A NEW INTERMEDIATE IN THE BIOSYNTHESIS OF THIAMINE

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Received 29 May 1970

1. Introduction

The final reactions in the biosynthesis of thiamine, as described by several research groups, include (1) a two-step pyrophosphorylation of 2-methyl-4-amino-5-hydroxymethyl-pyrimidine (pyrimidine moiety of thiamine), (2) a phosphorylation of 4-methyl-5-(2-hydroxymethyl)-thiazole (thiazole moiety of thiamine), (3) condensation of the two parts to thiamine monophosphate and, finally, its dephosphorylation [1]. Little is known, however, about the biosynthesis of the pyrimidine and thiazole moieties of thiamine. The pathway leading to the pyrimidine moiety separates from that of the purines on the level of 4-amino-imidazole ribonucleotide [2].

Three years ago, a collection of 21 mutants of *Saccharomyces cerevisiae* in the biosynthesis of the pyrimidine moiety of thiamine could be divided into two groups. With three exceptions, each individual of a group of 14 mutants (P2-mutants) fed each of a group of 7 (P1-) mutants, but not vice versa [3]. Recently, it has been possible to isolate and characterize a substance which accumulates in P2-mutant cells grown under limiting amounts of thiamine. This substance meets the growth requirements of a P1-mutant.

Its UV spectrum exhibits a maximum at 259 nm. This intermediate will be referred to as "compound 259" throughout this paper.

2. A biological assay for "compound 259"

A 0.03 ml sample from each fraction of the chromatogram was mixed with 5 ml minimal medium [4] and neutralized if necessary. About 10^7 carefully washed cells of the *S. cerevisiae* mutant HK 354 (P1-mutant) were then added to the autoclaved supplemented medium. Growth was checked turbidimetrically after 72 hr at 30°.

3. Preparation of "compound 259"

The mutant HK 210 of *S. cerevisiae* (P2-mutant) was grown in minimal medium [4] supplemented with 0.008 mg thiamine hydrochloride/l. After overnight incubation at 30°, the cells were harvested by centrifugation and suspended in minimal medium supplemented with (all 1 mM) glycine, sodium formate, glutamine, β-alanine, sodium acetate, methionine and aspartic acid. Glycine, formate and glutamate are known to be needed for the biosynthesis of 4-amino-imidazole ribonucleotide, the last common intermediate of the purine pathway and the pyrimidine moiety pathway [5]. β-Alanine etc. are possible canditates for precursor compounds in the biosynthesis of the pyrimidine moiety [2, 6–9].

After 15 hr at 30°, the cells were pelleted and the medium was passed through a column of granulated charcoal. The loaded column was washed with distilled water. "Compound 259" was then eluted with an ethanol-water mixture (1:1, v/v), the eluted solution was evaporated to dryness and the residue was dissolved in 0.5 N ammonia and applied to a column of Sephadex G-10 (2.5 × 96 cm). The thiamine precursor compound was eluted with 0.5 M ammonia. A distinct peak (II) was found to be associated with the biologically active compound (fig. 1). The fractions comprising this peak were again subjected to gel chromato-

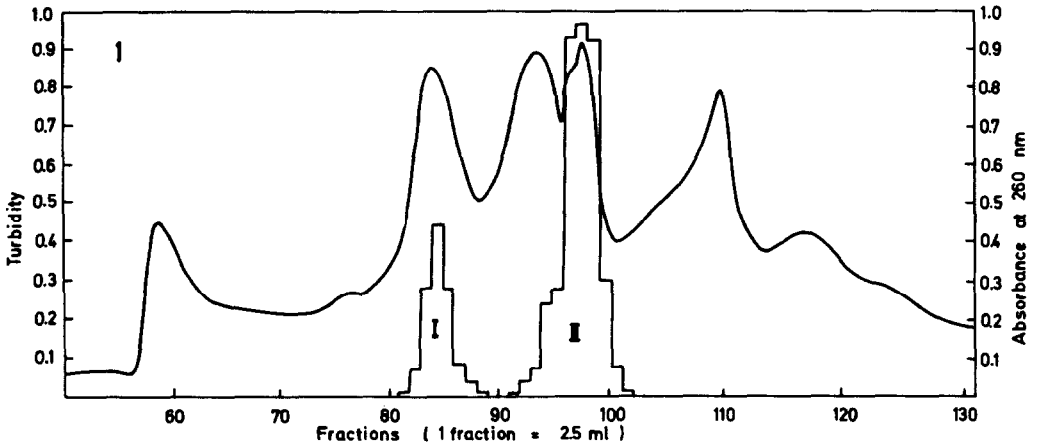

Fig. 1.

