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Transport overshoot during 2-methyl-4-amino-5-hydroxymethylpyrimidine uptake by *Saccharomyces cerevisiae*

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The transport overshoot during 2-methyl-4-amino-5-hydroxymethylpyrimidine (hydroxymethylpyrimidine) uptake by the thiamin transport system in *Saccharomyces cerevisiae* was investigated. The overshoot was found to be temperature- and energy-dependent and affected by the growth phase of the yeast. The efflux system for hydroxymethylpyrimidine appeared to be more sensitive to 2,4-dinitrophenol than the influx system, resulting in the loss of the overshoot of the pyrimidine in the presence of the uncoupler. Furthermore, the overshoot did not occur after the preincubation of yeast cells with inhibitors of protein synthesis such as cycloheximide and anisomycin. These results suggest that an active efflux system for hydroxymethylpyrimidine, which is rapidly synthesized, is involved in the overshoot of this pyrimidine during its transport in *S. cerevisiae*.

Introduction

In a previous study on the transport of 2-methyl-4-amino-5-hydroxymethylpyrimidine(hydroxymethylpyrimidine) in *Saccharomyces cerevisiae*, we found that hydroxymethylpyrimidine is taken up by a common transport system with thiamin; however, in contrast to thiamin, the accumulated hydroxymethylpyrimidine flows out of the cells [1]. This overshoot was further explored. In this paper we describe evidence indicating that the efflux of hydroxymethylpyrimidine is mediated by an active process specific for this pyrimidine which is synthesized during the early exponential phase of yeast growth.

Materials and Methods

Chemicals. [^{3}H]Hydroxymethylpyrimidine (6.5 Ci/mol) was prepared as previously described [1] and [*thiazole*-2-^{14}C]thiamin hydrochloride (24.3 Ci/mol) was obtained from Amersham International, Amersham, U.K. All other chemicals were purchased from commercial suppliers.

Organisms and growth conditions. The organism mainly used in this experiment was *S. cerevisiae*, which was isolated as previously described [2]. *S. cerevisiae* X2180-1A and *S. carlsbergensis (S. uvarum)* were also used. The growth conditions were the same as previously described [2] and the growth was measured turbidimetrically at 560 nm.

Assay of [^{3}H]hydroxymethylpyrimidine uptake. The uptake of [^{3}H]hydroxymethylpyrimidine was determined by a method previously described [1]. Washed yeast cells were suspended in 0.05 M potassium phosphate buffer (pH 5.0) containing 0.1 M glucose. The cell suspensions with an absorbance of 0.2 at 560 nm showed an average of 0.15 mg dry weight/ml. 5 ml of each cell suspension was preincubated for 15 min at 37°C with constant shaking and the uptake was then initiated by adding 50 μl of 0.1 mM [^{3}H]hydroxymethylpyrimidine (6.5 Ci/mol): the incubation was continued at 37°C. The sampling, filtration and counting were done as previously described [3]. The rate of hydroxymethylpyrimidine uptake at 37°C was expressed as nmol [^{3}H]hydroxymethylpyrimidine taken up per mg dry weight after subtracting the uptake at 0°C from that at 37°C, unless otherwise indicated. When the effect of temperature on [^{3}H]hydroxymethylpyrimidine uptake was tested, washed yeast cell suspensions in 0.05 M potassium phosphate buffer (pH 5.0) containing 0.1 M glucose were preincubated at 37°C for 15 min with constant shaking. The preincubated cells were collected by centrifugation at 4000 × *g* for 5 min and the cell

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pellets were washed once with cold water. The cell suspensions were added at a volume of 0.2 ml to 4.8 ml of 0.05 M potassium phosphate buffer (pH 5.0) containing 0.1 M glucose and 50 μl of 0.1 mM [^{3}H]hydroxymethylpyrimidine (6.5 Ci/mol) which was prewarmed at the indicated temperature and the incubation was continued with constant shaking. The radioactivity of 1 ml of the cell suspensions was measured at the indicated intervals.

Assay of [^{14}C]thiamin uptake. The uptake of [^{14}]thiamin was determined by a method previously described [3].

