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BIOSYNTHESIS OF RIBOFLAVIN IN VITRO

ISOTOPIC INCORPORATION STUDIES IN *PICHIA GUILLIERMONDII* EXTRACTS

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Summary

In the riboflavin-deficient mutant rib_3 and in wild-type cells of *P. guilliermondii*, GTP is transformed into 2,5-diamino-6-hydroxy-4-ribitylaminopyrimidine and riboflavin, respectively. We were able to demonstrate the partial in vitro synthesis of these compounds, including a reductive conversion step of the product of GTP cyclohydrolase II action upon labelled [^{14}C]GTP. In order to analyse the pyrimidine derivatives formed, 6,7-dimethyl-8-ribitylpterin and riboflavin were synthesized by addition of diacetyl. The data obtained indicate that the radiocarbon from the ribose moiety of GTP was transformed into the ribityl sidechain of 2,5-diamino-6-hydroxy-4-ribitylaminopyrimidine as well as riboflavin without any dilution. Therefore, the ribityl sidechain of the riboflavin formed originates from the ribose moiety of GTP.

Introduction

The biosynthesis of riboflavin starts with the removal of the carbon atom 8 of GTP by a guanosine triphosphate cyclohydrolase which was purified and characterized from *Escherichia coli* [1] and various yeasts [2—6]. The enzyme termed GTP cyclohydrolase II uses GTP as a substrate for the formation of formate and 2,4,5-triaminopyrimidine derivative as has been determined for instance in riboflavin overproducing yeast *Pichia guilliermondii* [6]. In rib_1 mutants of *P. guilliermondii* this enzyme is absent. Cells grown under iron deficiency which favours riboflavin overproduction have 20—40 fold increased enzyme activity when compared with nonoverproducing cultures (supple-

mented with iron). The product of cyclohydrolase II, presumably being 2,5-diamino-6-hydroxy-4-ribosylaminopyrimidine-5′-phosphate, was transformed by crude enzyme extracts of wild-type strains and riboflavin auxotrophs of *P. guilliermondii* to ribitylated pyrimidines in presence of NADPH, thus indicating a reductive step in the conversion of the ribose moiety of GTP into intermediates of the riboflavin biosynthetic path [7]. The triaminopyrimidine derivative formed from GTP by cyclohydrolase II was reacted with diacetyl to form either 6,7-dimethylpterin [2,6] or, after reduction with NADPH, 6,7-dimethyl-8-ribitylpterin and 6,7-dimethyl-8-ribityllumazine [7]. More recently, presence in *Escherichia coli* of a deaminase and a reductase has been shown involved in riboflavin biosynthesis. Purified deaminase (200-fold) only attacks the phosphorylated species of 2,5-diamino-6-hydroxy-4-ribosylaminopyrimidine, but not the dephosphorylated one. In the bacterial system, product of cyclohydrolase II action is deaminated before reduction occurs [8]. NADPH was about three times more effective than NADH. In yeast *Ashbya gossypii* the inverse reaction sequence exists, i.e., reduction is prior to deamination [9].

There is a mass of experimental evidence now in favour of the earlier assumption of Mailänder and Bacher [10] that the ribityl sidechain of riboflavin originates from the ribose moiety of a guanine compound. In earlier papers [11—13] a preferential role of ribitol in the biosynthesis of riboflavin in *Candida (Pichia) guilliermondii* and *Eremothecium ashbyii* was reported. The evidence available from such in vivo experiments was, however, not conclusive. Therefore, we did reinvestigate the problem using two different experimental approaches, first, a partial in vitro system of *P. guilliermondii* was built able to transform GTP into 2,5-diamino-6-hydroxy-4-ribitylaminopyrimidine and riboflavin, respectively, second, in riboflavin overproducing *P. guilliermondii* labelling patterns of riboflavin and GMP isolated from RNA were determined after feeding D-[1-^{14}C]ribose and D-[1-^{14}C]ribitol [14]. From distribution data of radiocarbon over the carbon atoms of the ribityl sidechain of riboflavin and the ribose moiety of GMP (RNA) follows that a common purine precursor of riboflavin formation and RNA synthesis exists being evidently identical with GTP [14]. D-Ribose as well as ribitol are consumed via D-ribose-5-phosphate [15] in nucleotide synthesis.

