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BIOSYNTHESIS OF RIBOFLAVIN: REDUCTASE AND DEAMINASE OF ASHBYA GOSSYPII

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Summary: Two enzymes involved in the biosynthesis of riboflavin have been found in Ashbya gossypii, an organism that produces and excretes riboflavin in large quantities. The first of these (called "reductase") functions to reduce the ribose group of 2,5-diamino-6-oxy-4-(5'-phosphoriboyslamino)pyrimidine (PRP, Compound II, Fig. 1) to the ribityl group of Compound IV (Fig. 1). The second enzyme (called "deaminase") catalyzes the deamination of Compound IV to Compound V. The evidence indicates that in A. gossypii the reductase functions before the deaminase, in contrast to the reaction sequence known to operate in Escherichia coli in which deamination takes place before reduction (Burrows, R.B. and Brown, G.M. (1978) J. Bacteriol., 136, 657-667).

Introduction: In Escherichia coli the first three enzymatic reactions of the pathway for the biosynthesis of riboflavin are those shown in Fig. 1 as Reactions 1, 2, and 3. The enzyme that catalyzes Reaction 1, named GTP cyclohydrolase II, was discovered and purified by Foor and Brown (1), and the enzymes that catalyze Reactions 2 and 3 were discovered by Burrows and Brown (2). Previous observations made by Bacher and coworkers (3) on the excretion products of a series of riboflavin-requiring mutants of yeast (Saccharomyces cerevisiae) suggested that the pathway in yeast differs from that in E. coli in that reduction appears to precede deamination in yeast; hence the pathway in yeast might be expected to include Reactions 1, 2a, and 3a shown in Fig. 1.

We have recently been studying the enzymatic reactions of the riboflavin biosynthetic pathway in Ashbya gossypii, a fungal-like yeast which produces riboflavin in large quantities. The results presented below provides enzymatic evidence that reduction precedes deamination in A. gossypii. In this respect this system resembles the pathway thought to

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operate in Saccharomyces cerevisiae, and is different from that known to operate in E. coli.

Materials and Methods: Sephadex G-25 and Sephadex G-200 were purchased from Pharmacia and Affi-Gel Blue from Bio-Rad. All other materials were obtained from the sources described in the paper by Burrows and Brown (2).

Cultural methods and the growth medium (0.5% proteose peptone, 0.3% Difco yeast extract, and 4% glucose, pH 5.6) for A. gossypii were adapted from Tanner et al. (4) and Ali and Al-Khalidi (5). A 15 ml 24 hour culture (incubated with shaking at 25°) was used to inoculate 1.5 liters of medium. Incubation was at 25°, with aeration, and riboflavin production during this time was followed by measurement of the absorbance of the liquid medium at 450 nm. When the absorbance reached 1.2 (80 to 100 hours of incubation), the mycelium-like material was harvested by filtration through cheese cloth. This material was frozen and stored at -20° until it was needed. To rupture the cells, the frozen material was put through a Hughes press. The resulting material was suspended in 50 mM Tris·HCl buffer, pH 8.0 (1 ml of buffer per 2 g of cells). The suspension was thoroughly mixed by homogenization and then subjected to centrifugation at 37,000 xg for 30 minutes at 4°. The resulting supernatant fluid contained approximately 7 mg per ml of protein, measured by the method of Lowry, et al. (6). Before use, this material was subjected to filtration through a column (1 x 50 cm) of Sephadex G-25 to remove small molecules such as NADPH from the extract. For convenience, this preparation will be called the "crude extract".

Since the possible products of enzymatic action (Compounds III, IV, and V, Fig. 1) are not very stable, the formation of these products was assessed by measurement of the more stable products formed by treatment of incubated reaction mixtures with butanedione and alkaline phosphatase. Details of this process have been described by Burrows and Brown (2). Briefly, this treatment would result (as shown in Fig. 1) in the conversion of Compound III to ribose-5-P and 6,7-dimethyllumazine (DML), Compound IV to 6,7-dimethyl-8-ribitylpterin (DMRPt), and Compound V to 6,7-dimethyl-8-ribityllumazine (DMRL). Thus, the detection of DML would be evidence for the enzymatic formation of Compound III via deamination by Reaction 2; detection of DMRPt would suggest the occurrence of Reaction 2a (reduction of the ribose group) to yield Compound IV as an enzymatic product; and detection of DMRL would indicate that Compound V was produced through reduction and deamination in no particular order.

The preparation and processing of reaction mixtures were as described earlier (2) except that no EDTA was added after the generation of PRP from GTP. Briefly, ^{14}C-labeled substrate [PRP (Compound II), Fig. 1], prepared enzymatically from [U-^{14}C]GTP, was incubated with enzyme preparations from A. gossypii, with and without NADPH, and after treatment of the incubated reaction mixtures with butanedione and alkaline phosphatase (except that treatment with phosphatase was not needed and, therefore, not included when the formation of DML was measured) followed by the addition of DML, DMRPt, and DMRL in small amounts as chromatographic standards, the mixtures were analyzed for the presence of radioactive DML, DMRPt, and DMRL by paper chromatographic and thin-layer chromatographic methods. For this purpose, material was first subjected to paper chromatography (Whatman 3 MM paper) with t-butyl alcohol: water (60:40, by volume) as solvent (development was by the descending fashion). The areas corresponding to the standards (detected as fluorescent zones) were eluted and the material was then subjected to two-dimensional thin-layer chromatography on cellulose plates. For analysis for DML the solvents were 20% ethyl alcohol (first dimension)

and n-butyl alcohol: pyridine:water (4:3:7, by volume) in the second dimension. In the analysis for DMRPt, n-propyl alcohol:1% ammonia (2:1, by volume) was used in the first dimension and 3% ammonium chloride in the second dimension. DMRL was detected with 20% ethyl alcohol (first dimension) and 3% ammonium chloride (second dimension). Zones of migration of the compounds were located as fluorescent areas, and radioactive areas were visualized by autoradiography (see (2) for the general procedure). To determine quantitatively the amount of radioactive compound produced, the appropriate radioactive area was scraped from the developed cellulose plate and the amount of radioactivity was determined in a scintillation counter (Packard model 3320).

