Ammonium Assimilation by *Candida albicans* and Other Yeasts: Evidence for Activity of Glutamate Synthase

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Activities and properties of the ammonium assimilation enzymes NADP⁺-dependent glutamate dehydrogenase (GDH), glutamate synthase (GOGAT) and glutamine synthetase (GS) were determined in batch and continuous cultures of *Candida albicans*. NADP⁺-dependent GDH activity showed allosteric kinetics, with an $S_{0.5}$ for 2-oxoglutarate of 7.5 mM and an apparent K_m for ammonium of 5.0 mM. GOGAT activity was affected by the buffer used for extraction and assay, but in phosphate buffer, kinetics were hyperbolic, yielding K_m values for glutamine of 750 μ M and for 2-oxoglutarate of 65 μ M. The enzymes GOGAT and NADP⁺-dependent GDH were also assayed in batch cultures of *Saccharomyces cerevisiae* and three other pathogenic *Candida* spp.: *Candida tropicalis*, *Candida pseudotropicalis* and *Candida parapsilosis*. Evidence is presented that GS/GOGAT is a major pathway for ammonium assimilation in *Candida albicans* and that this pathway is also significant in other *Candida* species.

INTRODUCTION

Two pathways for ammonium assimilation have been identified in bacteria (Tempest *et al.*, 1970; Miller & Stadtman, 1972), plants (Robertson *et al.*, 1975; Boland & Benny, 1977), yeasts (Roon *et al.*, 1974) and filamentous fungi (Lara *et al.*, 1982; Kusnan *et al.*, 1987). In the first pathway, glutamate dehydrogenase (GDH) catalyses the reaction:

Fungi possess both an NAD⁺-dependent and an NADP⁺-dependent GDH; the latter catalyses the anabolic reaction (Goldin & Frieden, 1974). The second pathway involves the combined action of glutamine synthetase (GS) and glutamate synthase (GOGAT):

The GDH in a number of organisms has considerably less affinity for ammonium than GS (Miller & Stadtman, 1972; Mifflin & Lea, 1977). Consequently, GDH activity may be insufficient for assimilation when the internal ammonium concentration is low. The GS/GOGAT pathway constitutes the main route of ammonium assimilation in plants (Robertson *et al.*, 1975; Mifflin & Lea, 1977), *Escherichia coli* (Miller & Stadtman, 1972) and *Rhizobium* (Osburne & Signer, 1980). The relative importance of the two pathways in fungi is less clear. The GS/GOGAT pathway is considered unimportant for ammonium assimilation in the yeasts *Saccharomyces cerevisiae* (Bogonez *et al.*, 1985), *Candida utilis* (Zwart & Harder, 1983)

Abbreviations: GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase; AAT, aspartate aminotransferase; MDH, malate dehydrogenase.

and *Candida boidinii* (Green & Large, 1984), NADP⁺-dependent GDH being the major route both in conditions of nitrogen excess and limitation. However, the GS/GOGAT pathway is utilized at low ammonium concentrations in the filamentous fungi *Aspergillus nidulans* (Kusnan *et al.*, 1987) and *Neurospora crassa* (Lomnitz *et al.*, 1987).

Recent studies (Holmes & Shepherd, 1987, 1988) have indicated that changes in nitrogen metabolism are involved in the control of morphogenesis in *Candida albicans*. The ammonium assimilation enzymes have a central role in nitrogen metabolism, and the aim of this study was to compare the nature and activities of these enzymes in *C. albicans* and other *Candida* spp. This study also confirms the presence of GOGAT in *S. cerevisiae* (Roon *et al.*, 1974).