The objection of the present paper was to give additional experimental evidence in favour of the view that the ribose moiety of GTP is transformed into the ribityl sidechain of 2,5-diamino-6-hydroxy-4-ribitylaminopyrimidine as well as of riboflavin. Using [^{14}C]GTP we were able to demonstrate in the presence of NADPH the partial in vitro synthesis of these compounds. In order to analyse the pyrimidine derivatives formed, 6,7-dimethyl-8-ribitylpterin and riboflavin were synthesized by addition of diacetyl (Fig. 1). The distribution of radiocarbon of [^{14}C]GTP was estimated prior to use by the enzymatic removal of the C-8 atom of the purine ring by a GTP cyclohydrolase II preparation and in a parallel manner by a hydrolytic decomposition of GTP to yield guanine and the ribose moiety. The distribution of ^{14}C over the sum of C atoms 1′—5′ of GTP was calculated. Distribution data thus obtained were compared with data obtained from chemical degradations of 6,7-dimethyl-8-ribitylpterin and riboflavin. This experimental approach strongly confirms the view that the ribityl sidechain of riboflavin originates from the ribose moiety of GTP.

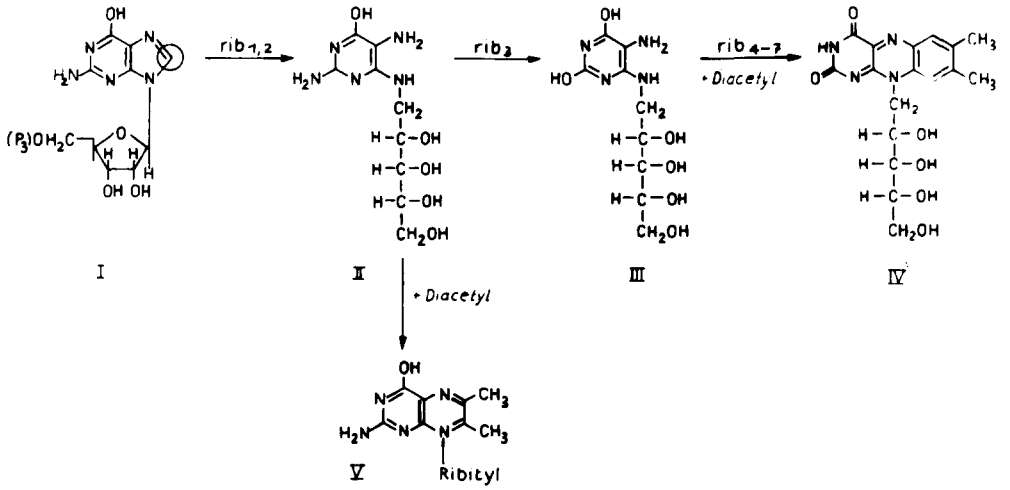

Fig. 1. Scheme of the riboflavin biosynthesis path as valid at present. Compound V was formed by reaction of intermediate II with diacetyl. Diacetyl was also necessary for the synthesis of compound IV. I, GTP; II, 2,5-diamino-6-hydroxy-4-ribitylaminopyrimidine; III, 5-amino-2,6-dihydroxy-4-ribitylaminopyrimidine; IV, riboflavin; V, 6,7-dimethyl-8-ribitylpterin.

Materials and Methods

Organisms and cultivation. Wild-type strains H-4 and ATCC 9058 (overproducing riboflavin in a iron-deficient culture medium) and a riboflavin-deficient mutant R-39 (rib_3, ade^-) of *P. guilliermondii* were used. The origin of the microorganisms as well as the cultivation procedure used has been described elsewhere [7,16].

