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CHARACTERISTICS OF PROLINE TRANSPORT IN NORMAL AND STARVED CELLS OF *CANDIDA ALBICANS*

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Summary

1. There was no apparent correlation between the rate of respiration and rate of accumulation of proline in *Candida albicans* cells.

2. In contrast to normal cells, the respiration in the starved cells became completely cyanide insensitive. The starvation of cells in the presence of cycloheximide prevented the cells from becoming cyanide insensitive. The addition of Fe(III), however, accelerated the process.

3. Oxidizable substrates e.g. NADH, acetate and glucose, when added to cyanide-insensitive starved cells, exhibited 40—280% stimulation in respiration rate. However, this enhancement in oxidation by various substrates was not coupled to a simultaneous increase in the proline uptake or in intracellular ATP levels.

4. There was 6-fold stimulation in proline uptake when cyanide-insensitive cells were preincubated with 50 mM glucose. The preincubation of starved cells resulted in a partial restoration of cyanide sensitivity and increased intracellular ATP levels. The preincubation of starved cells with other oxidizable substrates resulted in a partial restoration of cyanide sensitivity but had no stimulatory effect on intracellular ATP levels and proline accumulation.

5. Both the enhanced uptake and ATP levels in glucose preincubated cells were found to be completely abolished by iodoacetate.

6. It is proposed that the increased proline uptake in cells preincubated with glucose was mainly due to the production of glycolytic energy.

Introduction

Uptake of various amino acids in yeast cells is an energy-dependent process as shown by its stimulation by suitable sources of metabolic energy such as

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D-glucose and its inhibition by uncouplers of oxidative phosphorylation [1—6]. Ramos et al. [7] have demonstrated that various substrates, e.g. pyruvate, acetate, propionaldehyde and butanol, capable of activating mitochondrial electron transfer and oxidative phosphorylation can stimulate the concentrative uptake of leucine in *Saccharomyces cerevisiae*. Stubstrate-stimulated uptake of various amino acids and sugars is a common phenomenon in various systems [8—12]. In our earlier communication we have shown that proline uptake in *Candida albicans* is an energy-dependent process and is against a concentration gradient [13]. The present paper demonstrates that *C. albicans* cells acquire cyanide-insensitive respiration upon starvation, a phenomenon familiar among various bacterial and plant systems [14—20] and discusses its relevance to proline transport.

Materials and Methods

Yeast and growth conditions. C. albicans 3100, a wild type pathogenic yeast strain, was obtained from the National Chemical Laboratory, Poona, India. In each instance, cells were transferred from a slant into minimal medium containing 0.3% (w/v) KH_2PO_4, 0.3% (w/v) $(NH_4)_2SO_4$, 0.025% (w/v) $CaCl_2$; 0.025% $MgSO_4$ and 0.001% (w/v) biotin with the addition of a carbon source 0.5% (w/v) glucose. Cells were grown at 30°C for 16—17 h and the inoculum was then transferred to the same medium. Cell growth was monitored turbidimetrically by reading the absorbance at 470 nm in a Bausch and Lomb Spectronic 20 spectrophotometer. For uptake measurements cells growing in mid-exponential phase were harvested by centrifugation (1500 × *g* for 10 min), washed three times with sterile distilled water and were suspended in water before dividing into two portions. One portion of the washed cells (here termed 'normal cells') was immediately used for the extimation of intracellular ATP levels, proline and oxygen uptake. In order to deplete the endogenous energy pool, the other portion of the washed cells was aerated in a shaker for 14—15 h. Such aerated cells (here termed 'starved cells') were then used for the estimation of intracellular ATP levels, proline and oxygen uptake.

Measurement of L-[^{14}C]proline uptake. A reaction mixture containing normal or starved cells (160—180 μg protein/ml) were preincubated at 30°C for 10 min after the addition of cycloheximide (final concentration 200 μg/ml) to inhibit protein synthesis. The other additions specified in each case were also made along with the cycloheximide. Unless otherwise mentioned, the antibiotic was present in all the assay mixtures of proline uptake. The reaction was initiated by the additon of ^{14}C-labelled L-proline (1 mM, 5 μCi/ml). At indicated time intervals, 0.1-ml aliquots were removed with an Eppendorf pipette and immediately diluted in 5 ml chilled distilled water, or in 5 ml chilled phosphate buffer (0.05 M, pH 7.0). The diluted suspension was rapidly filtered through a 0.45 μm Millipore filter and radioactivity retained was counted in a Packard scintillation counter using a toluene-based scintillation fluid.

