KOR. JOUR. MICROBIOL. September. 1985 p. 167-171 Vol. 23, No. 3

The Origin of Ribityl Side Chain of Riboflavin in *Ashbya gossypii*

Choi, Won-Ja and Jeongbin Yim
Department of Microbiology, Seoul National University, Seoul, 151, Korea

Ashbya gossypii 에서의 리보플라빈 측쇄의 기원

최원자 · 임정빈
서울대학교 자연과학대학 미생물학과

In order to investigate the origin of the ribityl group of riboflavin and the involvement of GTP cyclohydrolase II in the riboflavin pathway, we studied the incorporation of ^{14}C-labeled guanosine using a well known riboflavin overproducer, *Ashbya gossypii.* Cells were grown in a media containing (U-^{14}C) guanosine and the riboflavin and GMP were isolated and purified by column chromatography. The isolated compounds, riboflavin and GMP were labeled in the ribityl and ribosyl side chain and the isoalloxazine and guanine moiety. By comparing the specific radioactivity of each compound we reached a conclusion that the ribose of guanosine is converted directly to the ribityl moiety of riboflavin. The results indicate that biosynthesis of the vitamin begins at the level of a guanosine compound and also support the involvement of GTP cyclohydrolase II in one of the early steps in the biosynthetic pathway.

The isolation of 2-aminopyrimidine-type compounds from riboflavin-deficient mutants of *Saccharomyces cerevisiae* suggested the specificd involvement of guanine-type precursor (Bacher and Lingens, 1970). This hypothesis was supported by the discovery of an enzyme, named GTP cyclohydrolase II, which catalyzes the conversion of GTP to 2,5-diamino-6-oxy-4-(5'-phosphoribosylamino) pyrimidine (Foor and Brown, 1975). However, little is known on the origin of the ribityl side chain of riboflavin. Plaut (1971) studied the incorporation of (^{14}C) glucose in *Ashbya gossypii* and obtained evidence for the involvement of a pentose intermediate. Supportive evidence was obtained by studies of Oltmanns and Bacher (1972) in experiments with *Saccharomyces cerevisiae.* The question remained whether the direct precursor of the ribityl side chain is the ribose moiety of a nucleotide or a free carbohydrate intermediate. In the present studies, we investigated the incorporation of ^{14}C-labeled guanosine and showed that the biosynthesis of riboflavin begins at the level of a guanosine compound whose ribose moiety is directly converted to the ribityl side chain of riboflavin.

MATERIALS AND METHODS

Chemicals

All reagents used were of the highest quality commercially available. (U-^{14}C) guanosine was purchased from New England Nuclear Corp. Dowex 50W-X8 and Dowex 1-X8 were purchased from Bio-Rad. Sephadex G-10, Sephadex LH-20 were purchased from Pharmacia, and florisil (activated magnesium silicate), GMP, guanosine,

This work was supported in part by the Basic Science Research Institute Program, Ministry of Education, 1983.

lumichrome, and lumiflavin were the products of Sigma Chemical Compamy.

Fungal Culture

Ashbya gossypii ATCC 10895, a well known riboflavin overproducer strain was purchased from American Type Culture Collection. The organism were grown at 25°C for 96 hours with vigorous aeration in a medium which contained, per 300ml: glucose, 12g; peptone (Difco), 1.5g; yeast extract (Oxoid), 0.5g; guanosine, 7mg (0.05 mCi).

Isolation of Riboflavin

Riboflavin was isolated from the culture fluid and purified to constant specific radioactivity as described previously (Bacher and Mailänder, 1973). Purification of riboflavin was performed in darkness to avoid decomposition of the vitamin.

Degradation of Riboflavin

Riboflavin was dissolved in 0.1 M sodium acetate, pH 4, and the solution was exposed to direct sunlight for 2 hours. The solution was evaporated to dryness. The residue was dissolved in 0.5 ml of 95% ethanol and the material was placed on a column of Sephadex LH-20 (0.6x 15cm). The column was developed with 95% ethanol and fractions of 1 ml were collected. Fractions containing lumichrome were combined and concentrated by rotary evaporation.