METHODS

Organisms. The strains used in this study were as follows: *C. albicans* strains ATCC 10261, MEN (Dr R. Cannon), NCPF 3153 and NCPF 3156 (Colindale, UK); *S. cerevisiae* strains DYC (brewers' yeast) and C31C (Dr C. Nombela); strains of *Candida tropicalis*, *Candida pseudotropicalis* and *Candida parapsilosis* were obtained from the National Health Institute, Wellington, New Zealand. All strains were propagated and maintained on yeast extract/peptone agar slopes. The identity of the *Candida* strains was confirmed using a commercial serological and assimilation kit (Iatron Laboratories, Tokyo).

Culture conditions. Batch cultures were grown in defined medium of glucose, salts and biotin (GSB) (Shepherd *et al.*, 1980) or yeast nitrogen base (YNB) (Difco) with glucose, at 30 °C in a rotary shaker. A chemostat with a capacity of 910 ml was used for continuous culture of *C. albicans* ATCC 10261. Growth was limited for either carbon or nitrogen using YNB medium without amino acids; the glucose concentration was reduced to 0.6 g l⁻¹ for carbon limitation and the ammonium sulphate concentration to 0.04 g l⁻¹ for nitrogen-limited growth. Temperature was maintained at 37 °C and pH was maintained at 4.5.

Cell extract preparation. Mid-exponential phase cells (200 ml) were harvested into ice, concentrated by centrifugation at 4 °C, washed once in ice-cold distilled water and resuspended in buffer (50 mM-phosphate, pH 7.0) to a final concentration of $1-4 \times 10^8$ cells ml⁻¹. Mercaptoethanol (2%, v/v) was added to the buffer for GDH and GOGAT determinations, and for GS determination 4.0 mM-EDTA was added. A volume (0.5 ml) of the cell suspension was added to 1.5 g of glass beads (400 µm; Sigma) in a 10 ml test tube cooled on ice. The suspension was vortexed for two 1 min periods with resting on ice. The extract was decanted, the beads washed with 1.0 ml buffer and the pooled material spun in a microfuge for 5 min to remove cell debris.

Enzyme assays. GS (EC 6.3.1.2) was measured using the biosynthetic assay (Farnden & Robertson, 1980). The reaction mixture contained 240 µl imidazole buffer (0.25 M, pH 7.0), 15 µl sodium glutamate (1.0 M, pH 7.0), 25 µl ATP (30 mM, pH 7.0), 25 µl MgSO₄ (0.5 M) and 50 µl cell extract; the reaction was started by the addition of 50 µl of freshly prepared hydroxylamine (equal volumes of 1.0 M-NH₂OH.HCl and 1.0 M-NaOH). After 15 min the reaction was stopped by the addition of 100 µl FeCl₃ reagent (Farnden & Robertson, 1980). The absorbance of γ-glutamyl hydroxamate was measured at 492 nm using a microtitre plate reader (volume per well 200 µl). One unit (U) of enzyme activity was defined as the amount of enzyme producing 1.0 nmol γ-glutamyl hydroxamate min⁻¹ (mg total protein)⁻¹ by reference to a standard curve. GOGAT (EC 1.4.1.14) was measured by following NADH oxidation at 340 nm in a Shimadzu recording spectrophotometer. The reaction mixture contained 920 µl phosphate buffer (50 mM, pH 7.0), 20 µl NADH (10 mM), 20 µl 2-oxoglutarate (100 mM, pH 7.0), 20 µl freshly prepared glutamine (100 mM) and 20 µl cell extract. The background rate of NADH oxidation was established before addition of glutamine, and this activity was subtracted from the total activity before determining NADH oxidation due to GOGAT. One unit (U) of activity was defined as the amount of enzyme causing oxidation of 1.0 nmol NADH min⁻¹ (mg total protein)⁻¹. GDH (NAD⁺- and NADP⁺-dependent; EC 1.4.1.2 and 1.4.1.4 respectively) was measured by following NAD(P)H oxidation as described above. The reaction mixture contained 920 µl phosphate buffer (50 mM, pH 7.0), 20 µl NAD(P)H (10 mM), 20 µl 2-oxoglutarate (0.1 M for NAD⁺-dependent and 1.0 M for NADP⁺-dependent, pH 7.0), 20 µl NH₄Cl (1.0 M), and 20 µl cell extract. Routinely the reaction was started by the addition of the ammonium chloride. One unit (U) of activity was defined as the amount of enzyme causing oxidation of 1.0 nmol NAD(P)H min⁻¹ (mg total protein)⁻¹.

