Polyamine and Ornithine Metabolism during the Germination of Conidia of *Aspergillus nidulans*

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1. The activities of ornithine decarboxylase, *S*-adenosylmethionine decarboxylase and ornithine-2-oxoglutarate aminotransferase were studied during the first 24 h of conidial germination in *Aspergillus nidulans*. 2. Increases (over 100-fold) in the activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase occurred during the emergence of the germ-tube and before the doubling of DNA and this was followed by a sharp fall in the activities of both enzymes by 16 h. 3. The increase in ornithine decarboxylase could be largely suppressed if 0.6 mM-putrescine was added to the growth medium. 4. Low concentrations of cycloheximide, which delayed germination by 2 h, caused a corresponding delay in the changes in ornithine decarboxylase activity. 5. Ornithine-2-oxoglutarate aminotransferase activity increased steadily during the first 24 h of germination. 6. Ornithine or arginine in the growth medium induced higher activity of ornithine-2-oxoglutarate aminotransferase, but did not affect ornithine decarboxylase activity. 7. The significance of these enzyme changes during germination is discussed.

There is much evidence to associate polyamine synthesis with the onset of growth in a wide variety of tissues and organisms (Williams-Ashman *et al.*, 1969; Stevens, 1970; Russell, 1973; Raina & Jänne, 1975). In many instances an increase in the rate of polyamine synthesis occurs concurrently with an increase in synthesis of RNA (Cohen, 1971; Raina & Jänne, 1970). The molecular basis of these interrelationships is unknown. In many cells and tissues in which polyamine synthesis has been studied, e.g., lymphocytes (Kay & Lindsay, 1973), hepatoma cells (McCann *et al.*, 1975), mouse fibroblasts (Clark, 1974), regenerating liver (Russell *et al.*, 1970) and hormone-stimulated tissues (Jänne *et al.*, 1968; Stastny & Cohen, 1972; Kaye *et al.*, 1971), the comparison is made between metabolizing but non-dividing cells and cells that are both metabolizing and dividing. The conidia (vegetative spores) of fungi such as *Aspergillus nidulans* have a very low rate of metabolism (Gottlieb, 1966) and germination results in both nuclear division and a large increase in metabolism. Growth of the conidia can be divided into two periods, the initial germinative phase and the proliferative phase. The initial germinative phase involves imbibition of water, the onset of respiration, protein synthesis and nucleic acid synthesis. The proliferative phase involves successive nuclear divisions and apical extension of the hyphae.

Hollomon (1970) has shown that concentrations of proflavine that are sufficient to inhibit RNA synthesis also inhibit germination of *Aspergillus nidulans*, suggesting that RNA synthesis is essential

for germination to occur. To assess the importance of polyamine synthesis in both the germinative and the proliferative phases, we have measured the activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, since these enzymes are considered to be the regulatory steps in polyamine synthesis (Morris & Fillingame, 1973). The activities of these enzymes are compared with that of ornithine transaminase, since although the latter is not directly concerned with polyamine synthesis it may nevertheless compete with ornithine decarboxylase for intracellular ornithine.

Materials and Methods

Chemicals

Spermine tetrahydrochloride, spermidine trihydrochloride, putrescine dihydrochloride, 1,4-dithiothreitol and *O*-aminobenzaldehyde were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. *S*-Adenosyl-L-methionine was purchased from Boehringer Corp. (London) Ltd., Ealing, London W5 2TZ, U.K. DL-[1-¹⁴C]Ornithine hydrochloride and *S*-adenosyl-L-[carboxyl-¹⁴C]methionine were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Sand (40–100 mesh), purified by acid washing, was obtained from BDH Chemicals, Poole, Dorset, U.K.

