Proline-induced Germ-tube Formation in *Candida albicans*: Role of Proline Uptake and Nitrogen Metabolism

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Proline-induced germ-tube formation and cell-cell aggregation in four strains of *Candida albicans* were completely inhibited when the pH of the medium was 5.0 or lower, whereas morphogenesis induced by *N*-acetylglucosamine (GlcNAc) was unaffected even at pH 4.5. The pH sensitivity of proline-induced germ-tube formation was not caused by a modulation of proline uptake, which was unchanged over the pH range 4.5–6.5. The proline uptake system was specific, constitutive and subject to ammonium repression, and only one permease was detected, with a K_m of 179 μ M. Cultures deprived of nitrogen in the presence of glucose were derepressed for proline uptake but the yeast-mycelial transition could not be mediated by either proline or GlcNAc. The inhibition of morphogenesis was reversed when the nitrogen starvation was relieved by the addition of ammonium ions, proline, or certain amino acids. These results indicate that the nitrogen status of the cells is critical for the morphogenesis of *C. albicans*.

INTRODUCTION

Candida albicans maintains a yeast morphology at temperatures below 34 °C. The transition to mycelial morphology requires incubation at higher temperatures and the presence of an inducer, such as serum, amino acids or *N*-acetylglucosamine (GlcNAc) (Shepherd *et al.*, 1985). Land *et al.* (1975) and Dabrowa *et al.* (1976) both found that of the small number of amino acids and the imino acid proline that promoted filamentation, proline was the most effective. The complex defined medium devised by Lee *et al.* (1975) for growth of both the yeast and mycelial forms contains a number of amino acids and proline in addition to glucose, salts and biotin. Mitchell & Soll (1979) showed that in this medium germ-tube formation is sensitive to external pH, and Herman & Soll (1984) have used pH regulation as a system to compare the properties of the yeast and mycelial forms of *C. albicans*. We have found that external pH also controls dimorphism in a simple buffered proline solution, whereas the response to another inducer of dimorphism, GlcNAc, is relatively insensitive to decreased pH. The present study was undertaken to determine whether the pH-sensitive event is related to proline uptake and metabolism.

Previously published work on the uptake of proline into *C. albicans* is contradictory. Dabrowa & Howard (1981) found that the uptake system was highly specific and not inducible, although uptake by mycelial cells was two to three times higher than that by yeast cells. In contrast, Jayakumar *et al.* (1979) concluded that uptake was both constitutive and inducible and not completely specific in that a number of amino acids partially inhibited uptake. In *Saccharomyces cerevisiae* two proline uptake systems have been described, neither of which is inducible (Lasko & Brandriss, 1981); in addition to a specific, high-affinity system (K_m 250 μ M) there is a low-affinity system (K_m 13 mM), probably the general amino-acid permease (GAP), which operates at high external concentrations of proline. Magaña-Schwenke *et al.* (1973) have also reported a specific high-affinity uptake system for imino acids in *S. chevalieri*, and showed that derepression operates under conditions of nitrogen starvation (Kuznar *et al.*, 1973). Verma &

Prasad (1983) reported that derepression of amino acid transport does not occur in *Candida* species.

This paper characterizes the proline uptake system in *C. albicans* under a variety of physiological conditions and presents evidence that proline uptake is unaffected at pH values which inhibit proline-induced germ-tube formation.

METHODS

Organism. *Candida albicans* strains (ATCC 10261, A72, 85/031 and a Dunedin hospital isolate) were propagated and maintained on malt extract agar slopes. Dry weight was determined from OD₅₄₀ in a Shimadzu spectrophotometer with reference to a standard curve.