Chromatographic identification of internal radioactive material following active transport of proline. Studies were undertaken to determine whether the accumulated proline was incorporated into protein or existed in the free from within the cells. The L-[^{14}C]proline was extracted and identified by paper and

thin layer chromatography as the free amino acid. More than 90% of the total radioactive proline was recovered in the free form.

Measurement of oxygen uptake. The rate of oxidation by normal or starved cells was measured polarographically at 30°C with an oxygen monitor (Yellow Spring Instruments Co., Ohio) according to Estabrook [21].

Determination of intracellular ATP levels in normal and starved cells. The samples to be assayed for ATP levels were extracted with cold perchloric acid (5%) for 45 min and then sonicated for 2 min and centrifuged. The supernatant was neutralized with the addition of 0.4 M triethanolamine/HCl (pH 7.4)/1.8 M KOH [22]. The precipitate was discarded by centrifugation and ATP content in the supernatant was evaluated by spectrophotometric measurements of NADPH produced by the addition of 15 mM glucose to the incubation medium containing 8 mM $MgCl_2$, 0.1 mM NADP, 1 unit glucose-6-phosphate-dehydrogenase and 2 units hexokinase in 100 mM Tris-HCl (pH 8.0) [23].

Protein was estimated according to Lowry et al. [24].

Chemicals. L-[^{14}C]Proline (specific activity: 100 mCi/mmol) was purchased from the BARC, Bombay, India. L-Proline, iodoacetic acid, antimycin, sodium azide, sodium arsenate, cycloheximide, glucose-6-phosphate dehydrogenase and hexokinase were purchased from Sigma Chemical Company, U.S.A. Other chemicals were of analytical grade.

Results

Proline uptake in normal cells. Fig. 1 shows the uptake of L-[^{14}C]proline in the absence of exogenous substrates in normal cells. An increase in the uptake was observed up to 20 min, after which a steady state was attained. Under the assay conditions, the total incorporation of proline into trichloroacetic acid-precipitable proteins was less than 10% of the total accumulation. The kinetic

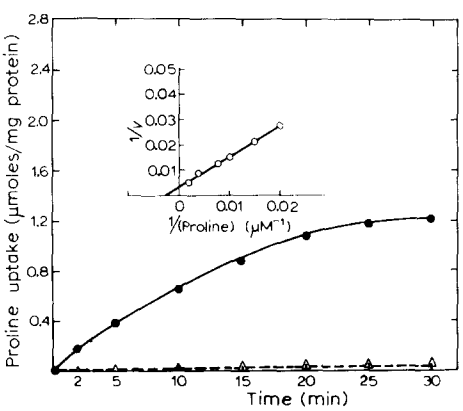


Fig. 1. Transport of L-[^{14}C]proline in normal *C. albicans*. Transport of L-[^{14}C]proline was assayed as described in Materials and Methods. For the determination of L-[^{14}C]proline into trichloroacetic acid-precipitable proteins, an aliquot of sample was diluted 25 times in 5% trichloroacetic acid and boiled for 15 min and then filtered through a Millipore filter. ●———●, L-[^{14}C]proline uptake in normal cells; △- - - - -△, L-[^{14}C]proline incorporation into trichloroacetic acid-precipitanle proteins. Inset shows kinetics of proline uptake; ○———○, normal cells.

studies showed that the apparent K_m for proline uptake was 250 μM and the V was 333 nmol/mg protein per min (Fig. 1, inset).

Effect of various respiratory inhibitors on proline uptake, oxygen uptake and ATP levels in normal cells. The inhibition of proline uptake with various respiratory inhibitors was much higher (40—90%) compared to their effect on oxygen uptake (30—60%) (Table I). Sodium arsenate, a known inhibitor of protein biosynthesis and phosphorylation, did not show any inhibition on oxygen uptake; however, proline uptake was reduced to 66%. All the inhibitors studied except *m*-chlorocarbonylcyanide phenylhydrazone reduced the intracellular ATP levels (35—80%); however, in most of the cases there was no parallel drop in the proline uptake. These results suggest that besides ATP, some other form of mitochondrial energy is probably required for the uptake of proline. In order to understand the form and nature of such an energy, cells were starved to deplete their endogenous energy reserves so that the energy input can easily be regulated.