Results and Discussion: No evidence could be obtained for the formation of Compound III (see Fig. 1) by incubation of PRP with crude extract in the

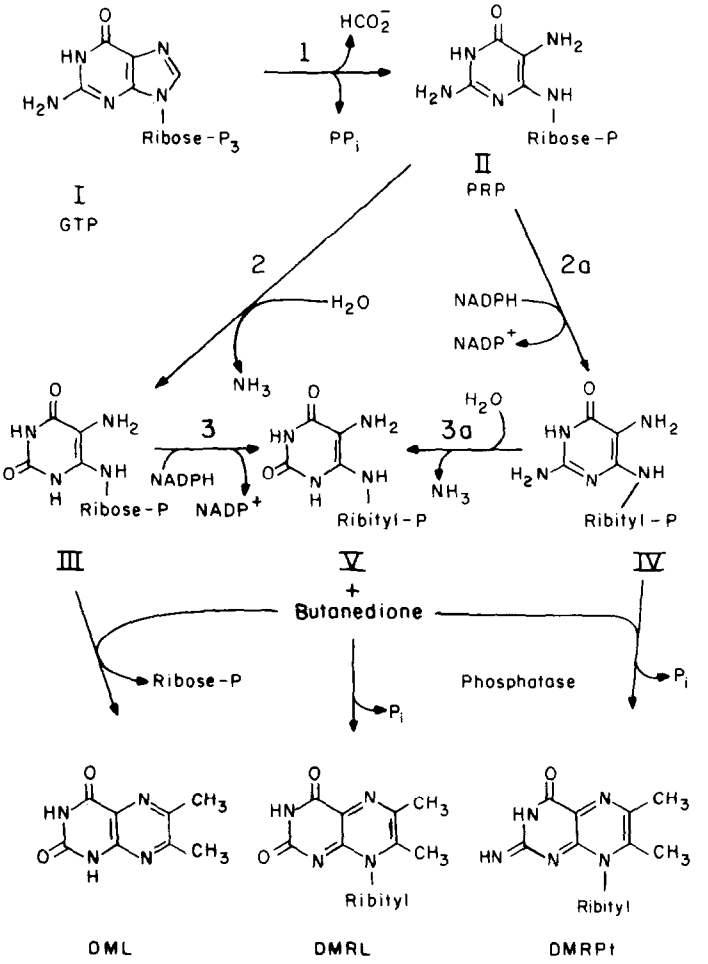

Fig. 1. Possible enzymatic reactions involved in the biosynthesis of riboflavin and the nonenzymatic reaction of butanedione with the products to yield either 6,7-dimethyllumazine (DML), or 6,7-dimethyl-8-ribityl-lumazine (DMRL), or 6,7-dimethyl-8-ribitylpterin (DMRPt).

Table 1. Enzymatic Production of Compounds which React with Butanedione to Yield DMRPt and DMRL

	Radioactive compound detected, cpm			
	DMRPt		DMRL	
	+NADPH	-NADPH	+NADPH	-NADPH
Experiment 1	626	72	549	37
Experiment 2	415	29	3187	44

absence of NADPH. This was established by the fact that no radioactive DML could be detected after the incubated reaction mixture had been treated with butanedione. However, the results presented in Table 1 indicate that radioactive DMRPt and DMRL were both produced and that the presence of NADPH in the enzymatic reaction mixture was essential for these transformations. These observations indicate that in the presence of the extract and NADPH, PRP was converted to Compounds IV and V. Although the total yield of the two compounds, IV plus V, was relatively low (1% to 3.5%, based on the specific radioactivity of the [U-^{14}C]GTP used to generate PRP), the results are nevertheless convincing since virtually none of these products could be detected when NADPH was omitted from the reaction mixtures.

From the results presented above, one can deduce that Compound V was produced via Reactions 2a and 3a with Compound IV as an intermediate. The fact that no evidence could be obtained for the formation of Compound III in the absence of NADPH (i.e., no radioactive DML was detected) would seem to rule out the production of Compounds III and V via Reactions 2 and 3, the reaction pathway that is known to operate in E. coli (2). Additional evidence to support the conclusion that in A. gossypii reduction precedes deamination was provided with the finding that fractionation of an enzyme preparation (that contained reductase and deaminase activities) on a column of Affi-Gel Blue allowed the recovery of reductase activity (eluted from the column with 3 mM NADPH in the developing buffer, 50 mM Tris·HCl at pH 8.1

which also contained 10 mM mercaptoethanol and 10% glycerol) for the conversion of PRP to Compound IV, but no deaminase activity was recovered (i.e., Compound V was not produced). The deaminase activity either was destroyed by this treatment or it was not eluted from the column.

These experimental observations indicate that Ashbya gossypii differs from E. coli in that reduction precedes deamination in the biosynthetic pathway for the production of riboflavin. Thus, in this respect, the pathway in Ashbya gossypii resembles that thought to function in Saccharomyces cerevisiae, as deduced from the excretion products of mutants blocked at different steps in the pathway (3).

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