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Transport of 2-methyl-4-amino-5-hydroxymethylpyrimidine in *Saccharomyces cerevisiae*

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The transport of 2-methyl-4-amino-5-hydroxymethylpyrimidine (hydroxymethylpyrimidine) was studied in resting cells of *Saccharomyces cerevisiae*. Hydroxymethylpyrimidine uptake was an energy- and temperature-dependent process which has an optimal pH at 4.5. The apparent K_m for hydroxymethylpyrimidine uptake was 0.37 μM, and the uptake was inhibited by 2-methyl-4-amino-5-aminomethylpyrimidine, thiamin and pyrithiamin. Furthermore, hydroxymethylpyrimidine uptake was inhibited by 4-azido-2-nitrobenzoylthiamin, a specific and irreversible inhibitor of the yeast thiamin transport system and it was greatly impaired in a thiamin transport mutant of *S. cerevisiae*. Thus, hydroxymethylpyrimidine is taken up by a common transport system with thiamin in *S. cerevisiae*, but in contrast to thiamin transport, accumulated hydroxymethylpyrimidine is released from yeast cells showing an overshoot phenomenon.

Introduction

Thiamin uptake by *Saccharomyces cerevisiae* has been established to be mediated by an active transport mechanism [1,2]. It is also known that 2-methyl-4-amino-5-hydroxymethylpyrimidine, the pyrimidine moiety of thiamin, and 4-methyl-5β-hydroxyethylthiazole, the thiazole moiety of thiamin, are quickly taken up to form thiamin by resting yeast cells [3] and that the separate moieties of thiamin can be assayed by the use of yeast [4]. In a previous study we presented evidence suggesting that hydroxyethylthiazole is transported into yeast cells by diffusion, followed by metabolic trapping due to hydroxyethylthiazole kinase-catalyzed phosphorylation [5]. On the other hand, no information regarding the transport of hydroxymethylpyrimidine in yeast has been available so far. In this paper we describe evidence indicating that hydroxymethylpyrimidine is taken up by a common transport system with thiamin in resting cells of *S. cerevisiae*.

Materials and Methods

Chemicals. [^{3}H]Hydroxymethylpyrimidine (6.5 Ci/mol), tritiated by catalytic exchange, was from Du Pont-New England Nuclear Research Products (95% pure as determined by paper and thin-layer chromatography). [*thiazole*-2-^{14}C]Thiamin hydrochloride (24.3 Ci/mol) was obtained from the Radiochemical Center, Amersham, U.K. Pyrithiamin hydrobromide and oxythiamin hydrochloride were the products of Sigma Chemicals Co. Hydroxymethylpyrimidine, 2-methyl-4-amino-5-aminomethylpyrimidine and 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine were gifts from Takeda Chemical Industries Ltd. (Osaka). 4-Azido-2-nitrobenzoylthiamin was prepared as previously described [6]. All other chemicals were purchased from commercial suppliers.

Organisms and growth conditions. S. cerevisiae obtained as a clonal isolate of commercial baker's yeast (Oriental's) and a thiamin transport mutant of *S. cerevisiae* (PT-R2) isolated by the procedure previously described [7] were used. Yeast cells were grown at 30 °C for 16 h in Wickerham's synthetic medium [8] except that thiamin was omitted. After harvesting, cells were washed once with cold water.

Assay of [^{3}H]hydroxymethylpyrimidine uptake. 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 at 560 nm of 0.2 showed an average of 0.15 mg dry weight/ml. 5 ml of the cell suspensions were 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. Sampling, filtration and counting were

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done as previously described [1]. The rate of hydroxymethylpyrimidine uptake at 37 °C is 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.

Assay of [^{14}C]thiamin uptake. The uptake of [^{14}C]thiamin was determined by the method previously described [1].

Photoinactivation procedure. Photoinactivation studies were carried out as previously described [6].

Results

Time dependence of hydroxymethylpyrimidine uptake

The time course of hydroxymethylpyrimidine uptake in *S. cerevisiae* is shown in Fig. 1. The uptake was linear for about 1 min, reaching a maximum at 5 min, followed by a loss of accumulated pyrimidine. There was a linear relationship between the initial rate of hydroxymethylpyrimidine uptake and the mass of cells at least up to 0.3 mg dry weight under the conditions employed.

Effect of pH and temperature

The pH dependence of hydroxymethylpyrimidine showed a maximum at 4.5 and no significant uptake was observed when the experiments were conducted at 0 °C (Fig. 1).

