Note

The Biosynthesis of the Pyrimidine Moiety of Thiamin in *Halobacterium salinarum*

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Summary The biosynthetic pathway of the pyrimidine moiety of thiamin was studied in the archaean *Halobacterium salinarum*. Thiamin is biosynthesized from 4-amino-5-hydroxymethyl-2-methylpyrimidine (pyrimidine) and 5-(2-hydroxyethyl)-4-methylthiazole (thiazole). The pyrimidine and the thiazole are biosynthesized de novo in microorganisms. The biosynthetic routes of pyrimidine in microorganisms differ between eukaryote and eubacteria. In the eukaryote *Saccharomyces cerevisiae*, histidine and pyridoxine are the precursors of pyrimidine, while in the eubacterium *Escherichia coli*, pyrimidine is biosynthesized from 5-aminoimidazole ribonucleotide (AIR), an intermediate of purine biosynthesis. Tracer investigations revealed that [¹⁵N]-, [1-¹³C]- and [2-¹³C] glycine, precursors of AIR, were incorporated into the pyrimidine in *H. salinarum*. These results suggested that the biosynthetic route of the pyrimidine in *H. salinarum* is similar to that of *E. coli*.

Key Words thiamin, biosynthesis, pyrimidine, archaea, *Halobacterium salinarum*

Thiamin (vitamin B₁) is an essential nutrient for all living organisms. The diphosphate derivative is involved in carbohydrate metabolism as a cofactor. Thiamin is biosynthesized from 4-amino-5-hydroxymethyl-2-methylpyrimidine (pyrimidine) and 5-(2-hydroxyethyl)-4-methylthiazole (thiazole) (1). These heterocyclic compounds are synthesized de novo in microorganisms and plants. The biosynthetic pathway of the pyrimidine differs between these organisms as shown in Fig. 1 (2). In *Escherichia coli* (eubacterium) and *Arabidopsis thaliana* (plant), the pyrimidine is formed by ring expansion of imidazole, accompanied by insertion of the C-4', C-5' fragment of the ribose of 5-aminoimidazole ribonucleotide (AIR), an intermediate of purine biosynthesis (Fig. 2) (3–7). The imidazole ring and the C-3', -4' and -5' of ribotide are used to construct the pyrimidine ring, and the C-3' of ribotide is a methyl group attached at C-2 of pyrimidine and C-4' and -5' are inserted into the imidazole ring of AIR. This complicated reaction is catalyzed by phosphomethylpyrimidine synthase (ThiC). On the other hand, glycine with glutamine and 10-formyltetrahydrofolate are the precursor of the imidazole ring of AIR in the biosynthesis of purine nucleotides. Hence, the C-1, C-2 and N atoms of glycine are incorporated into the C-4, C-6, and N-1 of the pyrimidine, respectively (2) (Fig. 2).

In *Saccharomyces cerevisiae*, we have shown that N-3, C-4 and the amino-N atom attached at C-4 of the pyrimidine are derived from N-1, C-2 and N-3 of the histidine imidazole ring (8–10), while the residual unit of the

pyrimidine originates from pyridoxine or its phosphate (11–13). Recently, it has been shown that the histidine66 of THI5p, a pyrimidine synthase in *Candida albicans*, is the donor for pyrimidine (14).

Archaea, the third domain of organisms, grow in extreme environmental conditions such as ultra-high temperatures or high-concentration saline solutions. Their metabolism has much in common with prokaryotes while their transcription and translation systems are closer to those of eukaryotes. We have previously reported on the characterization of thiamin phosphate synthase (15) and the biosynthetic route of the thiazole moiety of thiamin (16) in archaea. The thiazole biosynthesis in archaea seems to be similar to that in eukaryotes. However, a biosynthetic route of pyrimidine in archaea has not yet been elucidated experimentally. The genome of *H. salinarum* has been elucidated by Ng et al. (17) and the archaeon has been shown to possess ThiC. When the protein homology of *H. salinarum* ThiC was compared with those of *E. coli*, *Bacillus subtilis* and *A. thaliana* using NCBI (<http://www.ncbi.nlm.nih.gov>) Blast, it was shown to be 55%, 63% and 55% homologous with that of *E. coli*, *B. subtilis* and *A. thaliana*, respectively. This suggests that *H. salinarum* might have the same biosynthetic pathway as *E. coli* (eubacteria and plants). In this paper, we describe an incorporation study with stable isotope-labeled glycine to determine the biosynthetic route of the pyrimidine moiety of thiamin in *H. salinarum*.