Protein determination. Samples (10 µl) were precipitated with 5% (w/v) TCA and then subjected to the Lowry assay; BSA standards were included with each assay.

RESULTS AND DISCUSSION

Characterization of the ammonium assimilating enzymes in C. albicans ATCC 10261

Demonstration of GOGAT activity. Standard assays can be used for the determination of GDH and GS activities in crude extracts. However, there are a number of factors that could give rise to

Fig. 1. pH profiles of GOGAT (●), NADP⁺-dependent GDH (▲) and NAD⁺-dependent GDH (■) in *C. albicans* ATCC 10261.

spurious GOGAT activity. Reduced activity could result from inactivation during extraction; the enzyme is unstable, with a reported activity loss of 2% h⁻¹ at 0 °C (Robertson *et al.*, 1975). Farnden & Robertson (1980) recommend the inclusion of high levels of reducing agents, and Boland & Benny (1977) reported that the use of Tris buffers decreased the stability of their preparation. False positive GOGAT activity could arise if ammonium was present acting as a substrate for NAD⁺-dependent GDH activity in the crude extract. Ammonium could be present either as a contaminant of the glutamine preparation (even when freshly prepared) or if generated by glutaminase activity in the crude extract. In addition, amino acids as contaminants would be substrates for the combined activities of an aminotransferase and a dehydrogenase, the latter causing the oxidation of NADH in this assay. For example, in a crude extract of *C. albicans* there was considerable combined activity of aspartate aminotransferase (AAT) and malate dehydrogenase (MDH), as measured by NADH oxidation in the presence of aspartate and 2-oxoglutarate.

The GOGAT activity detected in extracts of *C. albicans* cells was NAD⁺-dependent, and no oxidation of NADPH was observed. The addition of mercaptoethanol reduced background NADH oxidase activity by 50–70%. GOGAT activity was unstable; in the absence or presence of 2% mercaptoethanol, activity decreased by 80% and 50% respectively after overnight incubation on ice. In all the experiments in this study, enzyme activities were measured within 4 h of extraction and mercaptoethanol was added to the extraction buffer except for the assay of GS. Mercaptoethanol had no effect on the other enzyme activities. Fig. 1 shows that the pH profile for GOGAT markedly differed from that of NAD⁺-dependent GDH activity, indicating that the observed activity did not result from ammonium contamination or *de novo* production. Furthermore, the GOGAT activity was inhibited by the glutamine analogue azaserine, indicating that glutamine was a substrate. Azaserine at a concentration of 0.5 mM inhibited the observed activity by 80%, whereas 1.0 mM-azaserine had no effect on the other NADH-dependent enzyme activities. We also found that GOGAT activity was significantly affected by the buffer used for both extraction and assay. When either phosphate or HEPES buffer was used for extraction and assay, similar activities were obtained. However, as shown in Table 1, the use of imidazole as an assay buffer abolished the GOGAT activity of a phosphate buffer extract; furthermore, cells extracted into imidazole buffer had no detectable GOGAT activity in a phosphate assay. NADP⁺-dependent GDH and the combined AAT/MDH activities were unaffected by the nature of the buffer used for extraction or assay, as were the NADH oxidase and NAD⁺-dependent GDH activities (results not shown). Tris buffer was also inhibitory of GOGAT but not GDH activity when used for extraction or assay. These results provided further

Table 1. *Effect on GOGAT activity of the buffer used for cell extract preparation or for enzyme assay*

C. albicans ATCC 10261 was grown to mid-exponential phase in GSB medium and cell extracts prepared as described in Methods using either phosphate buffer (50 mM, pH 7.0) or imidazole buffer (50 mM, pH 7.0). GOGAT and NADP⁺-dependent GDH were assayed as described in Methods. The combined aspartate aminotransferase and malate dehydrogenase (AAT/MDH) activity was measured by following NADH oxidation in the presence of 2-oxoglutarate (2.0 mM) and sodium aspartate (2.0 mM). The results are from a representative experiment repeated twice.

