A Constitutive Thiamine Metabolism Mutation, *thi80*, Causing Reduced Thiamine Pyrophosphokinase Activity in *Saccharomyces cerevisiae*

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We identified a strain carrying a recessive constitutive mutation (*thi80-1*) with an altered thiamine transport system, thiamine-repressible acid phosphatase, and several enzymes of thiamine synthesis from 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-β-hydroxyethylthiazole. The mutant shows markedly reduced activity of thiamine pyrophosphokinase (EC 2.7.6.2) and high resistance to oxythiamine, a thiamine antagonist whose potency depends on thiamine pyrophosphokinase activity. The intracellular thiamine pyrophosphate content of the mutant cells grown with exogenous thiamine (2×10^{-7} M) was found to be about half that of the wild-type strain under the same conditions. These results suggest that the utilization and synthesis of thiamine in *Saccharomyces cerevisiae* is controlled negatively by the intracellular thiamine pyrophosphate level.

Resting cells of *Saccharomyces cerevisiae* grown in thiamine-omitted Wickerham's synthetic medium (19) can accumulate a large amount of external thiamine by an active transport system (4), whereas the thiamine transport activity was repressed by thiamine in our growth medium (7). Our previous study showed that thiamine-repressible acid phosphatase (T-rAPase) encoded by the *PHO3* gene (17) and the enzymes (hydroxymethylpyrimidine kinase [EC 2.7.1.49], phosphomethylpyrimidine kinase [EC 2.7.4.7], hydroxyethylthiazole kinase [EC 2.7.1.50], and thiamine-phosphate pyrophosphorylase [EC 2.5.1.3]) involved in thiamine synthesis from 2-methyl-4-amino-5-hydroxymethylpyrimidine (hydroxymethylpyrimidine) and 4-methyl-5-β-hydroxyethylthiazole (hydroxyethylthiazole) were repressible by exogenous thiamine (9). It was also found that a *pho6* mutant defective in a regulatory gene for the synthesis of periplasmic T-rAPase (16) was auxotrophic for thiamine and that the activities of enzymes involved in thiamine synthesis described above were markedly low in the crude extract from the *pho6* mutant (9). These results indicate that both positive and negative regulatory mechanisms would function in thiamine metabolism of *Saccharomyces cerevisiae*.

In this paper, we describe the isolation of a *thi80* mutant of *S. cerevisiae*, in which mutation gives rise to a recessive constitutive phenotype concerning activities of thiamine transport, T-rAPase, and enzymes involved in thiamine synthesis from hydroxymethylpyrimidine and hydroxyethylthiazole. The *thi80* mutant showed a lower level of thiamine pyrophosphokinase activity, accompanied by the decrease of the intracellular concentration of thiamine pyrophosphate to about a half of that of the wild-type cells, when cultured in the presence of thiamine (2×10^{-7} M). These results suggest that thiamine pyrophosphate acts as a negative effector for the regulatory mechanism in thiamine metabolism of *S. cerevisiae*.

NTR7 (*MATa thi80-1 his4-519 gal2*) with a lower level of

the thiamine pyrophosphokinase activity was obtained from a parent strain, IFO 10482 (*MATa his4-519 gal2*), after ethyl methanesulfonate mutagenesis by the method of Lindegren et al. (11). X2180-1Btrp (*MATα trp1 gal2*) was also used in this study. Yeast cells were grown at 30°C in thiamine-omitted Wickerham's synthetic medium, which was used for a minimal medium (19). The medium was generally supplemented with histidine (20 μg/ml). For repression studies, thiamine was added at various concentrations as described below. YPD medium contained Difco yeast extract (10 g/liter), Difco Bacto-Peptone (20 g/liter), and glucose (20 g/liter). SD medium contained Difco Bacto-Yeast nitrogen base without amino acid (6.7 g/liter) and glucose (20 g/liter). Genetic techniques used were those described by Sherman et al. (15). Thiamine transport and T-rAPase activities on the plate were detected by the staining method based on the uptake and reduction of triphenyltetrazolium chloride (5) and diazo coupling reaction (18), respectively. [14C]thiamine ([*thiazole*-2-14C]thiamine hydrochloride, 24.3 Ci/mol) was purchased from Amersham International (Buckinghamshire, United Kingdom). Oxythiamine hydrochloride was a product of Sigma Chemical Co. All other chemicals were purchased from commercial suppliers.