Proline uptake in starved cells. As shown in Table II, the 10 min accumulation of proline in starved cells was reduced to 75% when compared to normal cells. Various inhibitors e.g. iodoacetate, antimycin A, azide and cyanide inhibited the proline uptake approximately to the same extent as that of normal cells (Table II). The kinetic studies revealed that the K_m for proline uptake in normal and starved cells was same. The V, which was reduced in starved cells, was further reduced in the presence of potassium cyanide (data not shown).

Effect of various inhibitors on oxygen uptake and ATP levels in starved cells. Potassium cyanide at 10 mM concentration exhibited 50—60% inhibition on oxygen uptake in normal cells (100% with 20 mM KCN). However, a complete insensitivity towards oxygen uptake in starved cells was observed (Tables I and

TABLE I

EFFECT OF VARIOUS RESPIRATORY INHIBITORS AND IONOPHORES ON PROLINE UPTAKE, OXYGEN UPTAKE AND ATP LEVELS IN NORMAL CELLS

L-[^{14}C]proline uptake and intracellular ATP levels were measured as described in Materials and Methods. Oxygen uptake was measured according to Estabrook [21] using a Clark oxygen electrode. For the effect of various inhibitors on ATP levels the cells were preincubated for 10 min with the inhibitor prior to the addition of perchloric acid. Freshly prepared buffered solutions of cyanide and azide were used.

Addition	Concentration	Proline uptake (μmol/mg protein per 10 min	O_2 uptake (nmol/mg protein per min)	Cellular concentration of ATP (nmol/mg protein)
None		0.68	178	15
Cyanide	10 mM	0.05	83	3.2
Antimycin A	10 μM	0.23	59	9.6
Iodoacetate	1 mM	0.41	118	9.0
Arsenate	25 mM	0.23	178	4.6
Azide	5 mM	0.09	60	9.6
Valinomycin + K^+	10 μM	0.18	178	n.d.
m-Chlorocarbonylcyanide phenylhydrazone	60 μM	0.20	178	14.1

n.d., not determined.

TABLE II

EFFECT OF VARIOUS INHIBITORS ON PROLINE UPTAKE, OXYGEN UPTAKE AND ATP LEVELS IN STARVED CELLS

Cells were starved as described in Materials and Methods. Assay conditions for proline uptake, oxygen uptake and ATP levels were similar to those described for Table I.

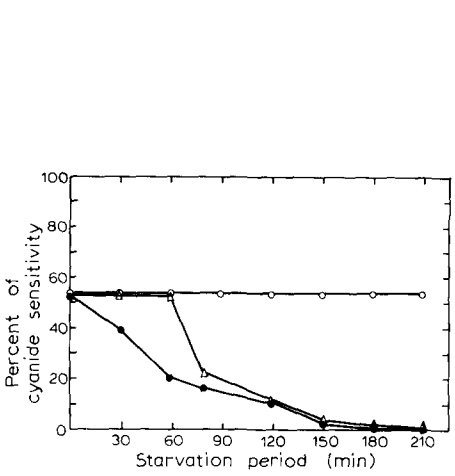
Addition	Concentration	Proline uptake (μmol/mg protein per 10 min)	O_2 uptake (nmol/mg protein per min)	Cellular concentration of ATP (nmol/mg protein)
None		0.20	60	6
Cyanide	10 mM	0.03	60	6
Antimycin A	10 μM	0.08	24	3
Azide	5 mM	0.02	20	3
Iodoacetate	1 mM	0.12	40	3.6

II), whereas other respiratory inhibitors such as antimycin A, azide and iodoacetate inhibited the oxygen uptake to an extent similar to normal cells. The insensitivity of starved cells to potassium cyanide may be due to the synthesis of a mitochondrial alternative respiration (by-pass) as has been observed in the case of *Saccharomyces lipolytica* [25].