Buffer		Enzyme activity [U (mg protein) ⁻¹]		
Extraction	Assay	GOGAT	NADP ⁺ -dependent GDH	AAT/MDH
Phosphate	Phosphate	28.0	407	74.6
	Imidazole	0.0	407	89.6
Imidazole	Phosphate	0.0	411	74.8
	Imidazole	0.0	411	103.7

Table 2. *Specific activities of the enzymes of ammonium metabolism in C. albicans*

Four strains of *C. albicans* were grown to mid-exponential phase in GSB medium. Cell extraction procedures and enzyme assays were as described in Methods. Values given are the mean of at least three experiments carried out in duplicate. Standard errors were within 15%.

Strain	Enzyme activity [U (mg protein) ⁻¹]			
	GOGAT	NADP ⁺ -dependent GDH	NAD ⁺ -dependent GDH	GS
ATCC 10261	29.6	382	5.5	5.0
MEN	45.3	472	6.4	2.8
NCPF 3156	38.7	387	6.8	3.6
NCPF 3153	40.0	387	5.0	3.7

evidence that the observed GOGAT activity was not the result of the combined activity of an aminotransferase with either GDH or MDH. GOGAT activity in the other *Candida* strains and in *S. cerevisiae* was also sensitive to Tris and imidazole buffers. Consequently, phosphate buffer was used routinely for both extraction and assay in all subsequent experiments.

Specific enzyme activities. The activities of the four enzymes involved in ammonium metabolism were measured in extracts of batch cultures grown to mid-exponential phase in GSB medium. NADP⁺-dependent GDH and GS had a pH optimum of 7.0, GOGAT of 6.85 and NAD⁺-dependent GDH of 7.5. Table 2 shows the activities obtained at saturating substrate concentrations in four strains of *C. albicans*. We found no evidence for the modification of either GDH activity by divalent cations or by ATP or AMP. The ratio NADP⁺-dependent GDH:GOGAT activity was between 10 and 13 in the four strains under these growth conditions. If cells were transferred for a short period (3 h) to ammonium-free medium, or medium containing an amino acid (leucine, proline or glutamate) as a nitrogen source, GS activity increased two- to threefold. The detection of significant GOGAT activity relative to GDH, and the response of GS activity to ammonium concentration, suggests the operation of the GS/GOGAT pathway in this organism. The following experiments comparing the kinetics of the enzymes involved in the two pathways, and the effect of controlled growth conditions on their activities, were performed in order to obtain information on the route of ammonium assimilation in this organism.

Kinetic parameters. We investigated the kinetics of the GOGAT and NADP⁺-dependent GDH activities in extracts of batch-culture cells. NAD⁺-dependent GDH has a catabolic function in yeasts (Large, 1986) and therefore was not subjected to kinetic analysis in this study. GS kinetics was not examined in detail as initial experiments revealed that maximal velocity, *V*, was obtained at < 20 µM-ammonium. For a three-substrate enzyme such as GOGAT or GDH, ideally kinetic parameters should be calculated from rates of reaction at a variety of concentrations of two of the substrates for each concentration of the third. However, as reported

Fig. 2

Fig. 2. Reaction velocity of *C. albicans* NADP⁺-dependent GDH activity in response to variation in concentrations of the substrates 2-oxoglutarate (▲) and ammonium (●). The $S_{0.5}$ value for 2-oxoglutarate (7.1 mM) was obtained by plotting $\log [v/(V-v)]$ versus $\log S$ (Hill plot); the K_m for ammonium (5.0 mM) was obtained from a reciprocal plot.