Enzyme assays. After being harvested, yeast cells were washed once with cold water and then suspended in 0.05 M potassium phosphate buffer (pH 7.5) containing 1 mM 2-mercaptoethanol and 1 mM EDTA. The cell suspensions were sonicated, and after centrifugation at 28,000 × g for 30 min, the supernatant was used as a crude extract. Overall thiamine-synthesizing activities of hydroxymethylpyrimidine, hydroxyethylthiazole, hydroxymethylpyrimidine kinase, phosphomethylpyrimidine kinase, hydroxyethylthiazole kinase, and thiamine-phosphate pyrophosphorylase were assayed as previously described (9). Thiamine pyrophosphokinase activity was measured by the procedure of Kaziro (10) with some modifications as follows: the assay mixture contained 0.02 M Tris hydrochloride buffer (pH 8.6), 1 mM thiamine, 1 mM ATP, 2 mM MnSO₄, and the crude extract (0.2 mg of protein) in a final volume of 1.5 ml. After
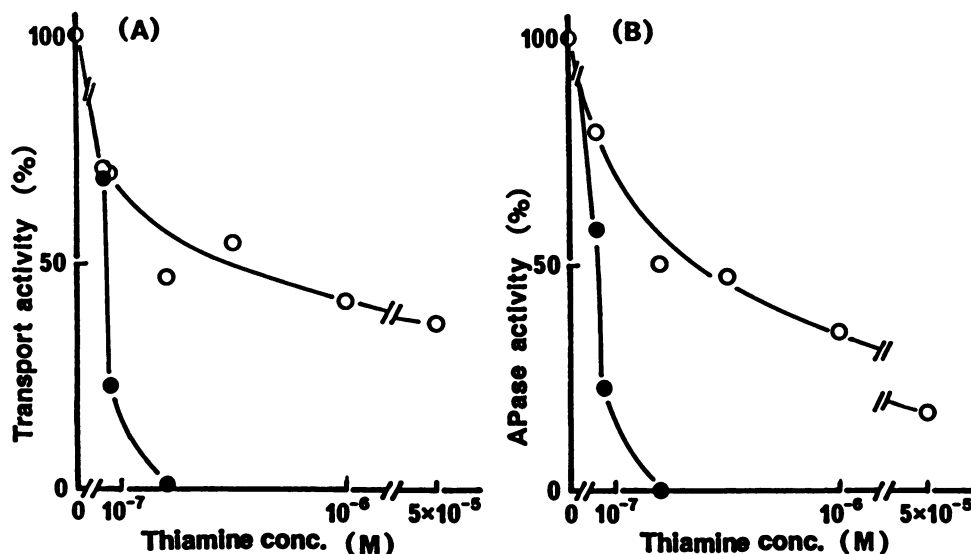
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FIG. 1. Effect of thiamine in growth medium on activities of thiamine transport and T-rAPase in the wild-type strain and NTR7. Thiamine transport and T-rAPase activities were measured as described in the text. (A) Thiamine transport activity in the wild-type strain (●) and NTR7 (○). (B) T-rAPase activity in the wild-type strain (●) and NTR7 (○). Each value is the mean from two experiments.

incubation for 15 min at 37°C, the reaction was stopped with 0.3 ml of 30% trichloroacetic acid. After centrifugation, trichloroacetic acid was removed twice by extraction with double volumes of ethyl ether. The amounts of thiamine and thiamine phosphates in the sample solution (water layer) were determined by high-pressure liquid chromatography after conversion to the corresponding thiochromes by alkaline oxidation with cyanogen bromide as previously described (13). T-rAPase activity with p-nitrophenylphosphate as a substrate was determined from the amount of p-nitrophenol produced as described earlier (14). The transport of thiamine was determined by the method described previously (4). Protein contents were determined by the method of Lowry et al. (12).

Measurement of intracellular contents of thiamine and thiamine phosphates. After being harvested, yeast cells were washed twice with cold water and 0.05 M sodium acetate buffer (pH 4.5) and then resuspended in the same buffer. The cell suspension (2 ml) was heated at 85°C for 15 min, and 0.4 ml of 30% trichloroacetic acid was added. After centrifugation, trichloroacetic acid was removed twice by extraction of ethyl ether. The content of thiamine and thiamine phosphates were measured by high-pressure liquid chromatography as previously reported (13).

