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SARCOSINE AND IMINO ACID UPTAKE IN *SACCHAROMYCES CHEVALIERI*

DEREPRESSION BY NITROGEN STARVATION

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SUMMARY

1. When grown in a complex medium, *Saccharomyces chevalieri* cells display a reduced uptake capacity for sarcosine.

2. Sarcosine uptake is greatly increased by incubating the yeast cells in a buffered nitrogen-free medium, the increase being practically identical under aerobic and anaerobic conditions. This increase is inhibited by cycloheximide, 5-methyltryptophan and proflavine.

3. The increase in sarcosine uptake capacity is paralleled by an increase in L-proline, D-proline and DL-azetidin carboxylic acid uptakes. This increase is blocked by cycloheximide. The uptake capacity for DL-hydroxyproline remains at a very low level. The capacity for the accumulation of DL-phenylalanine, DL-leucine and L-isoleucine increases only to a minor extent and is not affected by cycloheximide.

4. Evidence is presented which is consistent with the idea that the enhancement in sarcosine-accumulating capacity is mediated by the biosynthesis of a specific mRNA.

5. The results indicate that the uptake of sarcosine is mediated by a general imino acid transport system which is derepressed by nitrogen starvation.

INTRODUCTION

Amino acid uptake in fungi may be regulated by either the regulation of the activity or of the biosynthesis of the transport system. Crabeel and Grenson[1] have found that preloading with histidine immediately inhibits the activity of the histidine transport system. Pall[2] has reported that amino acids of the internal pool inhibit the activity of a methionine transport system. Also, Grenson *et al.*[3] have proposed that the loss of arginine transport activity observed in yeast after cycloheximide addition, is probably due to inhibition by free amino acids which accumulate as a result of the inhibition of protein synthesis.

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There is also evidence that amino acid uptake in fungi is regulated at the level of protein biosynthesis[4–6]. In some cases the amino acid uptake may be regulated both at the level of activity and at the level of protein biosynthesis[2].

These studies are further complicated by the fact that, in fungi, two different, independently regulated types of transport systems for amino acids have been reported[7].

Previous studies from our laboratory have supported the idea that proline transport in *Saccharomyces chevalieri* may be regulated by derepression[6]. It was also reported that sarcosine (*N*-methylglycine) was, among a number of analogs, the best competitive inhibitor for proline uptake[8].

If the proline transport system is able to recognize the structure of *N*-methylglycine it may be possible that, by measuring the increase in proline uptake capacity, one is in fact detecting the biosynthesis of a general imino acid transport system. If this assumption is correct, one would expect that the capacity to accumulate sarcosine should be increased by nitrogen starvation in a way analogous to that found for L-proline. Also, the uptake of D-proline and other imino acids should be comparably enhanced while the uptake of amino acids should remain practically unaltered.

Our results are in agreement with this hypothesis. We also present data indicating that the increase in uptake capacity for imino acids is mediated by the synthesis of mRNA.

MATERIALS AND METHODS

Yeast strain, growth conditions

Galactose-adapted cells of *S. chevalieri*, strain C_1, were used. The strain was obtained from culture NRRL-Y of the Northern Regional Laboratories (U.S.A.). The cells were grown at 30 °C in standing test tubes in complete medium with galactose as the carbon source, as previously described[6].

Radiochemicals

D-[U-^{3}H]Proline and [*carboxy*-^{14}C]sarcosine were purchased from Amersham. L-[*carboxy*-^{14}C]Alanine, DL-[4-^{14}C]azetidin carboxylic acid, DL-[*carboxy*-^{14}C]phenylalanine, DL-[*carboxy*-^{14}C]leucine and L-[*carboxy*-^{14}C]isoleucine were products from Calbiochem. DL-[2-^{14}C]hydroxyproline was obtained from The Radiochemical Centre. L-[U-^{14}C]Proline and ^{14}C-labeled reconstituted protein hydrolysate mixture No. 3122-08 were purchased from Schwarz/Mann. The specific activity of all amino and imino acids was adjusted to 0.12 Ci/mole with the unlabeled molecule. The activity of the ^{14}C-labeled protein hydrolysate was adjusted to 1 μCi/ml with the addition of the corresponding nonradioactive amino acids.

Compounds

Ingredients for the media were purchased from Difco Laboratories. Galactose was a product from Pfanstiehl. Proflavine hemisulfate, a B.D.H. product, was purified as the free base[9]. Cycloheximide was a gift from the Upjohn Co. DL-5-Methyltryptophan was obtained from Koch-Light. Non labeled amino acids and imino acids were all obtained from Calbiochem in their purest grade.

