Vol. 118, No. 3, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1984 Pages 814-820

BIOSYNTHESIS OF RIBOFLAVIN. ENZYMATIC FORMATION OF THE XYLENE MOIETY FROM [^{14}C]RIBULOSE 5-PHOSPHATE

Peter Nielsen*, Gerhard Neuberger*,
Heinz G. Floss#, and Adelbert Bacher*

*Lehrstuhl für Organische Chemie und Biochemie,
Technische Universität München, Lichtenbergstraße 4,
D-8046 Garching, German Federal Republic

#Department of Chemistry, The Ohio State University,
Columbus, Ohio 43210, USA

Received December 27, 1983

We have studied the enzymatic formation of the xylene ring of riboflavin using cell extracts from the flavinogenic yeast Candida guilliermondii. 5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione or its 5'-phosphate could serve as substrates. In addition, a pentose phosphate or pentulose phosphate was required. Experiments with [^{14}C]ribulose 5-phosphate gave evidence for the incorporation of the ribulose carbon atoms except C-4 into the xylene ring of the vitamin.

The terminal step in the biosynthesis of riboflavin (**3**) (Fig. 1) is well understood and involves a dismutation of 6,7-dimethyl-8-ribityllumazine (**2**) (for review see Refs. 1, 2). Thus, the xylene ring of the vitamin is ultimately formed by a duplication of carbon atoms 6α, 6, 7, and 7α of the lumazine. The biosynthetic origin of these 4 lumazine carbon atoms has been under investigation since the pioneering work by Plaut (3, 4). Eventually a considerable number of precursors have been discussed such as diacetyl, acetoin, a tetrose, a pentose and the ribityl side chain of the pyrimidine **1** (for review see Refs. 5-7). We have recently performed incorporation studies with a variety of ^{13}C-labeled precursors (7-10). The results were consistent with the incorporation of carbons 1, 2, 3 and 5 from a pentose into the pyrazine ring of **2**. However, carbon atom 4 of

0006-291X/84 $1.50

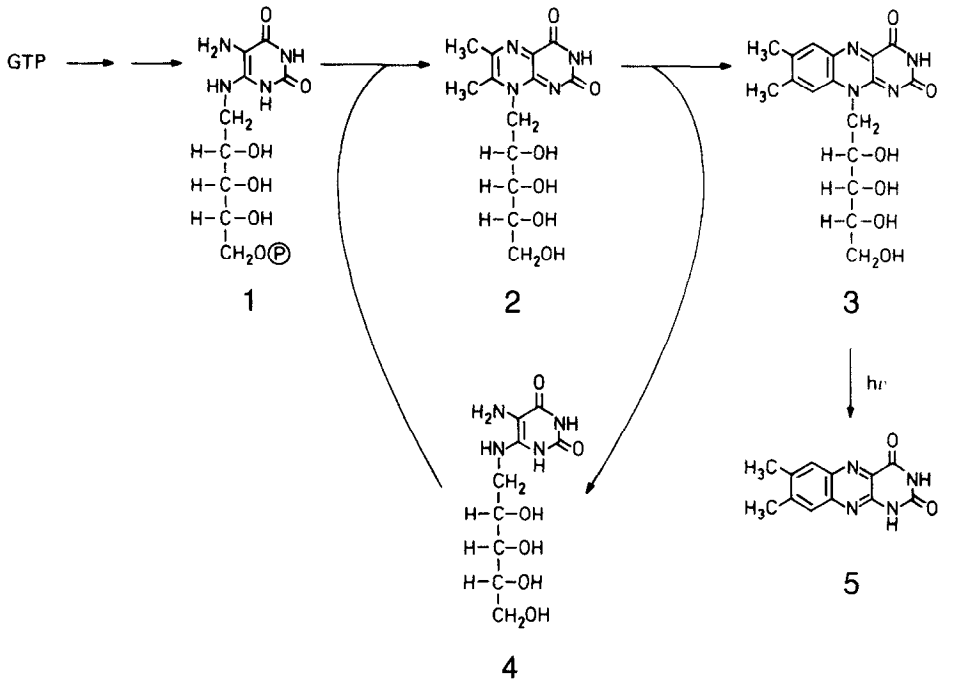

Fig. 1 Biosynthesis and photochemical degradation of riboflavin

the pentose had no equivalent in the pyrazine ring. It was further shown that this carbon atom is eliminated in the course of an intramolecular rearrangement. Whether this reaction actually occurs at the biosynthetic level of a pentose or some biochemical equivalent is not yet known.

Logvinenko and coworkers (11) have recently reported on the *in vitro* formation of riboflavin from GTP and ribose 5-phosphate by cell extracts from the flavinogenic yeast *Candida guilliermondii*. Glucose 6-phosphate and fructose 6-phosphate were less efficient substrates, and unphosphorylated sugars were inactive. Nothing is known about the chemical details of this reaction. We present evidence on the enzymatic formation of riboflavin from ribulose 5-phosphate with ^{14}C-label in different positions. The data confirm the elimination of carbon atom 4 of the pentose during the formation of the xylene ring of the vitamin.