In the previous paper (5), we reported that triphenyltetrazolium chloride, a basic dye, is taken up by yeast cells via the thiamine transport system, and the yeast cells grown without thiamine changed their color to red. On the other hand, the colonies of yeast cells grown in the presence of thiamine and of a thiamine transport mutant of *S. cerevisiae* remained unchanged in color after the addition of this dye (5). This suggested that the reduction of triphenyltetrazolium chloride by yeast cells is applicable to the selection of thiamine transport-constitutive mutants. After the treatment of the parent strain IFO 10482 with ethyl methanesulfonate, the presence of a colony colored red by the addition of triphenyltetrazolium chloride to agar plates of Wickerham's synthetic medium containing thiamine (5 × 10⁻⁷ M) led to the isolation of NTR7. NTR7 was also found to produce T-rAPase constitutively at the same time. Genetic analysis

was carried out to characterize the mutant NTR7. As a dominance-recessiveness test, NTR7 was crossed to the wild-type strain X2180-1Btrp. Both thiamine transport and T-rAPase activities in the resultant diploid cells were repressed by exogenous thiamine, indicating that this mutation is recessive. The obtained diploid was then sporulated, and four-spored asci were dissected. The phenotypes for thiamine transport and T-rAPase activities were cosegregated and showed a 2+:2− pattern in 100 asci as tested, indicating that both phenotypes were the result of the same mutation of a single gene in the nucleus. The mutation in NTR7 is designated *thi80-1*.

As previously described (2, 3, 6, 14), the activities of thiamine transport system and T-rAPase were repressible by exogenous thiamine. As shown in Fig. 1A, thiamine transport activity in the wild-type cells was repressed completely by exogenous thiamine at a concentration of 2 × 10⁻⁷ M. Approximately 50% of the transport activity in *thi80-1* mutant cells remained after growth with thiamine at this concentration. The activity was fairly detectable even at a concentration of 5 × 10⁻⁶ M. Similarly, Fig. 1B indicates that T-rAPase activity in *thi80-1* mutant cells is also constitutively expressed.

We have previously reported that activities of T-rAPase and the four enzymes involved in the formation of thiamine monophosphate from hydroxymethylpyrimidine and hydroxyethylthiazole, namely hydroxymethylpyrimidine kinase, phosphomethylpyrimidine kinase, hydroxyethylthiazole kinase, and thiamine-phosphate pyrophosphorylase, in a *pho6* mutant were markedly low or undetectable (9). Therefore, we further determined the activities of the four enzymes described above in the crude extract from the parent strain cells and *thi80-1* mutant cells cultured in the absence or presence of thiamine (2 × 10⁻⁷ M). As shown in Table 1, the enzyme activities in the crude extract prepared from the wild-type cells were significantly repressed by exogenous thiamine at a concentration of 2 × 10⁻⁷ M. On the other hand, the enzyme activities in *thi80-1* mutant cells were highly constitutive after cells were grown with thiamine at a concentration of 2 × 10⁻⁷ M compared with activities in

TABLE 1. Effect of thiamine added to the growth medium on activity of thiamine-synthesizing enzymes in the wild-type strain and NTR7

Enzyme	Enzyme activity[a] with thiamine concn for:			
	Wild-type strain		NTR7	
	None	0.2 μM	None	0.2 μM
Hydroxymethylpyrimidine kinase	9.4	0.2	8.3	3.3
Phosphomethylpyrimidine kinase	30.5	0	32.1	9.5
Hydroxyethylthiazole kinase	26.6	2.9	28.6	25.1
Thiamine-phosphate pyrophosphorylase	37.2	0.9	35.7	14.1
Thiamine pyrophosphokinase	4.6		1.1	

[a] Nanomoles per milligram of protein per 30 min. Each value is the mean from two experiments.

cells of the wild-type strain. The activity levels of hydroxymethylpyrimidine kinase, phosphomethylpyrimidine kinase, and thiamine-phosphate pyrophosphorylase in mutant cells grown with thiamine were 39.6, 29.6, and 39.5%, respectively, of those grown without thiamine, whereas the activity of hydroxyethylthiazole kinase was much more retained (87.8%) in the thi80-1 mutant cells.

Although there are several possibilities for altered repressibility of thiamine transport, T-rAPase, and thiamine biosynthesis by exogenous thiamine, the activity of thiamine pyrophosphokinase (which is responsible for the formation of a final product of thiamine biosynthesis, thiamine pyrophosphate) in the thi80-1 mutant was about 24% of that of the wild-type strain (Table 1). This suggests that the constitutive phenotype of the thi80-1 mutant in the activities of thiamine transport, T-rAPase, and the four enzymes involved in thiamine synthesis might be due to an insufficient intracellular concentration of thiamine pyrophosphate, which may be a negative effector, to repress these activities even after the cells are grown with thiamine.

