Biosynthesis of Thiamin under Anaerobic Conditions in *Saccharomyces cerevisiae*

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We studied the biosynthetic route of thiamin in *Saccharomyces cerevisiae* to see whether the route differed under aerobic and anaerobic conditions.

Histidine and pyridoxine are the precursors of the pyrimidine moiety of thiamin under aerobic conditions. Formate is incorporated into the pyrimidine via histidine. The incorporation of [^{13}C]formate and [$5\text{'-}^2\text{H}_2$]pyridoxine into the pyrimidine was examined under anaerobic conditions. The labels from [^{13}C]formate and [$5\text{'-}^2\text{H}_2$]pyridoxine were not incorporated into the pyrimidine under anaerobic conditions, indicating that the biosynthetic pathway of the pyrimidine differed from that under aerobic conditions.

On the other hand, [^{15}N]glycine was incorporated into the thiazole under both anaerobic and aerobic conditions. The biosynthetic pathway of the thiazole was therefore unaltered by the O_2 concentration.

Key words thiamin; biosynthesis; anaerobic conditions; *Saccharomyces cerevisiae*

Thiamin is generated by the union of two subunits, 4-amino-5-hydroxymethyl-2-methylpyrimidine (the pyrimidine) and 5-(2-hydroxyethyl)-4-methylthiazole (the thiazole). We reported earlier that the biosynthetic routes of the pyrimidine differ between eukaryotes and prokaryotes.¹⁾ In eukaryotes, we also reported that the N-1, C-2 and N-3 atoms of the imidazole ring of histidine were incorporated into the N-3, C-4 and amino-N of the pyrimidine as a unit,²⁾ while the C-2', C-2, N-1, C-6, C-5 and C-5' of pyridoxine were incorporated into C-2', C-2, N-1, C-6, C-5 and C-5' of the pyrimidine, also as a unit.^{3,4)}

The biosynthetic routes of the thiazole moiety of thiamin in facultative anaerobic bacteria differ from those of aerobic bacteria and eukaryotes.⁵⁾ The precursors of the thiazole in *Saccharomyces cerevisiae* are glycine⁶⁾ and an intact C5 unit derived from glucose.⁷⁾ In *Candida utilis*, we showed that a C5 unit derived from glucose was a precursor.⁸⁾

As thiamin pyrophosphate (TPP) is essential for energy metabolism under aerobic conditions, these biosynthetic studies were carried out under such conditions. However, TPP is also the essential cofactor for the fermentation in *S. cerevisiae*, but study of the biosynthesis has not yet been worked out under anaerobic conditions.

Downs studied auxotrophic mutants of *Salmonella typhimurium* by genetic analytical methods and reported that the biosynthetic route of the pyrimidine differed between aerobic and anaerobic conditions.^{9,10)} In *Escherichia coli*, we also confirmed a difference among the precursors between aerobic and anaerobic conditions.^{11,12)}

We studied the biosynthetic route of thiamin in a eukaryote, *S. cerevisiae*, to test whether there were differences in the route under aerobic and anaerobic conditions.

MATERIALS AND METHODS

Materials [^{13}C]Sodium formate (99 ^{13}C atom%) was purchased from ICON(NY, U.S.A.), and [^{15}N]glycine (99 ^{15}N atom%) was purchased from Amersham International plc Amersham UK. [$5\text{'-}^2\text{H}_2$]Pyridoxine was synthesized by the method reported previously.¹³⁾ All other chemicals were of analytical grade.

Growth Conditions *S. cerevisiae* IFO 1234 was grown in synthetic media under anaerobic conditions. The medium consisted of glucose 90.0 g, $(\text{NH}_4)_2\text{HPO}_4$ 6.0 g, KH_2PO_4 1.0 g, sodium citrate 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 g, pantothenate Ca 0.5 mg, inositol 10.0 mg, $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ 0.15 mg, biotin 20.0 mg, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 25.0 mg, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 25.0 mg, water 1 l. The pH of the medium was adjusted to 5.0 by the addition of H_3PO_4 . Before and after the inoculation of *S. cerevisiae*, the medium was bubbled with N_2 gas for 5 min. *S. cerevisiae* was grown in a stationary state in an Erlenmeyer flask equipped with a balloon for control, producing CO_2 gas pressure for 20 h at 30 °C. The cells were harvested by centrifugation, washed with saline and grown under the same conditions described above, with the tracer, 1 mM, for 14 h at 30 °C.

Isolation and Degradation of Thiamin The isolation and degradation of thiamin to the pyrimidine and the thiazole were carried out as described previously.^{5,14)}

After cultivation, the cells were harvested by centrifugation and washed with water. The cells were suspended in HCl solution, 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-diastase. Thiamin was purified by Amberlite CG-50 column chromatography. Thiamin was degraded with ethanethiol to 4-amino-5-(ethylthio)methyl-2-methylpyrimidine (pyrimidine).