The KH_2PO_4 used for the nitrogen starvation medium was the product No. 7100 from Mallinckrodt. We have noted that the purity and origin of this reagent is important to obtain derepression in short incubation times. In our experience, only the Merck product No. 4871 effectively replaces the Mallinckrodt product.

Derepression

Exponentially growing cells were collected and derepressed by nitrogen starvation aerobically and anaerobically as described[6].

Uptake experiments

The assay of sarcosine and imino acids uptake was run in the same way as previously described for L-proline[8]. The internal concentration of the accumulated imino acid, was calculated considering the amount of intracellular water volume calculated as reported by Cirillo[20]. Our values for uptake velocity are identical to the expression: nmoles/mg dry weight per min, because the dry weight of $1 \cdot 10^7$ cells is 0.200 mg. All radioactive compounds were adjusted to specific activities of 0.12 Ci/mole, and their final concentration in the uptake mixture was 50 μM.

Measurement of total protein synthesis

Cells, adjusted to $2 \cdot 10^7$ cells/ml in phosphate–galactose buffer, were incubated at 30 °C for 5 min; at zero time a synthetic mixture of ^{14}C-labeled amino acids was added. The final concentration of the amino acid mixture was 1.9 μg/ml and the final radioactivity 0.05 μCi/ml. The cells were incubated in a shaker at 30 °C and 2-ml samples were taken at intervals. Protein was precipitated by heating the cells for 10 min at 90 °C in 5% trichloroacetic acid. Samples were allowed to cool, filtered through nitrocellulose filters (Sartorius, 0.45 μm, pore size), washed 3 times with 5-ml portions of phosphate–galactose buffer, dried, glued onto planchets and counted.

Cycloheximide pretreatment

These experiments were carried out in two steps: a preincubation period of 90 min in the presence of cycloheximide followed by washing of the cells and then reincubation in fresh medium. For the preincubation period cells, adjusted to $2 \cdot 10^7$ cells/ml in phosphate–galactose buffer, to which 0.6 μg/ml cycloheximide had been added, were incubated at 30 °C in a rotatory shaker. The cultures were then filtered through nitrocellulose filters (Sartorius, 0.45 μm) and washed in the filter 3 times with equivalent volumes of phosphate–galactose buffer. For the reincubation, the filters with the cells were resuspended in phosphate–galactose buffer preheated at 30 °C and samples for measuring sarcosine uptake were taken at the appropriate intervals. All steps were done under carefully controlled sterile conditions.

RESULTS

Kinetics of sarcosine derepression in N-free medium

As shown in Fig. 1, the sarcosine-uptake capacity of the cells increased up to 200 min after commencement of nitrogen starvation, after an initial lag period

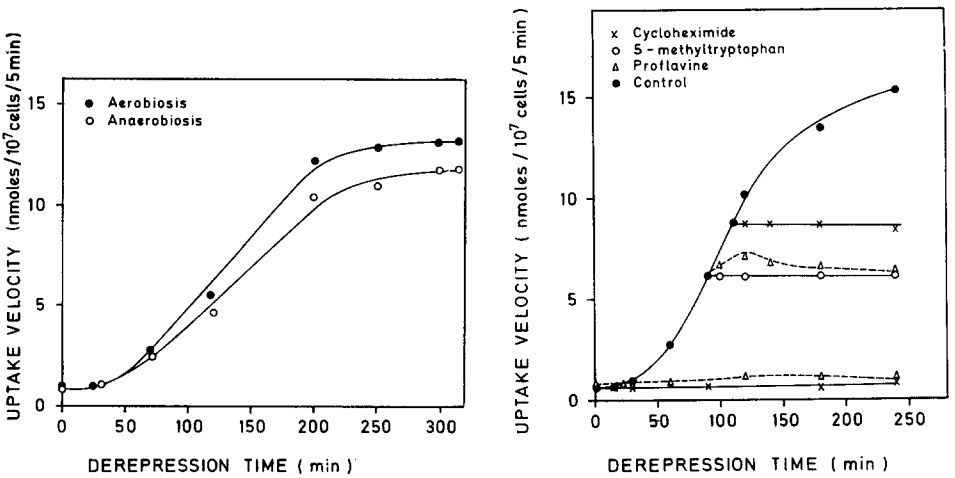

Fig. 1 Kinetics of sarcosine derepression by nitrogen starvation. Samples of cell suspension in phosphate–galactose buffer were taken at intervals and their capacity to accumulate [^{14}C]-sarcosine was assayed as described under Materials and Methods.