Organism and growth conditions

Aspergillus nidulans BWB 272 was maintained on nutrient agar at 37°C for 3 days. The conidia were washed off the nutrient agar by using 0.1% (v/v)

Tween 80. Conidial suspensions were used to inoculate 1-litre flasks containing 200 ml of minimal medium having the following composition (g/litre): D-glucose, 50; NaNO₃, 1; Na₂SO₄, 1.6; KCl, 0.5; K₂HPO₄, 0.56; KH₂PO₄, 0.93; MgSO₄·7H₂O, 0.25; ZnSO₄·7H₂O, 0.01; CuSO₄·5H₂O, 0.005; FeSO₄·7H₂O, 0.01. The final conidial density was between 5×10^6 and 20×10^6 /ml. The flasks were shaken in an orbital incubator at 160 rev./min at 37°C. Germinating conidia and mycelia were harvested on 47 mm membrane filters (pore size 0.45 µm). Conidia were counted on an improved Neubauer haemocytometer.

DNA determination

Germinating conidia or mycelia on membrane filters were ground in a mortar with one-half their weight of acid-washed sand and then washed twice with 5 ml of 0.3 M-HClO₄. DNA was twice extracted from the residue with 2 ml of 0.45 M-HClO₄ at 70°C for 15 min. DNA was then measured in the combined supernatants (Burton, 1956).

Ornithine decarboxylase and S-adenosylmethionine 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. All subsequent steps in the enzyme extraction were carried out at 0°C. The suspensions were centrifuged at 25000g for 15 min. For preparation of extracts containing ornithine decarboxylase, 270 mg of (NH₄)₂SO₄ was added to each 1 ml of supernatant. The precipitates formed were redissolved in 1 ml of the buffer and dialysed against 100 vol. of the buffer. For preparation of extracts containing S-adenosylmethionine decarboxylase, 430 mg of (NH₄)₂SO₄ was added to each 1 ml of supernatant and the precipitates were redissolved in 1 ml of buffer. Ornithine decarboxylase and S-adenosylmethionine decarboxylase were assayed by measurement of ¹⁴CO₂ released after incubation with [1-¹⁴C]ornithine and S-adenosyl-[1-¹⁴C]methionine respectively. The incubation mixture for ornithine decarboxylase was: 50 mM-Tris/HCl, pH 8.0, 0.05 mM-L-ornithine, 0.031 mM-pyridoxal phosphate, 0.125 µCi of DL-[1-¹⁴C]ornithine monohydrochloride (58 mCi/mmol), 0.1 ml of enzyme extract, in a total volume of 0.4 ml. The incubation mixture for S-adenosylmethionine decarboxylase was: 0.1 M-sodium phosphate, pH 7.4, 0.2 mM-S-adenosyl-L-methionine, 1.0 mM-putrescine, 0.025 µCi of S-adenosyl-L-[1-¹⁴C]methionine (45 mCi/mmol) and 0.1 ml of enzyme extract, in a total volume of 0.4 ml. Incubations were carried out in test tubes in a shaking water bath at 37°C. Small vials (12 mm diam.) containing 50 µl of 2-methoxyethanol/ethanol-

amine (2:1, v/v) were suspended by thread near the top of the test tubes and the test tubes were sealed with self-sealing caps. After 30 min, 0.1 ml of 50% (w/v) trichloroacetic acid was injected into the test tubes and they were incubated for a further 60 min to trap the liberated CO₂ completely. The small vials were then removed, 2.5 ml of scintillation mixture [31.5 g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole, 500 ml of methanol, 3 litres of toluene, 1.5 litres of Triton X-100] was added and the samples were counted for radioactivity in a Philips liquid-scintillation analyser. Measurements were corrected to d.p.m. by the channels-ratio method. To measure the protein concentration in the enzyme extracts, the extracts were dialysed against water to remove pyridoxal phosphate and dithiothreitol and then assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Ornithine transaminase assay

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 0.1 M-Tris/HCl/0.1 mM-pyridoxal phosphate/0.5 mM-EDTA buffer, pH 7.6. The enzyme was assayed in the supernatant after centrifugation at 30000g for 15 min as described previously (Stevens & Heaton, 1973). The millimolar extinction coefficient for the condensation product between O-amino-benzaldehyde and Δ¹-pyrrolidine-5-carboxylic acid was assumed to be 2.71 litre·mmol⁻¹·cm⁻¹ (Strecker, 1965).