As shown in Table 2, the intracellular contents of thiamine and thiamine phosphates in thi80-1 mutant cells grown in minimal medium without thiamine, in spite of the lower level of thiamine pyrophosphokinase activity, are the same as those of the wild-type cells, and the mutant is not auxotrophic for thiamine. However, after growth in minimal medium supplemented with thiamine $(2 \times 10^{-7}$ M), the thiamine content in thi80-1 mutant cells was remarkably higher than that in the wild-type cells, whereas the intracellular content of thiamine pyrophosphate was appreciably lower. By addition of exogenous thiamine at a concentration

TABLE 2. Effect of thiamine added to the growth medium on intracellular concentration of thiamine and thiamine phosphates in the wild-type strain and NTR7

Thiamine or thiamine phosphate	Intracellular concentration (μM)[a] with thiamine concn for:			
	Wild-type strain		NTR7	
	None	0.2 μM	None	0.2 μM
Thiamine	9.7	65.5	6.5	239.6
Thiamine monophosphate	2.4	8.0	2.4	5.4
Thiamine pyrophosphate	41.1	106.7	37.0	59.8

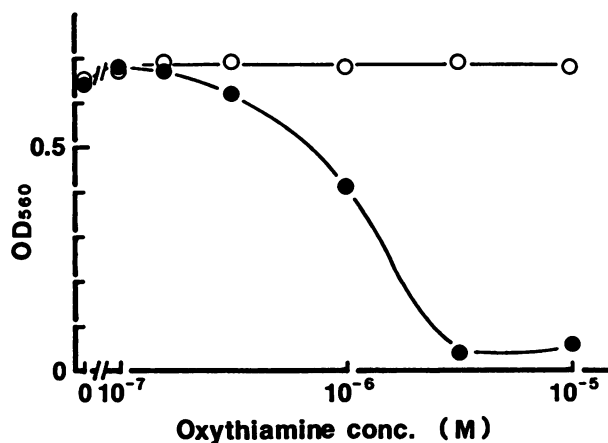[a] Each value is the mean from two experiments.

FIG. 2. Effect of oxythiamine on growth of the wild-type strain (●) and NTR7 (○). The growth was measured at an optical density of 560 nm after 16 h of growth. Each value is the mean from two experiments.

of 2×10^{-7} M, the intracellular content of thiamine in the mutant cells reached about fourfold that of thiamine pyrophosphate in the same cells. These results suggest that the expression of structural genes for thiamine transport system, T-rAPase, and the enzymes involved in thiamine monophosphate synthesis in S. cerevisiae is controlled negatively by the thiamine pyrophosphate level. As described previously (9), these activities were found to be correlatedly decreased with the increase of the concentration of intracellular thiamine pyrophosphate to about 150 μM in yeast cells.

Although it was reported that oxythiamine, a thiamine antagonist, shows only a slight inhibitory effect on thiamine pyrophosphokinase purified from S. cerevisiae (10), the antagonist can inhibit the growth of S. cerevisiae (8). Since it is presumed that the growth inhibition of yeast cells by oxythiamine occurs after its conversion to oxythiamine pyrophosphate by thiamine pyrophosphokinase (1), we examined the effect of oxythiamine on the growth of the thi80-1 mutant with a lower level of thiamine pyrophosphokinase activity. As shown in Fig. 2, the growth of the wild-type strain was almost completely inhibited by oxythiamine at a concentration of 5×10^{-6} M. On the other hand, no inhibition of the growth of the thi80-1 mutant with oxythiamine up to a concentration of 10 μM was observed. These results also indicate that the lower level of thiamine pyrophosphokinase activity in thi80-1 mutant cells may lead to the oxythiamine resistance phenotype of this mutant.

This is a first report on the isolation of a mutant of S. cerevisiae with a decreased activity of thiamine pyrophosphokinase. Although total deficiency of thiamine pyrophosphokinase in yeast cells is lethal, the thi80-1 mutant with a partial deficiency of this enzyme isolated in this study showed a constitutive phenotype of the activities of thiamine transport, T-rAPase, and enzymes involved in thiamine synthesis from hydroxymethylpyrimidine and hydroxyethylthiazole. Although it seems likely that a negative regulatory mechanism by thiamine pyrophosphate as an effector participates in the control of thiamine metabolism, the detailed mechanism of the regulation remains to be clarified further at the molecular level.

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