Results and Discussion

Time course of hydroxymethylpyrimidine transport. In a preceding paper [1], we demonstrated that the influx of hydroxymethylpyrimidine into *S. cerevisiae* is mediated by the same transport system as thiamin. As shown in Fig. 1, hydroxymethylpyrimidine as well as thiamin was taken up quickly by the yeast cells. However, the most unusual kinetic feature of hydroxymethylpyrimidine transport in contrast to thiamin transport was the overshoot which occurred after incubation for 5 min. The overshoot of hydroxymethylpyrimidine was also observed in other yeast strains such as *S. cerevisiae* X2180-1A and *S. carlsbergensis (S. uwarum)* (data not shown).

Effect of temperature on transport overshoot. When the incubation mixture was cooled in ice-water after incubation for 5 min at 37 °C the overshoot was hardly

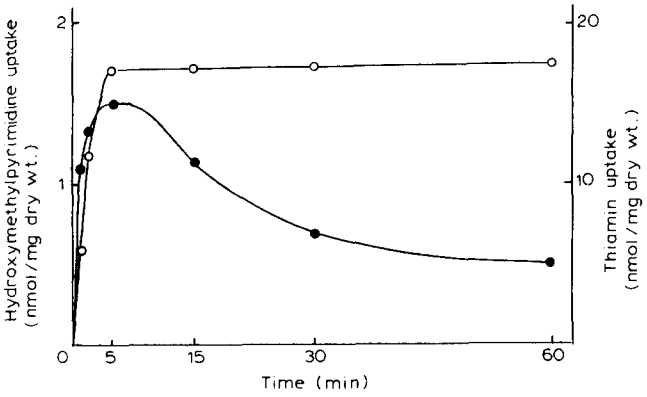

Fig. 1. Time course of [^{3}H]hydoxymethylpyrimidine uptake and [^{14}C]thiamin uptake. 10 ml of each yeast cell suspension (0.15 mg dry weight/ml) in 0.05 M potassium phosphate buffer (pH 5.0) containing 0.1 M glucose, was preincubated for 15 min at 37 °C, and then [^{3}H]hydroxymethylpyrimidine was added to the medium at a concentration of 1 μM, followed by further incubation at 37 °C. The uptake of [^{3}H]hydroxymethylpyrimidine (●) was measured as described in Materials and Methods, at the indicated times. The assay of [^{14}C]thiamin uptake (○) was determined by the same method as that of [^{3}H]hydroxymethylpyrimidine uptake described above except for the use of yeast cell suspensions (30 μg dry weight/ml). Each point represents the mean of two experiments.

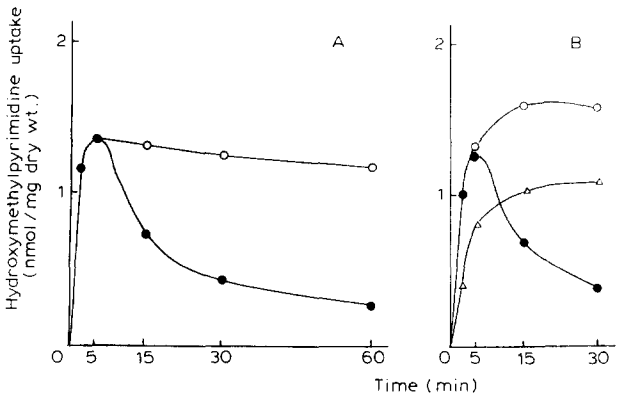

Fig. 2. Effect of temperature on [^{3}H]hydroxymethylpyrimidine uptake. The time course of [^{3}H]hydroxymethylpyrimidine uptake was examined as described in Materials and Methods. ●, control incubated with [^{3}H]hydroxymethylpyrimidine at 37 °C; ○, the incubation mixture was kept in ice-water after incubation with [^{3}H]hydroxymethylpyrimidine for 5 min at 37 °C (Fig. 2A). In Fig. 2B the uptake of [^{3}H]hydroxymethylpyrimidine by yeast cells preincubated at 37 °C was carried out at 37 °C (●, control), 45 °C (○) and 50 °C (△), respectively. Each point represents the mean of two experiments.

observed (Fig. 2A). Since the process involving overshoot seemed to be temperature-dependent, the effect of temperature on hydroxymethylpyrimidine uptake was further investigated. As shown in Fig. 2B, the initial rate of hydroxymethylpyrimidine uptake was almost the same at 37 °C and 45 °C and it decreased at 50 °C, whereas the overshoot was not observed in either the incubation at 45 °C or 50 °C. This finding, together with the results described above, indicates that the efflux process of hydroxymethylpyrimidine is temperature-dependent, like the influx process.