Results

Germination of *Aspergillus nidulans*

In submerged culture, 80% of conidia had visible germ tubes after 7.5 h incubation, whereas less than 10% were visible at 4.5 h. By 10 h the growing conidia clumped to form mycelial pellets. This is a slower rate of germination than that described by Bainbridge (1971), but slightly faster than that described by Rosenberger & Kessel (1967). The total DNA remained nearly constant for the first 6 h (Fig. 1), but had doubled by 9 h, so the S phase must lie somewhere between 6 and 9 h under these conditions.

Ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17)

When the 25000g supernatants obtained from germinating conidia were assayed for ornithine decarboxylase, the activity detected was very low. The activity was greatly increased when the supernatant was dialysed against three changes of 10 mM-potassium phosphate buffer, pH 7.6, containing 0.1 mM-EDTA, and the presence of a thiol-protecting agent, preferably dithiothreitol, was necessary to obtain maximum activity (Table 1).

The enzyme extract obtained after precipitation by (NH₄)₂SO₄ and dialysis against 10mM-potassium phosphate/0.1mM-EDTA/2mM-dithiothreitol had a pH optimum of 7.9. The enzyme reaction was linear with time for 60min and the K_m for L-ornithine was 0.06mM. Putrescine is a competitive inhibitor (K_i = 0.06mM); spermidine and spermine inhibit the reaction, but to a lesser extent. CuSO₄, ZnSO₄ or HgCl₂ (all 1mM) completely inhibited enzyme activity. MgCl₂, MnCl₂, D-ornithine, AMP, GMP, UMP and CMP (all 1mM) had no effect on the reaction rate. Isonicotinic hydrazide (1mM) caused 50% inhibition of the reaction. When the enzyme extract was prepared in buffer lacking pyridoxal phosphate and assayed in the absence of added

pyridoxal phosphate, the rate was 18% of that obtained in the presence of optimum concentrations (over 0.3 μM) of pyridoxal phosphate.

When conidia germinated in minimal medium, there was a rapid increase in ornithine decarboxylase for the first 8 h followed by a rapid fall in activity from 8 to 21 h (Fig. 2). This was seen whether total activity or specific activity was considered. If putrescine was included in the growth medium it decreased the induction of ornithine decarboxylase at 8 h (Fig. 3), but it did not affect the time at which germination occurred. When conidia were grown in the presence of 0.6mM-putrescine, the peak of ornithine decarboxylase activity was still apparent at 8h, but it was substantially decreased (Fig. 4). When either 2.5mM-

Fig. 1. DNA content of germinating conidia

DNA was estimated in HClO₄ extracts of germinating conidia as described in the Materials and Methods section.

Fig. 2. Change in total activity and specific activity of ornithine decarboxylase during germination of conidia

The total activity (●) and specific activity (○) of ornithine decarboxylase was measured in extracts of conidia as described in the Materials and Methods section.

Table 1. Effect of dialysis in the presence and absence of thiol reagents on ornithine decarboxylase activity

Supernatants were prepared and assayed as described in the Materials and Methods section. Mercaptoethanol was included in both the initial suspending buffer and the dialysing buffer in Expt. B, and dithiothreitol was included in the buffers used in Expt. C. Maximum activity is assumed to be when dithiothreitol is included in the buffers and the extract is dialysed.

Extract	Thiol reagent	Activity (μmol of CO ₂ /30min)	Activity (% of maximum)
Expt. A. Undialysed supernatant	—	0.003	0.06
Dialysed supernatant	—	0.110	2.5
(NH ₄) ₂ SO ₄ precipitation followed by dialysis	—	0.757	17
Expt. B. Undialysed supernatant	10mM-2-Mercapto-ethanol	0.165	3.7
Dialysed supernatant		1.23	27
(NH ₄) ₂ SO ₄ precipitation followed by dialysis		1.44	32
Expt. C. Undialysed supernatant	2mM-Dithiothreitol	0.19	4.0
Dialysed supernatant		4.52	100
(NH ₄) ₂ SO ₄ precipitation followed by dialysis		4.15	92

Fig. 3. Effect of polyamines on the development of ornithine decarboxylase activity in 8 h germinating conidia

Putrescine (●), spermidine (■) or spermine (▲) were added to the growth medium before inoculation. Ornithine decarboxylase activity was measured in 8 h germinating conidia as described in the Materials and Methods section.

