Some Properties of a *Saccharomyces cerevisiae* Mutant Resistant to 2-Amino-4-methyl-5- β -hydroxyethylthiazole

By AKIO IWASHIMA,* KAZUTO NOSAKA, HIROSHI NISHIMURA
AND YUKO KIMURA

Department of Biochemistry, Kyoto Prefectural University of Medicine, Kamikyo-ku,
Kyoto 602, Japan

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A mutant of *Saccharomyces cerevisiae* highly resistant to 2-amino-4-methyl-5- β -hydroxyethylthiazole (2-aminohydroxyethylthiazole), an antimetabolite of 4-methyl-5- β -hydroxyethylthiazole (hydroxyethylthiazole), has been isolated. Its resistance to 2-aminohydroxyethylthiazole was about 10^4 times that of the sensitive parent strain. The amount of thiamin synthesized in the cells of the resistant strain grown in minimal medium was less than half of that of the sensitive strain. The ability to synthesize thiamin from 2-methyl-4-amino-5-hydroxymethylpyrimidine (hydroxymethylpyrimidine) and hydroxyethylthiazole in the resistant strain was low compared with that of the sensitive strain. These results were found to be due to a deficiency of hydroxyethylthiazole kinase in the resistant strain: in sonic extracts of cells the enzyme activity was only 0.67% of that of the sensitive strain. Although the cells of the sensitive strain could accumulate exogenous hydroxyethylthiazole in the form of hydroxyethylthiazole monophosphate, no significant uptake of hydroxyethylthiazole by the cells of the resistant strain was observed. The possibilities that 2-aminohydroxyethylthiazole monophosphate may be the actual inhibitor of the growth of *Saccharomyces cerevisiae*, and that hydroxyethylthiazole may not be involved in the pathway of *de novo* synthesis of thiamin via hydroxyethylthiazole monophosphate, are discussed.

INTRODUCTION

2-Amino-4-methyl-5- β -hydroxyethylthiazole (2-aminohydroxyethylthiazole) inhibits the growth of micro-organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* (Nakayama, 1956; Nose & Iwashima, 1966). Since this inhibition was antagonized completely by 4-methyl-5- β -hydroxyethylthiazole (hydroxyethylthiazole), a thiazole moiety of thiamin, and noncompetitively by thiamin, it was presumed that 2-aminohydroxyethylthiazole blocks some stages in the condensation reaction between thiazole and pyrimidine moieties to produce thiamin (Nakayama, 1956). From enzymic studies with extracts of baker's yeast we previously proposed that the inhibition of thiamin synthesis by 2-aminohydroxyethylthiazole could involve two successive inhibitory reactions: (i) inhibition of hydroxyethylthiazole kinase (EC 2.7.1.50) by 2-aminohydroxyethylthiazole, and (ii) inhibition of thiamin phosphate pyrophosphorylase (EC 2.5.1.3) by 2-aminohydroxyethylthiazole monophosphate which was formed from 2-aminohydroxyethylthiazole by hydroxyethylthiazole kinase (Nose & Iwashima, 1966).

In this paper we describe the isolation of a mutant of *S. cerevisiae* highly resistant to 2-aminohydroxyethylthiazole and present evidence that the mutant is deficient in hydroxyethylthiazole kinase.

METHODS

Organisms and growth experiments. The micro-organism used was *S. cerevisiae* obtained as a clonal isolate of commercial baker's yeast (Oriental Yeast Co.) (Iwashima *et al.*, 1975). A mutant of this strain resistant to 2-aminohydroxyethylthiazole was isolated after treatment with ethylmethane sulphonate (Hawthorne, 1969). For

growth studies the yeasts were incubated at 30 °C for 16 h without shaking in 5 ml thiamin-free synthetic medium (Wickerham, 1951) in the presence and absence of 2-aminohydroxyethylthiazole. The growth rate of the resistant strain in thiamin-free synthetic medium was almost the same as that of the sensitive parent strain, and no reversion occurred even after many subcultures in the absence of 2-aminohydroxyethylthiazole. Growth was measured as the optical density at 560 nm.

