Proline Uptake in *Candida albicans*

By NINA DABROWA AND DEXTER H. HOWARD*

Department of Microbiology and Immunology, UCLA School of Medicine,
Los Angeles, California 90024, U.S.A.

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L-Proline entered both mycelial and yeast cells of *Candida albicans* by an active transport system of high specificity at low (<0.1 mM) external concentrations of substrate. The apparent K_m value of this system was 0.1 mM for both types of cells, while the V value was 4 nmol min⁻¹ (mg dry wt)⁻¹ for mycelial cells and 1.4 nmol min⁻¹ (mg dry wt)⁻¹ for yeast cells. At L-proline concentrations greater than 0.1 mM, the amino acid appeared to enter both morphological forms by diffusion as well as active transport. As saturation was approached diffusion became increasingly important. The higher uptake rate of mycelial cells seemed not to be the result of an inducible system. The optimal pH and temperature for transport of L-proline were 7.0 and 37 °C, respectively. Sodium azide and the proline analogues sarcosine and L-azetidine-2-carboxylic acid inhibited L-proline uptake, while L-thiazolidine-4-carboxylic acid was less effective. The active transport system was highly specific for L-proline since neither ammonium ions, which inhibit the general amino acid transport system of fungi, nor 16 different amino acids interfered substantially with uptake.

INTRODUCTION

Blastospores of the pathogenic fungus *Candida albicans* (Robin) Berkhout may initiate a mycelial form of growth by germination (Odds, 1979). The formation of germ tubes is fostered by a variety of environmental factors but an incubation temperature of 37 °C seems mandatory (Odds, 1979).

Among amino acids that induce germination, those that enter the metabolism by conversion to α -ketoglutarate are generally the more active (Land *et al.*, 1975), and in our experience proline is one of the most efficient (Dabrowa *et al.*, 1976). A long-term goal of our work is to study the role of proline in germ tube formation by *C. albicans*. In this paper we describe the characteristics of L-proline transport by blastospores (yeast cells) and germings (mycelial cells) of *C. albicans*.

METHODS

Organism. The strain of *Candida albicans* used was no. 304 from the Fungus Collection, University of California, Los Angeles, U.S.A. Stock cultures were maintained on glucose peptone agar in a refrigerator and were subcultured every month.

Media and buffers. Glucose peptone agar (GPA), glucose biotin salts medium (GBS) and glucose yeast extract broth (GYE) have been described previously (Dabrowa *et al.*, 1976). The STM-Tris buffer contained 10 mM-KCl, 2.4 mM-CaCl₂, 2.5 mM-MgCl₂ and 50 mM-Tris, and was titrated to the desired pH with 6 M-HCl. L-Proline (10 mM) was added to STM-Tris buffer to provide the medium in which the germings were prepared.

Growth and preparation of the fungal cells. Cells from GPA slants harvested after 24 h at room temperature were used to inoculate flasks of GBS. These were incubated on a gyrotory shaker (New Brunswick Scientific Co.) for 24 h at room temperature. Cells were harvested by centrifugation, washed twice and resuspended in STM-Tris buffer (pH 7.2). Such cells were standardized and used as yeast cells in uptake experiments. Germings, which represented the mycelial cells, were prepared by incubating yeast cells at 37 °C for 3 h in STM-Tris buffer

containing 10 mM-L-proline on a gyrotory shaker. The germlings were centrifuged, washed twice and resuspended in STM-Tris buffer. Standardized suspensions were prepared and used in the uptake experiments.