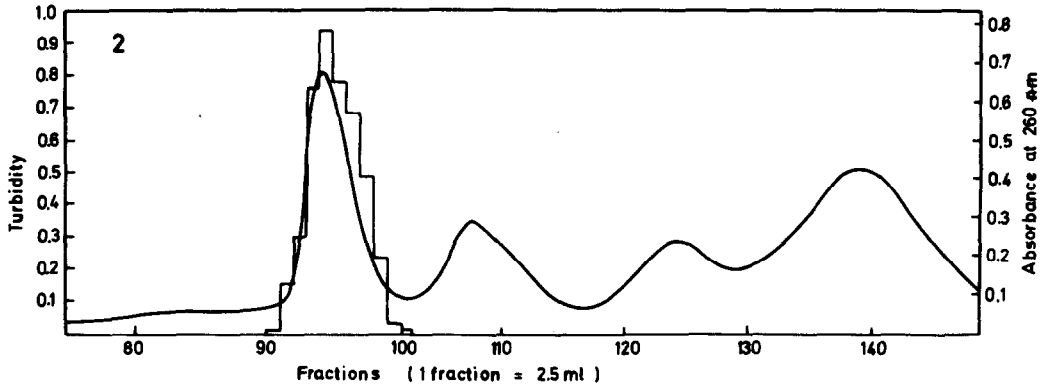

Fig. 1 and 2. Gel chromatography of "compound 259" on Sephadex G-10 with 0.5 N ammonia and 0.5 N acetic acid, respectively.

graphy on a column of Sephadex G-10 (2.5 X 96 cm) with 0.5 N acetic acid. The most prominent ultraviolet light absorbing peak in this fractionation corresponded to the biologically active material (fig. 2). These fractions were combined and evaporated to dryness. The residue was dissolved in 1 ml of mixture of methanol-water (80:20, v/v), and the solution was applied to a column of Sephadex G-25 fine. The elution was performed with the same solvent. "Compound 259" was eluted with fractions 51–58 (1 fraction = 20 ml).

Instead of partition chromatography on a column of Sephadex G-25, the precursor compound could be purified by thick layer chromatography. The solution was spotted quantitatively on a 0.5 mm thick layer of Kieselgel HF_{254} (Merck), and the material was subjected to chromatography with methanol-water (80:20, v/v). Inspection of the chromatogram developed under an ultraviolet lamp revealed one strong UV-absorbing compound (R_f, 0.45) and one faint band below the strong UV absorbing zone. Material at R_f 0.45 was eluted from Kieselgel with methanol. Supplementation with 0.005 or 0.010 mg thiamine hydrochloride/l during cultivation of mutant HK 210 gave similar results. However, cells grown in a complete medium [4] synthesized during the accumulation period only very small amounts of the precursor compound.

4. Properties of "compound 259"

The data indicate that in the biosynthetic pathway of the pyrimidine moiety, 2-methyl-4-amino-5-hydroxymethyl-pyrimidine is synthesized from 4-amino-imidazole ribonucleotide via "compound 259". The UV spectrum of the biologically active compound showed a maximum at 259 nm, both in 0.1 N HCl and 0.1 N NaOH. No significant shift was observed in the pH range from 1 to 13.

"Compound 259" is heat stable in aquous solution (pH 5–7) at temperatures up to 120°. Therefore it was possible to autoclave the precursor compound in minimal medium before testing its biological activity. However, it loses biological activity after several hr in 1 N NaOH at 100°; treatment with 1 N HCl at 100° converted the precursor compound to a biologically inactive substance with an altered spectroscopic and chromatographic behaviour. The maximum of the UV spectrum shifted from 259 nm to 261 nm (in 0.1 N HCl) and approximately 268 nm (in 0.1 N NaOH), respectively. The R_f value of the derivative in the above solvent system was 0.66.

The NMR spectrum of purified "compound 259" in D_2O shows a singulett at $\delta = 2.00$ ppm (rel. int. 3), a dublett at $\delta = 6.15$ ppm (rel. int. 2) and a triplett at $\delta = 8.5$ ppm (rel. int. 3, TMS, extern). The singulett corresponding to the methyl group in 2-methyl-4-amino-5-hydroxymethyl-pyrimidine was found at $\delta = 2.67$ ppm. From the shifted signal one may draw the conclusion that the methyl group in "compound 259" is bound at a double bond, probably $H_3C-\dot{C}=N-$, but not at an aromatic ring system. Elution of "compound 259" is retarded by the same degree on Sephadex G-10 in the solvent systems 0.5 N acetic acid and 0.5 N ammonia, respectively. From this it may be concluded, that if "compound 259" has a ring structure, it is not yet a completely unsaturated 4-amino-pyrimidine system. This is supported by the UV spectrum.

Acknowledgements

The thiamine mutants were generously provided by Prof. Dr. F.Lingens (University Hohenheim, Germany). The author is indebted to the Varian AG, Zürich, for the NMR spectrum.

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