Preparation of crude extracts. *S. cerevisiae* was grown as described above in 500 ml thiamin-free synthetic medium. The cells were harvested by centrifugation at 2600 *g* for 5 min, washed with distilled water, and then resuspended in 5 ml 0.05 M-potassium phosphate buffer (pH 7.5) containing 1 mM-2-mercaptoethanol and 1 mM-EDTA. The resulting cell suspensions were sonicated at 9 kHz for 20 min below 4 °C. The supernatant after centrifugation at 80000 *g* for 60 min was used as a crude extract.

Assay of thiamin-synthesizing activity. The reaction mixture contained 20 mM-potassium phosphate buffer (pH 7.0), 10 μ M-hydroxyethylpyrimidine, 10 μ M-hydroxyethylthiazole, 0.1 M-glucose and 1 ml cell suspension (8.0 mg dry wt cells) in a final volume of 10 ml. It was incubated at 37 °C for 1 h without shaking. Thiamin was extracted from the cells by heating them at 85 °C for 15 min after adjusting the pH to 4.5 with 1 M-HCl; total thiamin was estimated by thiochrome assay after Takadiastase hydrolysis (Fujita, 1955). Thiamin-synthesizing activity was expressed as nmol thiamin formed (mg dry wt cells)⁻¹. The amount of thiamin formed at 37 °C was about 1.3 times more than that formed at 30 °C.

Hydroxyethylthiazole kinase assay. The reaction mixture contained 0.02 M-potassium phosphate buffer (pH 7.5), 10 μ M-hydroxyethyl[2-¹⁴C]thiazole (892 MBq mmol⁻¹), 2 mM-ATP, 2 mM-MgCl₂ and 20 μ l crude extract (0.3–0.4 mg protein) in a final volume of 0.5 ml. After incubation at 37 °C for 30 min the reaction was terminated by heating at 90 °C for 5 min, followed by centrifugation at 2000 *g* for 15 min to remove denatured protein. Samples (20 μ l) of deproteinized reaction mixtures were chromatographed (ascending) with authentic hydroxyethylthiazole monophosphate standard on Toyo filter paper (no. 50, 2 × 40 cm with 2-propanol/0.5 M-sodium acetate buffer (pH 4.5)/water (65:15:20, by vol.) as the solvent system. After development, the UV(254 nm)-absorbing spots of hydroxyethylthiazole monophosphate (*R_F* 0.72) were cut out, dried and put into 10 ml scintillation fluid. The radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. The enzyme activity was expressed as nmol hydroxyethylthiazole monophosphate formed (mg enzyme protein)⁻¹; the amount of protein was estimated by the biuret method.

Assay of hydroxyethyl[2-¹⁴C]thiazole uptake. *S. cerevisiae* cells were cultured at 30 °C and harvested at an OD₅₆₀ of 0.35. They were washed once with distilled water and suspended to a final OD₅₆₀ of 0.20 in 0.05 M-potassium phosphate buffer (pH 5.0) containing 0.1 M-glucose. The cell suspension (10 ml) was preincubated at 37 °C for 15 min, unless otherwise stated. Uptake was initiated by the addition of 0.1 ml 0.1 mM-hydroxyethyl[2-¹⁴C]thiazole, and incubation was continued at 37 °C with constant shaking. At appropriate intervals, the cells in 1.0 ml were quantitatively filtered on a nitrocellulose filter (0.65 μ m pore size), followed by one wash with 10 ml 0.05 M-potassium phosphate buffer (pH 5.0). The filters were removed immediately from the suction apparatus, dried, put into scintillation vials containing 10 ml scintillation fluid, and the radioactivity was measured. The rate of hydroxyethyl[2-¹⁴C]thiazole uptake was expressed as pmol hydroxyethyl[2-¹⁴C]thiazole (mg dry wt cells)⁻¹.

