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BIOSYNTHESIS OF RIBOFLAVIN. AN ALIPHATIC INTERMEDIATE IN THE FORMATION OF 6,7-DIMETHYL-8-RIBITYLLUMAZINE FROM PENTOSE PHOSPHATE

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6,7-Dimethyl-8-ribityllumazine synthase deficient mutants of *Candida guilliermondii* were divided into two groups on the basis of *in vitro* complementation. Mutants of complementation group I produce an intermediate X from ribose 5-phosphate in a reaction requiring Mg^{++} ions. Compound X was partially purified and was shown to be a phosphoric acid ester. 6,7-Dimethyl-8-ribitylluma-zine can be formed from Compound X by cell extracts from mutants of complementation group II. The reaction requires 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione or its 5'-phosphate as second substrate. No divalent cations are required.

The xylene ring of riboflavin (**3**) originates by dismutation of 6,7-dimethyl-8-ribityllumazine (**2**) (Fig. 1; for review see Refs. 1, 2). Thus, all xylene C-atoms of **3** are derived from C-6α, C-6, C-7 and C-7α of **2**. The lumazine **2** is formed by the addition of a 4-carbon unit to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimi-dinedione (**4**) or its 5'-phosphate (**1**) (3-6). The biosynthetic origin of this 4C-moiety has been highly controversial for seve-ral decades. Acetoin, diacetyl, tetroses, pentoses, hexoses, and the ribityl moiety of the pyrimidine **4** have been proposed as precursors (for a recent review see Ref. 7). We were able to show that the lumazine atoms C-6α, C-6, C-7 and C-7α are biosynthetically equivalent to the carbon atoms 1, 2, 3 and 5 of pentose pool intermediates (7-9). C-4 of the pentose pool has no equiva-

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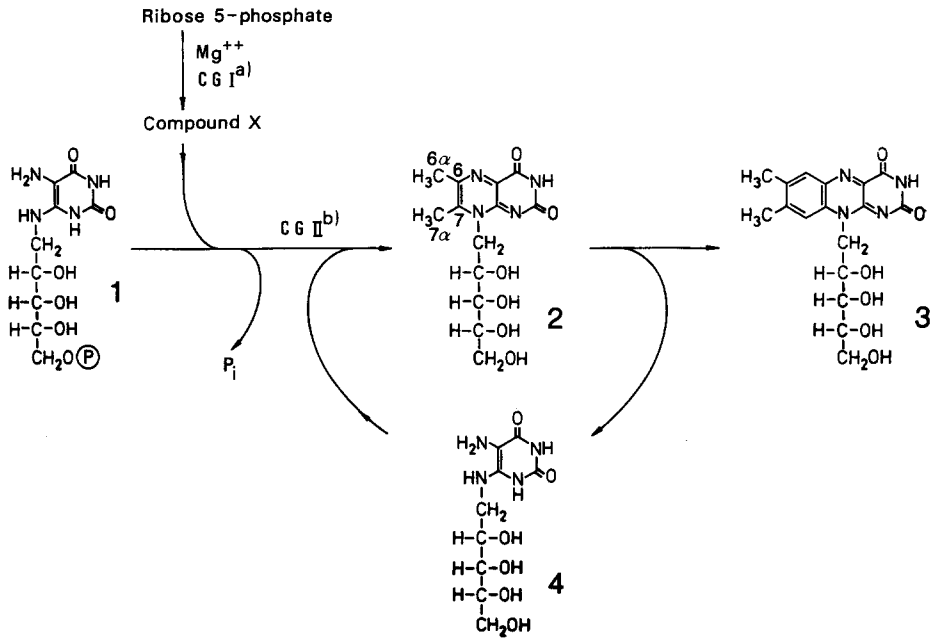

Fig. 1 Biosynthesis of riboflavin.
a) CG I, cell extract of complementation group I mutants
b) CG II, cell extract of complementation group II mutants

lent in the lumazine **2**. More specifically, this atom is eliminated by an intramolecular skeletal rearrangement as shown by *in vivo* studies with ^{13}C-labeled precursors (10).

Logvinenko et al. have observed the formation of **2** from **1** and ribose 5-phosphate by cell extract from *Candida guilliermondii* (11, 12). It was subsequently shown that this reaction follows the pattern observed *in vivo*, i. e. loss of C-4 from the carbohydrate precursor, and that the pyrimidines **1** and **4** can both serve as substrates (13).