Dependence of overshoot on hydroxymethylpyrimidine concentration. Since it was thought that hydroxymethylpyrimidine accumulated in the cells inhibits its influx reaction, decreasing the influx rate relative to the outflow after a short time, thus creating the overshoot, it was examined whether a relatively high concentration of hydroxymethylpyrimidine was required to create the overshoot. When the yeast cells were exposed to 0.1 μM [^{3}H]hydroxymethylpyrimidine rather than the usual concentration of 1 μM, the pyrimidine was transported without overshoot (data not shown). This observation suggests that a high hydroxymethylpyrimidine concentration is necessary to create conditions for diminished transport.

Effect of 2.4-dinitrophenol on transport overshoot. To investigate the energy dependence of the efflux of hydroxymethylpyrimidine, the effect of 2,4-dinitrophenol, which was found to inhibit the active transport of thiamin and hydroxymethylpyrimidine in *S. cerevisiae* [1,3], added to the preincubation medium on overshoot was examined. As shown in Fig. 3, no overshoot of hydroxymethylpyrimidine was observed in the presence of 0.2 mM 2,4-dinitrophenol, although the uncoupler

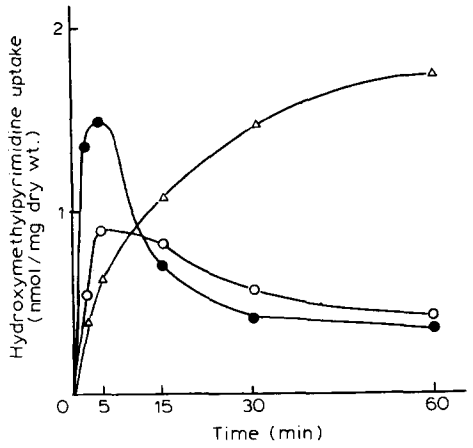

Fig. 3. Effect of 2,4-dinitrophenol on [^{3}H]hydroxymethylpyrimidine uptake. Yeast cell suspensions (0.15 mg dry weight/ml) were preincubated for 15 min at 37°C with 2,4-dinitrophenol of 0.1 mM (○) and 0.2 mM (△), respectively, and without the uncoupler (●, control). [^{3}H]Hydroxymethylpyrimidine was added to the medium at a 1 μM concentration, followed by further incubation for 60 min at 37°C. The uptake of [^{3}H]hydroxymethylpyrimidine was measured as described in Materials and Methods. Each point represents the mean of two experiments.

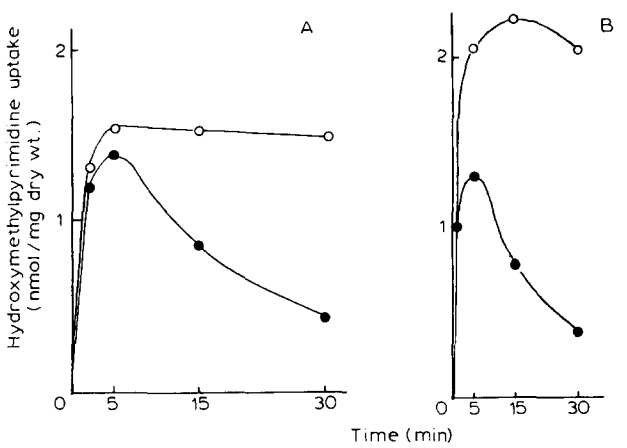

Fig. 4. Effect of cycloheximide and anisomycin on [^{3}H]hydroxymethylpyrimidine uptake. Yeast cell suspensions (0.15 mg dry weight/ml) were preincubated for 15 min at 37°C with cycloheximide (1 μg/ml, ○) in Fig. 4A and anisomycin (0.2 μg/ml, ○) in Fig. 4B, respectively and without inhibitor (●). [^{3}H]Hydroxymethylpyrimidine was added to the medium at a 1 μM concentration, followed by further incubation for 30 min at 37°C. The uptake of [^{3}H]hydroxymethylpyrimidine was measured as described in Materials and Methods. Each point represents the mean of two experiments.