Fig. 3

Fig. 3. Activities of *C. albicans* NADP⁺-dependent GDH (▲), GOGAT (●) and GS (■) at a range of growth rates in continuous culture under nitrogen limitation (—) or glucose limitation (---).

by Boland & Benny (1977), it was not possible to measure activity at low concentrations of NADH because the absorbance at 340 nm was too low. Hence, for the experiments in this study, the dependence of the rate of reaction on glutamine and 2-oxoglutarate (in the case of GOGAT) and ammonium and 2-oxoglutarate (in the case of NADP⁺-dependent GDH), was determined in the presence of saturating concentrations of NADH and NADPH respectively. Double reciprocal plots for GOGAT activity against both glutamine and 2-oxoglutarate concentration, for a range of concentrations of each, were linear, and the kinetic parameters were obtained from secondary plots of these results. K_m values of 750 μ M for glutamine and 65 μ M for 2-oxoglutarate were obtained. Glutamate, malate and pyruvate were competitive inhibitors with respect to 2-oxoglutarate. Kinetic analysis of NADP⁺-dependent GDH was limited because of its allosteric nature. Fig. 2 shows the allosteric behaviour of the enzyme with respect to

Table 3. Comparison of NADP⁺-dependent GDH and GOGAT activities in four *Candida* species and *S. cerevisiae*

Cells were grown to mid-exponential phase in YNB medium. Cell extraction procedures and enzyme assays were as described in Methods. Values given are the mean of at least three experiments carried out in duplicate. Standard errors are given in parentheses.

Organism	Enzyme activity [U (mg protein) ⁻¹]		
	GOGAT	GDH	GDH : GOGAT ratio
<i>C. albicans</i> (ATCC 10261)	33.3 (±9.0)	519 (±140)	15.6
<i>C. pseudotropicalis</i>	26.0 (±4.4)	157 (±36)	6.0
<i>C. parapsilosis</i>	18.0 (±4.7)	558 (±121)	31.0
<i>C. tropicalis</i>	34.0 (±4.9)	1318 (±172)	38.0
<i>S. cerevisiae</i> (DYC)	13.5 (±1.0)	1200 (±146)	89.0
<i>S. cerevisiae</i> (C31C)	11.4 (±3.2)	1292 (±307)	113.0

2-oxoglutarate, whereas a hyperbolic curve was obtained when ammonium concentration was varied at a saturating concentration of 2-oxoglutarate. An $S_{0.5}$ value of 7.5 mM for 2-oxoglutarate and an apparent K_m of 5.0 mM for ammonium were obtained. In the three other strains of *C. albicans* GDH activity was allosteric with respect to 2-oxoglutarate; activity at 2.0 mM-2-oxoglutarate was <5% of the apparent V value.

Comparative studies with other *Candida* species and *S. cerevisiae*

The enzymes NADP⁺-dependent GDH and GOGAT were assayed in three other *Candida* spp. and in *S. cerevisiae*. All cultures were grown to mid-exponential phase in YNB medium. Extraction and assays were as described in Methods; the pH optima for both enzymes was between 7.0 and 7.3, and therefore all assays were performed at pH 7.0. GOGAT activity was found in all the yeasts including *S. cerevisiae*. Table 3 compares the apparent V values (estimated by measurement of activity at saturating concentrations of all three substrates) for both enzymes in these yeasts and *C. albicans* ATCC 10261. The high GDH : GOGAT ratio in *S. cerevisiae* is in agreement with the data of Roon *et al.* (1974), who concluded that the GS/GOGAT pathway had only limited importance in ammonium assimilation in this yeast. However, in *C. albicans* and *C. pseudotropicalis* the ratio was significantly lower (Tables 2 and 3). Analysis of kinetic data for NADP⁺-dependent GDH in *S. cerevisiae*, *C. tropicalis*, *C. pseudotropicalis* and *C. parapsilosis* revealed Michaelis-Menten kinetics for both 2-oxoglutarate and ammonium, in contrast to the allosteric nature of the enzyme in *C. albicans*. However the K_m apparent for 2-oxoglutarate in *S. cerevisiae* (0.3 mM) was significantly lower than those observed in *Candida* (1.2, 3.2, and 5.6 mM for *C. tropicalis*, *C. pseudotropicalis* and *C. parapsilosis* respectively). The K_m apparent for ammonium was similar in all the yeasts (1.0–4.0 mM).