Measurement of L-proline uptake. Uniformly ^{14}C -labelled proline [$294 \text{ mCi mmol}^{-1}$ ($10.88 \text{ GBq mmol}^{-1}$); New England Nuclear] was used. The radioactive amino acid was diluted with unlabelled L-proline (Calbiochem) to achieve the desired molar concentrations of the amino acid. The uptake was measured by the method of Gupta & Howard (1971). Equal volumes of 2 μM -proline and of cell suspension (2 mg dry wt cells ml^{-1}) in STM-Tris buffer were placed in separate 50 ml flasks in a water bath shaker (New Brunswick Scientific Co.) and maintained at 37 °C for 15 min. After temperature equilibration the proline was added to the cell suspension and the flasks were shaken throughout the experiment. At intervals, samples (1.0 ml) were taken and filtered through membrane filters (0.45 μm), and portions (0.5 ml) of the filtrate were used to measure the radioactivity remaining. The decrease in radioactivity at various times compared with the whole suspension represented the uptake of L-proline. The scintillation fluid contained the following chemicals per litre of dioxan: naphthalene, 70 g; 2,5-diphenyloxazole, 7 g; 1,4-bis-2-(5-phenyloxazolyl)benzene, 0.05 g. The vials were counted for 10 min in a Beckman scintillation counter, model LS 3133T, and the counts were corrected for background. Other details of the procedures involved in uptake measurements have been published previously (Dabrowa & Howard, 1976; Gupta & Howard, 1971; Tang & Howard, 1973).

RESULTS

Initiation of blastospore germination

L-Proline triggers the germination of *Bacillus megaterium* spores but neither uptake nor metabolism of the amino acid is required for it to function as an inducer (Rossignol & Vary, 1979). Preliminary experiments were conducted to see whether or not such a triggering mechanism was operative in the L-proline-induced germination of *C. albicans* blastospores. Washed yeast cells from cultures in GBS medium incubated at 23 °C for 24 h were exposed to 10 mM-L-proline in STM-Tris buffer (pH 7.2) at 37 °C. Samples were withdrawn at intervals, washed once and resuspended in buffer at 37 °C. The percentage of germination among buffer-resuspended cells is shown in Table 1. Cells harvested from GBS did not germinate in buffer which did not contain the inducer. Virtually no germination was seen in cells harvested after 15 min exposure to proline. After 30 min a substantial number of the exposed cells germinated, and by 45 to 60 min most of the cells completed germination in the absence of the inducer. In fact, a large number (80%) of the cells harvested at 60 min had very small germ tubes that continued to elongate in the buffer medium. Thus, proline did not act as a simple trigger device in the germination of *C. albicans* blastospores. A substantial period of exposure to, and presumably metabolism of, the amino acid was required for germination to take place.

Table 1. *Initiation of germination of Candida albicans blastospores after exposure to L-proline for various times*

Cells grown at 23 °C in GBS were harvested, washed, and resuspended in 10 mM-L-proline in buffer and incubated at 37 °C. Cells were harvested at the indicated times, washed and resuspended in buffer at 37 °C. The percentage of the blastospores which germinated was recorded after 3 h.

Time of exposure to L-proline (min)	Germination (%)
0	0
15	2
30	33
45	71
60	80
75	80
180	95

Table 2. Germination of the blastospores of *Candida albicans* at different stages of growth tested in two media

Cells harvested from GYE broth were washed in saline and inoculated into flasks of fresh GYE broth. Cultures were incubated at 23 °C on a gyrotory shaker. The numbers of cells ml⁻¹ were determined by direct microscopic count in a haemocytometer. At the indicated times, a sample was removed and the cells were recovered by centrifugation. The cells were washed once and resuspended (5×10^6 cells ml⁻¹) in 10 mM-proline buffer or lamb's serum (Gibco, U.S.A.) at 37 °C. The percentage of the blastospores which germinated was recorded after 3 h.

