The Effect of 1,4-Diaminobutanone on the Stability of Ornithine Decarboxylase from *Aspergillus nidulans*

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1,4-Diaminobutanone, a competitive inhibitor of ornithine decarboxylase in *Aspergillus nidulans*, is able to increase the half-life of this enzyme and thus stimulate an increase in its activity *in vivo*. It also protects ornithine decarboxylase against proteolysis by chymotrypsin *in vitro*.

The activity of ornithine decarboxylase increases dramatically at the onset of growth and cell division in a variety of tissues (Raina & Jänne, 1975; Tabor & Tabor, 1976). It is an enzyme that turns over very rapidly in a number of eukaryotic tissues and has a very short half-life (Russell & Snyder, 1969; Williams-Ashman *et al.*, 1972; Kay & Lindsay, 1973; Clark, 1974; Hölttä, 1975). This rapid turnover is believed to be the main mechanism for regulating the synthesis of polyamines in eukaryotes, since ornithine decarboxylase is the rate-limiting step in the biosynthetic pathway in certain tissues (Morris & Fillingame, 1973; Jänne *et al.*, 1976). During the germination of conidia of *Aspergillus nidulans* there is a very large increase in ornithine decarboxylase activity (Stevens, 1975; Stevens *et al.*, 1976). 1,4-Diaminobutanone has been found to be a powerful competitive inhibitor of ornithine decarboxylase activity, but at the same time, when added to the growth medium, increases ornithine decarboxylase activity when the latter is assayed in dialysed conidial extracts (Stevens *et al.*, 1977). In the present paper we demonstrate that 1,4-diaminobutanone stabilizes ornithine decarboxylase against degradation.

Materials and Methods

Chemicals

Cycloheximide, phenylmethanesulphonyl fluoride and chymotrypsin (type II, salt-free) were from Sigma Chemical Co., St. Louis, MO, U.S.A. DL-[1-¹⁴C]Ornithine and L-[U-¹⁴C]lysine were from The Radiochemical Centre, Amersham, Bucks., U.K.

Organism and growth conditions

Aspergillus nidulans BWB 272 was maintained on nutrient agar and grown in submerged culture by using the basic medium described previously (Stevens *et al.*, 1976). When the conidia were grown in the

presence of 1,4-diaminobutanone, a 100 mM stock solution in sterile distilled water was made up immediately before use and added to the autoclaved medium to give a 0.5 mM final concentration.

Ornithine decarboxylase assays

Enzyme extracts were prepared by grinding frozen conidia or mycelia with one-half their weight of acid-washed sand in a precooled mortar and then extracting with 2–5 vol. of 10 mM-potassium phosphate/2 mM-1,4-dithiothreitol/1 mM-MgCl₂/0.1 mM-EDTA/0.1 mM-pyridoxal phosphate, pH 7.6 (extraction buffer). All subsequent steps in the enzyme extraction were carried out at 0°C. The suspensions were centrifuged at 25000g for 15 min. (NH₄)₂SO₄ (470 mg/ml) was added to the supernatant. The precipitated protein was redissolved in extraction buffer. Enzyme extracts from conidia grown in the presence of 1,4-diaminobutanone were dialysed against seven 100 vol. portions of extraction buffer, and those grown in the absence of 1,4-diaminobutanone were dialysed against two 100 vol. portions of extraction buffer. The enzyme assay, which is based on the production of ¹⁴CO₂ from [1-¹⁴C]ornithine, was as described previously (Stevens *et al.*, 1976).

Measurement of the effect of cycloheximide on protein synthesis and lysine uptake

Conidia were grown in submerged culture in 50 ml volumes. After 8 h incubation at 37°C cycloheximide was added at the concentrations indicated in the Results and Discussion section. After a further 15 min incubation 0.25 µCi of L-[U-¹⁴C]lysine (specific radioactivity 287 mCi/mmol) was added and incubated for a further 20 min, when 5 ml of 50% (w/v) trichloroacetic acid was added. The conidia were collected by centrifugation, washed twice in 25 ml of 5% trichloroacetic acid containing 0.1% lysine, resuspended in 10 ml of 5% trichloroacetic acid and incubated at 90°C for 15 min. The trichloroacetic acid-

insoluble material was quantitatively transferred to glass-fibre filters and the latter were dried under an i.r. lamp. The filters were placed in scintillation vials containing 10 ml of scintillation mixture {31.5 g of butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole], 500 ml of methanol, 3 litres of toluene and 1.5 litres of Triton X-100} and the radioactivities measured.