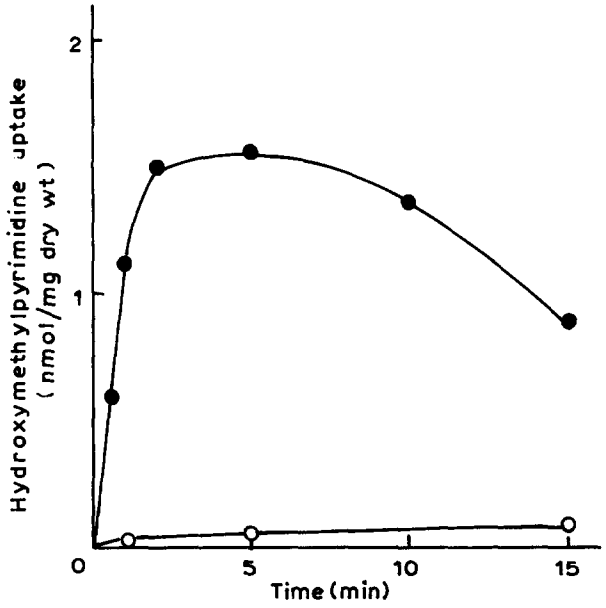

Fig. 1. Time course of [^{3}H]hydroxymethylpyrimidine uptake and effect of temperature. 10 ml of yeast cell suspensions (0.15 mg dry weight/ml) in 0.05 M potassium phosphate buffer (pH 5.0) containing 0.1 M glucose, were preincubated for 15 min at 37 °C, and then [^{3}H]hydroxymethylpyrimidine was added to the medium at a concentration of 1 μM, followed by further incubation at 37 °C (●) and 0 °C (○), respectively. The uptake of [^{3}H]hydroxymethylpyrimidine was measured as described in Methods at the indicated times. Each point represents the mean of two experiments.

TABLE I

Effect of glucose and 2,4-dinitrophenol on hydroxymethylpyrimidine uptake

Yeast cell suspensions (0.15 mg dry weight/ml) were preincubated for 15 min at 37 °C with or without the additions indicated. [^{3}H]Hydroxymethylpyrimidine was added to the medium at a concentration of 1 μM, followed by further incubation for 30 s at 37 °C. The uptake of [^{3}H]hydroxymethylpyrimidine was measured as described in Methods. Values are averages of two experiments.

Addition (mM)	Hydroxymethylpyrimidine uptake (nmol/30 s per mg dry wt.)	% Inhibition
None	0.88	
−Glucose	0.13	85.2
+2,4-Dinitrophenol (0.2)	0.35	60.2
+2,4-Dinitrophenol (0.4)	0.13	85.2

Energy requirement

As shown in Table I, after 30 s of incubation at 37 °C, yeast cells took up 6.8-times more labeled hydroxymethylpyrimidine in the presence of glucose than in its absence. The preincubation of yeast cells with 2,4-dinitrophenol caused a marked reduction in the amount of hydroxymethylpyrimidine taken up. These results suggest that the process of hydroxymethylpyrimidine permeation is an energy-dependent process.

Intracellular state of hydroxymethylpyrimidine transported and establishment of concentration gradients

When the intracellular [^{3}H]hydroxymethylpyrimidine was extracted from yeast cells, which had been allowed to accumulate for 5 min, and subjected to paper chromatography by the procedure previously described [1], a major radioactive peak (83.5%) was obtained with an R_F of 0.79, which corresponded to unlabeled hydroxymethylpyrimidine. The small peaks (R_F values: 0.38 and 0.14) represented hydroxymethylpyrimidine mono- and diphosphate, respectively. On the basis of 2.1 μl of intracellular water per mg dry yeast [9], the intracellular hydroxymethylpyrimidine concentration was approximately 610-fold the external hydroxymethylpyrimidine concentration.

Kinetics and substrate dependence

The hydroxymethylpyrimidine transport system showed dependence on substrate concentration and it was saturable (data not shown). Lineweaver-Burk plots of the transport as a function of hydroxymethylpyrimidine concentrations gave an apparent K_m for hydroxymethylpyrimidine of 0.37 μM.