inhibited the influx of hydroxymethylpyrimidine. These results suggest that both the influx and efflux processes of hydroxymethylpyrimidine are energy-dependent and the latter is more sensitive to the inhibitor than the former.

Effect of protein synthesis inhibitors on transport overshoot. Since an active efflux of intracellular hydroxymethylpyrimidine appeared to play an important role in the overshoot of this pyrimidine, the possibility was tested whether the synthesis of the efflux system occurred in resting yeast cells. Therefore, the uptake of [^{3}H]hydroxymethylpyrimidine was investigated in the presence of cycloheximide in the preincubation medium (1 μg/ml), which is the most commonly used inhibitor of protein synthesis in yeast. As shown in Fig. 4A, cycloheximide not only inhibited the overshoot but stimulated the influx of hydroxymethylpyrimidine. A similar result was obtained with anisomycin [4], another inhibitor of yeast protein synthesis (Fig. 4B). These results suggest that some protein component(s) involved in the active efflux of hydroxymethylpyrimidine is synthesized during preincubation for the uptake. On the other hand, the synthesis of the influx system appeared to be unaffected by these inhibitors, and the apparent stimulatory effect of the inhibitors on the influx could be attributed to their inhibitions on the synthesis of the efflux system of hydroxymethylpyrimidine.

Relation of the growth phase to transport overshoot. It has been shown that the activity of yeast thiamin transport system, namely the hydroxymethylpyrimidine transport system, is dependent on the growth phase of *S. cerevisiae* [5]. The uptake activity was highest during the early exponential phase of the yeast growth and then declined markedly with continued growth. Therefore, the relation of the growth phase to the overshoot of hydroxymethylpyrimidine was investigated. As in the results shown in Fig. 5, the overshoot became less prominent with the growth of *S. cerevisiae*, and disappeared in the yeast cells during the late exponential growth phase. This suggests that the efflux system of

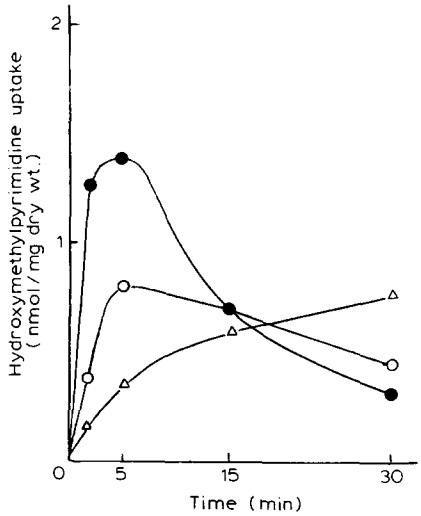

Fig. 5. Relation of yeast growth phase to [^{3}H]hydroxymethylpyrimidine uptake. Yeast cell suspensions (0.15 mg dry weight/ml) were prepared from three different batches of cultures. Yeast cells were harvested during the early exponential growth phase (●), midexponential growth phase (○) and late exponential growth phase (△), respectively. The uptake of [^{3}H]hydroxymethylpyrimidine by each of these cell suspensions was carried out as described in Materials and Methods. Each point represents the mean of two experiments.

hydroxymethylpyrimidine is synthesized before the mid-exponential phase of yeast growth.

It was finally concluded that yeast has an active efflux system specific for hydroxymethylpyrimidine which is synthesized mainly during the early exponential growth phase. The physiological role of this system remains to be further clarified, but a high level of intracellular hydroxymethylpyrimidine could be toxic in yeast, since this pyrimidine is known to act as a strong competitive inhibitor of pyridoxal kinase in living cells [6,7].

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