MATERIALS AND METHODS

5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (**4**) and its 5'-phosphate (**1**) were synthesized as described (12, 13). Radiochemicals were purchased from Amersham, enzymes from Boehringer, and ribulose 5-phosphate from Sigma.

[5-^{14}C]Glucose 6-phosphate and [4,6-^{14}C]glucose 6-phosphate were prepared from [2-^{14}C]glycerol and [1,3-^{14}C]glycerol, respectively (14). [2-^{14}C]Glucose 6-phosphate and [6-^{14}C]glucose 6-phosphate were obtained by enzymatic phosphorylation of the appropriate glucose with hexokinase. Each respective glucose 6-phosphate was converted to 6-phosphogluconate by glucose 6-phosphate dehydrogenase. The product was purified by chromatography on a column of Dowex 1 x 8 (formate form, elution with 30 mM ammonium formate containing 0.1 M formic acid pH 3.2). Each phosphogluconate sample was subsequently converted to ribulose 5-phosphate by 6-phosphogluconate dehydrogenase. The product was again purified by chromatography on columns of Dowex 1 x 8 as described above.

Lactobacillus casei ATCC 7469 was a gift of Dr. B. Mailänder, Pfizer Inc., Karlsruhe. Candida guilliermondii ATTC 9058 was obtained from the American Type Culture Collection and was grown as described previously (15). Cells were harvested and disrupted with an X-press. The cell extract was centrifuged and dialyzed against 25 mM Tris hydrochloride pH 8.0 containing 5 mM $MgCl_2$.

RESULTS

We have studied the formation of riboflavin from synthetic 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (**4**) and its 5'-phosphate **1**, respectively, by cell extracts from Candida guilliermondii. Riboflavin was monitored by bioassay with Lactobacillus casei.

The formation of **2** from the pyrimidine precursor requires a second substrate. The highest yield of riboflavin was observed with ribose 5-phosphate and xylulose 5-phosphate (Table 1). Ribulose 5-phosphate and arabinose 5-phosphate were less efficient precursors. Hexose phosphates and unphosphorylated carbohydrates gave values in the range of background level.

Riboflavin could be formed enzymatically from both the phosphorylated pyrimidine **1** and from unphosphorylated **4**. The yield of riboflavin was somewhat lower with **4** as substrate. Addition of

Table 1 Enzymatic formation of riboflavin by cell extract from Candida guilliermondii

Substrate (0.95 mM)	Riboflavin (uM)
none	1.6
Arabinose 5-phosphate	6.1
Fructose	1.7
Fructose 6-phosphate	1.9
Glucose	1.7
Glucose 6-phosphate	1.9
Ribose	1.6
Ribose 5-phosphate	10.6
Ribulose	1.5
Ribulose 5-phosphate	7.0
Xylulose 5-phosphate	9.8

The reaction mixture contained 0.72 mM **1**, 38 mM Tris hydrochloride pH 8.0, 3.8 mM $MgCl_2$, 10 mM dithioerythritol, 0.95 mM of the respective carbohydrate and 0.6 mg of protein; total volume, 400 µl. Incubation at 37 °C for 1 h.

EDTA to the assay mixture abolished the formation of riboflavin completely. Enzyme activity of cell extract dialyzed against buffer containing EDTA could be partially restored by the addition of 10 mM Mg^{++}. The addition of NAD, NADH, NADP, NADPH, thiamine pyrophosphate, or ATP did not enhance the formation of riboflavin.

The incorporation of radioactivity from various ^{14}C-labeled ribulose 5-phosphate samples into riboflavin was studied with the pyrimidine phosphate **1** as substrate. Riboflavin was isolated from the reaction mixture by adsorption on Florisil followed by HPLC (16). Riboflavin was photochemically converted to lumichrome (**5**) which was again purified by HPLC. Results are shown in Table 2. Radioactivity was efficiently incorporated into riboflavin from [1-^{14}C]ribulose 5-phosphate, [5-^{14}C]ribulose 5-phosphate, and [3,5-^{14}C]ribulose 5-phosphate. On the other hand, little radioactivity was incorporated from [4-^{14}C]ribulose 5-phosphate.