Culture conditions and germ-tube formation. Yeast cells were grown as shake cultures at 28 °C in glucose/salts/biotin medium (GSB) (Shepherd & Sullivan 1976). The nitrogen source was 10 mM-(NH₄)₂SO₄ unless otherwise stated. To prepare cells for optimal germ-tube formation, cultures were starved by aeration in distilled water (Shepherd *et al.*, 1980); germ-tube formation was induced by incubation at 37 °C in 10 mM-imidazole buffer at a cell concentration of $1.5\text{--}2.0 \times 10^7$ cells ml⁻¹ with either 2.5 mM-GlcNAc (Shepherd *et al.*, 1980) or 10 mM-proline (Dabrowa & Howard, 1981). Germ-tube formation was assessed by phase-contrast microscopy after dispersal of cell aggregates by mild sonication (10–15 s) in 0.1 M-NaOH containing 1% (w/v) SDS.

Assay of cell aggregation. The yeast cultures of strain ATCC 10261 do not form aggregates of cells, whereas germ-tube formation is accompanied by extensive flocculation. This flocculation is magnesium/calcium dependent and closely parallels germ-tube formation (unpublished results). We have found that an assay of cell-cell aggregation can be used routinely as an assessment of germ-tube formation. The degree of flocculation was determined by a sedimentation assay, as follows. A 3 ml sample of a vortexed cell suspension in a plastic cuvette was centrifuged briefly (0–1000 r.p.m. before braking). The OD₅₄₀ was measured in the cuvette immediately after centrifuging (OD_{540 initial}) and again after resuspending any sedimented cells (OD_{540 final}). The percentage aggregation was calculated from the formula $(\text{OD}_{540 \text{ final}} - \text{OD}_{540 \text{ initial}})/\text{OD}_{540 \text{ final}}$. Suspensions of non-flocculant yeast cells gave a background reading of $10\% \pm 4\%$.

Measurement of L-proline uptake. A final concentration of 50 µM uniformly labelled [¹⁴C]proline (Amersham) (specific activity 2.5 mCi mmol⁻¹, 92.5 MBq mmol⁻¹) was used in the experiments designed to examine the effects of nitrogen source, external pH and various inhibitors on proline uptake. A yeast cell suspension ($1.0\text{--}3.0 \times 10^7$ cells ml⁻¹) in either 50 mM-imidazole buffer or culture medium was preincubated at the required temperature for 15 min before addition of 10 µl 5.0 mM-[¹⁴C]proline. At 2 min intervals 50 µl samples were removed and washed on Whatman GFC filters, once with 10 mM unlabelled proline and three times with distilled water. The filters were dried at 60 °C and the radioactivity determined by scintillation counting.

Kinetic parameters were determined for different culture conditions by measuring uptake as above for a range of proline concentrations from 50 µM to 12.5 mM, achieved using three specific activities, 2.5 mCi mmol⁻¹ (50–125 µM), 0.25 mCi mmol⁻¹ (500–1000 µM) and 0.025 mCi mmol⁻¹ (5.0–12.5 mM). At the lowest specific activity 200 or 500 µl samples were taken from 2.0 ml cultures. In experiments in which cells were preincubated in proline, cells were washed three times in 2 vols buffer before assay.

For incorporation experiments, [¹⁴C]proline (20 µl, 0.25 µCi, 1.0 µmol), was added to a 2.0 ml suspension (1.5×10^7 cells ml⁻¹) of starved cells in imidazole buffer at 28 °C. At 30 min intervals 30 µl samples were removed into ice-cold 10 mM-proline for determination of uptake into whole cells; 120 µl samples were centrifuged, and after removal of 20 µl of the supernatant for assay of remaining radioactivity, the cell suspension was mixed with 100 µl ice-cold 20% TCA for determination of uptake into TCA-precipitable material (precipitated at 100 °C for 20 min).

Chemicals. Proline, GlcNAc, sarcosine, azetidine-2-carboxylic acid, hydroxyproline and amino acids were obtained from Sigma. All other chemicals were of analytical grade.