In order to understand if any new protein is involved in the biogenesis of the cyanide-insensitive pathway, the cells were starved both in the presence and in the absence of cycloheximide, a known inhibitor of protein synthesis. Cells which were starved in the absence of cycloheximide acquired cyanide insensitivity within 2—3 h. The addition of 2 mM Fe(III) to the starving cells (without cycloheximide) enhanced the rate of the appearance of cyanide-insensitive respiration (Fig. 2). However, when the cells were starved in the presence of cycloheximide, they did not acquire any cyanide insensitivity (Fig. 2). (Cycloheximide does not have any effect on oxygen uptake.) These results suggest that protein synthesis is necessary to bring about cyanide insensitivity during starvation.

Effect of various oxidizable substrates on oxygen uptake, proline uptake and ATP levels in starved cells. In contrast to normal cells, the immediate addition of various oxidizable substrates e.g. glucose, NADH and sodium acetate, accelerates the oxygen uptake (40—280%) in starved cells. However, the stimulation of oxidation was not accompanied by a simultaneous increase in the proline uptake or ATP levels (Table III). Furthermore, the stimulated oxidation by various oxidizable substrates was also insensitive to cyanide (data not shown). The ineffectiveness of various oxidizable substrates in stimulating proline uptake or ATP levels in starved cells may be due to the cyanide insensitivity acquired by the starved cells.

Proline uptake in starved cells preincubated with glucose. When cyanide-insensitive starved cells were preincubated with 50 mM glucose for 30 min, they exhibited a 6-fold stimulation in proline uptake (Fig. 3). However, the preincubation of such cells with other oxidizable substrates, e.g. NADH or acetate, did not influence the proline accumulation though they stimulated the oxygen uptake (data not shown). The preincubation of starved cells with glucose (with other substrate also) was found to be associated with a partial



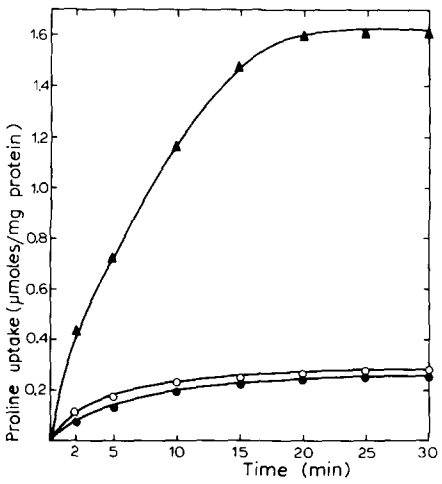


Fig. 2. The effect of cycloheximide, Fe(III) on cyanide insensitivity during starvation. The cells were starved as described in Materials and Methods. 200 μg/ml cycloheximide or 2 mM Fe(III) were added for observation of their effect on cyanide insensitivity. Normal cells exhibited 55% cyanide sensitivity which dropped to zero after starvation. The drop in cyanide sensitivity in presence or in the absence of cycloheximide or in the presence of Fe(III) has been plotted against the time of starvation. △———△, cyanide sensitivity in cells starved in water; ●———●, cyanide sensitivity in cells starved in water plus 2 mM $FeCl_3$; ○———○, cyanide sensitivity in cells starved in water plus 200 μg/ml cycloheximide.

Fig. 3. Effect of glucose and iodoacetate on L-[^{14}C]proline uptake in starved cells. Assay conditions were similar to those described for Fig. 1 and Table IV. ○———○, L-[^{14}C]proline uptake in starved cells; ▲———▲, starved cells plus 50 mM glucose; ●———●, starved cells plus 50 mM glucose plus 1 mM iodoacetate.

restoration of cyanide sensitivity (data not shown). The stimulated uptake of proline in the glucose preincubated cells was found to be completely sensitive to iodoacetate, a potent inhibitor of glycolysis [26]. There was approximately a 60% rise in cellular ATP levels in the cells preincubated with glucose which was abolished by the addition of iodoacetate (Table IV). When starved cells were preincubated with other oxidizable substrates like NADH and acetate there was no change in the intracellular ATP levels (data not shown). When cyclo heximide was added along with glucose to the starved cells, it did not

TABLE III

EFFECT OF VARIOUS SUBSTRATES ON OXYGEN UPTAKE, PROLINE UPTAKE AND ATP LEVELS IN STARVED CELLS

Oxygen uptake was measured with the immediate addition of various oxidizable substrates according to Estabrook [21] using a Clark electrode. Assay conditions for the uptake of L-[^{14}C]proline and ATP levels were similar to those described in Materials and Methods, with the exception that various oxidizable substrates were simultaneously added with L-[^{14}C]proline.