Materials and Methods

Materials. [¹⁵N]Glycine (98 ¹⁵N atom%) and [2-¹³C]-glycine (99 ¹³C atom%) were purchased from Shoko

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Fig. 1. The biosynthetic routes of pyrimidine in *Escherichia coli*, *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. PRPP: phosphoribosyl pyrophosphate, AIR: 5-aminoimidazole ribonucleotide, PLP: pyridoxal phosphate, TMP: thiamin monophosphate.

Fig. 2. The pyrimidine is synthesized from AIR. The C-1, C-2 and N atoms of glycine are incorporated into the C-4, C-6 and N-1 of the pyrimidine in eubacteria and plants, respectively.

Co., Ltd. (Tokyo, Japan). [$1\text{-}^{13}\text{C}$]Glycine (99 ^{13}C atom%) was purchased from Mass Trace, Inc. (Woburn, MA). Taka-diestase (thiamin-free) was from Sankyo Co., Ltd. (Tokyo, Japan). All other chemicals were of analytical grade.

Microorganisms and growth conditions. *Halobacterium salinarum* NBRC14715 was purchased from the Biological Resource Center, National Institute of Technology and Evaluation (Japan). The cells were pre-incubated in a growth medium based on Grey and Fitt's medium (18), L-alanine, 43 mg; L-arginine, 40 mg; L-cysteine hydrochloride monohydrate, 5 mg; L-isoleucine, 44 mg; L-leucine, 80 mg; L-lysine monohydrochloride, 85 mg; L-methionine, 37 mg; L-phenylalanine, 26 mg; L-proline, 5 mg; L-serine, 61 mg; L-threonine, 50 mg; L-tyrosine, 20 mg; L-valine, 100 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.07 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5 μg ; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.23 mg; KCl, 100 mg; KH_2PO_4 , 15 mg; K_2HPO_4 , 15 mg; NaNO_3 , 10 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g; $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 30 μg ; NaCl, 25 g; tri-sodium citrate dehydrate, 50 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 44 μg ; water, 100 mL; 0.1% w/v glycerol pH 6.6 with 50% w/v KOH. For the tracer investigation, the amino acids, alanine, cysteine, phenylalanine, proline, serine and tyrosine were eliminated from the growth medium and a tracer was added at a concentration of 1 mM. *H. salinarum* was incubated at 37°C for 96 h.

Extraction and purification of thiamin and analysis of the pyrimidine moiety and amino acids. After cultivation, the cells were harvested by centrifugation and suspended in 20 mL of purified water. The suspension was acidified with concentrated hydrochloric acid to pH 2 and heated for 10 min in a boiling water bath. The extracted phosphorylated thiamin was converted to free thiamin by treatment with Taka-diestase. Thiamin was purified by Amberlite CG-50(H^+ form, 20 $\times$ 100 mm) column chromatography. Thiamin was eluted with 1 mol/L HCl solution. The eluent (approximately 30 mL) was concentrated to 2 mL and the pH adjusted to 4.8. The same volume of ethanol and ethanliol was added to the solution and heated in a sealed tube at 100°C, for 15 h. Thiamin was degraded to 4-amino-5-(ethylthio)methyl-2-methylpyrimidine (ethylthiopyrimidine). The ethylthiopyrimidine was extracted with methylene chloride and was analyzed by GC-MS with a fused silica capillary column DB-5 (J & W, 30 m $\times$ 0.25 mm I.D.) as described previously (8, 9).

The cell debris was hydrolysed with 6 mol/L hydrochloric acid to its constituent amino acids. The amino acids were derivatized to ethoxycarbonyl ethyl esters and analyzed by GC-MS (19).