RESULTS

Conditions of germ-tube formation

The amino acid/salts medium of Lee *et al.* (1975) is commonly used for induction of germ-tube formation. The individual components of this medium were tested in various combinations for their ability to induce germ-tube formation in *C. albicans* strains ATCC 10261 and A72 (Table 1). Strain ATCC 10261 will not consistently form germ-tubes after growth in GSB unless it has been starved by aeration in distilled water (Shepherd *et al.*, 1980). In contrast, yeast cells of strain A72 could be used immediately after harvest from GSB and it is referred to as a high responder strain (Mattia *et al.*, 1982). The results demonstrated that of the constituents of Lee's

Table 1. Germ-tube formation in Lee's medium and in solutions of various amino acids

C. albicans strains A72 and ATCC 10261 were grown as yeast cultures in GSB, washed and resuspended in the test medium at a cell concentration of $1.5\text{--}2.0 \times 10^7$ cells ml^{-1} . Cells of strain A72 were used immediately after harvesting; ATCC 10261 cells were first starved by aeration. Germ-tube formation was induced and the percentage of germ-tube-forming cells was determined by microscopy as described in Methods. The amino acids were all added at 10 mM final concentration in 10 mM-imidazole buffer pH 6.8. Values shown are the range found for at least three different experiments.

Medium	Germ-tube formation (%)	
	ATCC 10261	A72
Lee's medium	> 90	> 90
Lee's medium minus glucose	> 90	> 90
Salts solution of Lee's medium	0	0
Amino acid mixture from Lee's medium	> 90	> 90
Glucose only	10–20	20–30
Methionine, lysine, phenylalanine	0	10–20
Alanine, lysine, leucine	> 10	10–20
Proline, alanine	60–80	> 90
Proline, leucine	60–80	> 90
Proline, ornithine	> 90	> 90
Proline, arginine	> 90	> 90
Ornithine, arginine	50–60	50–60
Proline	80–90	> 90
Arginine	40–60	50–60
Glutamine	40–60	60–80

medium, only proline is able to act as an effective inducer of germ-tube formation. Arginine and glutamine, which are not present in Lee's medium, could also induce germ-tube formation. Ornithine gave a partial induction but no other amino acid tested individually could effect the transformation. No germ-tubes were observed when either D-proline or L-proline immobilized onto agarose (Sigma) was used as inducer.

The time course of germ-tube induction by proline was similar to that observed with GlcNAc (Shepherd *et al.*, 1985). The first protuberances were observed 60–90 min after exposure to the proline at 37 °C. After 150 min germ-tubes three to five times the length of the mother cell had appeared in more than 90% of the yeast cells. Usually only one germ-tube was associated with each yeast cell but occasionally (<5%) two tubes developed on one yeast cell. Germ-tubes induced by proline were identical in appearance to those produced in GlcNAc. For both proline and GlcNAc a concentration of at least 0.5 mM was required for optimal germ-tube formation.

Since proline had been shown to be the active component of Lee's medium, it was of interest to determine whether proline-induced morphogenesis was also subject to pH regulation. Table 2 shows the effect of varying external pH on morphogenesis. With either proline or GlcNAc as inducer, at pH 6.5 or 5.6, germ-tube formation was >80% and accompanied by extensive cell-cell aggregation (>85%, see Methods). If the initial pH of the buffering solution (either 10 mM-imidazole or 50 mM-potassium phthalate) was 5.0 or 4.5, then proline-induced germ-tube formation was inhibited, whereas GlcNAc induction was unaffected. Similarly, cell-cell aggregation was <10% in proline at pH 4.5 but >80% in GlcNAc buffered to pH 4.5. The final pH was determined at the end of each experiment. The data shown are for strain ATCC 10261; similar results were obtained with the other three strains. The following experiments were undertaken to determine whether the observed pH regulation reflected an inhibition of proline uptake.

Proline uptake by yeast cells

At 4 °C, in a suspension of starved cells of strain ATCC 10261, no uptake was detectable for a range of proline concentrations (25 to 1000 μM), even when assays were extended up to 1 h. Uptake was, however, observed at 28 °C; at the lowest concentration tested (25 μM), the uptake

Table 2. *Effect of pH on GlcNAc-induced and proline-induced germ-tube formation*

Starved yeast cells of *C. albicans* ATCC 10261 were washed and resuspended in 5 ml of each buffer at the indicated pH values at a cell concentration of $1.5\text{--}2.0 \times 10^7$ cells ml^{-1} . The cultures were incubated at 37 °C for 3 h and germ-tube formation was assayed as described in Methods. The pH of the supernatants was measured after germ-tube formation. With phthalate buffer it was unchanged; in imidazole buffer the final pH was 5.1 when the initial pH was 4.5, and 5.8 when the initial pH was 6.5.

