Assimilation of Ammonia During Sporogenesis of *Saccharomyces cerevisiae*: Effect of Ammonia and Glutamine

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Comparison of the pools of glutamic acid and glutamine and of the specific activities of glutamine synthetase and glutamate dehydrogenases in sporulating a/α and non-sporulating α/α cells of *Saccharomyces cerevisiae* revealed a difference in their nitrogen metabolism. Glutamine synthetase and glutamine appeared to be necessary for the sporulation process, glutamine playing, at least, a catabolic role. However, exogenous glutamine as well as ammonia inhibited sporulation while glutamic acid did not. Glutamine seemed to act through its amino group. Both inhibitors had at least two sites of action, one effective early in sporulation and related to DNA synthesis and the other acting later and not related to it.

INTRODUCTION

Starvation of the yeast *Saccharomyces cerevisiae* induces a differentiation process leading to meiosis and sporulation of diploid (a/α) cells. This process is inhibited by ammonia and some other nitrogen compounds (Miller, 1963; Piñon, 1977) but very little is known about the metabolism of ammonia in sporulating cells. In this paper, we report our studies on the activities of enzymes involved in ammonia assimilation (glutamine synthetase, NAD- and NADP-linked glutamate dehydrogenases), the effect of the substrates of these enzymes on the sporulation process, and the variation in the sizes of pools of free glutamine and glutamic acid during the first hours after the transfer of cells to the sporulation medium with or without ammonia.

METHODS

Yeast strains, growth and sporulation. Diploid cells of *Saccharomyces cerevisiae* 532 a/α and 533 α/α , isogenic except for their mating type (Pellucier, 1973), were used. The a/α cells were grown and allowed to sporulate as described by Durieu-Trautmann & Delavrier-Klutcho (1977). The α/α cells, which are unable to sporulate, were used as a control.

Enzyme activities. Extracts were prepared at 4 °C by passing suspensions of cells in 100 mM-potassium phosphate buffer pH 7.8, containing 1 mM-2-mercaptoethanol, through a French press at 100 MPa. Cell debris was removed by centrifuging for 30 min at 30000 g and the supernatant was dialysed overnight at 4 °C against buffer similar to that used for extraction.

Activities of NAD-linked glutamate dehydrogenase (NAD-Gdh; EC 1.4.1.2) and of NADP-linked glutamate dehydrogenase (NADP-Gdh; EC 1.4.1.4) were estimated by determining the rate of NADH or NADPH oxidation at 25 °C (Boehringer, 1968). The rate of reaction with NAD-Gdh was corrected by subtracting the rate of NADH oxidation observed in the absence of 2-oxoglutarate.

The activity of glutamine synthetase (GS; EC 6.3.1.2) was measured by assaying transferase activity (Woolfolk *et al.*, 1966).

Protein was determined by the Folin method of Lowry, using bovine serum albumin as standard.

Glutamate and glutamine pools. Samples containing about 10^8 cells were collected on membrane filters

Fig. 1. Effect of ammonia and glutamine on sporulation. a/α cells were incubated in sporulation medium with the following additions: none (Δ); 1 mM-ammonia ($\square$); 2.5 mM-ammonia ($\blacksquare$); 5 mM-ammonia $\pm$ 40 mM-glutamic acid ($\blacksquare$); 2.5 mM-glutamine ($\circ$); 5 mM-glutamine ($\bullet$); 7.5 mM-glutamine ($\bullet$); 40 mM-glutamic acid (∇). Sporulation was measured by counting the number of asci formed. The viability of cells exposed to the inhibitors was only slightly reduced and was still 60% after 24 h.

(Millipore) and washed rapidly three times with 10 ml portions of ice-cold water. The filters were extracted by heating for 5 min with 5 ml water in a boiling water bath, the extracts were filtered and taken to dryness at 25 °C, and the residues were dissolved in water for electrophoretic analysis. Electrophoresis was performed at pH 6.5 on Whatman 3MM paper for 30 min at 80 V cm^{-1} . Amino acids were recovered by elution with 6 M-HCl and eluates were hydrolysed at 110 °C overnight and analysed using a Technicon TMS amino acid analyser (Spackman *et al.*, 1958).

RESULTS AND DISCUSSION

Addition of either ammonia or glutamine at the time when cells were transferred to sporulation medium inhibited sporulation and no growth was observed under the conditions used (Fig. 1): 5 mM-ammonium sulphate or 7.5 mM-glutamine completely suppressed ascus formation; at lower concentrations, there was a lag in the appearance of the first asci and a reduction in the final sporulation yield. In contrast, glutamic acid did not inhibit sporulation and did not relieve the glutamine- or ammonia-mediated inhibition even at concentrations as high as 40 mM (Fig. 1) (entry of glutamic acid into the cells was verified by using [^{14}C]-glutamic acid). As expected, cells homozygous for mating type (α/α) neither sporulated nor grew under sporulation conditions.