Effect of pyrimidine derivatives and thiamin analogs

2-Methyl-4-amino-5-aminomethylpyrimidine was found to inhibit hydroxymethylpyrimidine uptake,

TABLE II

Effect of pyrimidine derivatives and thiamin analogs on hydroxymethylpyrimidine uptake

Yeast cell suspensions (0.15 mg dry weight/ml) in the uptake medium were exposed simultaneously to 1 μM [^{3}H]hydroxymethylpyrimidine and analogs at the concentrations indicated. After incubation for 30 s at 37°C [^{3}H]hydroxymethylpyrimidine taken up by the cells was measured as described in Methods. Values are averages of two experiments.

Addition (μM)	Hydroxymethylpyrimidine uptake (%)
None	100
2-Methyl-4-amino-5-aminomethylpyrimidine (1.0)	59.8
2-Methyl-4-hydroxy-5-hydroxymethylpyrimidine (1.0)	0
Thiamin (1.0)	48.5
Pyrithiamin (1.0)	59.5
Oxythiamin (1.0)	0

whereas 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine had no effect on the uptake (Table II). It has been shown that the yeast thiamin transport system displays a high structural specificity for the pyrimidine moiety of thiamin, since [^{14}C]thiamin uptake was markedly inhibited by hydroxymethylpyrimidine as well as pyrithiamin, an antagonist of thiamin [10]. Therefore, the effect of thiamin and its analogs on hydroxymethylpyrimidine uptake by yeast cells was tested. As shown in Table II, both thiamin and pyrithiamin showed strong

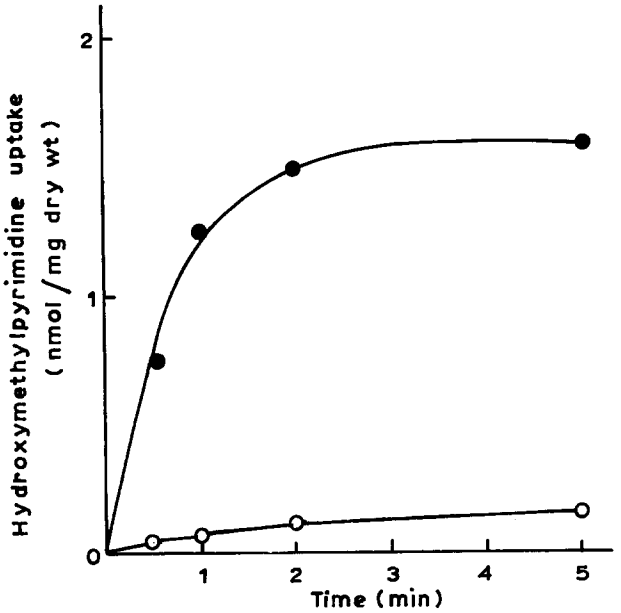

Fig. 2. Time course of hydroxymethylpyrimidine uptake by a thiamin transport mutant of *S. cerevisiae*. Uptake by the parent strain (●) and a thiamin transport mutant(PT-R2) of *S. cerevisiae* (○) was measured as described in Methods. Each point represents the mean of two experiments.

inhibitory effects on hydroxymethylpyrimidine uptake, whereas oxythiamin showed no effect. The inhibition by thiamin was competitive and the apparent K_i value of thiamin for the hydroxymethylpyrimidine transport system was 0.31 μM (data not shown).

Effect of 4-azido-2-nitrobenzoylthiamin-dependent photoinactivation of thiamin transport system on hydroxymethylpyrimidine uptake

Our previous study showed that 4-azido-2-nitrobenzoylthiamin is a specific and irreversible inhibitor of the thiamin transport system in *S. cerevisiae* [6]. When yeast cells were irradiated with visible light in the presence of 1 μM 4-azido-2-nitrobenzoylthiamin the uptake of hydroxymethylpyrimidine was remarkably reduced which correlated well with a decrease in thiamin uptake (Table III). These results strongly suggest that the thiamin transport system in *S. cerevisiae* also functions for the transport of hydroxymethylpyrimidine.

Hydroxymethylpyrimidine uptake by a thiamin transport mutant of S. cerevisiae

In order to obtain further evidence for a common transport system for thiamin and hydroxymethylpyrimidine the uptake of hydroxymethylpyrimidine by a thiamin transport mutant of *S. cerevisiae*(PT-R2), which was found to be almost totally defective in thiamin uptake [7], was investigated. Fig. 2 shows the time course of hydroxymethylpyrimidine uptake by *S. cerevisiae*. It can be seen that there was a remarkable decrease in the activity of hydroxymethylpyrimidine uptake by the mutant compared to the parent strain. The rate of hydroxymethylpyrimidine uptake by the mutant cells was less than 7% of that of the parent strain.