Chromatographic analysis of intracellular hydroxyethyl[2-¹⁴C]thiazole derivatives. The fate of hydroxyethyl[2-¹⁴C]thiazole taken up by *S. cerevisiae* was studied by paper chromatographic analysis. Cell suspensions (50 ml) prepared as described above were incubated with 1 μ M-hydroxyethyl[2-¹⁴C]thiazole at 37 °C; after 30 s and 2 min the cells in 25 ml were filtered followed by one wash with 25 ml 0.05 M-potassium phosphate buffer (pH 5.0). The filters were immediately put into 10 ml 0.01 M-sodium acetate buffer (pH 4.5) previously heated to 85 °C; they were then kept for 15 min to extract hydroxyethylthiazole derivatives. The supernatants obtained by centrifugation at 2000 *g* for 15 min were lyophilized independently and the material dissolved in 0.1 ml distilled water. A 20 μ l portion of each of the samples was developed with authentic hydroxyethylthiazole and hydroxyethylthiazole monophosphate by ascending paper chromatography as described above; the UV-absorbing spots of hydroxyethylthiazole (*R_F* 0.96) and hydroxyethylthiazole monophosphate (*R_F* 0.72) were cut out, and their radioactivities were measured in 10 ml scintillation fluid.

Chemicals. Hydroxyethyl[2-¹⁴C]thiazole was prepared and purified from [thiazole-2-¹⁴C]thiamin hydrochloride (899 MBq mmol⁻¹, Amersham) by the method of Williams *et al.* (1935). 2-Methyl-4-amino-5-hydroxymethylpyrimidine (hydroxymethylpyrimidine), hydroxyethylthiazole, hydroxyethylthiazole monophosphate and 2-aminohydroxyethylthiazole were kind gifts from the late Dr S. Yurugi, Takeda Pharmaceutical Industries, Osaka. All other chemicals used were of analytical grade.

RESULTS

Inhibition of growth by 2-aminohydroxyethylthiazole

The effect of 2-aminohydroxyethylthiazole on the growth of the parent and mutant strains of *S. cerevisiae* is shown in Fig. 1. The growth of the parent strain was almost completely inhibited

Fig. 1. Inhibition by 2-aminohydroxyethylthiazole of growth of *S. cerevisiae* sensitive (●) and resistant (○) to 2-aminohydroxyethylthiazole. The growth was measured as OD₅₆₀ after 16 h growth. Each experimental point is the mean of three separate measurements.

by this compound at a concentration of 1 μ M. This inhibition was completely abolished by addition of 0.1 μ M-hydroxyethylthiazole or thiamin (data not shown). On the other hand, the growth of the 2-aminohydroxyethylthiazole-resistant mutant was not greatly inhibited by 2-aminohydroxyethylthiazole up to a concentration of 0.1 mM, but was inhibited at a concentration of 1 mM. Thus it was about 10^4 times more resistant to 2-aminohydroxyethylthiazole than the parent strain.

The development of resistance to an antimetabolite has sometimes been attributed to overproduction of the metabolite, due to derepression of the controlled biosynthetic system or to loss of regulatory mechanisms. However, cellular thiamin contents of the sensitive and resistant strains were 0.12 and 0.05 nmol (mg dry wt cells)⁻¹, respectively. This result suggested that thiamin-synthesizing activity may be reduced in the mutant strain.

Thiamin production from hydroxymethylpyrimidine and hydroxyethylthiazole

Resting cells of *S. cerevisiae* can synthesize thiamin from hydroxymethylpyrimidine and hydroxyethylthiazole in the presence of glucose (Ashida, 1942). Since *de novo* synthesis of thiamin in the resistant strain was presumed to be impaired, thiamin production by resting cells in the presence of hydroxymethylpyrimidine and hydroxyethylthiazole was investigated. As shown in Table 1, no significant thiamin synthesis by the resistant strain was observed, whereas the sensitive strain synthesized 2.93 nmol thiamin (mg dry wt cells)⁻¹ for 1 h.