Determination of the distribution of ^{14}C in [U-^{14}C]GTP including carbon 8. GTP with and without labelling of radiocarbon used for enzymatic investigations and chemical degradation was purified by column chromatography on Sephadex G-10 or G-15 (0.7 × 55 cm, flow rate 15—16 ml/h) according to Wasternack [17]. The percentage of radioactivity in the ribose moiety of labelled GTP was estimated after acidic hydrolysis with 20 ml 1 M HCl containing a mixture of 10 mg GTP and 2.6 μg [^{14}C]GTP for 4 h at 100° C under reflux. The mixture was dried in vacuo, and guanine as a hydrolysis product was purified by column chromatography on Sephadex G-10 [17]. The identification of this compound was carried out by thin-layer chromatography in two solvent systems and by the column constant K_{av} (Table I). By monitoring the quantity and radioactivity of guanine the specific radioactivity of this substance was calculated. 31% of the radioactivity was found in guanine by comparison of the specific radioactivities of guanine with GTP (Table I). After that, the radioactivity of the ribose moiety of [^{14}C]GTP was calculated to 69%.

The carbon atom 8 of GTP was eliminated in vitro by cyclohydrolase II. The incubation mixture was centrifuged at 5600 × *g* and the supernatant was treated with charcoal in a small column [1,4] to eliminate pyrimidine compounds. Thus, the effluate contains only H^{14}COOH and [^{14}C]ribose derived from unstable ribosylpyrimidine which was removed as osazone by reaction

TABLE I

PURIFICATION AND HYDROLYSIS OF RADIOCARBON LABELLED GUANOSINE-5'-TRIPHOSPHATE

The [^{14}C]GTP used in the experiments and the guanine of hydrolysate were analysed by column chromatography. The quantity as well as radioactivity of guanine and its triphosphate were measured. From these data the percentage distribution of radiocarbon in [^{14}C]GTP was calculated by comparison of the specific activities of both substances. Three experiments given the average in the table.

Compound	K_{av}	R_f *	Quantity radioactivity			
			(μmol)	cpm	cpm/μmol	%
GTP-standard	1.10	0				
[^{14}C]GTP	1.10	0	0.360	151 760	421 556	100
Guanine-standard	3.48	0.57				
[^{14}C]guanine	3.47	0.57	0.550	71 947	130 812	31

* *n*-Propyl alcohol/25% NH_4OH, 6:3 (v/v); silica gel.

with 2,4-dinitrophenylhydrazine in acidic solution. This osazone was precipitated by centrifugation at 1000 × *g* and the radioactivity as well as quantity of H^{14}COOH was estimated after distillation of the supernatant in a microapparatus and by comparison with authentic H^{14}COOH solution. 2.3% of the radioactivity was found in the carbon atom 8 of [^{14}C]GTP by comparison of the specific radioactivities of H^{14}COOH with [^{14}C]GTP (Table II).

The data obtained from these experiments indicated that the [U-^{14}C]GTP purchased commercially was not a uniformly labelled molecule. [^{14}C]GTP used was shown to contain in the purine ring 31% of the radiocarbon. From this value the radiocarbon labelling of the ribose moiety was calculated to 69%. In the carbon atom 8, 2.3% of the radioactivity of [^{14}C]GTP was found. These data were the basis for comparison of radiocarbon distribution in the ribose moiety of GTP and the ribityl sidechain of 6,7-dimethyl-8-ribitylpterin and riboflavin, respectively, as synthesized in vitro from [^{14}C]GTP.

Enzyme assay. Crude enzyme preparation possessing GTP cyclohydrolase II and 2,5-diamino-6-hydroxy-4-ribosylamino-pyrimidine-5'-phosphate reductase activities [7] were added to the standard reaction mixture containing in a total

TABLE II

ANALYSIS OF RADIOCARBON IN THE CARBON ATOM 8 DERIVED FROM [^{14}C]GTP AFTER ENZYMATIC DEGRADATION WITH A CRUDE GTP CYCLOHYDROLASE II PREPARATION

Formic acid was measured after precipitation of ribose and distillation of charcoal column effluate. The percentage distribution of radiocarbon of carbon atom 8 was estimated by comparison of the specific radioactivities of GTP with formic acid (see Materials and Methods).