Buffer	Initial pH	Germ-tube formation (%) [*]	
		GlcNAc	Proline
Imidazole (10mM)	6.5	90	90
	4.5	90	< 10
Phthalate (50mM)	6.2	90	90
	5.6	90	90
	5.0	90	< 10
	4.6	90	0

^{*}Percentage of germ-tube forming cells (standard error $\pm 10\%$).

Table 3. *Effect of culture conditions on proline uptake rate*

Cultures of *C. albicans* ATCC 10261 yeast cells were harvested after 18 h growth and proline uptake was determined under the conditions described below. Cell growth, culture incubations and uptake assays were all performed at 28 °C. The growth medium was GSB; for experiments 6 and 7, bactopectone was added. The uptake $V_{\max}$ values were calculated from a typical experiment which was repeated three times.

Expt	Culture and assay conditions	$V_{\max}$ [nmol (mg dry wt) ⁻¹ min ⁻¹]
1	Cells incubated in fresh GSB medium for 3 h before assay	0.43
2	Cells incubated in fresh nitrogen-free GSB medium for 3 h before assay	2.2
3	Cells incubated in fresh glucose-free GSB medium for 3 h before assay	0.22
4	Starved [*] cells	2.87
5	Starved [*] cells; incubated for 1 h with proline (10mM) before assay [†]	5.88
6	Starved [*] cells	6.24
7	Starved [*] cells incubated for 1 h with proline (10mM) before assay [†]	9.18

^{*} Cells were starved by aeration (100 ml min^{-1} per 100 ml cell suspension) in distilled water for 18 h at room temperature before resuspending and incubation for 1 h at 28 °C in imidazole buffer with or without 10 mM-proline.

[†] Before the uptake assay cells were washed free of proline by centrifugation (three times) and resuspension in buffer.

rate was $0.34 \text{ nmol (mg dry wt)}^{-1} \text{ min}^{-1}$. In addition, prelabelled cells did not lose any significant amount of label when kept on ice for up to 3 h.

At 28 °C, in exponential-phase yeast cells (18 h in GSB), there was a low basal rate of proline uptake [$V_{\max} 0.04 \text{ nmol (mg dry wt)}^{-1} \text{ min}^{-1}$] which increased if proline (final concentration 10 mM) was added to the culture 1 h before washing in spent medium and assay [$V_{\max} 0.22 \text{ nmol (mg dry wt)}^{-1} \text{ min}^{-1}$]. The pH of spent medium was low (3.2–3.8) but proline uptake was unaffected if the pH was adjusted to that of fresh medium. The effect on uptake of altering the culture conditions is shown in Table 3. Transferring the cells to fresh medium increased uptake approximately ten-fold and this was increased a further five-fold if the fresh medium did not contain a nitrogen source. Omitting glucose from the medium did not result in an increase in uptake. An increased rate of uptake was also found if cells were starved of both carbon and nitrogen reserves by aeration in distilled water (Table 3, experiment 4); this was also observed for similarly treated cells which had been grown in the nitrogen-rich medium of GSB

Fig. 1

Fig. 2

Fig. 1. Proline uptake by *C. albicans* ATCC 10261. Proline uptake at 28 °C was determined for a range of proline concentrations in yeast cells cultured under the following conditions: starved by aeration and preincubated for 1 h in buffer (●); starved by aeration and preincubated for 1 h in 10 mM-proline (○); preincubated for 6 h and assayed in GSB without ammonium sulphate (□).