Hydroxyethylthiazole kinase activity

From the results described above, it was suspected that the alteration of the enzyme responsible for the synthesis of thiamin from hydroxymethylpyrimidine and hydroxyethylthiazole might be involved in the resistance of *S. cerevisiae* to 2-aminohydroxyethylthiazole. Micro-organisms convert hydroxyethylthiazole to hydroxyethylthiazole monophosphate, followed by incorporation into thiamin monophosphate. Therefore, the activity of hydroxyethylthiazole kinase, which catalyses the phosphorylation of hydroxyethylthiazole, was determined in sonic extracts of the two strains. As shown in Table 1, the hydroxyethylthiazole kinase activity of the resistant strain was very low. Thus, resistance to 2-aminohydroxyethylthiazole can be explained by a deficiency of hydroxyethylthiazole kinase in the mutant strain which results in a defect in phosphorylation of 2-aminohydroxyethylthiazole to 2-aminohydroxyethylthiazole monophosphate, a strong competitive inhibitor of thiamin phosphate pyrophosphorylase.

Fig. 2

Fig. 3

Fig. 2. Time course of hydroxyethyl[2- 14 C]thiazole uptake by *S. cerevisiae* sensitive and resistant to 2-aminohydroxyethylthiazole. Cell suspensions prepared as described in the text were incubated with $1\text{ }\mu\text{M}$ -hydroxyethyl[2- 14 C]thiazole; uptake was then determined as described in the text. ●, ○, Uptake by the sensitive strain at 37°C (●) and 0°C (○); ▲, uptake by the resistant strain at 37°C . Each experimental point is the mean of three separate measurements.

Fig. 3. Effect of 2-aminohydroxyethylthiazole or hydroxymethylpyrimidine on hydroxyethyl[2- 14 C]thiazole uptake. 2-Aminohydroxyethylthiazole ($1\text{ }\mu\text{M}$, ▲) or hydroxymethylpyrimidine ($10\text{ }\mu\text{M}$, ○) was added to yeast cell suspensions along with $1\text{ }\mu\text{M}$ -hydroxyethyl[2- 14 C]thiazole. The control (●) contained no 2-aminohydroxyethylthiazole or hydroxymethylpyrimidine. Each experimental point is the mean of three separate measurements.

Table 1. *Thiamin synthesis from hydroxymethylpyrimidine and hydroxyethylthiazole by resting cells and hydroxyethylthiazole kinase activity in cell extracts*

Thiamin-synthesizing activity and hydroxyethylthiazole kinase activity were expressed as nmol thiamin formed (mg dry wt cells) $^{-1}$ for 1 h and nmol hydroxyethylthiazole monophosphate formed (mg protein) $^{-1}$ for 1 min, respectively. Values shown represent means of at least two separate determinations.

Strain	Thiamin synthesis	Hydroxyethylthiazole kinase
Sensitive	2.93	0.448
Resistant	0.005	0.003

Hydroxyethyl[2- 14 C]thiazole uptake

In *E. coli* K12 evidence for direct participation of the thiamin-synthesizing enzymes in hydroxyethylthiazole uptake has been presented (Yamasaki *et al.*, 1973). Therefore, it was of interest to study hydroxyethylthiazole uptake by our hydroxyethylthiazole kinase-deficient *S. cerevisiae* mutant. Fig. 2 shows the time course of hydroxyethyl[2- 14 C]thiazole uptake by resting cells of *S. cerevisiae* sensitive and resistant to 2-aminohydroxyethylthiazole. The uptake of hydroxyethyl[2- 14 C]thiazole by the sensitive strain at 37°C proceeded linearly for 5 min and then reached a steady state at about 30 min; uptake was negligible at 0°C . No significant uptake of hydroxyethyl[2- 14 C]thiazole by the mutant strain was observed at 37°C . The fate of hydroxyethyl[2- 14 C]thiazole after entry into the cells of the sensitive strain was analysed by paper chromatography. After 2 min incubation hydroxyethylthiazole monophosphate was present in the cells to the extent of approximately 80% of the total radioactivity taken up by the cells,