Subsequent experiments were designed to determine if ammonia and glutamine exert their inhibitory action at a specific step (or steps) in the sporulation process, and if the inhibitory effect was reversible. As shown in Table 1, ammonia and glutamine blocked sporulation when added within 12 h of transfer to sporulation medium, but if the addition was made after 12 h, some asci were formed, and the later the addition the greater the number of asci observed. Inhibition was reversible up to about 12 h. If the inhibitor was removed after 12 h, some inhibition was observed, its extent being dependent on the time of removal of the inhibitor. Thus, 12 h appeared to be the point of commitment to spore formation for strain 532 a/α under the conditions used. These results agree with those of previous experiments (Durieu-Trautmann & Delavier-Klutchko, 1977; Delavier-Klutchko & Durieu-Trautmann, 1978) which showed that, in sporulating (a/α) as well in non-sporulating (α/α) cells, glutamine and ammonia prevent RNA, protein and glycogen synthesis from occurring in the sporulation medium after 4 h. RNA, protein and glycogen synthesis occurring within 4 h is insensitive to these inhibitors. This inhibitory effect is reversible. In addition, glutamine and ammonia reduce and slow down DNA synthesis (a specific event of sporogenesis), which starts at around 4 h (Durieu-Trautmann & Delavier-Klutchko, 1977; Piñon, 1977). Thus, these inhibitors have at least two sites of action, one at around 4 h and another at

Table 1. *Effect of glutamine and ammonia on sporulation when added or removed at different times*

The percentage of asci formed, relative to a control without inhibitor, was determined 40 h after transferring the cells to sporulation medium (the control contained 55% asci at this time). The inhibitor (5 mM-ammonium sulphate or 10 mM-glutamine) was either added to the sporulation medium at the time indicated and left in the medium or added at 0 h and removed at the time indicated by transferring cells to fresh sporulation medium. Similar results were obtained with both inhibitors.

Time of addition or removal of inhibitor (h)	Asci (% relative to control)	
	Addition of inhibitor	Removal of inhibitor
0	0	100
4	0	100
11	0	90
12	2	73
13-14	22	66
15-16	26	59
17-19	57	2
20	90	0

Fig. 2. NAD-linked (a) and NADP-linked (b) glutamate dehydrogenase activities of diploid *a/α* and *α/α* cells during incubation in sporulation medium with or without ammonia: *a/α* cells in sporulation medium (○); *α/α* cells in sporulation medium (△); *a/α* cells in sporulation medium + 5 mM-ammonia (●); *α/α* cells in sporulation medium + 5 mM-ammonia (▲). Initial specific activities [$\mu\text{mol NADH or NADPH oxidized h}^{-1} (\text{mg protein})^{-1}$]: NAD-Gdh in *a/α* cells, 9.8, and in *α/α* cells, 15.5; NADPH-Gdh in *a/α* cells, 4.8, and in *α/α* cells, 5.3. V indicates cells in the vegetative state.

Fig. 3. Glutamine synthetase activities of diploid a/α and α/α cells during incubation in sporulation medium (a) and in sporulation medium + 5 mM-ammonia (b). Symbols as in Fig. 2. Initial specific activities [$\mu\text{mol } \gamma\text{-glutamyl hydroxamate formed h}^{-1} (\text{mg protein})^{-1}$]: in a/α cells, 126, and in α/α cells, 114.

approximately 12 h, when DNA synthesis is completed. Piñon (1977) observed an inhibitory effect of ammonia and glutamine on sporulation and DNA synthesis of *S. cerevisiae*, but he reported that this effect was irreversible and that glutamate was able to prevent it to some extent, observations that do not agree with ours.