Fig. 2. Derepression of proline uptake by *C. albicans* ATCC 10261. Yeast cells from an 18 h culture (28 °C) in GSB were centrifuged and resuspended in either NH_4^+ -free medium (●) or fresh complete medium (○) and reincubated at 28 °C. At the times indicated 1 ml samples were removed and the proline uptake rate was determined at 28 °C as described in Methods.

supplemented with 1% (w/v) bactopectone (Table 3, experiment 6). In both cases a still higher uptake rate was observed after preincubation with proline (Table 3, experiments 5 and 7). The maximum uptake rate was achieved in cells which had been grown in nitrogen-rich medium, starved by aeration and then preincubated with proline for 1 h.

Double reciprocal plots of proline uptake for cells produced under a variety of conditions are shown in Fig. 1 (concentration range 50 μM to 2.0 mM). The V_{max} varied, and the K_m , calculated by a direct linear plot (Eisenthal & Cornish-Bowden, 1974), had a mean value of 179 μM (SD 12.7). Data for cultures containing ammonium ions could not be shown on the scale of Fig. 1 but the same K_m was obtained and the V_{max} values are given in Table 3 or in the text. To investigate the possibility that another permease operates at high proline concentrations, uptake rates were determined over the concentration range 2.0–12.5 mM. For starved cells, with or without proline preincubation, there was no significant increase in the proline uptake rate. Cells cultured in the presence of ammonium ions were not tested at these concentrations as the uptake rates at the lower specific activities were too low.

The results in Table 3 suggested that the proline uptake system in *C. albicans* is subject to ammonium repression. Fig. 2 shows the time course of derepression of proline uptake following removal of the nitrogen source. In cells transferred from an 18 h culture to fresh GSB the uptake rate increased, but the increase was five- to six-fold higher when the fresh medium lacked ammonium ions. A maximum rate was obtained within 2 h of transfer. Both ammonium ions and glutamine were found to mediate repression of proline uptake; the uptake rate for cells transferred from GSB to GSB containing 10 mM-glutamine as the nitrogen source was sevenfold lower (at 2 h) than that for cells transferred to nitrogen-free GSB. The repressive effect of ammonium ions or glutamine was not due to direct competition for the permease, since neither ammonium sulphate (10 mM) nor glutamine (5 mM) inhibited proline uptake when added to derepressed cultures immediately before assay. However, 13 min after the addition of ammonium sulphate or glutamine, proline uptake was reduced from 0.29 to 0.07 and from 0.25 to 0.13 $\text{nmol (mg dry wt)}^{-1} \text{ min}^{-1}$, respectively. Derepression also occurred in cells transferred from GSB to medium lacking both nitrogen and glucose (results not shown) and in cultures starved by aeration in distilled water (Table 3); however, in these cultures proline uptake could

not be repressed by the subsequent addition of ammonium sulphate. Cells transferred to GSB containing either alanine (doubling time 2.5 h) or leucine (doubling time 6.0 h) as the sole nitrogen source were derepressed for proline uptake [uptake rates 0.3 and 0.5 nmol (mg dry weight)⁻¹ min⁻¹ respectively after 6 h, proline concentration 50 µM].

Specificity of the proline uptake system

A number of analogues of proline and other compounds were tested for their effect on proline uptake, germ-tube formation and cell-cell aggregation. Proline uptake was assayed as described in Methods, the final concentration of proline was 50 µM and analogues or other compounds were added to a final concentration of 1.0 mM 5 min before addition of [¹⁴C]proline. D-Proline, sarcosine and azetidine-2-carboxylic acid all inhibited proline uptake to a similar degree (60–70%), but of these compounds only sarcosine and azetidine-2-carboxylic acid (10 mM) completely inhibited germ-tube formation and cell-cell aggregation induced by L-proline (1.0 mM). Azetidine-2-carboxylic acid, at concentrations greater than 0.1 mM, also inhibited germ-tube induction by GlcNAc, but sarcosine (10 mM) had no effect. Canavanine, an analogue of arginine, also inhibited both GlcNAc- and proline-induced germ-tube formation. Glutamine, glycine, alanine, histidine, arginine, hydroxy-L-proline and ammonium ions (as chloride or sulphate) had no significant effect on proline uptake, indicating the specificity of the permease.