Time (h)	10 ⁻⁷ × No. of cells ml ⁻¹	Germination (%)	
		In proline buffer	In serum
0	0.7	66	90
1	0.7	90	95
2	1.0	88	95
3	1.0	90	98
4	1.4	76	95
5	1.7	44	96
6	2.2	18	95
7	2.4	2	90
8	4.5	2	95
9	6.5	0	95
10	8.0	0	96
24	20.0	60	95

Germination of blastospores at different stages of growth

Soll & Bedell (1978) have confirmed an earlier observation of Chaffin & Sogin (1976) that a temperature shift (from 25 to 37 °C) induced germination of the blastospores of *C. albicans* in amino acid-containing defined media only after the cells had entered a stationary phase of growth. When cells began to proliferate at 25 °C, a temperature shift no longer induced germination (Soll & Bedell, 1978). Since such a commitment might influence our studies on amino acid uptake we re-examined this point. Table 2 shows that blastospores in stationary phase (0 time and 24 h) germinated when placed in proline buffer and that the percentage of germination decreased as the cells entered an exponential phase of growth (4–10 h). Interestingly, this commitment was only apparent in the synthetic medium. In serum, even rapidly dividing cells germinated promptly after shift to 37 °C. Accordingly, we germinated stationary phase cells in proline buffer to obtain the germlings used for comparative studies (see Methods).

Time course of uptake of L-proline

The time course of L-proline uptake by yeast and mycelial cells of *C. albicans* is shown in Fig. 1. Both types of cells accumulated the amino acid as a linear function of time until the exogenous supply became limiting. There was no lag in uptake and the amino acid in the soluble pool first increased and then rapidly decreased. The initial velocity was higher in mycelial cells than in yeast cells.

An inducible transport system for L-proline in *C. albicans* has been described (Jayakumar *et al.*, 1979). Since the mycelial phase cells were germlings induced by incubation in proline, the higher rate of uptake might have been due to this preliminary exposure to the amino acid. To investigate this possibility, cells germinated in glutamic acid were tested for proline uptake. Figure 2 shows there was no enhancement of the uptake of L-proline in proline-germinated cells as compared to glutamic acid-germinated cells. These results, together with the specificity of the proline transport system (see below), suggested that the increased uptake by the mycelial cells was not due to an induced transport system.

Fig. 1

Fig. 2

Fig. 1. Time course of uptake of L-proline by yeast and mycelial cells of *Candida albicans* at 37 °C. The system contained 1 nmol L-proline and 1.0 mg dry wt cells per ml STM-Tris buffer (pH 7.0). $\blacktriangle$, Total uptake by mycelial cells; $\triangle$, cold trichloroacetic acid (TCA)-soluble fraction in mycelial cells; $\bullet$, total uptake by yeast cells; $\circ$, cold TCA-soluble fraction in yeast cells.

Fig. 2. Time course of uptake of L-proline by mycelial cells of *Candida albicans* germinated in proline or in glutamic acid. The system contained 1 nmol L-proline and 1.0 mg dry wt cells per ml STM-Tris buffer (pH 7.0). $\bullet$, Cells germinated in 10 mM-L-glutamic acid; $\triangle$, cells germinated in 10 mM-L-proline; $\circ$, cells germinated in 10 mM-L-glutamic acid, and then kept for 40 min in 10 mM-L-proline and washed before use.

Kinetics of L-proline uptake

Neither of the two morphological forms showed complete saturation kinetics. At low external concentrations of L-proline (10 μ M to 0.1 mM) the rate of uptake of the amino acid by yeast and mycelial cells increased rapidly with increasing external concentration. This rapid increase started to diminish when the external concentration of L-proline was about 0.1 mM; any further increase in the external concentration resulted in a slow but linear increase in the initial rate of L-proline uptake. Although the transport system did not exhibit simple Michaelis-Menten kinetics, an apparent K_m value of 0.1 mM for both yeast and mycelial cells was obtained by calculation. The results indicated that L-proline was transported by two systems. At low external concentrations a permease system was operative; however, at higher external concentrations (greater than 0.1 mM) the amino acid was also entering the cells by diffusion. As saturation was approached, diffusion became increasingly important in uptake of the amino acid. The calculated V value for the permease-like system of mycelial cells was approximately threefold higher [$4 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$] than that of the yeast cells [$1.4 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$].