although the amount of the non-phosphorylated form of hydroxyethylthiazole (64%) exceeded the phosphorylated form (36%) after 30 s incubation. Intracellular hydroxyethylthiazole monophosphate increased rapidly with incubation time, whereas free hydroxyethylthiazole remained almost constant. These results suggest that exogenous hydroxyethylthiazole is accumulated in the form of hydroxyethylthiazole monophosphate after it enters yeast cells. The simultaneous addition of 2-aminohydroxyethylthiazole to the uptake medium at a concentration of 1 μM did not inhibit the initial rate of hydroxyethylthiazole uptake for 2 min, but inhibited subsequent uptake on further incubation, suggesting that the antimetabolite inhibited mainly the accumulation of hydroxyethyl[2- ^{14}C]thiazole monophosphate by inhibiting hydroxyethylthiazole kinase (Fig. 3). The addition of 10 μM -hydroxymethylpyrimidine did not stimulate the initial rate of hydroxyethyl[2- ^{14}C]thiazole uptake, but increased the uptake after incubation for 5 min or more, suggesting that accumulated hydroxyethylthiazole monophosphate was further converted to thiamin monophosphate, resulting in the increase in net inflow of exogenous hydroxyethylthiazole (Fig. 3).

DISCUSSION

In earlier work, we isolated a mutant of *E. coli* highly resistant to 2-aminohydroxyethylthiazole which showed no detectable difference in the properties of the enzymes for thiamin synthesis from the parent strain, but which required 100–200 times as much hydroxyethylthiazole for thiamin synthesis by resting cells compared with the amount required for synthesis by the sensitive strain (Nose & Iwashima, 1966; Iwashima & Nose, 1967). From these results we presumed that the impairment of the ability to take up hydroxyethylthiazole, and hence 2-aminohydroxyethylthiazole, was the probable mechanism for the resistance of *E. coli* to 2-aminohydroxyethylthiazole. On the other hand, the 2-aminohydroxyethylthiazole-resistant mutant of *S. cerevisiae* isolated in this study was deficient in hydroxyethylthiazole kinase, which phosphorylates 2-aminohydroxyethylthiazole as well as hydroxyethylthiazole. The presence of a hydroxyethylthiazole kinase-deficient mutant which can grow well on minimal medium strongly suggests that a genetic block of the phosphorylation of 2-aminohydroxyethylthiazole is a primary cause of the resistance of the mutant to 2-aminohydroxyethylthiazole. These findings also support the hypothesis that hydroxyethylthiazole may not be an obligatory intermediate in the pathway of *de novo* synthesis of hydroxyethylthiazole monophosphate in thiamin biosynthesis in *S. cerevisiae* (White & Spenser, 1982). It thus appears that the hydroxyethylthiazole kinase is involved in the salvage synthesis of hydroxyethylthiazole monophosphate from hydroxyethylthiazole which is formed by enzymic degradation of thiamin and hydroxyethylthiazole monophosphate in *S. cerevisiae*. The reduced intracellular thiamin content of the mutant strain may be due to the decrease in the synthesis of thiamin from hydroxyethylthiazole via hydroxyethylthiazole monophosphate.