Effect of pH on uptake and incorporation of [¹⁴C]proline

In two different buffer systems (imidazole and phthalate), uptake at pH 4.5 was similar to that at pH 6.5, irrespective of temperature and preincubation conditions (Table 4). Fig. 3 shows, for both pH values, the uptake of proline into whole cells, the incorporation of radioactivity into TCA-precipitable material, and the concomitant loss of radioactivity from the medium. This experiment confirms that lowering the pH does not reduce the uptake of proline into *C. albicans*, nor the intracellular incorporation of the radioactive label into macromolecules. Indeed there was slightly more incorporation in the cultures at pH 4.5 than in those at pH 6.5. At pH 4.5 approximately 25% of the radioactivity was lost from the cultures, presumably as carbon dioxide. A similar pattern of incorporation was observed at both pH values when the proline concentration was increased to 2 mM. However, after 3 h only 20% of the radioactivity had been removed from the medium.

Nitrogen status and germ-tube formation

It has been previously observed that in some strains of *C. albicans*, starvation is a necessary prerequisite for germ-tube formation (Shepherd *et al.*, 1980), and exponentially growing cells of strain ATCC 10261 do not reproducibly form germ-tubes when transferred directly to 37 °C with an inducer. However, when cells harvested at mid-exponential phase were first incubated in glucose-free medium for at least 3 h, germ-tube formation was consistently induced in >90% of the cells (Fig. 4). In contrast, cells starved of nitrogen in the presence of glucose for 3 h or more were not competent to undergo morphogenesis. The results shown are for the GlcNAc induction system and strain ATCC 10261. Similar results were obtained when proline was used as inducer, and nitrogen-starved cultures of the three other strains used in this study were also unable to form germ-tubes in the presence of either inducer. The ratio of viable count to OD₅₄₀ was unchanged after 24 h of either nitrogen or glucose starvation. If proline, glutamine, asparagine, arginine or ornithine replaced ammonium ions in the medium, cells remained competent to form germ-tubes. In contrast, cells transferred to medium containing leucine, alanine or phenylalanine as the nitrogen source did not, although these compounds can act as a sole source of nitrogen for growth. Nitrogen-starved cells regained the capacity to form germ-tubes if they were transferred to GSB medium containing any one of ammonium ions, proline, arginine, ornithine, glutamine or asparagine as the nitrogen source for at least 3 h. Nitrogen-starved cells subjected to glucose or glucose/nitrogen starvation remained refractory for germ-tube formation. These results are summarized in Fig. 5. Batch cultures grown in glucose-limiting conditions (GSB in which the glucose concentration was reduced 10-fold) produced cells

Fig. 3

Fig. 3. Effect of pH on the incorporation of [^{14} C]proline into whole cells and TCA-precipitable material. Starved yeast cells of *C. albicans* ATCC 10261 were resuspended in 2.0 ml imidazole buffer (10 mM). The uptake of [^{14} C]proline into whole cells and TCA-precipitable material, and the disappearance of radiolabel from the medium, were determined as described in Methods. ○, ●, Uptake into whole cells, at pH 6.5 (○) and at pH 4.5 (●); □, ■, uptake into TCA-precipitable material, at pH 6.5 (□) and at pH 4.5 (■); △, ▲, radioactivity in culture supernatant, at pH 6.5 (△) and at pH 4.5 (▲).

Fig. 4

Fig. 4. Effect of nitrogen and glucose limitation on germ-tube formation. Cells of *C. albicans* ATCC 10261 were harvested by centrifugation from 18 h cultures in GSB, transferred to GSB lacking either glucose (●) or ammonium ions (○) and incubated at 28 °C. Samples (1.0 ml) were taken at 1 h intervals and washed cells assayed for the ability to form germ-tubes in imidazole buffer with GlcNAc at 37 °C at cell concentrations of $1.5\text{--}2.0 \times 10^7$ cells ml^{-1} .

Table 4. Effect of pH on proline uptake

Suspensions of *C. albicans* ATCC 10261 cells that had been starved by aeration were washed and resuspended in either imidazole (10 mM) or phthalate (5.0 mM) buffer at pH 4.5 or pH 6.5. The rate of proline uptake was measured as described in Methods.