For analysis of the thiazole moiety, the thiamin was degraded to 5-hydroxyethyl-4-methylthiazole with sodium bisulfite as described previously.¹⁵⁾

Isolation and Determination of the Pyrimidine The pyrimidine was extracted with methylene chloride. The extract was evaporated and the pyrimidine was analyzed by GC-MS QP-2000 with a fused silica capillary column DB-5 (J & W, 30 m $\times$ 0.25 mm i.d.) as described previously.¹⁴⁾

Isolation and Determination of the Thiazole The thiazole was extracted with chloroform. After evaporation of the chloroform extract, the thiazole was converted to a trifluoroacetic derivative by the addition of trifluoroacetic anhydride. The trifluoroacetic derivative was analyzed by GC-MS as described previously.⁵⁾

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a)

b)

Fig. 1. Mass Spectrum of a) the Pyrimidine and b) the Pyrimidine Derived from Thiamin Produced by *S. cerevisiae* in the Presence of $[5-^2H_2]$ Pyridoxine and $[^{13}C]$ Formate

The data of $[5-^2H_2]$ pyridoxine under aerobic conditions referred to previous results.⁴⁾

RESULTS

Incorporation of $[^{13}C]$ Formate and $[5-^2H_2]$ Pyridoxine into the Pyrimidine under Anaerobic Conditions The mass spectrum of the pyrimidine is given in Fig. 1a. Analysis of the fragmentation of the pyrimidine was based on a report by White & Rudolph.¹⁶⁾ Figure 1b shows a comparison of the

base peaks of the MS spectra of the pyrimidine produced under aerobic and anaerobic conditions. As we showed previously, under aerobic conditions, the base peaks were shifted from m/z 122 to 123 with $[^{13}C]$ formate¹⁷⁾ and to 124 with $[5-^2H_2]$ pyridoxine.³⁾ However, under anaerobic conditions, $[^{13}C]$ formate and $[5-^2H_2]$ pyridoxine were not incorporated into the pyrimidine. These results showed that, under anaero-

a)

b)

Fig. 2. Mass Spectrum of a) the Thiazole and b) the Thiazole Derived from Thiamin Produced by *S. cerevisiae* in the Presence of $[^{15}\text{N}]$ Glycine
The data of aerobic conditions referred to previous results.⁵⁾

bic conditions, the biosynthetic pathway for the pyrimidine moiety of thiamin in *S. cerevisiae* was different from the pathway under aerobic conditions.

The Incorporation of $[^{15}\text{N}]$ Glycine into the Thiazole under Anaerobic Conditions The mass spectrum of the thiazole is given in Fig. 2a. The molecular ion (M^+) is m/z 239 and the base peak is m/z 125. The base peak at m/z 125

and the fragment ion at m/z 112 include the N atom of the thiazole. The N atom of thiazole originates from the nitrogen atom of glycine under aerobic conditions.

Figure 2b shows the mass spectra of the thiazole synthesized under aerobic and anaerobic conditions. The label of $[^{15}\text{N}]$ glycine was incorporated into the thiazole under both aerobic and anaerobic conditions, suggesting that the biosyn-

thetic pathway of the thiazole is identical under aerobic and anaerobic conditions.

DISCUSSIONS

We confirmed that the biosynthetic pathway for the pyrimidine moiety of thiamin in *S. cerevisiae* under anaerobic conditions differed from that under aerobic conditions.

Nicotineamide adenine dinucleotide (NAD⁺) is an essential cofactor in alcoholic fermentation or pyruvate oxidative degradation by the TCA cycle. Niacin is a constitutive vitamin in NAD⁺. It is well known that the biosynthetic pathway of niacin under aerobic conditions differs from that under anaerobic conditions, in which niacin is synthesized from quinolinate, a tryptophan oxidative compound.¹⁸⁾ Under an anaerobic condition, niacin is formed *via* quinolinate from aspartate and glycerol.

The biosynthesis of the pyrimidine is different between prokaryotes and eukaryotes. Furthermore, the biosynthetic pathway of the pyrimidine under anaerobic conditions in *S. cerevisiae* is different from that under aerobic conditions. Formate is incorporated into the pyrimidine under aerobic conditions in prokaryotes and eukaryotes. Under anaerobic conditions, formate was not incorporated into the pyrimidine in *S. cerevisiae*. It is clear that the biosynthetic route under anaerobic conditions is not related to that in prokaryotes under aerobic conditions. Now, we are studying the precursors of the pyrimidine in anaerobic conditions.

It is very interesting how the biosynthetic pathways have been changed in the process of evolution.

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