Hydroxyethylthiazole is taken up by *E. coli* (Yamasaki *et al.*, 1973) and *Salmonella typhimurium* (Bellion *et al.*, 1983) and the uptake is markedly enhanced by the presence of hydroxymethylpyrimidine. Thus, the rate of hydroxyethylthiazole uptake appeared to be regulated by the thiamin-synthesizing enzyme system in these bacteria. In the present study hydroxyethylthiazole was accumulated in yeast cells in the form of hydroxyethylthiazole monophosphate. Hydroxymethylpyrimidine and 2-aminohydroxyethylthiazole did not affect the initial rate of hydroxyethylthiazole uptake but they respectively stimulated and inhibited the later stages of the uptake. These effects may be exerted on the step of enzymic conversion of hydroxyethylthiazole into thiamin monophosphate in yeast cells. In fact no appreciable uptake of hydroxyethylthiazole was observed in the hydroxyethylthiazole-kinase-deficient mutant of *S. cerevisiae*. The mechanism of hydroxyethylthiazole transport across the yeast cell membrane is not known, but it appears to be energy-independent, since the addition of *N,N'*-dicyclohexylcarbodiimide did not affect the initial rate of hydroxyethylthiazole uptake in *S. cerevisiae* (data not shown). It is therefore thought that hydroxyethylthiazole uptake by *S. cerevisiae* probably occurs by diffusion, simple or facilitated, followed by metabolic trapping due to hydroxyethylthiazole-kinase-catalysed phosphorylation.

REFERENCES

- ASHIDA, K. (1942). Synthesis of vitamin B₁ by microbes. I. *Bulletin of the Agricultural Chemical Society of Japan* **18**, 723–726.
- BELLION, E., LASH, T. D. & MCKELLAR, R. (1983). Transport of thiamine and 4-methyl-5-hydroxyethylthiazole by *Salmonella typhimurium*. *Biochimica et biophysica acta* **735**, 331–336.
- FUJITA, A. (1955). Thiaminase. *Methods in Enzymology* **2**, 622–628.
- HAWTHORNE, D. C. (1969). The selection of nonsense suppressors in yeast. *Mutation Research* **7**, 187–197.
- IWASHIMA, A. & NOSE, Y. (1967). An *Escherichia coli* mutant resistant to 2-amino-hydroxyethylthiazole. *Journal of Biochemistry* **62**, 537–542.
- IWASHIMA, A., WAKABAYASHI, Y. & NOSE, Y. (1975). Thiamine transport mutants of *Saccharomyces cerevisiae*. *Biochimica et biophysica acta* **413**, 243–247.
- NAKAYAMA, H. (1956). Studies on thiamine-requiring mutants of *Escherichia coli*. III. A modification of penicillin-technique for isolation of thiamine-requiring mutants. *Vitamins* **11**, 20–25.
- NOSE, Y. & IWASHIMA, A. (1966). Biosynthesis of thiamine and its inhibition by aminothiazole. In *Current Aspects of Biochemical Energetics*, pp. 343–350. Edited by N. O. Kaplan & E. P. Kennedy. New York: Academic Press.
- WHITE, R. L. & SPENSER, I. D. (1982). Thiamine biosynthesis in yeast. Origin of the five-carbon unit of the thiazole moiety. *Journal of the American Chemical Society* **104**, 4934–4943.
- WICKERHAM, L. J. (1951). Taxonomy of yeast. 1. Techniques of classification. 2. A classification of the genus *Hansenula*. *United States Department of Agriculture Technical Bulletin* **1029**, 1–56.
- WILLIAMS, R. R., WATERMAN, R. E., KERESZTESY, J. C. & BUCHMAN, E. R. (1935). Studies on crystalline vitamin B₁. III. Cleavage of vitamin with sulfite. *Journal of the American Chemical Society* **57**, 536–537.
- YAMASAKI, H., SANEMORI, H., YAMADA, K. & KAWASAKI, T. (1973). Hydroxyethylthiazole uptake in *Escherichia coli*. General properties and relationship between uptake and thiamine biosynthesis. *Journal of Bacteriology* **116**, 1280–1286.