Buffer	Temperature of assay (°C)	Uptake rate [nmol (mg dry wt) $^{-1}$ min $^{-1}$]	
		pH 4.5	pH 6.5
Imidazole	28	0.36	0.44
Imidazole	37	0.95	0.98
Phthalate	28	0.44	0.46
Phthalate	37	0.84	0.75

competent to form germ-tubes whereas cells cultured in nitrogen-limiting conditions (GSB in which the ammonium sulphate concentration was reduced 10-fold) to stationary phase were not.

DISCUSSION

Proline was found to be the sole effector of morphogenesis in Lee's medium; the carbon source (glucose), and vitamin (biotin), were only required to maintain growth (Table 1). The observations of Pollack & Hashimoto (1987), who report proline-induced germ-tube formation

Fig. 5. Schematic representation of the relationship between the nutritional status of the cells and morphogenesis.

at pH 3.7, are difficult to reconcile with our data and those of Mitchell & Soll (1979). Morphogenesis in Lee's medium is pH dependent (Mitchell & Soll, 1979). We have shown a similar sensitivity to pH for germ-tube formation in a simple buffered proline solution, whereas GlcNAc induction was unaffected. The proline transport system was examined to determine whether this differential sensitivity resulted from an inhibition of proline uptake at low pH.

Two characteristics of the proline transport system in *C. albicans* suggest that regulation of proline uptake is not related to morphogenesis. Firstly, uptake is unaffected at a pH inhibitory of germ-tube formation (Table 4, Fig. 3). Secondly, although uptake is relatively high [$V_{\max}$ 2.2 nmol (mg dry weight) $^{-1}$ min $^{-1}$] in cultures starved of nitrogen in the presence of glucose (Table 3), these cells did not form germ-tubes in the presence of either inducer (Fig. 4). Thus, although we have confirmed that L-proline can act, like GlcNAc (Simonetti *et al.*, 1974), as a specific inducer of germ-tube formation (Dabrowa *et al.*, 1976), uptake of proline does not necessarily promote the transition from the yeast to the mycelial form. Mattia *et al.* (1982) reported that responsiveness to germ-tube induction was not correlated with the capacity to take up and metabolize GlcNAc. Furthermore, GlcNAc immobilized onto agarose can act as an inducer for germ-tube formation in *C. albicans* (Shepherd & Sullivan, 1983). We found immobilized proline to be ineffective, but the possibility that proline acts as an external effector of morphogenesis cannot be excluded as GlcNAc-agarose is linked via the C6 of the sugar moiety, whereas proline is linked to agarose via the -NH group. The secondary amine sarcosine inhibited proline uptake and proline-induced germ-tube formation, but had no effect on GlcNAc induction of morphogenesis. Again, the possibility of proline action being extracellular cannot be excluded although any receptor system would be separate from that for GlcNAc. By contrast, another proline analogue, azetidine-2-carboxylic acid, inhibited both proline- and GlcNAc-induced germ-tube formation and cell-cell aggregation. Azetidine-2-carboxylic acid, like the arginine analogue canavanine, causes synthesis of defective proteins (Peterson & Fowden, 1963). Previous studies have shown that *de novo* protein synthesis is critical during

germ-tube formation (Shepherd *et al.*, 1980; Sullivan *et al.*, 1983), and azetidine-2-carboxylic acid may be incorporated into germ-tube-specific proteins rendering them defective.

Characterization of the proline uptake system showed it to be constitutive, as found by Dabrowa & Howard (1981) and Jayakumar *et al.* (1979), specific, confirming the results of Dabrowa & Howard (1981), and partially inducible in that preincubation in proline increased uptake, a finding also reported by Jayakumar *et al.* (1979). We also found that proline uptake was subject to ammonium repression; removal of nitrogen from the medium resulted in an increase of the proline uptake rate in both the presence and absence of glucose. Verma & Prasad (1983) found no evidence for ammonium repression of the uptake of a number of amino acids or the imino acid proline in *C. albicans*, but it has been demonstrated in *S. chevalieri* (Kuznar *et al.*, 1973), *S. cerevisiae* (Lasko & Brandriss, 1981) and *Aspergillus nidulans* (Arst *et al.*, 1980). The difference in uptake rates between yeast and mycelial cells found by Dabrowa & Howard (1981) may have reflected derepression occurring in the mycelial cultures before the uptake determination.