Isolation of RNA

The fungal cells were subjected to sonication in 0.02M Tris-HCl, pH8.0 containing 0.01M $MgCl_2$. Following centrifugation the supernatant solution was brought to pH 8 by careful addition of concentrated NH_4OH. Barium acetate (2ml of a 2.5% solution) and ethanol (150ml) were added, and the solution was kept at 4°C overnight. The precipitate was pelleted and dissolved in 8ml of 0.05 N HCl. Sodium sulfate (2ml of a 2.5% solution) was added and the precipitate was pelleted. The supernatant was placed on a column of Dowex 50W-X8 (H^+ form, 1.5×50cm). The column was develpoed with 100ml of 0.05 N HCl and subsequently with deionized water. Fractions of 10ml were collected throughout. UMP was eluted in Fractions 3 to 6, CMP in fractions 9 to 12, AMP and GMP in fractions 19 to 30. GMP was further purified by chromatography on a column of Sephadex G-10 (1 ×60cm). Elution was with deionized water.

Degradation of GMP

GMP was dissolved in 0.5 ml of 1 N HCl, and 1mg of unlabeled carrier was added. The solution was heated on a steam bath for 1 hour. It was subsequently passed through a column of Dowex 50W-X8 (H^+ form, 0.5×10cm). The column was washed with 50ml of deionized water. The effluent (ribose fraction) was collected and concentrated. The column was washed with 50ml of 0.5 N HCl. Subsequently, guanine was eluted with 2 N HCl.

Miscellaneous Methods

Radioactivity was determined in a scintillation counter in 0.4 % BBOT in toluene.

RESULTS

Isotope incorporation studies were performed

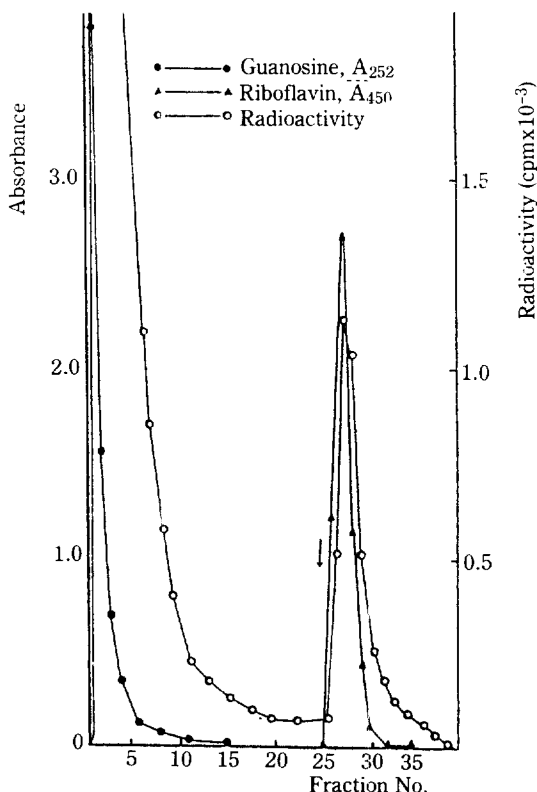

Fig. 1A *Chromatography of the cell free culture medium on a column of magnesium silicate (2×3cm). The column was washed with 300ml of H_2O. Arrow denotes the point of elution with 50% acetone.*

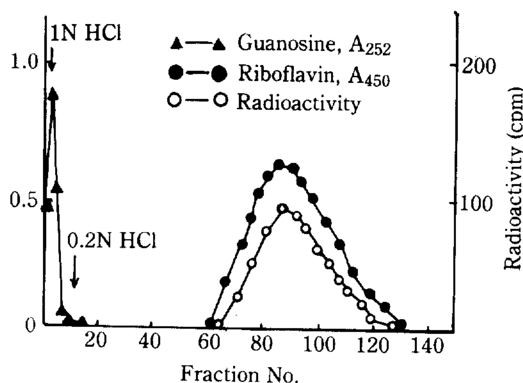

Fig. 1B *Purification of riboflavin on a column of Dowex 50W-X8 (1.2×30cm). Pooled peak fractions (Fraction Nos. 26-29) of the magnesium silicate column eluate were applied to the column which had been previously equilibrated with 1 N HCl. The column was washed sequentially with 1 N HCl and 0.2 N HCl.*