Fig. 2. Effect of inhibitors on derepression kinetics. Cycloheximide (0.7 μg/ml) was added at zero time and again at 110 min. Proflavine (100 μM) was added at zero time and also at 90 min; 5-methyl-tryptophan (1 mM) was added at 90 min. In each case, the inhibitors were added to a different portion of the cell suspension and thereafter samples were taken to assay their capacity to accumulate [^{14}C]sarcosine as described in Fig. 1.

of 30 min. The shape of the derepression curve remained practically the same when the nitrogen starvation was run under anaerobic conditions (see Materials and Methods). The initial velocity of uptake increases 10 to 15 times. Cells do not grow under this conditions.

Effect of inhibitors on the time course of derepression

The increase in sarcosine-uptake capacity during nitrogen starvation was prevented by cycloheximide, proflavine (free base) and 5-methyltryptophan. The concentrations used were those which inhibit the growth of our strain after 24 h by 80%. When either proflavine or cycloheximide were added at the onset of nitrogen starvation they completely prevented the increase in sarcosine uptake capacity. No lag in the action of proflavine was observed in this case (see Fig. 2).

Initial velocity of uptake during nitrogen starvation for other amino and imino acids

DL-Azetidin carboxylic acid (Fig. 3), D-proline and L-alanine (Fig. 4), show a variation in their initial velocity of uptake similar to that of sarcosine and L-proline (see also ref. 6). In all cases, the increase was completely prevented by cycloheximide.

No change in DL-hydroxyproline uptake capacity can be observed during nitrogen starvation (Fig. 5). DL-Phenylalanine uptake increased 5 times even when cycloheximide was added. The uptake of DL-leucine and L-isoleucine shows a similar behavior to that of DL-phenylalanine (Fig. 6).

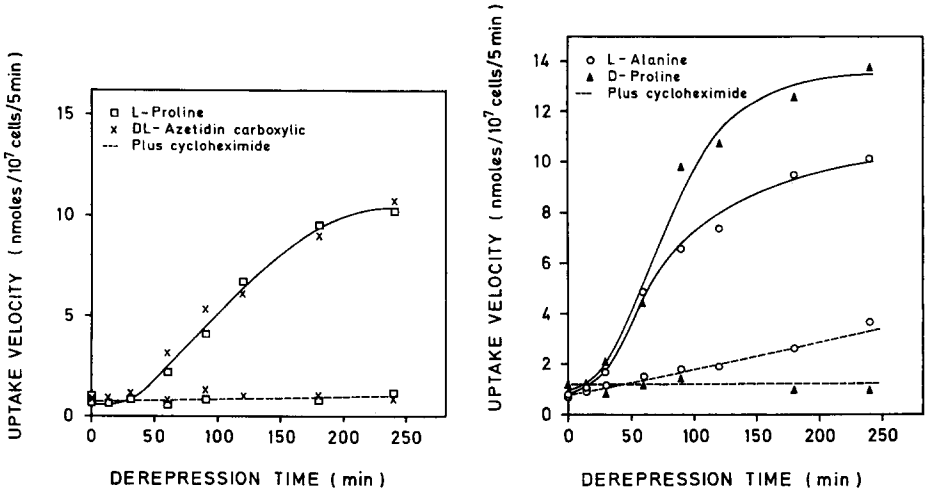

Fig. 3. Kinetics of L-proline and DL-azetidin carboxylic acid derepression in nitrogen-free medium. The accumulation of the imino acids was assayed as described for Fig. 1. To a portion of cell suspension 0.7 μg/ml of cycloheximide was added at zero time.

Fig. 4. Kinetics of L-alanine and D-proline derepression under nitrogen starvation. The variation in initial velocity of uptake in the presence and absence of 0.7 μg/ml cycloheximide was assayed as described in Fig. 3.

