine and (b) L-[¹⁴C]leucine in the presence and absence of PPG in the wild-type strain of *S. cerevisiae* and a PPG-resistant mutant of this strain. ○, Wild-type; ●, wild type, 1 mM-PPG present; △, PPG-resistant mutant. The results are from one representative experiment.

auxotrophy. Besides methionine, other amino acids restored growth when supplied at high concentrations. The same phenomenon of protection by amino acids at high concentrations was observed with regard to the effect of 1 mM-PPG on the wild-type (now shown).

When 10 mM-methionine was added to liquid cultures of wild-type *A. nidulans* together with 1 μ M-PPG, almost no inhibition of γ -cystathionase was observed.

In *S. cerevisiae*, methionine added to the medium together with PPG restored both growth and γ -cystathionase activity. The effect depended on the ratio of methionine to inhibitor (Table 2). About 90% of normal γ -cystathionase activity was found in cells grown in the presence of 100 μ M-PPG plus either 10 mM-cysteine or 50 mM-leucine, whereas 50 mM-proline had no effect.

We have already suggested that, in the *cysB* mutant of *A. nidulans*, PPG-induced inhibition of growth is the result of auxotrophy since it can be reversed by cysteine at low concentration. In *S. cerevisiae*, a higher concentration of cysteine is required to reverse inhibition, and in both organisms a high concentration of metabolically unrelated amino acids may reverse inhibition. These observations imply the existence of a second mechanism for the relief of inhibition, possibly at the level of transport.

Interference of PPG with amino acid transport: mutants resistant to PPG

In *A. nidulans* grown on minimal medium, neutral amino acids, including sulphur-containing ones, are taken up by a so-called neutral permease; a separate permease transports basic amino acids like arginine and lysine (Piotrowska *et al.*, 1976). In the experiment shown in Fig. 2, the direct effect of PPG on the uptake of a neutral amino acid (leucine) and a basic one (arginine) was studied. PPG at five times the concentration of leucine inhibited its uptake. In contrast,

PPG did not restrict the uptake of arginine. Thus PPG interferes with the uptake of neutral amino acids.

From a *cysB cysC* double mutant of *A. nidulans* sensitive to 1 μ M-PPG, several PPG-resistant mutants were isolated following UV mutagenesis. When two of them were tested for amino acid transport, they were found to be impaired in the uptake of two neutral amino acids: leucine and phenylalanine. No difference from the wild-type was found for the rate of transport of arginine (Fig. 2). These mutants are likely to be impaired in the neutral amino acid permease, and their simultaneous resistance to the inhibition of PPG supports the conclusion that PPG enters the cell by the neutral amino acid transport system.

In *S. cerevisiae* most of the amino acids (basic and neutral, but not proline) are transported by a high-velocity system, the so-called general amino acid permease (Gap) (Grenson *et al.*, 1970). The observed pattern of protection could suggest transport of PPG via Gap. In some yeast strains Gap is inactive in the presence of ammonia. Gap is responsible for transport of D-amino acids, highly toxic to yeast, which offers a simple test for Gap sensitivity to ammonia (Rytka, 1975). Inhibition of our strain by D-serine and D-histidine was examined on GO medium containing ammonia and on a medium with proline as sole nitrogen source. The strain was sensitive to D-amino acids on the proline medium only, and this thus of the ammonia-sensitive type. The next test was performed with citrulline, which according to Grenson *et al.* (1970) enters the cells only via Gap. When citrulline at high concentrations (10 mM and above) was included together with 100 μ M-PPG in the medium, no protection against PPG toxicity was observed whether proline or ammonia was used as nitrogen source. Hence Gap should probably be excluded as a transport system for PPG.

Besides Gap, several (at least 10) amino acid transport systems have been described in *S. cerevisiae*, each specific for only one or a small number of amino acids (Grenson *et al.*, as cited by Rytka, 1975). We checked, on GO medium, whether PPG interfered with the transport of methionine, leucine and arginine, each of which is transported by a separate permease (Fig. 3). The initial rates of transport of both leucine and methionine were markedly decreased by 1 mM-PPG. Transport of arginine was not affected even by 5 mM-PPG (now shown).

Mutants of *S. cerevisiae* resistant to PPG were obtained after UV mutagenesis on GO supplemented with 250 μ M-PPG. Activity of γ -cystathionase was checked in several of them and found to be unchanged. Two such mutants were chosen arbitrarily and transport of amino acids was assayed. They both displayed lowered transport rates for leucine and methionine (Fig. 3), but the normal rate for arginine.

We cannot therefore attribute PPG transport with certainty to any particular yeast amino acid permease, but the results of the uptake experiments, taken together, point to transport as the level of PPG-amino acid interference.

DISCUSSION

In bacteria, PPG inhibits cystathionine γ -synthase. An *A. nidulans* mutant blocked in this step of methionine synthesis is an auxotroph (Paszewski & Grabski, 1975). The fact that in the wild-type administration of 1 μ M-PPG does not result in methionine auxotrophy excludes cystathionine γ -synthase as being blocked by 1 μ M-PPG. On the other hand, γ -cystathionase is completely inhibited at this concentration of PPG. Thus the mode of action of PPG in *A. nidulans* resembles that found in mammals, rather than that in bacteria. At higher concentrations of PPG (1 mM) some inhibition of the growth of wild-type *A. nidulans* was observed, but no conclusion can be drawn concerning its nature. We suspect that, at this concentration, several nonspecific effects, as described in the literature, may be responsible for growth inhibition. In *A. nidulans* we did not observe an inverse correlation between the glutathione and cystathionine pools as found in mammalian cells (Shinozuka *et al.*, 1982), where γ -cystathionase inhibition by PPG administered *in vivo* results in parallel cystathionine accumulation and glutathione decrease. These results support earlier findings that the homocysteine to cysteine pathway does not operate in the wild-type of *A. nidulans* grown on sulphate as a sulphur source, in contrast to the *cysB* strain impaired in the main pathway of

cysteine synthesis. PPG-induced growth inhibition, presumed cysteine auxotrophy and the drastic decrease of the sulphur amino acid pool in the *cysB* strain indicate that the functioning of this pathway is indispensable for the latter strain.

The role of γ -cystathionase in *S. cerevisiae* is not clear. No mutants corresponding to *A. nidulans cysB* and *cysC* strains are known in this organism, and it is thus more difficult to say whether the effect of PPG on γ -cystathionase is mainly responsible for growth inhibition. No γ -cystathionase-less mutant was, until recently, known in yeasts. Ono *et al.* (1984) described a mutant with lowered γ -cystathionase activity. It accumulates cystathionine, which suggests that at least some part of cysteine is synthesized from cystathionine. This mutant is a prototroph, and the authors do not report any decrease in its growth rate, which in turn suggests that γ -cystathionase is not essential for *S. cerevisiae*.

Our data obtained with four fungal species indicate that γ -cystathionase sensitivity to PPG may differ among various fungi. Highly sensitive (*A. nidulans* and *N. crassa*) and highly resistant (*C. acremonium*) enzymes were found. In the case of a fungus with PPG-sensitive γ -cystathionase, the use of the antibiotic may give insight into the physiological role of this enzyme.

An important finding of this investigation is that, in fungi, PPG is transported into the cells by amino acid permeases, which has several implications for the use of this antibiotic in these organisms. It is worth noting that the interference of PPG with amino acid transport in mammalian cells has been ruled out (Beatty & Reed, 1980).

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