THE PROTEINASE INHIBITOR PHENYLMETHYLSULFONYL FLUORIDE PROTECTS XANTHINE TRANSPORT IN *SCHIZOSACCHAROMYCES POMBE* AGAINST INACTIVATION BY AMMONIUM IONS

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1. Introduction

Ammonium ions interfere with the uptake of purine bases in several fungi [1–4]. In *Saccharomyces cerevisiae*, they influence the transport of certain amino acids [5,6] and affect a number of enzymes involved in nitrogen catabolism [7–11]. In all instances the presence of ammonium ions results in reduced or lacking biosynthesis of the corresponding proteins, a phenomenon called 'nitrogen catabolite repression'.

Here, we indicate that xanthine transport in *Schizosaccharomyces pombe* is not only subject to nitrogen catabolite repression, but also may be irreversibly inactivated in the presence of ammonium ions. In contrast to repression, inactivation is largely prevented when the cells are pretreated with the proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF).

A general characterization of the xanthine transport system by means of steady state kinetics has been reported in [3].

2. Materials and methods

[2-^{14}C]Xanthine was supplied from the Commissariat à l'Energie Atomique, Gif-sur-Yvette. Cycloheximide and phenylmethylsulfonyl fluoride were obtained from Serva, Heidelberg. Cellulose nitrate filters (poresize 0.45 μm, diam. 25 mm) were purchased from Schleicher and Schüll, Dassel.

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Schizosaccharomyces pombe, strain 972 h$^-$, was a kind gift from Dr H. Heslot, Paris. Cells were cultivated in a semi-synthetic medium just to the beginning of the stationary phase and were then preincubated for 1 h in a buffered glucose solution (50 mM sodium citrate, pH 5.4, 100 mM glucose) as in [12].

Uptake was measured at room temperature by incubating 2 × 10^7 cells for different times in 1 ml glucose solution coutaining 2 μM [2-^{14}C]xanthine (16 or 48 Ci/mol). The cell suspensions were passed through cellulose nitrate filters using the Millipore 3025 sampling manifold. The filters were washed with ice-cold glucose solution, completely dried and assayed for radioactivity in a Beckman LS-233 liquid scintillation counter. The scintillation fluid consisted of 5 g 2,5-diphenyloxazole/1-toluene.

3. Results and discussion

When *Schizosaccharomyces pombe* is harvested from the stationary growth phase the cells have a very restricted uptake capability for xanthine which may be overcome by their preincubation in glucose solution (fig.1,2, curves A in each). The time necessary for obtaining maximum uptake activity depends on the age of the preculture: with cells just arriving in the stationary phase a preincubation of 1 h at 30°C is sufficient for full activation, whereas older cultures may need several hours [3].

The stimulatory effect of the glucose pretreatment may be interpreted in terms of: (i) the activation of a pre-existing uptake capacity due to a rapid increase in the pool of energy-rich compounds; or (ii) the de novo synthesis of some protein involved directly

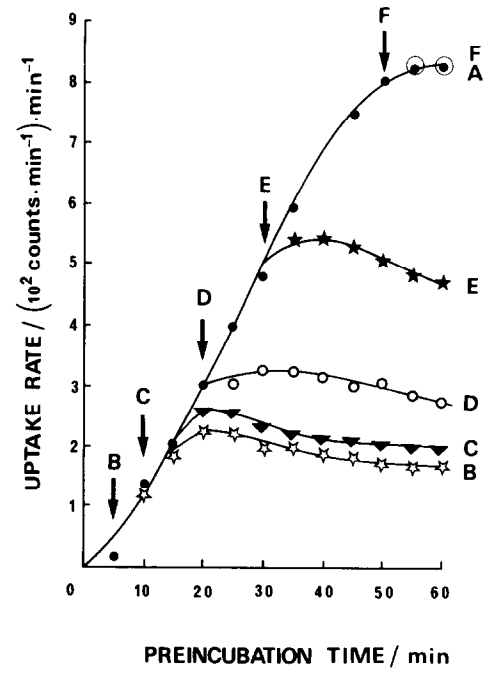

Fig.1. Stimulation of xanthine uptake during glucose pretreatment (curve A) and the effect of cycloheximide (100 mg/l) added at different times of the preincubation period (curves B–F).