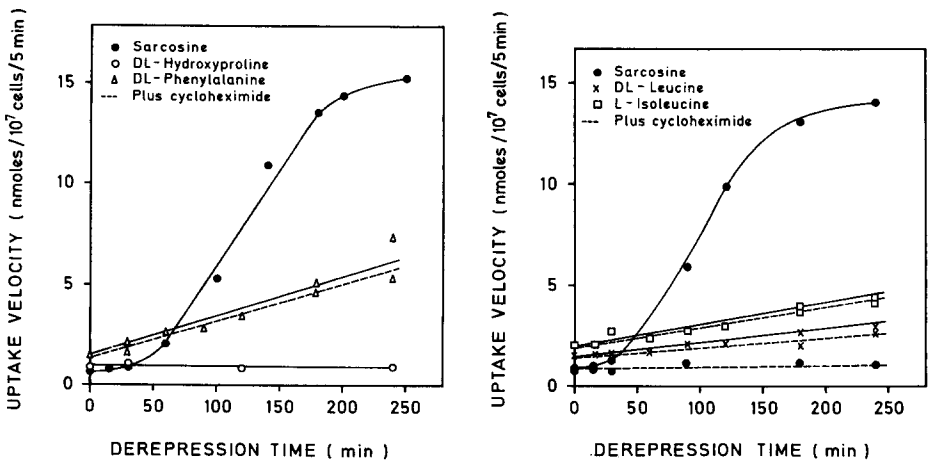

Fig. 5. Time-course variation of DL-hydroxyproline and DL-phenylalanine uptake during nitrogen starvation. Conditions are as those described for Fig. 3. For comparison, [^{14}C]sarcosine uptake was also measured.

Fig. 6. Time-course variation of DL-leucine, and L-isoleucine uptake under nitrogen starvation. Conditions are identical to those described for Fig. 3. For comparison, [^{14}C]sarcosine uptake was also measured.

Recovery of protein synthesis in S. chevalieri after preincubation with cycloheximide

The capacity of our yeast strain to recover its protein-synthesizing ability after 90 min of incubation with 0.6 μg/ml of cycloheximide is clearly demonstrated

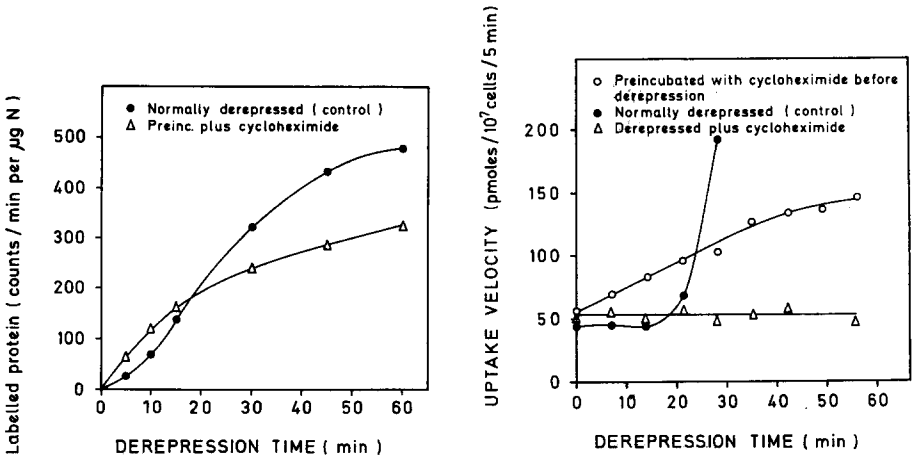

Fig. 7. The effect of cycloheximide preincubation on total protein synthesis. A synthetic mixture of ^{14}C-labeled amino acids were added to repressed cells which had been preincubated with 0.6 µg/ml of cycloheximide for 90 min, washed and resuspended in nitrogen-free buffer and thereafter samples were taken and the protein was precipitated by trichloroacetic acid. For comparison total protein synthesis in cells not preincubated with cycloheximide was also determined. For details see Materials and Methods.

Fig. 8. The effect of cycloheximide preincubation on sarcosine derepression kinetics. Cells subjected to the same conditions as those described for Fig. 7 were assayed for their [^{14}C]-sarcosine uptake capacity as indicated in Materials and Methods.

in Fig. 7. Furthermore, it may be seen that protein synthesis is initially accelerated after such treatment.

Time course of derepression for sarcosine-uptake capacity after preincubation with cycloheximide

From Fig. 8 it may be concluded that after pre-incubation with cycloheximide, washing and resuspension in nitrogen-free buffer, the cells show an immediate and linear increase in their sarcosine-uptake capacity. No lag is observed in this case. If cycloheximide is not removed, no increase in sarcosine uptake can be detected.

The effect of the preincubation time with cycloheximide on the subsequent sarcosine derepression

It may be seen from Fig. 9 that there is an optimun preincubation period of 90 min for cycloheximide. The curve suggests that a precursor for protein biosynthesis is accumulated in the presence of cycloheximide.