Results and Discussion

The mass spectrum and fragmentation of ethylthiopyrimidine from *H. salinarum* incubated with 1 mM non-labeled glycine is shown in Fig. 3. Analysis of the fragmentation of ethylthiopyrimidine is based on a report by White & Rudolph (20). The spectra of ethylthiopyrimidine with tracers are shown in Fig. 4. The isotopic peak (m/z 123) increased when *H. salinarum* was grown with labeled glycine ($[^{15}\text{N}]$ -, $[1\text{-}^{13}\text{C}]$ - or $[2\text{-}^{13}\text{C}]$ glycine). Glycine was incorporated as a complete structure into the pyrimidine. The incorporation of glycine in cell protein was measured as an internal indicator of tracer incorporation into the cell. As shown in Table 1, the incorporation of tracer into the pyrimidine and glycine in protein is very similar in the experiments with labeled

Fig. 3. The mass spectrum and fragmentation of pyrimidine derivative (4-amino-5-(2-ethylthio)methyl-2-methylpyrimidine) from *H. salinarum* incubated with 1 mM non-labeled glycine. The molecular peak m/z 183 and base peak m/z 122 contain all of the nitrogen and carbon atoms of the pyrimidine.

Fig. 4. The base peaks of mass spectra of pyrimidine biosynthesized from *H. salinarum* incubated with [^{15}N]-glycine (A), [$1\text{-}^{13}\text{C}$]-glycine (B) and [$2\text{-}^{13}\text{C}$]-glycine (C).

Table 1. Incorporation of labelled glycine into pyrimidine and cell protein in *H. salinarum*.

Tracer	Incorporation rate (%)		
	Pyrimidine	Glycine in cell protein	Histidine in cell protein
[^{15}N]-Glycine	47.2	50.4	6.18
[$1\text{-}^{13}\text{C}$]-Glycine	57.0	65.1	9.79
[$2\text{-}^{13}\text{C}$]-Glycine	64.3	58.0	18.5

The incorporation rate of tracer into pyrimidine and glycine in cell protein was measured by GC-MS. The natural abundance ratios of pyrimidine and amino acids were subtracted. The natural abundance ratios of pyrimidine, glycine and histidine in the cell were measured in experiments in which *H. salinarum* was incubated with non-labeled glycine.

glycine. On the other hand, the incorporation rates of labeled glycine into histidine were much lower than that of the pyrimidine. These results supported that histidine, a precursor of the pyrimidine in *S. cerevisiae*, is not the precursor in *H. salinarum*, and that archaea have the same biosynthetic route for pyrimidine as eubacteria and plants do.

It is noted that, in the case of [$2\text{-}^{13}\text{C}$]-glycine, the incorporation rate into pyrimidine (64.3%) was higher than that into glycine in cell protein (58.0%). Further, the mass spectrum (Fig. 4 (C)) showed that ^{13}C from [$2\text{-}^{13}\text{C}$]-

glycine was incorporated into not only m/z 123 but also m/z 124. These results indicate that two ^{13}C atoms were incorporated into the pyrimidine. The C-2 of glycine incorporated into the cell is metabolized to the C-1 unit of folate (21). The C-2 of the pyrimidine is derived from 10-formyl-tetrahydrofolate via AIR (4, 21). In the cell, the C-2 of histidine is derived from 10-formyl-tetrahydrofolate via the C-2 of the purine nucleotide (21). Both carbon atoms, the C-2 of the pyrimidine and the C-2 of histidine, are derived from 10-formyl-tetrahydrofolate. The incorporation rate of histidine in cell protein in the

experiment with [2-¹³C]glycine was higher than those with other tracers (Table 1). It is possible that [2-¹³C]glycine was mainly incorporated into the pyrimidine and partly metabolized to 10-formyl-tetrahydrofolate and incorporated into the pyrimidine via AIR. These results suggested that N, C-1 and C-2 of glycine were all incorporated into pyrimidine via AIR.

In conclusion, we have demonstrated that the pyrimidine moiety of thiamin in *H. salinarum* is synthesized by the same route as in eubacteria and plants, that is, via AIR.

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