in *Ashbya gossypii* with unifomly ^{14}C-labeled guanosine. Riboflavin was isolated from the culture medium and purified to constant specific activity by the use of column chromatographic techniques (Fig. 1A, 1B). The radiochemical purity of the compound was confirmed by cellulose thin layer chromatography (data not shown). Riboflavin was photochemically degraded to lumichrome and purified as described under "Materials and Methods" (Fig 2). Cellular RNA was hydrolyzed by dilute NaOH, and the resulting cyclic nucleotides were isolated and purified (Fig. 3). Radioactivity was incorporated into GMP, but UMP, AMP and CMP were practically unlabeled. The isolated 2',3' -cyclic GMP was then hydrolyzed by acid and the material was subjected to chromatography on a cationic exchanger (Fig. 4). Radioactivity associated with guanine moiety was determined and the specific radioactivities were compared with those obtained from riboflavin. The results are summarized in Table 1. Guanosine compound was incorporated into nucleic acid GMP without change in the labeling pattern as observed by chemical degradation. Incorporation of radioactivity into other ribonucleotides was minimal. Lumichrome obtained from the degradation of riboflavin was radioactive in agreement with the labeling pattern of the guanosine precursor. The results indicate that the ribose moiety of

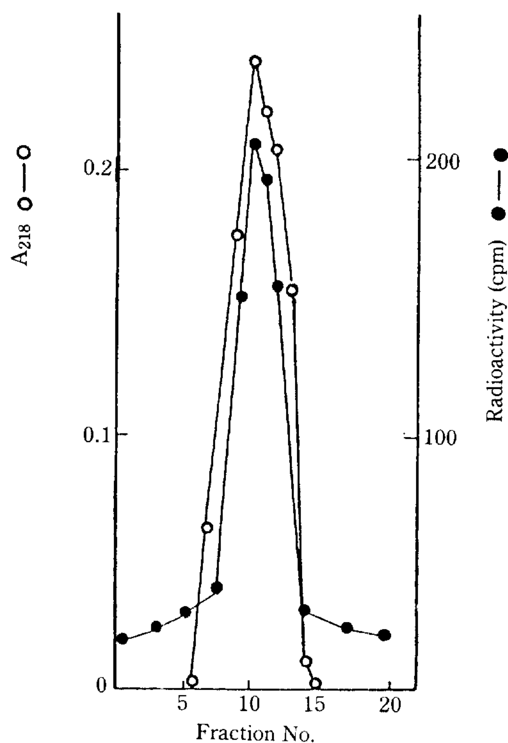

Fig. 2 *Purification of lumichrome on a column of Sephadex LH-20. Sample (3 ml in 95% ethanol) was loaded on the column and eluted as described in "Materials and Methods".*

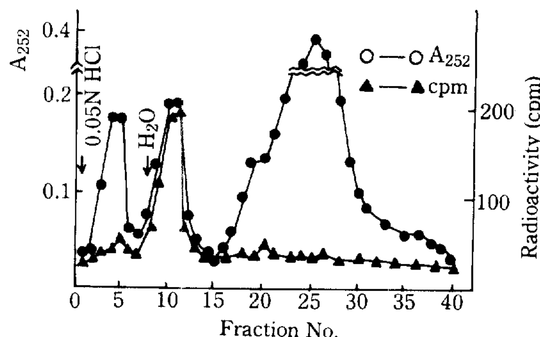

Fig. 3 *Elution profile of 2',3'-cyclic ribonucleotides of RNA on cation exchange chromatography. The column was developed with 100ml of 0.05 N HCl and subsequently with deionized water.*

the added guanosine was directly converted to the ribityl moiety of the vitamin. It also suggests that the biosynthesis begins with a guanosine compound and not with free guanine. However, our experiments yield no information with respect to the potential phosphorylation of the actual starting material.

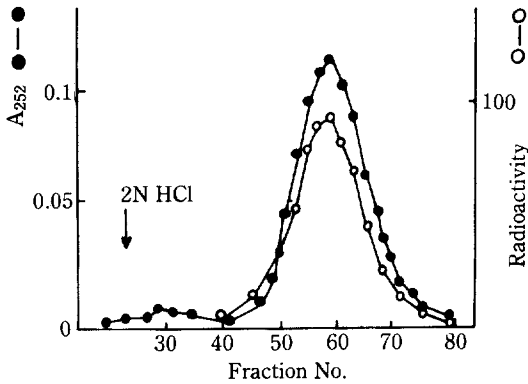

Fig. 4 *Chromatography of acid hydrolysate of 2',3'-cyclic GMP on a column of Dowex 50W-X8 column. The column was develped as described in "Materials and Methods".*

Table 1. *Incorporation of uniformly ^{14}C-labeled guanosine into guanine nucleotide and riboflavin in A. gossypii.*

Compounds	Concen-ration (nmole/ml)	Radioac-tivity (cpm/ml)	Spec. Act. (cpm/nmol)	No. of ^{14}C
from RNA				
GMP	9.6	256	27.5	10
Guanine	18.9	255	13.5	5
from Media				
Riboflavin	35.9	678	18.9	9+(8)*
Lumichrome	27.9	232	8.3	4+(8)*

*These carbons originate from "4C" unit of unknown source.