Effect of thiamin added to the growth medium on hydroxymethylpyrimidine uptake

It has been found that yeast cells are repressed for thiamin uptake when grown in the presence of exogenous thiamin [11]. As shown in Table IV the addition of thiamin to the growth medium also caused a marked decrease in hydroxymethylpyrimidine uptake, suggesting that the intracellular level of thiamin, possibly thiamin pyrophosphate, in growing yeast regulates a common transport system for thiamin and hydroxymethylpyrimidine, a direct precursor for thiamin biosynthesis in yeast.

Discussion

It was of interest to investigate whether yeast has a specific transport system for hydroxymethylpyrimidine, since this compound is not only the pyrimidine moiety of thiamin, but it has a structure similar to the pyrimidine bases of nucleic acids and vitamin B-6. Bellion and

TABLE III

Photoinactivation of hydroxymethylpyrimidine uptake with 4-azido-2-nitrobenzoylthiamin

Photoinactivation studies were carried out in a 20-ml glass beaker which was kept on ice. The washed yeast cells, suspended in 2 ml of 0.05 M potassium phosphate buffer (pH 5.0) at the concentration of 0.5 mg dry weight per ml, were irradiated in the presence of 1 μM 4-azido-2-nitrobenzoylthiamin (ANBT). The irradiated cells were washed with cold water and suspended in 1 ml of cold water. Aliquots of the cell suspensions were added to the uptake medium containing 1 μM [^{3}H]hydroxymethylpyrimidine or 1 μM [^{14}C]thiamin which was prewarmed at 37°C. After 30 s at 37°C the radioactivity was measured as described in Methods. Values are averages of two experiments.

Addition (μM)	Irradiation	Uptake (nmol/30 s per mg dry wt.) (%)	
		thiamin	hydroxymethylpyrimidine
None	+	2.42 (100)	0.75 (100)
ANBT (1.0)	−	2.49 (102.9)	0.76 (101.3)
ANBT (1.0)	+	1.01 (41.7)	0.30 (40.0)

Lash [12] reported that an active transport system specific for hydroxymethylpyrimidine exists in *Salmonella typhimurium*. The results described in the present paper indicate that *S. cerevisiae* also possesses an active transport system for hydroxymethylpyrimidine. It is dependent on the presence of an energy source, glucose, and shows saturation kinetics. The transport results in the accumulation of hydroxymethylpyrimidine against a large concentration difference and it is inhibited by an uncoupler. However, in contrast to the transport system in *Salmonella typhimurium*, hydroxymethylpyrimidine transport in *S. cerevisiae* was found to be competitively inhibited by thiamin. Since it has been shown that yeast has an active transport system for thiamin which is specific for the structure of the pyrimidine moiety of thiamin [10], we examined the possibility that thiamin and hydroxymethylpyrimidine share a common transport system in yeast. From the results obtained with specific inhibitors for thiamin transport and a thiamin transport mutant of *S. cerevisiae* it could be concluded that hydroxymethylpyrimidine is taken up by the same transport system as thiamin in yeast, but the presence of other transport systems for hydroxymethylpyrimidine can not be excluded, because a small amount of [^{3}H]hydroxymethylpyrimidine uptake was still observed in the thiamin transport mutant which is almost totally defective in the thiamin transport system. Finally, it was found in this study that there was a difference between the transport of thiamin and hydroxymethylpyrimidine in *S. cerevisiae*, which was a transport overshoot during hydroxymethylpyrimidine uptake. Since thiamin accumulated in yeast cells does not flow out of the cells, this overshoot phenomenon appears to be specific for this pyrimidine and it may work as a mechanism which rids yeast cells of excess hydroxymethylpyrimidine.

TABLE IV

Effect of thiamin added to the growth medium on the rate of hydroxymethylpyrimidine uptake

After 16 h of growth in Wickerham's minimal medium (50 ml) containing the indicated thiamin concentration, uptake of [^{3}H]hydroxymethylpyrimidine was measured as described in Methods. Average values of two independent experiments are given.

Addition (μM)	Hydroxymethylpyrimidine uptake (nmol/30 s per mg dry wt.)
None	0.67
Thiamin (0.2)	0.06
Thiamin (1.0)	0.02

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