Fig. 4. Development of ornithine decarboxylase activity during germination in the presence and the absence of putrescine

Ornithine decarboxylase was measured in extracts of conidia which had been grown either in the presence of 0.6 mM-putrescine (○) or in its absence (●).

Fig. 5. Effect of cycloheximide on germination and development of ornithine decarboxylase activity

(a) Percentage of conidia germinating in the presence (○) and absence (●) of cycloheximide. (b) Ornithine decarboxylase activity in conidia grown in the presence (○) and absence (●) of cycloheximide. Ornithine decarboxylase activity was measured as described in the Materials and Methods section. Cycloheximide (5 μ g/ml) was added to the growth medium 4 h after inoculation in both (a) and (b).

ornithine or 2.5 mM-arginine were included in the growth medium, neither changed the ornithine decarboxylase activity. Low concentrations (5 μ g/ml) of cycloheximide when added to the growth medium caused a delay in germination of about 2 h and there was a similar delay in the rise in ornithine decarboxylase activity (Fig. 5).

S-Adenosylmethionine decarboxylase (*S*-adenosyl-L-methionine carboxy-lyase, EC 4.1.1.50)

The 25000g supernatant from *A. nidulans* was assayed for *S*-adenosylmethionine decarboxylase and the properties of the enzyme were very similar to those from yeast (Pösö *et al.*, 1975) and animal tissues (Pegg & Williams-Ashman, 1968; Raina & Hannonen, 1970). The enzyme from *A. nidulans* was strongly stimulated by putrescine, 50% of the optimum stimulation occurring in the presence of 0.12 mM-putrescine. Its pH optimum was 7.4 and the K_m depends on the putrescine concentration; with 3.1 mM-putrescine $K_m = 0.063$ mM, and with 1.0 mM-putrescine $K_m = 0.16$ mM. There was a rapid increase in activity of the enzyme during germination for the first 10 h, followed by an equally rapid decrease, the time-course being very similar to that of ornithine

Fig. 6. Change in activity of *S*-adenosylmethionine decarboxylase during germination and the delay induced by cycloheximide treatment

S-Adenosylmethionine decarboxylase was measured in controls (●) and cycloheximide-treated cultures (○) as described in the Materials and Methods section. Cycloheximide (5 µg/ml) was added to the cultures at the time of inoculation.

Fig. 7. Ornithine transaminase activity during germination

Ornithine transaminase activity was measured in conidial extracts grown in minimal medium (●), in the presence of 2.5 mM-ornithine (▲), or in the presence of 2.5 mM-arginine (□).

decarboxylase (Fig. 6). A concentration of cycloheximide (5 µg/ml) that was sufficient to delay germination and induction of ornithine decarboxylase by 2 h also delayed the induction of *S*-adenosylmethionine decarboxylase. When 1.6 mM-spermidine was added to the growth medium, the activity at 10 h was decreased to less than 10% of that found in the

absence of spermidine. Putrescine (0.54 mM) added to the growth medium did not significantly affect the activity.

Ornithine transaminase (*L*-ornithine-2-oxoglutarate aminotransferase, EC 2.6.1.13)

The optimum conditions for assay of this enzyme in *A. nidulans* have been described previously (Stevens & Heaton, 1973). The following substances when included in the incubation mixture at a final concentration of 5 mM decreased the reaction rate to the following percentages of the control: spermine, 48%; α -*N*-acetylornithine, 24%; 4-aminobutyric acid, 47%; 5-aminopentanoic acid, 55%. Putrescine, D-ornithine, L-lysine and L-arginine (all 5 mM) did not affect the reaction rate. The total ornithine transaminase activity increased during germination and continued to increase during apical extension (Fig. 7). The increase occurred approximately concurrently with the total protein increase during that period. In contrast with their effect on ornithine decarboxylase activity when arginine or ornithine was included in the growth medium, there was a substantial increase in the activity of ornithine transaminase (Fig. 7). This effect has been reported to occur in 24 h mycelia (Cybis & Weglenski, 1969).