Genetic studies with yeasts had suggested earlier that the formation of **2** from **1** requires a minimum of two enzymes (14, 15). We present evidence for the formation of an aliphatic intermediate designated as Compound X from ribose phosphate. Compound X yields **2** in a second enzyme step requiring pyrimidine **1** or **4** as cosubstrate.

MATERIALS AND METHODS

5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (**4**) was prepared as published (13). Light riboflavin synthase from *Bacillus subtilis* was purified as described earlier (16).

Riboflavin deficient mutants were obtained after treatment of *C. guilliermondii* ATCC 9058 with ethylmethane sulfonate (15, 16). Mutants deficient in the conversion of **1** to **2** were recognized by their ability to grow alternatively with riboflavin or with diacetyl (50 mg/l) (17). *C. guilliermondii* cells were grown as described (13). Mutants were supplemented with riboflavin (200 mg/l). Cell extracts were prepared and dialyzed as described (13).

Assay mixtures for the determination of 6,7-dimethyl-8-ribityllumazine synthase activity contained 1.0 mM **4**, 2.0 mM ribose phosphate or approximately 5 µM Compound X, 0.1 M Tris hydrochloride pH 7.8, 2.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM dithioerythritol, 150 U of riboflavin synthase, and cell extract in a total volume of 1.0 ml. The mixture was incubated for 1 h at 37 °C. Riboflavin was monitored by bioassay with *Lactobacillus casei* ATCC 7469.

Assay mixtures for the preparation of Compound X contained 0.1 M Tris hydrochloride pH 7.8, 5 mM ribose phosphate, 10 mM dithioerythritol, 2 mM $MgCl_2$, and dialyzed cell extract from *C. guilliermondii* mutant E 20 (50 mg of protein) in a total volume of 6.25 ml. The mixture was incubated at 37 °C for 1 h. The solution was centrifuged in a Spinco Ti 60 rotor (40,000 rpm, 4 °C, 14 h). The pellet was discarded. The supernatant was passed through an Amicon PM 10 ultrafiltration membrane. The filtrate was placed on a column of DEAE cellulose (0.8 x 55 cm). The column was developed with a linear gradient of 0-0.5 M ammonium acetate pH 6.0 (total volume, 200 ml). Elution of Compound X was monitored by assay of 6,7-dimethyl-8-ribityllumazine formation using cell extract from mutant E 9 and the pyrimidine **4** as the second substrate. Fractions were pooled and lyophilized.

RESULTS

We have isolated 13 mutants of *C. guilliermondii* which can grow alternatively with riboflavin or diacetyl. These mutants excrete the pyrimidine **4** into the culture medium, and their cell extracts are devoid of 6,7-dimethyl-8-ribityllumazine synthase activity. Catalytic activity was restored in certain pairwise combinations of mutant cell extracts by *in vitro* complementation. On this basis, the mutants were assigned to two complementation

Table 1 *In vitro* complementation of *C. guilliermondii* mutants deficient of 6,7-dimethyl-8-ribityllumazine synthase activity

CG[a)]		I						II						
	Strain	E12	E18	E20	E23	E24	E25	E7	E8	E9	E11	E13	E19	E22
I	E12	0	0	0	0	0	0	0	+	++	0	+	+	+
	E18	0	0	0	0	0	0	+	++	+++	++	++	++	++
	E20	0	0	0	0	0	0	+	++	+++	+	+	++	++
	E23	0	0	0	0	0	0	++	++	++	++	++	++	++
	E24	0	0	0	0	0	0	++	++	++	++	++	++	++
	E25	0	0	0	0	0	0	+	0	++	0	++	++	+
II	E7	0	+	+	++	++	+	0	0	0	0	0	0	0
	E8	+	++	++	++	++	0	0	0	0	0	0	0	0
	E9	++	+++	+++	++	++	++	0	0	0	+	0	0	0
	E11	0	++	+	++	++	0	0	0	+	0	0	0	0
	E13	+	++	+	++	++	++	0	0	0	0	0	0	0
	E19	+	++	++	++	++	++	0	0	0	0	0	0	0
	E22	+	++	++	++	++	+	0	0	0	0	0	0	0

6,7-Dimethyl-8-ribityllumazine synthase activity was measured in pairwise mixtures of mutant cell extracts as described under Methods. Concentration of riboflavin produced: 0, < 0.4 µM; +, 0.4-1.0 µM; ++, 1.0-3.0 µM; +++, > 3.0 µM.
a)CG, complementation group.

groups (Table 1). These findings confirm that a minimum of two enzymes is required for the production of **2** from **1** as suggested by earlier genetic data (14, 15).