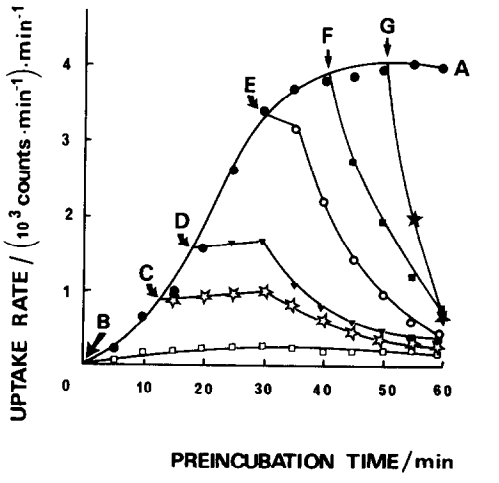

Fig.2. Stimulation of xanthine uptake during glucose pretreatment (curve A) and the effect of ammonium sulfate (5 g/l) added at different times of the preincubation period (curves B–G).

or indirectly in the transport process; or (iii) both. To decide between these alternatives, cycloheximide, a potent inhibitor of protein biosynthesis in yeasts [13] was added at different times to the preincubation mixture (fig.1). When the inhibitor is added at the beginning of the preincubation period stimulation continues for a certain time giving rise to an evidently higher uptake rate than obtained at the time of addition (curves B, C), whereas addition at a later time stops the stimulatory process more or less immediately keeping the uptake rate at the level attained thus far (curves D,E). This result argues for alternative (iii). It should be mentioned, however, that the pre-existing uptake capacity decreases with the age of the culture.

The stimulatory effect of the glucose pretreatment is also suppressed in the presence of ammonium ions [3]. The suppression is almost complete when ammonium sulfate is applied at the beginning of the preincubation period (fig.2, curve B). Addition at a later time leads, after a time-lag of distinct duration, to an exponential decrease in the initial uptake velocity (fig.2, curves C–G). The later the ammonium ions are added the shorter is the time-lag and the more impressive is the decrease.

The result of fig.2 compared with fig.1 may most conveniently be explained by the assumption that the presence of ammonium ions leads to the repression of some protein involved in the transport process and, beyond that, causes the inactivation of protein molecules already synthesized, whereby inactivation occurs faster than repression. Accordingly, the time-lag in the inhibition pattern of fig.2 reflects the period during which protein synthesis still continues but inactivation already takes place.

Routinely, we performed the ammonium experiments with the rather high concentration of 5 g ammonium sulfate (= 76 mequiv. NH_4^+)/1. Almost the same result may be obtained with concentrations as low as 0.1 mequiv. NH_4^+/1 (fig.3) indicating the high sensitivity of the cells to these ions when they are harvested from the early stationary phase. With old cultures which need several hours of glucose preincubation for complete recovery of their uptake capacity we still found the repression mechanism but failed to observe an inactivation by ammonium ions [3].

The question arises whether the inactivation of xanthine uptake by ammonium ions is reversible or irreversible. Following washing-out experiment should

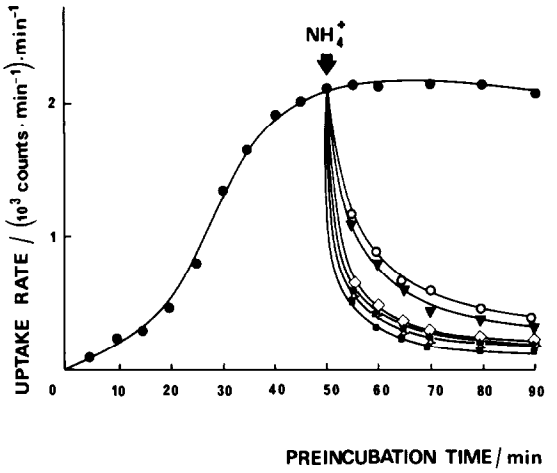

Fig.3. Inactivation of xanthine uptake by different ammonium concentrations added after 50 min of the preincubation period. Final concentrations were (mequiv. NH_4^+/1): 0 (●); 0.1 (○); 1 (▼); 5 (◇); 10 (★); 20 (☆); and 38 (■).

reveal an irreversible mechanism, if existent.

Cells of a preculture were divided into 4 parts and preincubated for 1 h in glucose solution with no addition (tube A) and with the addition of ammonium sulfate at the beginning (tube D) or after 50 min of the preincubation period (tubes B,C). Then the cells where centrifuged, carefully washed with glucose solution and resuspended in glucose solution containing labeled xanthine. In addition, tubes C, D contained cycloheximide to suppress protein de novo synthesis during the uptake experiment. In intervals, aliquots were removed from the tubes to measure the radioactivity incorporated into the cells.

The result presented in fig.4 shows that the inactivation of xanthine transport is not abolished by the attempt to wash out the ammonium ions (curves B–D). However, the transport activity recovers to a large extent when the cells are allowed to perform protein biosynthesis (curve B). Curve B indicates a steady increase in the uptake velocity, whereas curve A exhibits an approximately constant high uptake rate over the first 20 min of the experiment. Later on the velocity curves decline, probably due to the accumulation of inhibiting substrate molecules or their derivatives within the cell and/or a decrease in the energy supply.