Results and Discussion

When *A. nidulans* was grown in submerged culture in the presence of 0.5 mM-1,4-diaminobutanone, the germination time was delayed by about 30 min when compared with the control cultures. The ornithine decarboxylase activity measured in dialysed conidial extracts was considerably increased when compared with control cultures at all the times investigated (Fig. 1). The extent of the stimulation ranged between 2- and 10-fold in four successive experiments. The reason for this variation is almost certainly because 1,4-diaminobutanone has to be completely removed to obtain maximum enzyme activity. This was performed by dialysis against extraction buffer seven times. At the same time, once the 1,4-diaminobutanone has been removed, the enzyme becomes less stable.

Fig. 1. Ornithine decarboxylase activity in germinating conidia grown in the presence (●) and absence (○) of 0.5 mM-1,4-diaminobutanone

Ornithine decarboxylase activities were measured in enzyme extracts as described in the Materials and Methods section.

To test whether 1,4-diaminobutanone stabilized ornithine decarboxylase and increased its half-life it was necessary to find a suitable concentration of cycloheximide to inhibit protein synthesis. Low concentrations (5 $\mu\text{g/ml}$) of cycloheximide cause a delay in germination (Stevens *et al.*, 1976), but the organism subsequently recovers and grows normally. Various concentrations of cycloheximide were added to the growth medium at 8 h, and after a further 15 min 0.25 μCi of [^{14}C]lysine was added. The conidia were harvested after a further 20 min and the radioactivity was measured in the protein precipitated by trichloroacetic acid. The [^{14}C]lysine incorporated after the addition of 5, 20, 50, 100 and 200 μg of cycloheximide/ml (final concn.) was decreased by 95, 97.5, 98, 98.6 and 99% respectively in comparison with the control.

The concentration of cycloheximide used to determine the half-life of ornithine decarboxylase was 100 $\mu\text{g/ml}$. A check was made to determine that this concentration of cycloheximide was not inhibiting lysine uptake into the spores, and it was found that more than 90% of the [^{14}C]lysine was taken up within 15 min from the medium of both cycloheximide-inhibited cultures and control cultures. The activity of ornithine decarboxylase was measured up to 2 h after the addition of cycloheximide. 1,4-Diaminobutanone appreciably increases the half-life of the enzyme (Fig. 2). By 1 h after the addition of cycloheximide the conidia are beginning to recover and the decay of ornithine decarboxylase is no longer first-order.

Fig. 2. Ornithine decarboxylase activity in conidial extracts after the addition of cycloheximide

Cycloheximide (100 $\mu\text{g/ml}$) was added to 8 h-germinating conidia grown in the presence (●) and absence (○) of 0.5 mM-1,4-diaminobutanone. Ornithine decarboxylase activities were measured as outlined in the Materials and Methods section, and are expressed relative to the activity at 8 h (see Fig. 1). ----, Expected activity if loss of activity continued with first-order kinetics.

To confirm that 1,4-diaminobutanone has a stabilizing effect on ornithine decarboxylase, the susceptibility of the enzyme to chymotrypsin was investigated. An 8 h conidial extract was divided into four 1 ml samples, which were subjected to the following treatments. Sample 1 was mixed with 1,4-diaminobutanone (1 mM final concn.). Sample 2 was mixed with 1,4-diaminobutanone (1 mM final concn.) and phenylmethanesulphonyl fluoride (0.3 mg/ml final concn.). Sample 3 was mixed with 0.1 mg of chymotrypsin and incubated for 15 min at 37°C, then phenylmethanesulphonyl fluoride (0.3 mg/ml final concn.) and 1,4-diaminobutanone (1 mM final concn.) were added. Sample 4 was mixed with 1,4-diaminobutanone (1 mM final concn.) and 0.1 mg of chymotrypsin and incubated for 15 min at 37°C, then phenylmethanesulphonyl fluoride (0.3 mg/ml) was added.

Each sample was dialysed against seven 100 ml portions of extraction buffer. The ornithine decarboxylase activities remaining in the four samples were 2.99, 2.98, 1.30 and 2.03 mmol of CO₂/30 min respectively. Ornithine decarboxylase is thus less readily degraded by chymotrypsin in the presence of 1,4-diaminobutanone. Also the phenylmethanesulphonyl fluoride used to inhibit chymotrypsin does not inhibit ornithine decarboxylase activity in the extracts.

1,4-Diaminobutanone therefore stabilizes ornithine decarboxylase against degradation *in vitro*.

Ornithine decarboxylase turns over rapidly in germinating conidia of *A. nidulans*. Higher activities of ornithine decarboxylase are found when *A. nidulans* is grown in the presence of 1,4-diaminobutanone and this seems to be due to the increased half-life of the enzyme.

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