Relative activities of the two ammonium assimilation pathways

Continuous culture has been used to indicate whether an enzyme has an assimilating role by comparison of activities under nitrogen- and carbon-limited growth (Zwart & Harder, 1983). Fig. 3 shows that the activities of NADP⁺-dependent GDH, GOGAT and GS in *C. albicans* ATCC 10261 all increased with increasing nitrogen limitation. At high dilution rates approaching wash-out, activities were similar to those in batch culture. Under nitrogen limitation, at a low dilution rate (0.1 h⁻¹), both GDH and GOGAT were five- to sixfold higher than the activities under glucose limitation at the same dilution rate and three- to fourfold higher than batch culture activity. GS activity varied to a greater extent; at a dilution rate of 0.1 h⁻¹ under nitrogen limitation, activity was eightfold higher than that in batch culture, whereas under glucose limitation at this dilution rate activity was not detected. The dependence of all three enzyme activities on ammonium suggests that both pathways operate. The low level of GS in glucose-limited cultures compared with nitrogen-limited or with batch culture possibly reflects modification, degradation or decreased synthesis to conserve carbon as reported for *N. crassa* (Marzluf, 1981). Alternatively, GS activity may be affected by ammonium

concentration; the ammonium concentration in the glucose-limited medium is fivefold higher than that in the GSB medium used for the batch cultures. Our results are in contrast to those obtained by Zwart & Harder (1983), using continuous cultures of *Candida utilis*. In their study the activity of NADP⁺-dependent GDH was 10-fold higher under nitrogen limitation than with glucose limitation, whereas there was little change in either the GOGAT or the GS activities.

Comparative analysis of the kinetic data for the two enzymes has also been used to determine the relative significance of the two pathways (Mifflin & Lea, 1977). The GDH : GOGAT ratio in *C. albicans* was low in comparison to that in *S. cerevisiae* (Tables 2 and 3). Furthermore, the $S_{0.5}$ of GDH for 2-oxoglutarate in *C. albicans* was 7.1 mM, more than 10-fold higher than that in *S. cerevisiae*. This is considerably higher than the reported internal concentration of 2-oxoglutarate in *S. cerevisiae* of 0.12 mM (Bogonez *et al.*, 1985). The relative *in vivo* activities of GOGAT and NADP⁺-dependent GDH in each yeast can be estimated using a modified formula for a three substrate enzyme (Dixon & Webb, 1979):

$$\text{Velocity at intracellular concentration [C]} = \frac{V}{1 + K_m/[C]}$$

where C is the intracellular 2-oxoglutarate concentration, and is assumed to be 0.12 mM (other substrates in excess), and the V and K_m ($S_{0.5}$ for GDH) values for both enzymes are those given in Tables 2 and 3 and in the text. A K_m of 0.14 mM for GOGAT in *S. cerevisiae* was used (Roon *et al.*, 1974). The relative percentage velocities of GOGAT and GDH for *S. cerevisiae* would be 1.6% and 98.4%, respectively. This contrasts with values of 78% for GOGAT and 22% for GDH in *C. albicans*. Similar calculations made for the other *Candida* species indicate that the relative contribution of GOGAT was significant (13–70%). The actual ratio may be more in favour of GOGAT in *C. albicans* since the GDH K_m for ammonium (5 mM) is likely to exceed the *in vivo* concentration.

Collectively, these data indicate a significant role for GOGAT in *Candida* spp.

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