Considerable variation was observed in the uptake rate under different culture conditions, but over a wide range of proline concentrations (50 μM to 12.5 mM), only one uptake system was detected, with a K_m of 179 μM . We could not detect uptake by either diffusion, as suggested by Dabrowa & Howard (1981), or the operation of a separate low-affinity permease at high external concentrations as shown for *S. cerevisiae* (Lasko & Brandriss, 1981).

The observation that either the amino sugar GlcNAc or the imino acid L-proline is sufficient to effect morphogenesis led us to examine the role of the nitrogen status of the cell in the yeast-to-mycelial transformation. Fig. 5 summarizes the effect of altering nitrogen status on the competence of yeast cells to form germ-tubes. Essentially, limitation of the carbon source for strain ATCC 10261 produced cells competent for morphogenesis, but in the presence of glucose, removal of the nitrogen source reversibly abolished the ability of cells to form mycelial elements for the four strains tested. In batch culture, the ability of stationary-phase cells to undergo morphogenesis depended on the nature of the limiting nutrient. Cells limited for nitrogen were unable to form germ-tubes whereas with stationary-phase cells from glucose-limited cultures germ-tubes could be induced by either GlcNAc or proline. This was confirmed using nitrogen-limited cells from chemostat cultures (A. Collings & A. R. Holmes, unpublished results). Several workers have observed that mycelial induction is favoured by using stationary phase cells (Chaffin & Sogin, 1976; Soll & Bedell, 1978; Shepherd *et al.*, 1980), which under the conditions used would have been glucose limited. It has been postulated that germ-tube formation occurs only when the cells are blocked at an early stage (G1) of the cell cycle (Soll & Bedell, 1978). This was challenged by Mattia & Cassone (1979), who demonstrated that in the presence of a suitable inducer cells at any stage of growth are able to form germ-tubes at 37 °C. The present study would suggest that it is the nutritional status of the cells rather than the stage of the cell cycle that determines the competence to undergo morphogenesis.

It is tempting to speculate that the two known simple chemical inducers of morphogenesis, proline and GlcNAc, act in a similar manner via catabolism to a common intermediate. Proline is catabolized via glutamate to 2-oxoglutarate (Land *et al.*, 1975). During GlcNAc metabolism, glutamine is produced (Gopal *et al.*, 1982), from which 2-oxoglutarate could be formed, via glutamate. Glutamate synthase activity has been demonstrated in *S. cerevisiae* (Roon *et al.*, 1974). The amino acids, such as arginine, that can give partial induction of germ-tube formation and which can also reverse the inhibitory effects of nitrogen starvation on morphogenesis, are also metabolized via 2-oxoglutarate. During nitrogen starvation, in spite of an increased uptake of proline, there is no induction of germ-tube formation. Using low cell concentrations, Pollack & Hashimoto (1987) reported that glucose can inhibit germ-tube formation, and suggested that the action of glucose is mediated by catabolite repression. We speculate that under conditions of nitrogen starvation, which may produce a glucose repression effect, yeast morphology is favoured and for morphogenesis to occur the tricarboxylic acid cycle must be stimulated. This would result in an increased production of CO_2 , and recently, Sims (1986) has reported that elevated CO_2 partial pressure enhances the mycelial growth of *C. albicans* at the normally suboptimal temperature of 30 °C.

This study has shown that pH-regulated morphogenesis is not the result of modulation of the inducer uptake. We have demonstrated that the nutritional status of the cell is a critical factor in determining whether cells of *C. albicans* will undergo morphogenesis and suggest that proline and GlcNAc may act in a similar manner through a common intermediate of catabolism.

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