Xanthine transport in *Schizosaccharomyces pombe* is therewith obviously irreversibly inactivated in the presence of ammonium ions and becomes regenerated

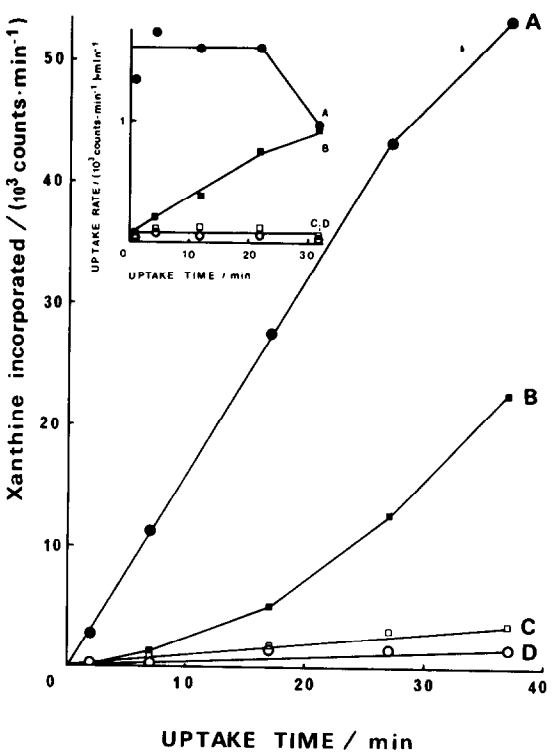

Fig.4. Progress of xanthine uptake in the absence (curves A,B) and in the presence (curves C,D) of 1 mg cycloheximide/10 ml glucose solution containing 2 μM [^{14}C]xanthine. Prior to the uptake measurement the cells have been preincubated for 1 h in glucose solution without (curve A) or with the addition of ammonium sulfate (5 g/l). Addition occurred at the beginning (curve D) and after 50 min preincubation period (curves B,C). Insert: Replot of the slopes from curves A–D of the main figure.

by way of protein de novo synthesis under derepression conditions.

The ammonium effect described here resembles closely to the phenomenon of catabolite inactivation which is caused by glucose and its metabolites and which is discussed in terms of a proteolytic mechanism ([1] cf. [14]). Among other lines of evidence this hypothesis is supported by the finding [15] that inactivation of α-isopropylmalate synthase from yeast is partially protected against catabolite inactivation when the serine proteinase inhibitor PMSF is present in the culture medium. In this context it was worth to test the possibility whether inactivation by ammonium ions might also be the consequence of proteolytic attack.

Cells of a stationary preculture were divided into 4

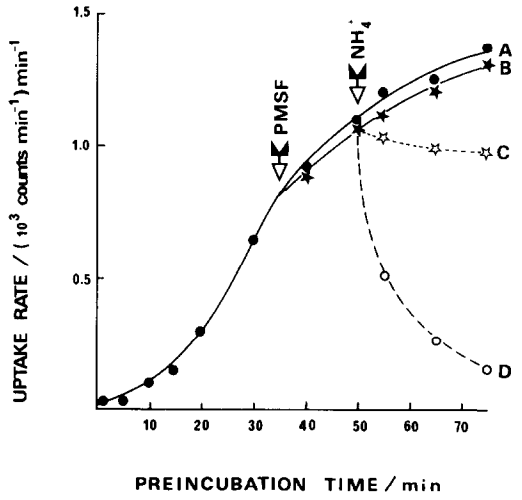

Fig.5. Protection of xanthine transport against ammonium inactivation by the proteinase inhibitor phenylmethylsulfonyl fluoride. PMSF (2 mM) was added after 35 min (curves B,C) and ammonium sulfate (5 g/l) after 50 min of the preincubation period (curves C,D).

parts and preincubated for 60 min in glucose solution with no addition (tube A), with the addition of PMSF after 35 min (tubes B,C), and with the addition of ammonium sulfate after 50 min preincubation period (tubes C,D). In intervals, aliquots were removed from the tubes to measure the uptake activities of the cells.

The result (fig.5) shows definitely that the presence of the proteinase inhibitor protects the xanthine transport system for the most part against ammonium inactivation, whereas PMSF alone does not influence significantly the uptake capability of the cells.

In conclusion, catabolite inactivation by proteolytic enzymes appears to be a general regulatory mechanism that not only exists in carbohydrate catabolism but may also be found in nitrogen metabolism.

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