Effect of temperature and pH on L-proline uptake

Further evidence that a permease-like system was operative at low external concentrations of substrate was derived from the studies on the effect of temperature and pH on uptake. Only yeast cells were studied in this way. The optimum pH was 7.0 and the rate of uptake was fastest at 37 °C.

Inhibition of L-proline uptake by metabolic inhibitors, analogues and ammonium ions

Sodium azide markedly inhibited proline uptake by both morphological forms of *C. albicans*, but 2,4-dinitrophenol and arsenate were far less efficient in inhibition of uptake (Table 3). Sarcosine and L-azetidine-2-carboxylic acid were effective analogue inhibitors of L-proline uptake, while L-thiazolidine-4-carboxylic acid had much less of an effect at the

Table 3. Inhibition of L-proline uptake of *Candida albicans* by metabolic inhibitors, analogues and ammonium ions

Inhibitor (1 mM)	Rate of uptake of L-proline (% of control)*	
	Yeast cells	Mycelial cells
Sodium azide	12	25
Sodium arsenate	71	86
2,4-Dinitrophenol	82	89
Sarcosine	24	19
L-Azetidine-2-carboxylic acid	53	61
L-Thiazolidine-4-carboxylic acid	71	86
Ammonium chloride	106	97

* In controls (i.e. in the absence of inhibitor), the initial rates of uptake of L-proline (1 μ M) were 0.17 nmol min⁻¹ (mg dry wt)⁻¹ for yeast cells and 0.36 nmol min⁻¹ (mg dry wt)⁻¹ for mycelial cells.

Table 4. Effect of different amino acids on L-proline uptake by *Candida albicans*

Unlabelled competitor (1 mM)	Rate of uptake of L-proline (% of control)*	
	Yeast cells	Mycelial cells
L-Alanine	70	68
L-Arginine	80	ND
L-Cysteine	72	90
L-Cystine	86	85
L-Glutamic acid	73	90
Glycine	96	80
L-Histidine	89	95
L-Isoleucine	94	90
L-Leucine	71	80
L-Lysine	108	110
L-Methionine	80	95
L-Phenylalanine	82	90
L-Serine	126	100
L-Tryptophan	100	108
L-Tyrosine	130	110
L-Valine	108	120

ND, Not determined.

* In controls (i.e. in the absence of competitor amino acid), the initial rates of uptake of L-proline (1 μ M) were 0.13 nmol min⁻¹ (mg dry wt)⁻¹ for yeast cells and 0.37 nmol min⁻¹ (mg dry wt)⁻¹ for mycelial cells.

concentration tested (1 mM). Ammonium ions, which are known to inhibit general transport systems, did not affect the uptake of L-proline by *C. albicans*.

Effect of other amino acids on L-proline transport

The addition of any one of 16 different amino acids had very little effect on the uptake of L-proline by yeast or mycelial cells (Table 4). Modification of uptake varied from 30% inhibition to 30% enhancement among the different amino acids. The failure of other amino acids to inhibit substantially L-proline uptake emphasizes the specificity of the active transport system.

DISCUSSION

Proline triggers the germination of *Bacillus megaterium* spores (Rossignol & Vary, 1979). However, neither uptake nor metabolism of the amino acid was necessary to initiate

germination because (i) only a brief exposure (30 s) to the trigger was required, (ii) the enzymes required for proline metabolism were not present in the spores, (iii) several non-metabolizable analogues of L-proline were effective triggers, (iv) germination occurred in the presence of inhibitors of oxygen uptake and metabolism, and (v) uptake of proline occurred after germination had been triggered. Proline does not function as such a trigger in the germination of the blastospores of *Candida albicans* where several minutes of exposure to proline, and thus some metabolism of it, is required for the amino acid to sponsor germination (Table 1).