Discussion

In many cells and tissues the onset of rapid growth is accompanied by a dramatic increase in ornithine decarboxylase activity, and if growth is accompanied by synchronous cell division then fluctuations in ornithine decarboxylase activity are observed at different stages of the cell cycle (Friedman *et al.*, 1972; Kay & Lindsay, 1973; McCann *et al.*, 1975). But the changes in activity during the cell cycle are much less pronounced than the initial increase at the onset of growth. In regenerating rat liver, for example, which undergoes four fairly synchronous cell divisions in 96 h (Canellakis *et al.*, 1959), the initial increase in ornithine decarboxylase is about 20-fold, but the changes occurring between 20 and 96 h are much less marked (Russell & Snyder, 1968; Raina *et al.*, 1970). A very similar pattern is observed with germinating conidia of *A. nidulans*. There is more than a 100-fold increase in ornithine decarboxylase during the first 8 h. This precedes the DNA doubling. The specific activity of the ornithine decarboxylase then falls to a value that is less than one-twentieth of that at 8 h. The large rise in activity coincides with the onset of germination, and a much lower activity is maintained during the phase of atypical extension, although the hyphal mass and total DNA is still increasing rapidly. The large rise occurs in parallel with the increase in total RNA (Stevens, 1975). The changes in *S*-adenosyl-

methionine decarboxylase during germination are very similar to those of ornithine decarboxylase. These results suggest that there is a special requirement for polyamines at the onset of germination over and above that required for subsequent nuclear division. A similar change has been observed during the germination of spherules of *Physarum polycephalum* (Mitchell & Rusch, 1973).

Preliminary determination of intracellular putrescine suggests that the concentration is insufficient to influence the activity of ornithine decarboxylase but that it is sufficient to stimulate *S*-adenosylmethionine decarboxylase.

From a comparison of the $V_{\max}$ and K_m of ornithine decarboxylase ($V_{\max}$ = 2 nmol/30 min per 10^8 conidia, K_m = 0.06 mM), *S*-adenosylmethionine decarboxylase ($V_{\max}$ = 4 nmol/30 min per 10^8 conidia, K_m = 0.1 mM) and ornithine transaminase ($V_{\max}$ = 80 nmol/30 min per 10^8 conidia, K_m = 7.0 mM) in 10 h germinating conidia it is apparent that both ornithine decarboxylase and *S*-adenosylmethionine decarboxylase activities are low compared with that of ornithine transaminase. In *A. nidulans* we have found that both ornithine transaminase and ornithine decarboxylase are in the 30000g supernatant, suggesting that both enzymes are present in the cytosol. This is in contrast with liver ornithine transaminase, which is a mitochondrial enzyme (Peraino & Pitot, 1963). Preliminary estimates show that intracellular ornithine concentration in 5 h germinating conidia is about 5–20 mM (M. Tait & L. Stevens, unpublished work). At these concentrations ornithine decarboxylase is likely to be saturated, whereas ornithine transaminase is not. If these enzymes are in the same cell compartment they are both likely to compete for intracellular ornithine. The equilibrium constant for ornithine transaminase favours conversion of ornithine into glutamic semialdehyde (Strecker, 1965). The K_m values for ornithine decarboxylase and ornithine transaminase suggest that decarboxylation of ornithine is likely to be independent of the intracellular ornithine concentration, whereas transamination may vary with the intracellular concentration.

The presence of either ornithine or arginine in the growth medium induces higher activity of ornithine transaminase, but ornithine decarboxylase is unaffected. The roles of the two enzymes in germination are therefore likely to be different. Ornithine decarboxylase with its higher affinity for ornithine ensures that an adequate supply of putrescine for germination of the conidia is maintained. The role of ornithine transaminase, on the other hand, is probably to convert ornithine via glutamic semialdehyde into glutamate, proline and other intermediates for biosynthesis. Thus when there is an exogenous supply of ornithine or arginine, higher amounts of ornithine transaminase are induced to

enable the organism to make optimum use of these amino acids.

The changes in *S*-adenosylmethionine decarboxylase are very similar to those in ornithine decarboxylase. It is concluded that the two decarboxylases maintain the supply of spermidine that appears to be necessary for the conidia during an early stage in germination. This is also supported by the observation that the putrescine auxotroph, *A. nidulans* puA, requires putrescine for germination, but not for subsequent apical extension (Stevens, 1975).

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