DISCUSSION

A considerable amount of evidence shows that the biosynthesis of all pteridines and related compounds studied so far begins at the level of GTP (Yim and Brown, 1976; Kim and Yim, 1982). The initial step consists of the removal of carbon atom 8 from the imidazol ring of GTP. It has been suggested that the biosynthesis of riboflavin may follow the same general path (Oltmanns and Bacher, 1972).

Foor and Brown (1975) recently isolated an enzyme designated GTP cyclohydrolase II from *E. coli*. The enzyme catalyzes the release of formate and pyrophosphate from GTP and yields a ribose phosphate derivative of hydroxytriamino pyrimidine. The reaction product of GTP cyclohydrolase II reacts nonenzymatically with diacetyl under loss of the ribose moiety with formation of 2-amino-6, 7-dimethyl-4-hydroxypteridine. The latter compound was isolated previously from cultures of *rib* 7 mutants of *Saccharomyces cerevisiae* incubated with diacetyl. (Bacher and Lingens, 1971). If follows that the gene *rib* 7 codes for the enzyme activity subsequent to the GTP cyclohydrolase. We propose that this enzyme catalyzes the Amadori rearrangement and subsequent reduction of the ribose moiety yielding 2,5-diamino-6-hydroxy-4-ribitylamino pyrimidine or the corresponding monophosphate. Although direct experimental evidence for the role of GTP cyclohydrolase II is not available, but the suggested involvement in the first step of riboflavin is in full agreement with our present data.

적 요

*E. coli*에서 ribosyl-HTP(hydroxytriamino pyrimidine)를 GTP로 부터 합성하는 효소 GTP cyclohydrolase II가 발견된 뒤 riboflavin의 ribityl group이 guanine nucleotide의 ribosyl group에서 직접 유래한다는 가설이 제안되었다. 본 연구에서는[U-^{14}C] guanosine을 media에 첨가하여 배양한 riboflavin overproducer 균주 ***Ashybya*** 에서 추출 정제한 riboflavin과 RNA에 각각 incorporate된 guanosine label의 specific radioactivity를 비교 측정함으로써 ribityl group이 guanosine에서 기원한다는 결과를 얻을 수 있었다. 이는 GTP cyclohydrolase II가 riboflavin생합성의 초기 단계에서 직접 관여한다는 가설을 지지해 주는 것이다.

REFERENCES

1. Bacher, A. and F. Lingens (1970) Biosynthesis of riboflavin: Formation of 2.5-diamino-6-hydroxy-4-(1' -ribitylamino) pyrimidine in a riboflavin auxotroph, *J. Biol. Chem.*, **245,** 4647-4652.
2. Bacher, A., and F. Lingens (1971) Biosynthesis of riboflavin: Formation of 2,5-diamino-6-hydroxy-4-(1'-ribitylamino) pyrimidine in a riboflavin auxotroph, *J. Biol. Chem.*, **245,** 4647-4652.
3. Bacher, A., and B. Mailänder (1973) Biosynthesis of riboflavin: The structure of the purine precursor, *J. Biol. Chem.*, **248,** 6277-6231.
4. Foor, F., and G.M. Brown (1975) Purification and properties of GTP cyclohydrolase II from *Escherichia coli*, *J. Biol. Chem.*, **250,** 3545-3551.
5. Kim, W., and J. Yim (1982) Partial purification and some properties of guanosine triphosphate cyclohydrolase from *Pseudomonas putida*, *Kor. J. Microbiol.*, **20,** 201-209.
6. Oltmanns, O., and A. Bacher (1972) Biosynthesis of riboflavin in *Saccharomyces cerevisiae*: The role of genes ribl and rib7, *J. Bacteriol.*, **110,** 818-822.
7. Plaut, G.W.E. (1971) Metabolism of water-soluble vitamins, *Comprehensive Biochem.*, **21.** 11-45.
8. Yim, J., and G.M. Brown (1976) Characteristics of GTP cyclohydrolase I purified from *Escherichia coli*, *J. Biol. Chem.*, **251,** 5087-5094.

(Received August 1, 1985)