The activities of NAD-Gdh, NADP-Gdh and GS under sporulation conditions were measured in extracts of a/α and α/α cells in order to distinguish specific sporulation events. A very rapid loss of activity of NAD-Gdh occurred in both a/α and α/α cells in the absence of ammonia (Fig. 2a). In the presence of ammonia, the enzyme activities of a/α and α/α cells were lost at a much slower rate and to a lesser extent. The rapid decrease of NAD-Gdh activity was due to proteolytic degradation, as suggested by Betz & Weiser (1976) and as shown in our laboratory (unpublished results), and ammonia at least partly prevented this degradation. The pattern of change in NADP-Gdh in the absence of ammonia was different in strains a/α and α/α (Fig. 2b): in sporulating cells, its activity was unaffected for at least 5 h, whereas in cells unable to sporulate, its activity increased by 50% within the first hour and then remained constant. Addition of ammonia to the sporulation medium promoted a rapid decrease in the activity of NADP-Gdh in both cell types (Fig. 2b). The patterns of variation of activity of GS in a/α and α/α cells transferred to a nitrogen-free medium were identical (Fig. 3a): the enzyme activities fell after the shift and then increased reaching 130 to 160% of their original values after 5 h. When the sporulation medium contained ammonia or glutamine (Fig. 3b), a similar decrease in the GS activities of both a/α and α/α cells was observed at 0 h, as described above, followed by a slow rise; activities reached the initial level within 2 h and then declined slowly. The initial loss of activity might be due either to a rapid proteolytic degradation of GS, or to the conversion of the active form to a less active one, as in *Candida utilis* (Sims *et al.*, 1974) and in *Neurospora crassa* (Kapoor *et al.*, 1969).

In parallel with the assays of enzyme activities, the changes in the sizes of the pools of free glutamic acid and glutamine were followed in cells transferred to sporulation medium (Table 2). In both a/α and α/α cells, the glutamine concentration decreased rapidly and

Table 2. *Changes in glutamic acid and glutamine contents of cells exposed to sporulation medium with or without ammonia*

Medium	Time (min)	Intracellular concn (nmol per 10 ⁸ cells)			
		Strain <i>a/α</i>		Strain <i>α/α</i>	
		Glutamic acid	Glutamine	Glutamic acid	Glutamine
Sporulation medium	0	168.8	29.0	127.3	39.0
	15	114.1	14.1	369.5	40.9
	30	74.0	9.3	—	1.6
	60	71.6	8.8	347.7	1.3
	90	76.0	9.8	—	2.6
	120	84.5	8.2	—	3.8
	360	80.4	18.8	368.0	2.2
Sporulation medium + 5 mM-ammonia	15	226.6	38.0	369.5	—
	30	226.7	39.7	—	1.8
	60	310.2	38.0	547.0	1.3
	90	290.0	38.1	—	1.6
	120	269.6	42.2	—	4.3
	360	319.1	49.5	513.0	2.3

—, Not determined.

remained low, while, as noted above, the activity of GS increased. This suggests that GS and/or glutamine or some product of its metabolism, are necessary, though not specific, for sporulation. This hypothesis is confirmed by the recent findings of E. Dubois & J. M. Wiame (personal communication) who observed that a glutamine synthetase-less strain of *S. cerevisiae* was unable to sporulate. The glutamic acid pool differed in *a/α* and *α/α* cells. In *a/α* cells, the concentration of free glutamic acid declined (the activity of the anabolic glutamate dehydrogenase was constant), whereas in *α/α* cells, it increased and reached a value almost five times higher than that in *a/α* cells. At the same time NADP-Gdh activity also increased. This result shows a disparity in the glutamate metabolism of the two strains which could relate to the sporulation process, but we do not yet know how glutamate acts. When ammonia was added to the sporulation medium, a difference in the nitrogen metabolism of the two strains was also observed: the glutamine pool decreased in *α/α* cells (as in the absence of inhibitor) but it increased slightly in *a/α* cells; the glutamic acid pool behaved in a similar way in both types of cells but was larger in *α/α* than in *a/α* cells.

The observation that glutamic acid did not inhibit sporulation whereas glutamine and ammonia did was not expected since glutamic acid is the first compound of ammonia assimilation in growing cells of *S. cerevisiae* (Jones *et al.*, 1969; Witt & Holzer, 1964) and is, through GS, a precursor of glutamine. Moreover, in the presence of ammonia, both the glutamine and glutamic acid pools were increased. One explanation could be that the glutamine formed endogenously is accumulated in the vacuoles (Wiemken & Dürr, 1974) and is not available as an inhibitor. Another hypothesis is that glutamine, rather than glutamic acid, is the first product of ammonia assimilation and GS is the first enzyme involved in this process, rather than NADP-Gdh. This would suggest the involvement of an enzyme such as glutamate synthase. This enzyme operates in bacteria (Elmerich & Aubert, 1971; Tempest *et al.*, 1973). Although the existence of glutamate synthase is controversial in *S. cerevisiae* (Brown *et al.*, 1973), Roon *et al.* (1974) have reported evidence for it in cells growing on non-limiting concentrations of ammonia. Further studies using mutants in ammonia metabolism would help to resolve these problems.

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