We could further characterize the reaction sequence by the following approach. Cell extracts of mutants from one complementation group were incubated with ribose phosphate in the absence of pyrimidine cosubstrate. Protein was removed by precipitation with trichloroacetic acid, and the supernatant was lyophilized. The residue was then treated with a mixture containing cell extract from a mutant of the other complementation group, pyrimi-

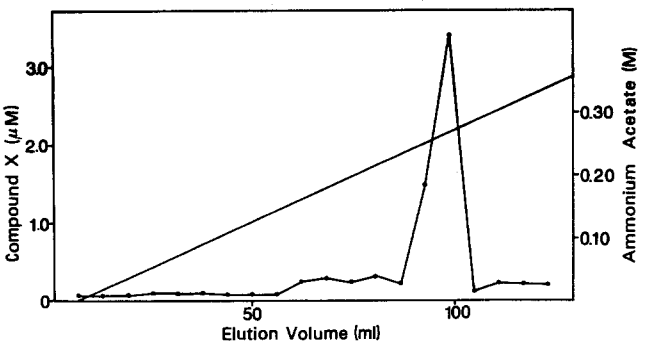

Fig. 2 Chromatographic separation of Compound X. For experimental details see Methods.

dine **4**, and riboflavin synthase. Riboflavin was formed only when cell extract from a complementation group I mutant acted in the first incubation step and cell extract from a complementation group II mutant acted in the second step.

This indicates that mutants of complementation group I convert pentose phosphate to an intermediate subsequently designated as Compound X. The formation of Compound X requires Mg^{++} and is completely inhibited by EDTA.

Compound X was isolated and partially purified by chromatography on DEAE cellulose as described under Methods (Fig. 2). Treatment of partially purified Compound X with a mixture of pyrimidine **4**, mutant E 9 cell extract and riboflavin synthase yields riboflavin as shown in Table 2. The reaction is not inhibited by EDTA. Compound X is inactivated by treatment with alkaline phosphatase.

DISCUSSION

Work with *Saccharomyces cerevisiae* had shown that the products of at least two unlinked genes are required for the biosynthesis of the lumazine **2** from its pyrimidine precursor (14). Genetic studies with a large number of mutants of *C. guilliermondii* moreover have suggested the involvement of three gene products in this biosynthetic step (15). Only two *in vitro* com-

Table 2 Enzymatic formation of riboflavin from partially purified Compound X

Addition	Riboflavin formed[a] (μM)
none	2.0
Mg^{++}	1.9
EDTA	1.7
phosphatase[b]	< 0.02

Compound X was purified as described under Methods. Assay mixtures contained 1.0 mM **4**, approximately 5.0 μM Compound X, 0.1 M Tris hydrochloride pH 7.8, 10 mM dithioerythritol, 220 U of riboflavin synthase, and cell extract from mutant E 9 (16 mg) in a total volume of 1.9 ml. Assays were incubated for 1 h. Riboflavin was monitored by bioassay with *L. casei*.

[a] Values are corrected for blank (0.15 μM)

[b] Compound X was preincubated with 7 U of alkaline phosphatase for 60 min at 37 °C.

plementation groups were found in the present study. However, since the number of mutants studied was relatively small, it is possible that not all potential complementation groups were found.

Cell extracts of complementation group I mutants produce a stable intermediate, Compound X, from ribose phosphate. The reaction requires Mg^{++}, but no other cofactors. Compound X is rapidly destroyed by alkaline phosphatase and thus appears to be a phosphoric acid ester. The compound is relatively stable and can be purified by chromatographic procedures.

Compound X is converted to the lumazine **2** by cell extracts from complementation group II mutants in a reaction requiring one of the pyrimdines **1** or **4**, but no cofactor. This reaction can proceed in the presence of EDTA and thus does not require divalent cations. Experiments to elucidate the structure of Compound X are in progress.

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