Table 2 Enzymatic formation of riboflavin from ^{14}C-labeled ribulose 5-phosphate samples

Substrate	Specific activity (dpm/nmol)		
	Ribulose 5-phosphate	Riboflavin (3)	Lumichrome (5)
[1-^{14}C]Ribulose 5-phosphate	403	516	504
[3,5-^{14}C]Ribulose 5-phosphate	457	535	499
[4-^{14}C]Ribulose 5-phosphate	290	9.6	9.1
[5-^{14}C]Ribulose 5-phosphate	194	238	220

Assay mixtures contained 38 mM Tris hydrochloride pH 8.0, 3.8 mM $MgCl_2$, 10 mM dithioerythritol, 0.95 mM ribulose 5-phosphate, 0.82 mM **1**, and 3 mg of protein; total volume, 2 ml. Samples were incubated at 37 ^{o}C for 1 h.

DISCUSSION

The formation of **1** as an intermediate of the biosynthesis of riboflavin in yeast has been shown conclusively (17). On the other hand it is known that the last biosynthetic step requires unphosphorylated **2** as substrate (18). This reaction yields dephosphorylated **1** as second product. In the present study, formation of riboflavin was observed with both **1** and **4** as substrates. It is thus conceivable that the enzyme can process both the free pyrimidine and the phosphoric acid ester. This opens the possibility for the recycling of dephosphorylated **1** which is produced as a byproduct of the riboflavin synthase reaction. Our results do not rule out the possibility that the unphosphorylated pyrimidine **4** is the only substrate for the formation of **2**, and that the phosphate **1** is hydrolyzed by the action of phosphatase present in the cell extract prior to the conversion to **2**.

Formation of **2** from **4** has been reported with enzymes from Escherichia coli (18). This enzyme system required pyridine nucleotides and **4** as the sole substrate. It was therefore proposed that the ribityl side chain of **4** served as the source of the 4C-unit by dismutation. Our data suggest a pentose or pentulose phosphate as a second substrate in agreement with the data of Logvinenko et al. (11). The possibility that fungi and bacteria may use different pathways should be considered.

We have shown that carbons 1, 3 and 5, but not C-4 from ribulose phosphate can be incorporated into the isoalloxazine moiety of riboflavin. This finding is in excellent agreement with the results of in vivo studies with ^{13}C-labeled precursors suggesting the loss of C-4 from a pentose precursor or its biochemical equivalent by an intramolecular rearrangement (7, 10). The details of this intriguing reaction are presently under study.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

REFERENCES

1. Plaut, G. W. E. (1971) in: Comprehensive biochemistry (Florkin, M., and Stotz, E. H., eds.) vol. 21, pp. 11-45, Elsevier, Amsterdam, New York.
2. Plaut, G. W. E., Smith, C. M., and Alworth, W. L. (1974) Annu. Rev. Biochem. 43, 899-922.
3. Plaut, G. W. E. (1954) J. Biol. Chem. 211, 111-116.
4. Plaut, G. W. E., and Broberg, P. L. (1956) J. Biol. Chem. 219, 131-138.
5. Brown, G. M., and Williamson, J. M. (1982) Adv. Enzymol. 53, 345-381.
6. Alworth, W. L., Dove, M. F., and Baker, H. N. (1977) Biochemistry 16, 526-531.
7. Bacher, A., Le Van, Q., Keller, P. J., and Floss, H. G. (1983) J. Biol. Chem., in press.
8. Bacher, A., Le Van, Q., Bühler, M., Keller, P. J., Eimicke, V., and Floss, H. G. (1982) J. Amer. Chem. Soc. 104, 3754-3755.
9. Keller, P. J., Le Van, Q., Bacher, A., Kozlowski, J. F., and Floss, H. G. (1983) J. Amer. Chem. Soc. 105, 2505-2507.
10. Floss, H. G., Le Van, Q., Keller, P. J., and Bacher, A. (1983) J. Amer. Chem. Soc. 105, 2493-2494.

11. Logvinenko, E. M., Shavlovskii, G. M., Zakal'skii, A. E., and Zakhodylo, I. V. (1982) Biokhimiya 47, 931-936.
12. Plaut, G. W. E., and Harvey, R. A. (1971) Methods Enzymol. 18B, 515-538.
13. Bacher, A., and Nielsen, P. in: Chemistry and biology of pteridines (Blair, A. J., ed.) pp. 705-709, Walter de Gruyter, Berlin, New York.
14. Longenecker, J. P., and Williams, J. F. (1980) J. Labelled Compounds Radiopharm. 18, 309-317.
15. Klein, G., and Bacher, A. (1980) Z. Naturforsch. 35b, 482-484.
16. Nielsen, P., Rauschenbach, P., and Bacher, A. (1983) Anal. Biochem. 130, 359-368.
17. Nielsen, P., and Bacher, A. (1981) Biochim. Biophys. A. 662, 312-317.
18. Harzer, G., Rokos, H., Otto, M. K., Bacher, A., and Ghisla, S. (1978) Biochim. Biophys. A. 540, 48-54.
19. Hollander, I. J., Braman, J. C., and Brown, G. M. (1980) Biochem. Biophys. Res. Commun. 94, 515-521.