Fraction	Distribution of radioactivity				
	cpm	MBq	μmol	TBg/mol^{-1}	%
Supernatant of incubation mixture	$19.8 \cdot 10^6$	19.79	1.26	15.72	100
Effluate of charcoal column	$70.8 \cdot 10^4$				
Formic acid	$15.3 \cdot 10^4$	0.152	0.42	0.361	2.3

volume of 4.0 ml 80 μmol Tris-HCl buffer, pH 8.2, 16.4 μmol $MgCl_2$, 20 μmol dithioerythritol, 2.0 μmol NADPH, 2.0 μmol [U-^{14}C]GTP and 4.0 mg protein. The reaction mixture was incubated for 1 h at 30°C, subsequently 0.3 ml diacetyl (10 mg/ml water) was added, and after 5 min the tubes were heated at 70°C for 30 min in a waterbath. After centrifugation at 5000 × *g* for 10 min, the supernatant was used to isolate labelled 6,7-dimethyl-8-ribitylpterin and riboflavin.

The carbon atom 8 of [U-^{14}C]GTP was eliminated by GTP cyclohydrolase II in an incubation mixture containing in a total volume of 4.0 ml 2.8 μmol [U-^{14}C]GTP, 40 μmol dithioerythritol, 41.8 μmol Tris-HCl buffer, pH 8.5, and 552 μg protein. Incubation of the test-tubes was carried out at 30°C for 1 h, after which it was stopped by the addition of 0.5 ml formic acid.

Purification of riboflavin and 6,7-dimethyl-8-ribitylpterin. Both riboflavin (wild-type strains ATCC 9058, H-4) and 6,7-dimethyl-8-ribitylpterin (rib_3-mutant R-39) derived from [^{14}C]GTP were isolated from the incubation mixture by passing the solution successively through small columns of Sephadex G-10 and DEAE-cellulose (riboflavin) or two times through a column of CM-Sephadex C-25 (6,7-dimethyl-8-ribitylpterin) according to Bacher et al. [18] and Miersch et al. [12] to their constant specific radioactivities. The purification of riboflavin and 6,7-dimethyl-8-ribitylpterin synthesized in vitro was controlled by paper chromatography, ultraviolet and fluorescence spectra [7].

Degradation of riboflavin and 6,7-dimethyl-8-ribitylpterin. Riboflavin and 6,7-dimethyl-8-ribitylpterin were degraded by ultraviolet irradiation in methanolic solution to yield lumichrome and 6,7-dimethylpterin, respectively [12]. Hydrolysis with 5 M HCl of 6,7-dimethyl-8-ribitylpterin gives 6,7-dimethyl-8-ribityllumazine [19].

Chemicals. [U-^{14}C]GTP (spec. act. 15.725 TBq/mol) was purchased from UVVVR, Prague (Czechoslovakia). The origin of the other materials has been described previously [7].

Results and Discussion

In vivo experiments with wild-type cells of *P. guilliermondii* with regard to the origin of the ribityl sidechain of riboflavin are very difficult because of the extremely high randomization of radiocarbon. Therefore, Mailänder and Bacher [10] used the 3-fold blocked mutant BM 2 of *Salmonella typhimurium.* In parallel to this incorporation studies in vivo we were able to clarify the introduction of radiocarbon in the ribityl sidechain of intermediary pyrimidines and riboflavin derived from [^{14}C]GTP in vitro.

Crude enzyme preparations from *P. guilliermondii* catalyse the transformation of [^{14}C]GTP into 2,5-diamino-6-hydroxy-4-ribitylaminopyrimidine (mutant rib_3) or into riboflavin (wild-type cells). From the specific radioactivities of riboflavin and of 2,5-diamino-6-hydroxy-4-ribitylaminopyrimidine converted to 6,7-dimethyl-8-ribitylpterin by reaction with diacetyl as well as from the specific radioactivities of their degradation products lumichrome and 6,7-dimethylpterin, respectively, the percentage of radioactivity in the ring moiety of these substances was measured to 28.2% (average of the number in

TABLE III

Percentage of radiocarbon distribution in the pyrimidine and isoalloxazine ring of in vitro synthesized 2,5-diamino-6-hydroxy-4-ribitylaminopyrimidine and riboflavin, resp. The pyrimidine was isolated as 6,7-dimethyl-8-ribitylpterin (DMRP). 6,7-Dimethyl-8-ribitylpterin and riboflavin were purified and photochemically degraded to yield 6,7-dimethylpterin (DMP) and lumichrome (LC), respectively.