The effect of preloading the cells with imino acids

Preloading with either sarcosine or L-proline produces an increase in sarcosine-uptake velocity (Fig. 10).

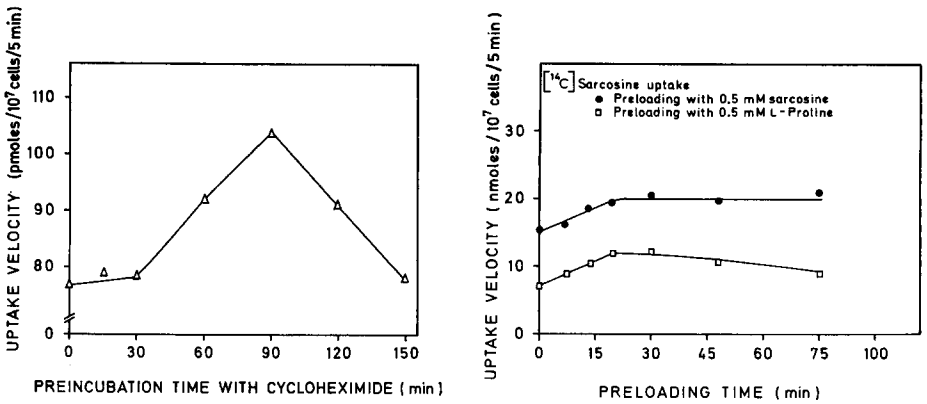

Fig. 9. The effect of preincubation time with cycloheximide on the sarcosine uptake capacity. 0.6 μg/ml of cycloheximide was added to the repressed cells. Samples were taken at intervals, washed, resuspended in fresh phosphate–galactose buffer, nitrogen-starved for 15 min and their capacity to accumulate [^{14}C]sarcosine assayed as described under Materials and Methods.

Fig. 10. Effect of preloading the cells with imino acids on the initial velocity of sarcosine uptake. Derepressed cells were preloaded with the indicated concentration of unlabeled sarcosine and L-proline. Samples were removed at intervals, centrifuged, washed, resuspended in phosphate–galactose medium and their initial velocity of uptake of [^{14}C]sarcosine measured as indicated in Materials and Methods.

DISCUSSION

From the data presented above, it is clear that sarcosine uptake greatly increases by subjecting yeast cells to the nitrogen starvation conditions described for L-proline uptake[6]. Also, DL-azetidin carboxylic acid and D-proline uptakes show a similar phenomenon. DL-Hydroxyproline uptake did not increase under nitrogen-starvation conditions. This is consistent with the previous finding that hydroxyproline is not recognized by the proline transport system[8]. L-Alanine uptake also increases but the increase is not completely prevented by cycloheximide. This result may indicate that L-alanine is accumulated by two different systems.

Phenylalanine, isoleucine and leucine uptakes show a completely different time-course variation in nitrogen-starvation conditions, even in the presence of cycloheximide. Thus, the uptake of these amino acids in yeast may be regulated by a completely different mechanism, probably retroinhibition as described for histidine[1] or methionine in fungi[2]. The fact that preloading with unlabeled sarcosine or L-proline does not inhibit subsequent [^{14}C]sarcosine uptake argues against a regulation by specific retroinhibition for imino acid uptake. Further characterization of the sarcosine uptake supports this idea[19].

The results obtained for sarcosine uptake with inhibitors of protein synthesis such as cycloheximide[11,12] and 5-methyltryptophan[13] or with inhibitors of RNA synthesis such as proflavine[14], are entirely analogous to those found for proline derepression[6]. Furthermore, the effect of cycloheximide is completely different from that reported for arginine uptake, which has been reported to be regulated by feed-back by internally accumulated amino acids[3].

When our yeast cells were preincubated with cycloheximide, washed and then subjected to nitrogen starvation, the lag period normally observed for the increase in sarcosine uptake was not detected. The elimination of the latency period in these conditions can be explained by the accumulation of an mRNA necessary for the synthesis of one or more proteins indispensable for the operation of the sarcosine transport system. This is in line with the finding that cycloheximide reversibly inhibits protein biosynthesis in yeast, but allows the accumulation of RNA with a DNA-like composition[10,15]. Similar experiments have been described for the elimination of the lag period during enzymatic induction[16,17]. There is also an optimum preincubation period with cycloheximide for the posterior translation of the specific mRNA information.

These results are in agreement with our hypothesis that the transport of sarcosine and of other imino acids in *S. chevalieri* is regulated at the level of protein biosynthesis.

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