Addition	Concentration	O_2 uptake (nmol/mg protein per min)	Proline uptake (μmol/mg protein per 10 min)	Cellular concentration of ATP (nmol/mg protein)
None		60	0.20	6
Glucose	50 mM	231	0.20	6
NADH	6.6 mM	118	0.21	5.5
Sodium acetate	50 mM	93	0.19	5.5

TABLE IV

EFFECT OF PREINCUBATION WITH GLUCOSE ON PROLINE UPTAKE AND ATP LEVELS IN STARVED CELLS

L-[^{14}C]proline uptake and its incorporation into trichloroacetic acidprecipitable proteins and ATP levels were determined as described in Materials and Methods and Fig. 1. Glucose alone or with the additions indicated below were preincubated with the starved cells for 30 min before the addition of L-[^{14}C]proline or perchloric acid.

Addition	Proline uptake (μmol/mg protein per 10 min)		Cellular concentration of ATP (nmol/mg protein)
	Total	Trichloroacetic acidprecipitable fraction	
None	0.20	0.03	6.6
Glucose (50 mM)	1.11	0.05	10.6
Glucose (50 mM) + cycloheximide (200 μg/ml)	1.00	0.009	n.d.
Glucose (50 mM) + iodoacetate (1 mM)	0.21	n.d.	6.4

n.d., not determined.

show any effect on glucose stimulated proline uptake excluding the possibility of proline being incorporated into proteins (Table IV).

Discussion

In the starved cells of *C. albicans* there is no stimulated uptake of proline in presence of various oxidizable substrates as has been demonstrated in various *Saccharomyces* and bacterial systems [2,4,5,6—12]. Normal *C. albicans* cells do not show any enhancement in oxygen utilization upon the addition of oxidizable substrates. However, when cells were starved to deplete their endogenous energy pool, various oxidizable substrates, e.g. sodium acetate, NADH and glucose, exhibited a 40—280% stimulation in the respiration rate. But contrary to various reports on *Escherichia coli* and *Saccharomyces* species, *C. albicans* cells do not shown any simultaneous increase in the proline uptake. It should be mentioned here that proline uptake has been shown to be an energy-dependent process in these cells [13]. The observation suggests that either the substrate oxidation in starved cells is not coupled to phosphorylation of ADP and P_i, or there is an uncoupling between inextricably associated oxidation and proline uptake.

Starved cells were found to respire at a slow rate as compared to the normal cells, however, the oxygen utilization was completely insensitive to cyanide whereas in normal cells 100% cyanide sensitivity is obtainable. A mitochondrial alternative respiration insenstive to cyanide and antimycin A has already been reported in a large number of organisms [14—20]. Furthermore, a cyanide-insensitive respiration has also been induced in *S. lipolytica* [25].

When oxidizable substrates were added to cyanide-insensitive starved cells, the stimulated oxidation was not coupled to a simultaneous increase in the intracellular ATP levels. Since we could not seen any increase in the proline uptake, it was assumed that probably a restoration of cyanide sensitivity might

enhance the proline upon the addition of oxidizable substrates. When starved cells were preincubated with glucose or with other oxidizable substrates, a partial restoration in cyanide sensitivity was achieved; however, glucose alone could stimulate the proline accumulation. This glucose-stimulated uptake was accompanied by a simultaneous rise in the intracellular ATP levels. Both the levels of proline accumulation and intracellular ATP were completely sensitive to iodoacetate in these preincubated cells. Other substrates could not enhance the intracellular ATP levels, but they restored cyanide sensitivity. It seems probably that an uncoupling between an energy-requiring process (proline transport) and an energy yielding process (ATP production) takes place when cells are starved to deplete their endogenous energy pool. These two uncoupling processes could not be restored even when cells acquire cyanide sensitivity. It is proposed that the stimulated uptake of proline in glucose-preincubated cells is mainly due to the production of energy via the glycolytic pathway.

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