Experiment	Specific radioactivity GBq/mol	Degradation to DMP or LC			
		cpm	μmol	GBq/mol	%
1 [^{14}C]DMRP	28.1	1 630	0.002	9.10	32.4
2 [^{14}C]DMRP	1010.6	38 790	0.002	289.9	28.7
3 [^{14}C]Riboflavin	0.259	1 395	0.251	0.093	35.7
4 [^{14}C]Riboflavin	2.33	4 550	0.143	0.44	19
5 [^{14}C]Riboflavin	0.074	310	0.307	0.018	25

Table III). In the carbon atom 8 of the purine precursor of riboflavin was located 2.3% of radiocarbon and, consequently, the percentage of radioactivity in the ribityl sidechain of the molecules was calculated to 69.5% (Table IV). From the data obtained we conclude that the ribityl sidechain is derived from the ribose moiety of GTP without any breakdown of the carbon skeleton of the sugar moiety. We found 28.2% of radioactivity in the isoalloxazine ring of [^{14}C]riboflavin and of the pteridine portion of ^{14}C-labelled, 6,7-dimethyl-8-ribitylpterin synthesized in vitro (Tables III, IV). This correspond to 69.5% of the radioactivity in the ribityl sidechain of riboflavin or of 6,7-dimethyl-8-ribitylpterin exactly as in the ribose moiety to 69% of added [^{14}C]GTP (Tables II, IV). The ratio of the radioactivity of ribose (GTP)/ribityl group (riboflavin or pterin) and of guanine minus carbon atom 8 (GTP)/lumichrome or 6,7-dimethylpterin are 1 : 1 (Table IV). The distribution of the radiocarbon in the ribityl sidechain of riboflavin or 2,5-diamino-6-hydroxy-4-ribitylamino-

TABLE IV

COMPARISON OF THE PERCENTAGE DISTRIBUTION OF RADIOCARBON IN [^{14}C]GTP WITH THE [^{14}C]DISTRIBUTION IN 6,7-DIMETHYL-8-RIBITYLPTERIN (DMRP) AND RIBOFLAVIN

DMRP and riboflavin were isolated from the incubation mixture and after purification and chemical degradation the percentage distributions of radiocarbon were estimated in 6,7-dimethylpterin and lumichrome.

[^{14}C]compound	Distribution of radiocarbon (%)
Precursor	
GTP	100
Guanine	31
Ribosyl moiety	69 *
C-8	2.3
Purine ring minus C-8	28.7
Products	
DMRP/riboflavin	100
6,7-Dimethylpterin/lumichrome	28.2
6,7-Dimethylpterin/lumichrome/atom 8 of the precursor GTP	30.5
Ribityl-moiety of DMRP/riboflavin	69.5 *

* Calculated.

pyrimidine and of the ribosyl moiety of GTP described in the present experiments was calculated in a indirect manner whereas the the distribution of radiocarbon of the ribose moiety of GMP isolated from RNA and the ribityl group of riboflavin were measured directly in our laboratory, also [14]. The data or our isotopic incorporation studies in vitro are in agreement with the in vivo studies done with using wild-type cells of *P. guilliermondii* which were fed with D-[1-^{14}C]ribose and D-[1-^{14}C]ribitol after which the incorporation of radiocarbon into the ribose moiety of 3′(2′)-GMP isolated from RNA and the ribityl sidechain of excreted riboflavin, respectively, was compared [14].

In the in vitro studies, the addition of diacetyl to the incubation mixture for 5 min at 30°C heated than to 70°C is a prerequisite for riboflavin formation. Diacetyl is incorporated into riboflavin in vivo [20], but it might be in vitro a protector of riboflavin synthetase, too.

Thus, obviously, the biosynthesis of riboflavin starts from GTP and the ribityl sidechain of riboflavin originates from the ribose moiety of GTP.

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