Chaffin & Sogin (1976) presented evidence, derived from a study of yeast cell size, which indicated that germination of blastospores could be induced only in stationary phase cells incubated at 37 °C in a defined medium consisting of amino acids (Lee *et al.*, 1975). Budding cells in an exponential phase of growth did not germinate when incubated at 37 °C in the same medium. Soll & Bedell (1978) have expanded the observations of Chaffin & Sogin and have established that a 'commitment point' is passed by cells growing at 25 °C after which they cannot be induced to form germ tubes by incubation at 37 °C until they again enter the stationary phase. However, actively budding cells can be induced to germinate by certain substances such as serum (Mattia & Cassone, 1979). Our results confirm the findings of both Soll & Bedell (1978) and Mattia & Cassone (1979), but it should be noted that a complex medium such as serum may have effects other than induction of germination. For example, serum may inhibit bud formation and thus evoke stationary phase conditions in the yeast cells which then respond to the effects of amino acids or other inducers in the medium. The factors involved in germination are diverse and much still needs to be done to sort out seemingly conflicting observations.

Inducible proline transport systems have been described in both bacteria and yeasts (Jayakumar *et al.*, 1978, 1979; Kay & Gronlund, 1969; Schwencke & Magaña-Schwencke, 1979). The kinetics of proline uptake in *C. albicans* studied by Jayakumar *et al.* (1979) showed that the K_m for preincubated or proline-grown cells was similar to that of normal cells. In contrast, the V values of proline-grown or preincubated cells were 7–13 times higher than those of the normal cells. The germlings used for mycelial cells in our work were prepared in proline buffer. Thus, one could argue that the threefold greater V value obtained with mycelial cells might have been due to induction of a different proline permease. However, this appeared not to be the case, because both glutamic acid-germinated cells, and glutamic acid-germinated cells subsequently incubated in proline, exhibited virtually the same velocity of initial uptake as did proline-germinated cells.

Many multiple transport systems have been described in bacteria (Guardiola *et al.*, 1974; Menzel & Roth, 1980) and in fungi (Breton & Surdin-Kerjan, 1977; Tang & Howard, 1973). For example, among fungi, *Cryptococcus albidus* takes up L-glutamic acid by two saturable systems: a high-affinity system (K_m 11.5 μ M) and a low-affinity system (K_m 2.5 mM) (Tang & Howard, 1973). L-Leucine and L-proline enter the cells of *Histoplasma capsulatum* by high-affinity systems (K_m 20 μ M and 17 μ M) and by simple diffusion (Dabrowa & Howard, 1976; Gupta & Howard, 1971). Low-affinity systems may be difficult to distinguish from diffusion (Tang & Howard, 1973). *Blastomyces dermatitidis* transports L-leucine by a high-affinity system (K_m 11 μ M) and by diffusion (Bowdon & Garrison, 1974). In the present study, L-proline was noted to enter the cells by two systems. At low substrate concentrations (10 μ M to 0.1 mM) an active transport system which was (i) highly specific for proline, (ii) uninhibited by ammonium ions, (iii) temperature and pH dependent, and (iv) inhibited by sodium azide was demonstrable. At high substrate concentrations (0.2 to 0.5 mM) the active transport system was accompanied by diffusion. As saturation was approached diffusion became increasingly important.

Among the analogues studied, sarcosine strongly inhibited proline uptake. This suggests that the accumulation of L-proline and sarcosine are mediated by the same uptake system. Moreover, the somewhat smaller but demonstrable effects of L-azetidine-2-carboxylic acid

and L-thiazolidine-4-carboxylic acid suggest there is a general imino acid transport system in *C. albicans* similar to that observed in *Saccharomyces chevalieri* (Kuznar *et al.*, 1973; Magaña-Schwencke & Schwencke, 1969; Magaña-Schwencke *et al.*, 1973). The fact that glycine and alanine were transported by the system in *S. chevalieri* but not by the one reported in this paper indicates some differences between the two systems. The inhibition of germination of *C. albicans* in proline-buffer medium exerted by some of these analogues (Dabrowa *et al.*, 1976) could be related to antagonism of uptake of L-proline.

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